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Article

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Disulfide bond-containing ajoene analogs as novel quorum sensing inhibitors of *Pseudomonas aeruginosa*

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ABSTRACT

Since its discovery 22 years ago, the bacterial cell-to-cell communication system, termed quorum sensing (QS), has shown potential as anti-pathogenic target. Previous studies reported that ajoene from garlic inhibits QS in opportunistic human pathogen *Pseudomonas aeruginosa*. In this study, screening of an in-house compound library revealed two sulfur-containing compounds which possess structural resemblance with ajoene and inhibit QS in bioreporter assay. Following a quantitative structure-activity relationship (SAR) study, 25 disulfide bond-containing analogs were synthesized and tested for QS inhibition activities. SAR study indicated that the allyl group could be replaced with other substituents, with the most active being benzothiazole derivative (IC_{50} = 0.56 μM). The compounds were able to reduce QS-regulated virulence factors (elastase, rhamnolipid and pyocyanin), and successfully inhibit *P. aeruginosa* infection in murine model of implant-associated infection. Altogether, the QS inhibition activity of the synthesized compounds is encouraging for further exploration of novel analogs in antimicrobial drug development.

INTRODUCTION

Infectious diseases are the direct cause of 16 million deaths annually.[1] Among those, an estimated 65% fatalities are associated with biofilm infections, where a chronic inflammatory phase with increased resistance towards antibiotic treatments are often observed.[2] It has been reported that bacteria in the biofilm mode are orders of magnitude more tolerant to antibiotics in compared with their planktonic counterparts.[3] For two decades, academia has proposed a paradigm shift in the development of new antimicrobial agents which includes anti-virulence and anti-pathogenic strategies.[4] According to our previous work, "anti-pathogenic" refers to an

antibacterial strategy that doesn't kill bacteria *per se* but rather attenuates virulence and the capacity to cause diseases.[5] Most commonly available antibiotics primarily focus on the bactericidal properties which inherently stimulate bacterial evolution.[6] Instead of targeting components essential for growth, the anti-pathogenic strategy relies on attenuating cascades of bacterial gene expression leading to pathogenicity (i.e. microbial attachment, invasion, and biofilm formation, secretion of toxins, chemical signaling and immune evasion, etc.).[7]

For some pathogenic bacterial species, it has been shown that central processes in biofilm development are regulated by quorum sensing (QS), in particular those involved in shielding the biofilm bacteria from antibiotics and phagocytic neutrophils.[8] In *P. aeruginosa,* the two main QS system are regulated by *N*-acyl homoserine lactones (AHLs)-based signaling molecules *las* and *rhl* systems. In the *las* system, LasI synthase directs the synthesis of *N*-(3-oxododecanoyl)-$_L$-homoserine lactone (3-oxo-C12-HSL), which triggers the expression of LasR to induce virulence genes such as *lasB, apr* and *toxA*.[9] The LasIR system also positively regulates RhlIR system, which directs the synthesis of *N*-butanoyl-$_L$-homoserine lactone (C4-HSL) and enhances the transcription of *lasB* and rhamnolipid synthesis genes *rhlAB*.[10] The *rhl* system also directs the synthesis of rhamnolipid, pyocyanin and hydrogen cyanide synthase.[11] The third intercellular signal is 3-heptyl-3-hydroxy-4-quinolone, which is also referred as *Pseudomonas* quinolone signal (PQS). PQS system provides regulatory link and intertwined between the two AHL-based QS systems, where LasR and RhlR additionally regulate the PQS system.[12] On top of that, it also autoinduces its own production via interaction with transcriptional regulator PqsR and positively modulates several virulence genes expression.[12a] Altogether, the QS signals control 5-10% of the *P. aeruginosa* genome through its complex yet finely regulated mechanism.[13] Recently, QS inhibitors (QSI) have been proposed as a promising agent for controlling persistent infections.[4b]

Deletion of one or more QS genes in *P. aeruginosa* result in lower virulence factors as compared to the wild type and render biofilm bacteria more susceptible towards antibiotic treatment.[4a]

Till date, non-native AHLs remain the most extensively studied class of synthetic QSIs,[14] with several SAR studies been carried out around AHLs backbone and halogenated furanones been reported.[14-15] However, the HSL is susceptible to hydrolysis at physiological pH and degradable by mammalian lactonases,[16] and the halogenated furanones suffer from inherent reactivity.[17] The resulting side products are either inactive against QS or toxic, thus limiting the use of such molecules as pharmaceutical agents.[18] The exploration of new compound classes that are structurally distinctive from native AHLs is needed to ultimately deliver clinical candidates.

In the present study, we report a SAR study on novel QSIs identified through the screening of in-house chemical libraries against a well-established QS reporter strain *P. aeruginosa* PAO1-*lasB-gfp*.[19] Four of these compounds were discovered to inhibit *lasB, rhlA, and pqsA* expression as judged from bioreporter assay based on expression of green fluorescence protein (GFP) in *P. aeruginosa* PAO1 (*lasB-gfp; rhlA-gfp; pqsA-gfp*). Moreover, we also discovered important scaffolds of the asymmetric disulfide that could improve the bioactivities to sub-micromolar range. The structure of these small organosulfur compounds had been previously reported as potent modulator of several mammalian thiol and selenium-containing enzymes.[20] In the case of mammalian thioredoxin-1 (Trx), selective inhibition of an asymmetric disulfide could be achieved via cysteine residues on the active sites.[21] Nevertheless, to our knowledge, no precedence of QSIs based on this class of compounds has ever been reported. A small focused library consisting of compounds carrying disulfide bonds was then constructed to provide insights into features important for bioactivities. As potential QSI candidates, we also tested the most promising compounds on QS-activated virulence phenotypes in *P. aeruginosa*, including

elastase, pyocyanin and rhamnolipid production. In addition, a mouse implant-biofilm infection experiment was performed to evaluate the bioactivities of compounds *in vivo*.

RESULTS AND DISCUSSION

Screening of Quorum Sensing Inhibitors. Four hundred and eighty compounds were screened against reporter strain, PAO1-*lasB*-*gfp* for the QS inhibition activities. Induction of the *lasB* gene (encoding elastase) is mainly controlled by LasR and PAI-1 (the Las QS system),[22] hence it is a good indicator for LasR activity. In this reporter system, a decrease in GFP fluorescence would indicate the presence of an antagonist of 3-oxo-C12-HSL, which inhibits the expression of *lasB*. Growth of the reporter strain (optical density of 600 nm (OD_{600})) was monitored to ensure that the compounds would not affect the growth rate (Supporting Information Figure S1). The GFP expression was normalized by dividing the GFP values with the growth measured at the respective time points. Four compounds were found to inhibit *lasB*-*gfp* expression in a dose-dependent manner with micromolar IC_{50} values without affecting growth (Figure 1B).

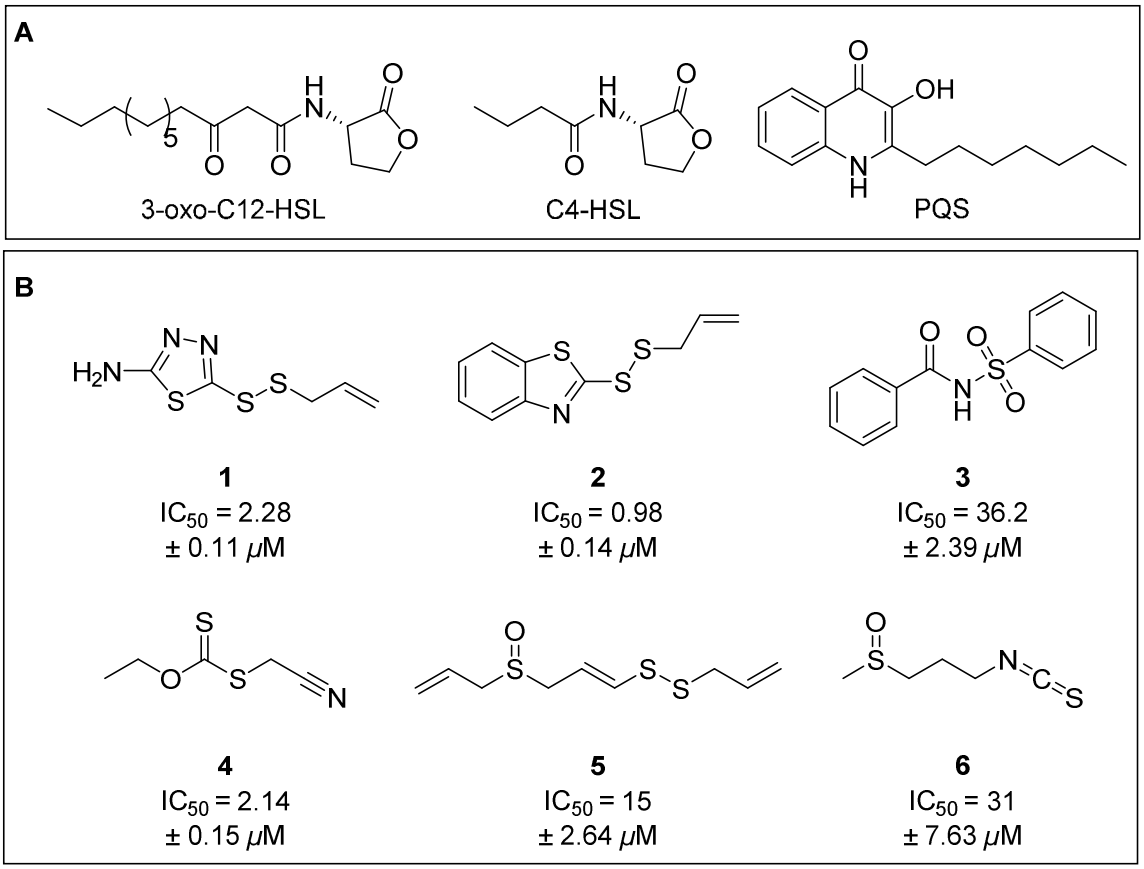


Figure 1. (A) Natural AHL signal molecules used by *P. aeruginosa*. (B) Structure of hit compounds **1** – **4**, nature-derived ajoene **5** and iberin **6** with antagonistic activity lowering the expression of PAO1-*lasB-gfp*.

Compounds **1** - **4** were further tested with the *rhlA-gfp* and *pqsA-gfp* bioreporters in PAO1. Both *rhlA* and *pqsA* are the first genes of the *rhl* operon and *pqs* operon that code for the production of rhamnolipid and PQS molecule.[23] To ensure that the compounds indeed target corresponding QS genes and not the GFP, we also tested the compounds with PAO1-*gfp* as control and did not observe any reduction in the fluorescence output signals (Supporting Information Figure S2). Promising results were obtained for compound **2** with all three reporter strains (Figure 2), which indicated the importance of an allyl disulfide bond in the structure.

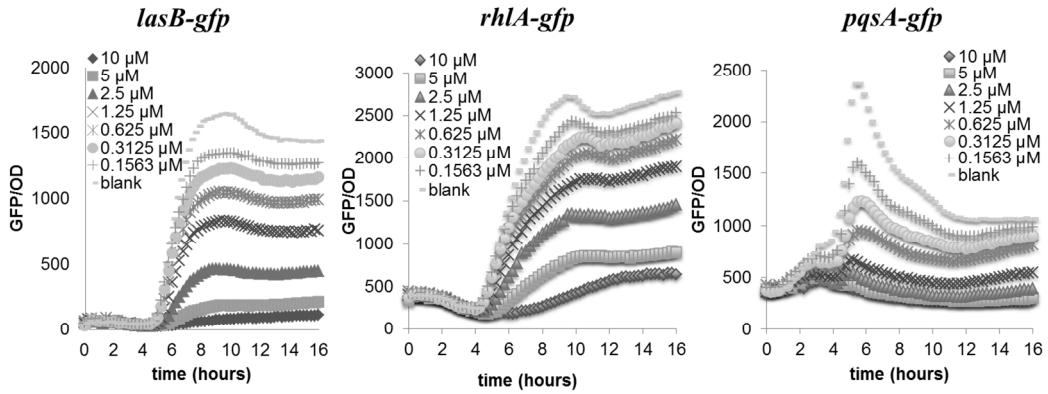


Figure 2. Dose-dependent inhibition curves of compound **2** incubated with the following QS monitors PAO1-*lasB-gfp, rhlA-gfp*, and *pqsA-gfp.* The experiments were done in triplicate manner.

IC_{50} values were calculated from the dose-response curves when inhibition started to occur, usually in the range from 4-8 hours. The IC_{50} values represent half of the concentration required to inhibit the biosynthesis rate of GFP due to AHL induction.[24] Promising results were obtained from our first screening with IC_{50} values in the low micromolar range (< 40 μM). The most potent inhibitory activity was seen for compound **2** with an IC_{50} value at 0.98 μM for *lasB-gfp* bioreporter strain (Figure 3).

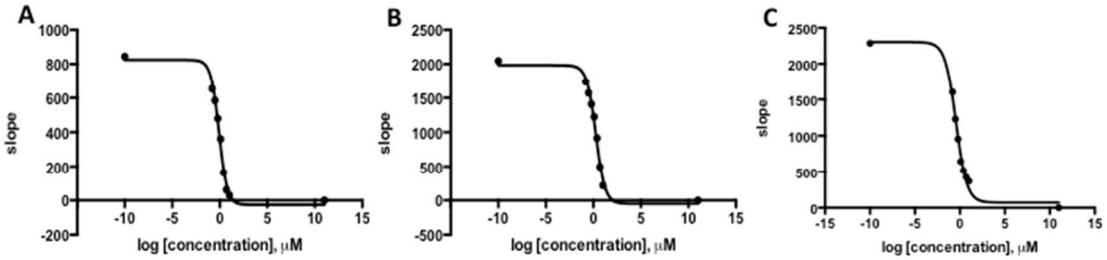


Figure 3. IC_{50} values of compound **2** (A) 0.98 μM for PAO1-*lasB-gfp,* (B) 2.03 μM for PAO1-*rhlA-gfp,* (C) 0.33 μM for PAO1-*pqsA-gfp*. IC_{50} calculation was based on three biological replicates and performed using Graphpad Prism 6 software.

Structural-Activity Relationship (SAR) Study. As shown in Figure 1B, two of the six compounds (**1** and **2**) with good activity in the QS reporter screenings have disulfide (S-S) bonds

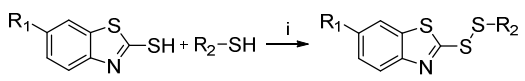
with different heterocyclic functional groups. Disulfide compounds are known to exhibit a wide range of bioactivities, such as antitumor, enzyme-inhibitory, antibiotic and antifungal properties.[25] Ajoene, one of the active ingredients extracted from garlic also contains a disulfide moiety. Previous report by Jakobsen et al. indicated that ajoene can inhibit a subset of QS-controlled virulence genes in *P. aeruginosa* and successfully clear infection in a pulmonary mouse model.[26] Based on our knowledge, no synthetic compounds carrying a disulfide backbone have been identified as QSIs, aside from the natural derivative ajoene. With facile synthetic transformations, a series of compounds with disulfide backbone were synthesized for a SAR study.

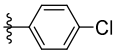
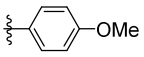
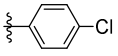
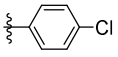
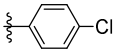
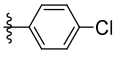
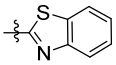
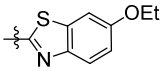
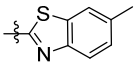
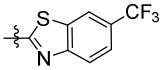
Compounds **1** and **2** share the same allylic disulfide bond as a structural feature with ajoene, thus it was hypothesized that the bioactivities of these compounds are related to the allyl-disulfide functional group. To test this hypothesis, initial chemical transformation started with the replacement of allyl group with different aliphatic and aromatic groups while retaining the benzothiazole disulfide moiety. There are various approaches available for the synthesis of disulfide compounds, and DDQ oxidation was selected for this study (Scheme 1). The reaction proceeded smoothly and relatively fast to give the benzothiazole-functionalized disulfide compounds **7 - 19** in moderate yields. During the synthesis, 1.5 eq. of DDQ was necessary to ensure full conversion of the starting materials. In most cases, homo-coupling of 2-mercaptobenzothiazole was observed as the major side-reaction.

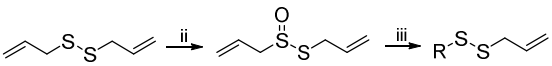
For the modification of the benzothiazole aromatic side-chain, the effects of heteroatoms other than S (i.e. O and N) in the benzothiazole ring or simple benzyl group were investigated. In addition, substituents with diverse electronic properties were examined to evaluate the effect on bioactivity. The synthesis started with the oxidation of diallyl disulfide with slow addition of *m*-

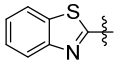
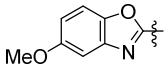
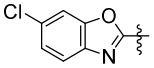
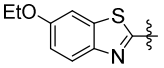
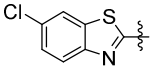
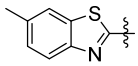
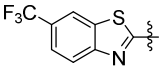
CPBA (meta-chloroperbenzoic acid). The resulting intermediate allicin was directly treated with heterocyclic thiols to give compounds **2**, **20** - **26** (Scheme 1). Lastly, several analogs of monosulfide were synthesized to study the importance of disulfide functional group. The monosulfides **27** - **30** were readily synthesized through alkylation of 2-mercaptobenzothiazole with various halides (Scheme 2).

Scheme 1. **Preparation of unsymmetrical disulfides**[a]



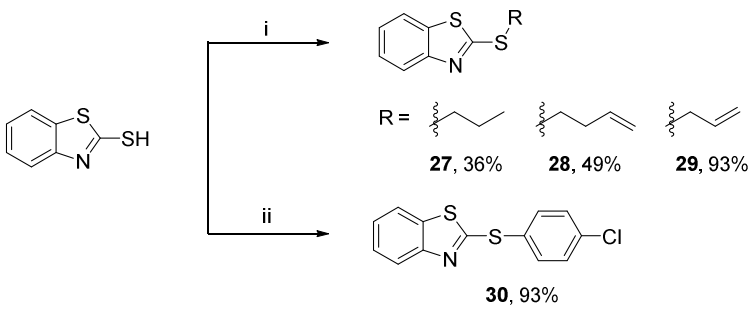
Compound	R_1	R_2	Yield (%)
7	H		40
8	H		36
9	H		15
10	H		54
11	H		76
12	OEt		15
13	Cl		10
14	Me		25
15	CF_3		40
16*	H		26
17*	OEt		5
18*	Me		5
19*	CF_3		45



Compound	R	Yield (%)
2		90
20		31
21		40
22		30
23		95
24		40
25		90
26		97

[a]Reagents and conditions: (i) DDQ, CH_2Cl_2, 0 °C; (ii) *m*-CPBA, CH_2Cl_2, 0 °C; (iii) RSH, MeOH
*Compounds isolated as minor products.

Scheme 2. **Synthesis of 2-mercaptobenzothiazole derivatives**[a]



[a]Reagents and conditions: (i) RBr, K_2CO_3 or NEt_3, H_2O; (ii) CuI, 1,10-phenanthroline, 1-chloro-4-iodobenzene, K_2CO_3, DMF, 120 ^oC.

QSI activities of the novel synthetic S-S disulfides bond compounds. The synthesized compounds were tested for their QS inhibitory activity against the *P. aeruginosa* PAO1-*lasB-gfp* strain and compared with the natural product QSIs iberin and ajoene. Twenty of the synthesized compounds inhibited the expression of *lasB* gene in *P. aeruginosa* without affecting the growth of bacteria (Table 1).

From the preliminary screening with the *P. aeruginosa* PAO1-*lasB-gfp* strain, it could be deduced that the bioactivity was not strictly contingent with the presence of allyl group. For example, the saturated analog **10** displayed excellent activity towards inhibiting the expression of *lasB* gene. Interestingly, the *p*-chlorophenyl derivative **7** improves the bioactivity as compared to phenyl or *p*-methoxyphenyl analogs **(8, 9).** However, the cyclohexyl **11** and benzothiazole moieties in compounds **16-19** were not efficient due to the hydrophobicity and bulky groups.

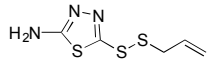
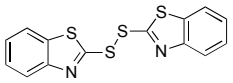
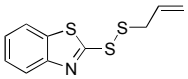
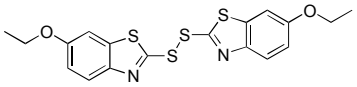
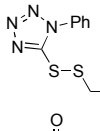
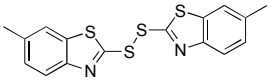
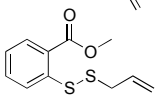
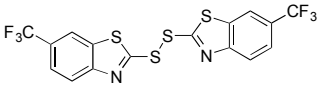
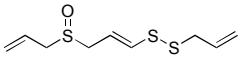
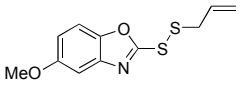
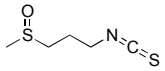
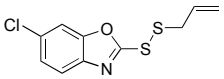
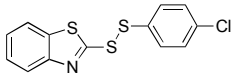
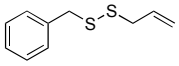
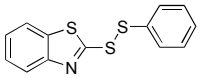
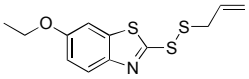
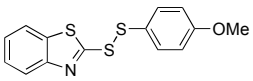
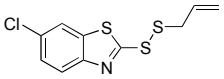
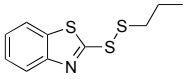
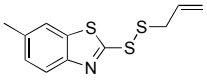
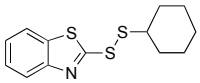
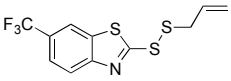
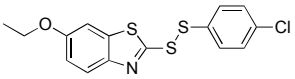
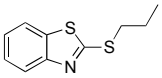
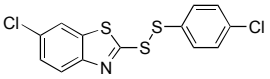
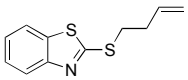
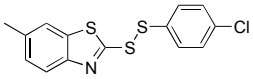
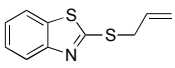
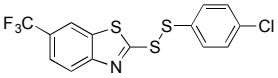
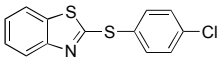
Based on the structure of compound **7**, we further explored the presence of different functional groups on the benzothiazole core while retaining the *p*-chlorophenyl moiety. This heterocycle is present in many compounds that display a range of pharmacologically interesting properties, such as anticancer, antifungal, anti-inflammatory, analgesic and antipyretic activities.[27] Compounds **12**, **14** and **15** displayed excellent bioactivities with IC_{50} values in the sub-micromolar range (0.56 – 0.72 μM). For those compounds without the benzothiazole functional group such as **31** and **32**, *lasB* expression was not observed when tested at 10 μM. Based on the

experimental results, it could be deduced that the presence of a benzothiazole (or analogous benzoxazole) system was essential for antagonist activity towards expression of the *lasB* gene in *P. aeruginosa.*

To confirm that the absence of disulfide bond will render the QSI ineffective, several analogues without the disulfide bonds (**27** - **30**) were synthesized and tested against *PAO1-lasB-gfp.* Starting material bearing the 2-mercaptobenzothiazole moiety was also included as part of this mini-SAR study. Although there have been numerous reports on the bioactivities of benzothiazole,[28] we did not observe any significant reduction of *lasB* with the *P. aeruginosa* PAO1-*lasB-gfp.* The results confirm our hypothesis that the presence of disulfide bonds is important for bioactivities.

In summary, our initial studies indicate that both disulfide and benzothiazole moieties are highly important for QSI activity, as determined by the reduction in *lasB* expression. Most of the compounds synthesized showed encouraging results with QSI activity higher than that of ajoene (Table 1). The IC_{50} values calculated for ajoene was 15 μM and 50 μM for *lasB-gfp* and *rhlA-gfp* reporter strains.[26] By simply changing the substituents to *p*-chlorophenyl moiety, the synthetic ajoene-derived analogs displayed excellent QSI activity without affecting the growth of bacteria.

Table 1. Summary of QSI activity against PAO1-*lasB-gfp*[a]

Compound	Inhibitor Structure	IC_{50} (uM)	Compound	Inhibitor Structure	IC_{50} (uM)
1*		2.28 ± 0.11	16		14.48 ± 3.63
2*		0.98 ± 0.14	17		45.38 ± 7.71
31*		-	18		16.9 ± 0.99
32*		-	19		12.13 ± 1.44
5**		15 ± 2.64	20		3.45 ± 1.40
6**		31 ± 7.63	21		4.08 ± 1.25
7		0.56 ± 0.11	22		20.92 ± 4.29
8		1.34 ± 0.36	23		3.58 ± 0.62
9		1.66 ± 0.24	24		0.78 ± 0.07
10		2.20 ± 0.46	25		1.12 ± 0.24
11		6.00 ± 1.27	26		1.35 ± 0.22
12		0.72 ± 0.12	27		-
13		3.25 ± 0.61	28		-
14		0.68 ± 0.12	29		-
15		0.58 ± 0.08	30		-

[a]IC_{50} values calculations were based on three biological replicates and performed using Graphpad Prism 6 software. *Compounds from in-house chemical library. **QSIs isolated from natural sources (**5**: ajoene from garlic; **6**: iberin from horseradish)[26, 29]

Effect of QSIs on *P. aeruginosa* virulence. Based on the experimental results, compounds **2** and **7** displayed excellent inhibition activity on genes that are expressed in response to QS signal molecules (*lasB, rhlA,* and *pqsA).* In this study, the three virulence factors (elastase, pyocyanin and rhamnolipid) were quantified to assess the effects of these lead compounds on virulence. Elastase or LasB protease is the major virulence factor of *P. aeruginosa* and capable to cause extensive tissue damage during infection in the human host.[30] Rhamnolipids have the properties of biosurfactants and are known as key component in the protection against innate immunity.[31] Rhamnolipid is believed to be prerequisite for development of chronic infections in CF patients, whereby production of rhamnolipids has led to killing of polymorphonuclear (PMN) leukocytes of host organism.[31a] This virulence phenotype is directly controlled by RhlR, which governs expression of the essential enzymes responsible for rhamnolipid biosynthesis (*rhlAB*).[32] Pyocyanin is a blue redox-active secondary metabolite produced by *P. aeruginosa* and one of the key virulence factors that plays a major role in cystic fibrosis (CF) patients.[33] It has been shown that a high level of pyocyanin (27 μg/mL) is present in the sputum of CF patients infected with *P. aeruginosa*.[33-34] Recent studies have shown the roles of pycoyanin in *P. aeruginosa*-mediated pathogenesis.[35] Pyocyanin was shown to be critical for lung infection in mice by mediating tissue damage and necrosis during infection.[34]

To further evaluate the QSI activities, the abilities of the lead compounds on elastase, rhamnolipid and pyocyanin were tested. The elastase assay was done using a standard enzymatic assay to measure the elastase activities of *P. aeruginosa* cultures. The Δ*lasI*Δ*rhlI* double mutant of PAO1 was used as negative control, where rhamnolipid production and elastolysis were almost absent.[22] Elastase production was found to be suppressed by compounds **2** and **7** within 3-hours cultivation period, which was comparable to the levels of PAO1 Δ*lasI*Δ*rhlI* (Figure 5A).

There was also apparent reduction in pyocyanin level in the treated samples (Figure 5B). Rhamnolipid production was also reduced to almost 50% as compared to the wild type PAO1. In QS mutant strain, no significant rhamnolipid production was detected (Figure 5C). These results showed that compounds suppressing QS genes indeed lowered the production of virulence factors.

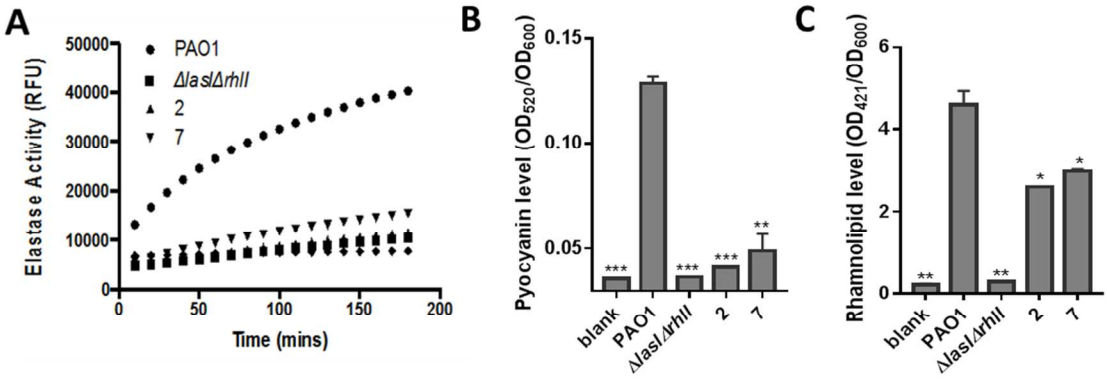


Figure 5. Effects of compounds **2** and **7** on virulence. (A) Elastase activity, (B) pyocyanin production, and (C) rhamnolipid production. Compounds were tested at final concentration of 10 μM. PAO1 Δ*lasI*Δ*rhlI* was used as negative control. Error bars are means ± SDs. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, Student's *t* test.

Effect of serum albumin on QSI activity of the novel compounds. Serum albumin is one of the most abundant proteins in blood plasma and often interferes and binds to drug molecules. To investigate the QSI effects of compounds in the presence of serum albumin, compounds were incubated with bovine serum albumin (BSA) up to 50 mg/mL. For this assay, *P. aeruginosa* with the *lasB-gfp* reporter strain was used as bioreporter. No apparent reduction in QSI activity of compounds **2** and **7** was observed at 10 μM concentration with BSA concentration up to 50 mg/mL (Supporting Information Figure S3).

Cytotoxicity of the novel compounds. Murine macrophage RAW264.7 cell line was used to evaluate toxicity. As compared to DMSO, compounds **2** and **7** showed cytotoxic effects only at higher concentrations of 100 μM. No cytotoxic effect was observed at concentration of 10 μM

and lower (Figure 6). This indicates that compounds **2** and **7** were not toxic to eukaryotic cells at QSI-relevant concentrations and could be used for subsequent mouse experiments.

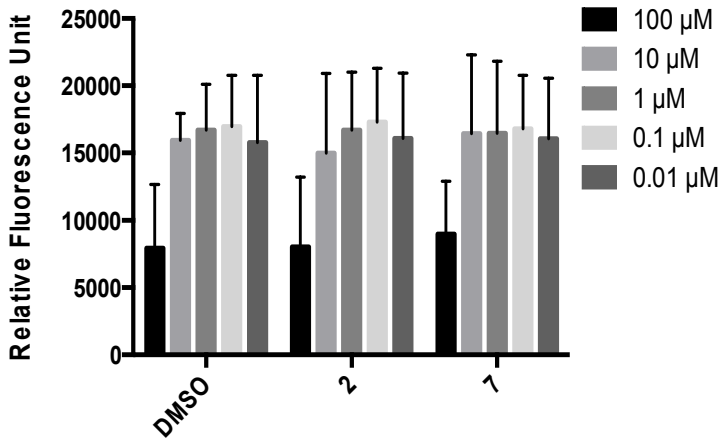


Figure 6. Cell toxicity assay. Macrophages were treated with DMSO control and compounds **2** and **7** at various concentrations (100 μM, 10 μM, 1 μM, 0.1 μM, and 0.001 μM). Data represent the average of three measurements. Error bars are means ± SDs.

Mouse implant infection model. Biofilms formed on the surfaces of infected foreign bodies like caterers, implants, and heart valves further increase the susceptibility to infections and resistance towards conventional antibiotics treatment.[36] To assess the QS effects of the identified compounds *in vivo*, a murine model for an implant-associated infection was conducted.[37] This assay would allow us to study the efficacy of compounds used *in vivo* as it mimics the foreign body-related infections.

The QS activity of compounds appears to weaker the biofilm formation on implants and hence facilitate host immune system in clearing the biofilm infections. There was apparent reduction in bacteria counts for treated groups, which is consistent with the physical appearance and well-being of the mice. Treatment of mice with compounds **2** and **7** after 2 h of implantation was able to significantly reduce the development of bacterial biofilms on the implants as well as to

prevent bacterial dissemination to the spleen (Figure 7). We are further optimizing the dosing strategy for applying these QSIs in treatment of infections in combination with antibiotics.

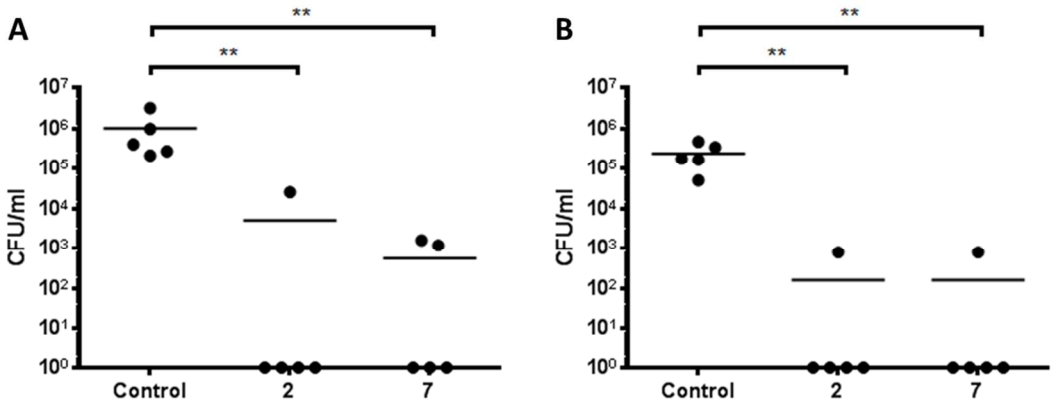


Figure 7. Foreign body mouse model of infection. Each mouse was challenged with a silicone implant coated with *P. aeruginosa* that would develop into biofilms. 2 h.p.i, the two groups of mice were treated with compounds **2** and **7** at 10 μM. Mice were sacrificed on 24 h.p.i and the bacterial loads on (A) implants and (B) spleen were quantified. The statistical difference in CFU/mL was tested by Mann-Whitney U test using GraphPad Prism 6 software. ** = $p < 0.01$.

CONCLUSION

The discovery of small molecules as QSIs is an attractive approach to develop resistant-robust drugs and new antimicrobial treatments. Toward this goal, many research groups have tried to identify and develop QSIs from natural sources as well as *in silico* study. In this study, we screened an in-house chemical library with *P. aeruginosa* PAO1-*lasB-gfp* bioreporter strain. Four hit compounds were identified and found to have dose-dependent inhibition of QS-related gene expression (*lasB, rhlA* and *pqsA*). Sulfur-containing compounds appear as interesting class of molecules capable of inhibiting QS, since they have little structural similarity to AHLs and yet possess excellent QSI properties. We performed quantitative structure-activity relationship (QSAR) studies to extend the library and assess their effects of QS modulation, with the main

focus on virulence factors. The results presented here, from high-throughput screening (HTS) to SAR studies, could provide important scaffolds for future chemical development, in both design and discovery of novel QSIs.

We have shown that 10 μM compounds **2** and **7** efficiently inhibit *las-rhl-pqs* system in a dose-dependent manner. Among the synthesized compounds, compound **7** has the lowest IC_{50} values at 0.56 μM for PAO1-*lasB-gfp* bioreporter. IC_{50} values on *rhlA-gfp* was 3.49 μM and 5.63 μM for *pqsA-gfp* bioreporter strain. The difference in IC_{50} values of compound **7** against the three QS systems suggests that these compounds might not target a specific QS pathway. Instead, our compounds might interact with multiple targets involved in the QS process. We also showed that the compounds could reduce the production of important QS-controlled virulence factors in wild-type *P. aeruginosa.* Elastase, pycoyanin and rhamnolipid levels were reduced upon addition of compounds **2** and **7** at 10 μM. Several potent synthetic QSIs have been identified to date, however, only few of these inhibit QS regulated genes in the sub-micromolar range.[18, 24, 38]

Lastly, we evaluated the effectiveness of our compounds *in vivo*. The compounds were not eliminated by BSA binding up to 50 mg/mL and found to be non-toxic in murine macrophage cell lines at 10 μM. Treatment of the *P. aeruginosa* foreign-body implants with compounds **2** and **7** showed significant bacterial clearance effect, compared to the non-treated control group. Our data showed encouraging results for our compounds to be developed as potential QSI and antivirulence drugs for treatment of *P. aeruginosa* infections in future.

It is yet unclear on the underlying mechanism and mode of inhibition of our identified compounds. Previously, microarray and transcriptomic analysis of ajoene indicated that the inhibition occurs at the posttranscriptional level, where ajoene targets small regulatory RNAs in the lower part of the QS hierarchy.[26] As the compounds have structural similarity with ajoene, it

could be speculated that our compounds have similar working mechanism to ajoene. Future work in our laboratory is aiming to provide molecular mechanisms of these compounds for the QS inhibition, and exploring other chemical scaffolds as potential QS-interfering compounds in the treatment of bacterial infections.

EXPERIMENTAL SECTION

1. Biological Assays. Bacterial strains and media. Bacterial strains and plasmids used in this study are described in Table 2. Bacteria were cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and ABTGC (B-medium (0.1% $MgCl_2$, 0.1% $CaCl_2$, 0.1% $FeCl_3$) supplemented with 10% A10, 0.2% glucose and 0.2% casamino acids). Media were solidified with 1.5% Bacto Agar (Difco) and 300 μg/mL Carbenicillin was used when appropriate. Kings medium (MilliQ water supplemented with proteose peptone (20 g/L), potassium sulphate (10 g/L), magnesium chloride, anhydrous (1.640 g/L) and glycerol (10% v/v)) were used for pyocyanine quantification assay.

Table 2. Strains and plasmid used in this study

Strains or plasmids	Relevant genotype and/or characteristics[a]
Strains	
PAO1	ATCC *Pseudomonas aeruginosa.*[39]
PAO1-*gfp*	GFP-tagged wild type *Pseudomonas aeruginosa.*[40]
PAO1 *lasB-gfp*	PAO1 containing *lasB-gfp*(ASV) translational reporter fusion.[39]
PAO1 Δ*lasI*Δ*rhlI*	Gm[a]; PAO1 *lasI and rhlI* mutant.[4b]
Plasmid	
p_{rhlA}-*gfp*	Gm[a]/Carb[a]; pUCPNotI-based plasmid carrying RlhR-regulated *rhlA-gfp*(ASV) translational

	fusion.[41]
p_{pqsA}-*gfp*	Gm[a]/Carb[a]; pUCP22NotI-based plasmid carrying *pqsA-gfp*(ASV) transcriptional fusion.[40]

[a]Description of the strains' antibiotic resistance. Gm, gentamicin; Carb, carbenicillin resistance.

1.1. Screening of Quorum Sensing Inhibitors. All screening compounds were acquired from the Nanyang Chemical Library, Chemistry and Biological Chemistry Department, School of Physical and Mathematical Science at Nanyang Technological University in Singapore. The purity of all hit compounds was assessed via ^{1}H and ^{13}C NMR spectroscopy. Compounds were then weighed and dissolved in DMSO to make 10 mM stock solutions, before transferring to 96-well plates for subsequent screening steps.

1.2. *P. aeruginosa* QS inhibition assays.[24] The first batch of screening involved 480 compounds prepared in 96-well microtiter plate (Nunc, Denmark) at concentration of 10 mM in 100% DMSO. Test compounds were then mixed with ABTGC medium and serial diluted to give final concentration of 20 μM in the first well. An overnight culture of PAO1-*lasB-gfp* strain (grown in LB medium at 37 °C, 200 rpm) was diluted in ABTGC medium to a final optical density at 600 nm (OD_{600}) of 0.02 (2.5 x 10^8 CFU/mL). An equal amount of the bacterial suspension was added to the wells to reach final inhibitor concentration of 10 μM. DMSO control (0.1% final concentration) and blank control were used. The microtiter plate was incubated at 37 °C in Tecan Infinate 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland) to measure the cell density (OD_{600}) and GFP fluorescence (excitation at 485 nm, emission at 535 nm) with 15-min intervals for at least 12 hours. The inhibition assay for all test compounds and controls were done in triplicate manner.

For *P. aeruginosa* Rhl and Pqs inhibition assay, similar method was performed as the LasB inhibition assay. The bacterial strains and plasmids used for respective test are shown in Table 2.

1.3. Elastase assay. Overnight culture of *P. aeruginosa* wild type and Δ*lasI*Δ*rhlI* mutant strain were diluted in 5 mL of ABTGC medium to a final optical density at 600 nm (OD_{600}) of 0.01. Compounds were added to give final concentration of 10 μM. The cultures were grown for 24 hours at 37 ˚C, shaking condition (200 rpm). 0.8 mL culture supernatants were sampled and centrifuged at 10,000 rpm for 10 mins. Elastase activity was measured by using EnzChekElastase assay kit (Invitrogen, USA), which consisted of BODIPY fluorophore (FL)-labeled DQ elastin conjugate and yields fluorescent fragments upon cleavage by elastase enzyme.[24] Fluorescence signal was recorded every 10 mins with Tecan Infinate 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland; excitation 490 nm, emission 520 nm) for three hours.

1.4. Pyocyanin Quantification Assay. Overnight culture of *P. aeruginosa* wild type and Δ*lasI*Δ*rhlI* mutant strain was standardized to OD_{600} of 0.1 and diluted 100 times into 25 mL of fresh Kings Medium in 250 mL flask. Compounds were added to give final concentration of 10 μM. The cultures were grown for 48 hours at 37 ˚C, shaking condition (200 rpm). The cultures were monitored every 24 hours to observe any colour changes from light yellow to greenish yellow solution in untreated flask. Mutant strain Δ*lasI*Δ*rhlI* was used as negative control. The final cell density was measured at 600 nm (OD_{600}) using Tecan Infinate 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). The cultures were then centrifuged for 10 mins at 10,000 rpm and 7.5 mL of the supernatants were transferred into new Falcon tubes. Pyocyanin extraction was conducted by extracting with chloroform (3 mL) and 0.2 M HCl (1.5 mL). The top aqueous layer of HCl containing pyocyanin was pipetted into microtiter plate and absorbance

was measured at 520 nm. The data was normalized by dividing OD_{520} reading with the final OD_{600} values.

1.5. Rhamnolipid Quantification Assay. Rhamnolipid production was quantified using Orcinol according to original protocol by Koch et al. with modifications.[42] Subculture of *P. aeruginosa* was diluted 100 times from overnight culture into fresh ABTGC medium (OD_{600} = 0.1). Treated cultures were added with test compounds to give final concentration of 10 μM. The cultures were grown for 24 hours at 37 °C, shaking condition (200 rpm). The final cell density was measured at 600 nm (OD_{600}) using Tecan Infinate 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Supernatants were collected by centrifuging at 10,000 rpm for 10 mins and extracted with diethyl ether (twice). Organic fractions were concentrated to yield white solids. It was then re-suspended in deionized water and added with 0.19% (w/v) orcinol in 50% H_2SO_4. The resulting mixture was incubated at 80 °C for 30 mins to give yellow-orange solution. After cooling to room temperature, the absorbance was measured at 421 nm and results were normalized with final OD_{600} values.

1.6. Effects of serum albumin on QSI activity of the compounds. Bovine serum albumin was dissolved in ABTGC medium to a concentration of 100 mg/mL. In a 96-well microtiter dish (Nunc, Denmark), 200 μL of the serum albumin was added to the first row and 2-fold serial dilution was made to the rest of the rows. Compounds were then added into each well to give final concentration of 20 μM. An overnight culture of PAO1-*lasB-gfp* strain (grown in LB medium at 37 °C, shaking condition) was diluted in ABTGC medium to a final optical density at 600 nm (OD_{600}) of 0.02. An equal amount of the bacterial suspension was added to the wells to reach final inhibitor concentration of 10 μM. After incubation for 18 hours, GFP/OD readings

were monitored with Tecan Infinate 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland) at 37 °C. Similar protocol was done as described in previous section.

1.7. Cytotoxicity assay. The murine macrophage RAW264.7 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies), supplemented with 10% fetal bovine serum (Gibco) at 37 °C and 5% CO_2. For experiment, 1×10^5 RAW264.7 macrophages were first grown in each well of 96-well culture plates (Nunc, US) for 16 hrs. The cells were then washed with phosphate-buffered saline (PBS) and treated with varying concentrations of compound (0, 0.01, 0.1, 1, 10, 100 μM) in 200 μL DMEM. The cells were incubated with the compound at 37 °C and 5% CO_2 for 16 hrs. Alamar Blue Cell Viability Reagent (Life Technologies) was added to the final concentration of 25 μM to each well of cells. Cells were incubated at 37 °C and 5% CO_2 for 3 hrs. If the cells are alive, there will be a color change of Alamar Blue from blue to red, whereas dead cells are unable to convert the dye to red. OD_{595} of each well was measured with Tecan Infinate 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were performed in triplicates, and the results are shown as the mean±s.d.

1.8. Implant infection model. Female BALB/c mice were purchased from InVivos Singapore at 8 weeks of age. Mice are maintained on water and standard mouse chow *ad libitum* for 7 days before the experiment. All animal experiments were conducted in compliance with the guide for the Care and Use of laboratory animals (National Research Council) under Nanyang Technological University Institutional Animal Care and Use Committee (IACUC) protocol number ARF SBS/NIE-A0192, which was approved by the Nanyang Technological University-IACUC. The experiment was conducted as previously reported.[37, 43] Silicone tubes (inner diameter 4.0 mm, outer diameter 6.0 mm, wall thickness 1.0 mm) were cut to a length of 4 mm.

The implants were placed in 20 mL 0.9% NaCl in the Erlenmeyer flasks with overnight culture of *P. aeruginosa (*OD_{600} of 0.1) and incubated for 18 h at 37 °C, shaking condition (110 rpm).

Mice were anesthetized by subcutaneous injections in the groin area with 100 μL cocktail of Ketamine HCl and Xylazine HCl (2 mL Ketamine HCl (100 mg/mL), 0.5 mL Xylazine HCl (20 mg/mL) and 7.5 mL saline). The implant was inserted into the right side of the peritoneal cavity near the groin area. The drugs were prepared by dissolving compounds in 96% EtOH to give final concentration of 10 mM before further diluted 1000 times in a 20% vehicle solution [(2-hydroxypropyl)-β-cyclodextrin dissolved in 0.9% NaCl] to a final concentration of 10 μM. 200 μL of this solution was then injected at the site of implantation after 2 h of infection. In the control group, only the vehicle solution was given. One day post infection, the mice were sacrificed to remove the implants and also the spleens to investigate the spread of systemic infection. CFU/mL determination of implants and spleens was conducted as follows: implants were placed in Eppendorf tube containing 1 mL 0.9% NaCl and sonicated in ice water bath for 10 mins (5 mins of degassing followed by 5 mins of sonication) to disrupt the biofilm. The spleens were removed from the mouse peritoneum and homogenized with homogenizer (Bio-Gen Pro200, Pro Scientific) to disrupt the tissues and release any bacterial cells. Next, the samples were serially diluted, plated on LB plates and incubated at 37 °C overnight. CFU/mL was calculated by multiplying the average number of colonies by the dilution factor and divided by the volume in mL.

2. Chemical Synthesis. General Information. ^{1}H NMR spectra were recorded using a Bruker AV-300 (300 MHz), BrukerAvance III 400 (400 MHz) and Bruker AMX500 (500 MHz) spectrometer. ^{13}C NMR spectra were recorded using Bruker AV-300 (75.6 MHz) and BrukerAvance III 400 (100 MHz) spectrometer. Chemical shifts (ppm) were recorded with

residual solvent peak as internal reference. Mass spectrometric data were recorded on a ThermoFinnigan PolarisQ MS / ThermoFinnigan LCQ Fleet MS. The values were reported in the unit of mass to charge ratio (m/z). Purification of products via column chromatography was done on Merck 60 (0.040 – 0.063 mm mesh silica gel). Thin layer chromatography (TLC) was performed using Merck 60 F254 silica gel plates. Visualization was carried out with UV lamp and KMnO4 stain. Melting points (mp) were recorded on Buchi B-54 melting point apparatus and uncorrected. Purity of all final compounds was 95% and higher. Purity of compounds was tested with elemental analysis and HPLC. Elemental analysis was done using Vario MICRO cube elemental analyzer, Elementar Analysensysteme GmbH, Hanau Germany. If necessary, purity of compounds was determined by high performance liquid chromatography (HPLC) using Shimadzu LC-20AD workstation with SPD-M20A photodiode array detector. The column was Venusil MP C18(2) (Agela Technologies), 5 μm particle size (250 mm x 4.6 mm), eluent CH_3CN/H_2O with 0.1% trifuluoroacetic acid, with a flow rate of 1 mL min^{-1} and detection at 260 nm; column temperature of 25 °C.

2.1. Synthesis of unsymmetrical disulfide via oxidation with DDQ[44]: 2-mercaptobenzothiazole (1.0 mmol) and thiol (1.5 mmol) were dissolved in 3 mL of dichloromethane (DCM) at 0 °C. DDQ (1.0 mmol) was then slowly added in portions to the solution and the reaction mixture was stirred for 0.5 – 1 hour on an ice bath. Progress of the reaction was monitored with TLC and LCMS. Upon completion, solvents were removed via rotary evaporation, and the crude compounds were purified via column chromatography (ethyl acetate:hexane, 1:50) to give the desired disulfide reaction products.

2-((4-chlorophenyl)disulfanyl)benzothiazole (7). Yellow solid; 40% yield; mp 59 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.90 (d, J = 8.2 Hz, 1H), 7.79 (d, J = 8.0 Hz, 1H), 7.61 – 7.54 (m,

2H), 7.48 – 7.42 (m, 1H), 7.39 – 7.28 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8, 154.7, 135.9, 134.9, 133.5, 130.6, 129.6, 129.4, 129.3, 126.5, 125.0 , 122.4, 121.2; MS (ESI, m/z) calc for $[M+H]^+$ = 310.84, found $[M+H]^+$ = 310.06, $[M+Na]^+$ = 333.10. Elemental analysis calc (%) for $C_{13}H_8ClNS_3$: C 50.39, H 2.60, N 4.52, found: C 50.45 H 2.92 N 4.39.

2-(phenyldisulfanyl)benzothiazole (8). Yellow oil; 36% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.89 (d, J = 8.1 Hz, 1H), 7.82 – 7.74 (d, 8.1, 1H), 7.64 (dt, J = 4.3, 2.4 Hz, 2H), 7.50 – 7.40 (m, 1H), 7.39 – 7.27 (m, 4H); ^{13}C NMR (75.6 MHz, $CDCl_3$): δ 174.5, 155.0, 135.9, 135.0, 129.4 (2C), 129.0 (2C), 128.5, 126.3, 124.7, 122.3, 121.2; MS (ESI, *m/z)* calc for $[M+H]^+$ = 276.40, found $[M+H]^+$ = 276.10. HPLC analysis: retention time = 21.280 min; peak area, 99.21%; eluent CH_3CN/H_2O with 0.1% TFA over 30 min with a flow rate of 1 mL min^{-1} and detection at 260 nm; column temperature, rt.

2-((4-methoxyphenyl)disulfanyl)benzothiazole (9). White solid; 15% yield; mp 60 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.89 (d, J = 8.1 Hz, 1H), 7.81 (d, J = 7.3 Hz, 1H), 7.67 – 7.59 (m, 2H), 7.48 – 7.40 (m, 1H), 7.39 – 7.31 (m, 1H), 6.90 – 6.83 (m, 2H), 3.79 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.2, 161.0, 155.1, 136.1, 133.4, 126.3, 124.6, 122.5, 121.1, 115.0 (2C), 55.4. HPLC analysis: retention time = 21.973 min; peak area, 99.62%; eluent CH_3CN/H_2O with 0.1% TFA over 30 min with a flow rate of 1 mL min^{-1} and detection at 260 nm; column temperature, rt.

2-(propyldisulfanyl)benzothiazole (10). Light yellow oil; 54% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.92 – 7.73 (m, 2H), 7.38 (dddd, J = 8.5, 7.9, 7.3, 1.3 Hz, 2H), 2.99 – 2.87 (m, 2H), 1.87 – 1.70 (m, 2H), 1.03 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.2, 155.2, 135.9, 126.2, 124.5, 122.1, 121.1, 41.5, 22.4, 13.1; MS (ESI, *m/z)* calc for $[M+H]^+$ = 242.38, found $[M+H]^+$ = 242.09. HPLC analysis: retention time = 20.917 min; peak area, 99.17%; eluent

CH_3CN/H_2O with 0.1% TFA over 30 min with a flow rate of 1 mL min^{-1} and detection at 260 nm; column temperature, rt.

2-(cyclohexyldisulfanyl)benzothiazole (11). Light yellow oil; 76% yield. ^1H NMR (300 MHz, $CDCl_3$): δ 7.82 (ddd, J = 21.6, 8.1, 0.6 Hz, 2H), 7.52 – 7.19 (m, 1H), 3.03 (tt, J = 10.9, 3.7 Hz, 1H), 2.19 – 2.04 (m, 2H), 1.88 – 1.72 (m, 2H), 1.61 (dd, J = 10.0, 4.4 Hz, 1H), 1.55 – 1.39 (m, 2H), 1.28 (qdd, J = 11.4, 8.5, 2.7 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.5, 155.2, 135.8, 126.2, 124.5, 122.1, 121.7, 50.8, 32.6 (2C), 26.0 (2C), 25.4. HPLC analysis: retention time = 24.277 min; peak area, 97.01%; eluent CH_3CN/H_2O with 0.1% TFA over 30 min with a flow rate of 1 mL min^{-1} and detection at 260 nm; column temperature, rt.

2-((4-chlorophenyl)disulfanyl)-6-ethoxybenzo[d]thiazole (12). White solid; 15% yield; mp 96 °C. ^1H NMR (300 MHz, $CDCl_3$) δ 7.77 (d, J = 9.0 Hz, 1H), 7.60 – 7.50 (m, 2H), 7.34 – 7.28 (m, 2H), 7.23 (d, J = 2.5 Hz, 1H), 7.02 (dd, J = 9.0, 2.5 Hz, 1H), 4.06 (q, J = 7.0 Hz, 2H), 1.43 (t, J = 7.0 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.5, 156.9, 149.0, 137.4, 134.7, 133.8, 130.5 (2C), 129.5 (2C), 123.0, 115.9, 104.8, 64.2, 14.8; MS (ESI, *m/z*) calc for $[M+H]^+$ = 354.90, found $[M+H]^+$ = 354.08. Elemental analysis calc (%) for $C_{15}H_{12}ClNOS_3$: C 50.91 H 3.42 N 3.96. Found C 50.70 H 3.52 N 3.88.

6-chloro-2-((4-chlorophenyl)disulfanyl)benzo[d]thiazole (13). White solid; 10% yield; mp 155 °C. ^1H NMR (300 MHz, $CDCl_3$) δ 7.78 (t, J = 5.8 Hz, 2H), 7.57 (d, J = 8.5 Hz, 2H), 7.40 (dd, J = 8.8, 1.8 Hz, 1H), 7.33 (d, J = 8.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.8, 153.2, 134.7, 133.2, 131.5, 130.2 (2C), 129.5 (2C), 126.9, 122.8, 120.6; MS (ESI, *m/z*) calc for $[M+H]^+$ = 345.29, found $[M+H]^+$ = 345.08. HPLC analysis: retention time = 24.736 min; peak area, 95.28%; eluent CH_3CN/H_2O with 0.1% TFA over 30 min with a flow rate of 1 mL min^{-1} and detection at 260 nm; column temperature, rt.

2-((4-chlorophenyl)disulfanyl)-6-methylbenzo[d]thiazole (14). White solid; 25% yield; mp 100 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.72 – 7.61 (m, 2H), 7.60 – 7.52 (m, 2H), 7.35 – 7.27 (m, 2H), 7.16 (dd, J = 8.2, 1.0 Hz, 1H), 2.47 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.4, 155.2, 136.6, 133.7, 132.9, 130.5 (2C), 129.5 (2C), 126.5, 122.5, 120.7, 21.5; MS (ESI, *m/z*) calc for $[M+H]^+$ = 324.87, found $[M+H]^+$ = 324.11. Elemental analysis calc (%) for $C_{14}H_{10}ClNS_3$: C 51.92 H 3.11 N 4.32. Found C 51.54 H 3.37 N 4.12.

2-((4-chlorophenyl)disulfanyl)-6-(trifluoromethyl)benzo[d]thiazole (15). White solid; 40% yield; mp 86 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.96 (d, J = 8.6 Hz, 1H), 7.68 (dd, J = 8.6, 1.4 Hz, 1H), 7.62 – 7.52 (m, 2H), 7.38 – 7.29 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.9, 156.9, 135.9, 135.3, 133.0, 130.8 (2C), 129.7 (2C), 127.1, 125.1), 123.5 (d, J = 3.4 Hz), 122.6, 118.8 (d, J = 4.2 Hz); MS (ESI, *m/z*) calc for $[M+H]^+$ = 378.84, found $[M+H]^+$ = 378.14. HPLC analysis: retention time = 23.499 min; peak area, 95.71%; eluent CH_3CN/H_2O with 0.1% TFA over 30 min with a flow rate of 1 mL min^{-1} and detection at 260 nm; column temperature, rt.

1,2-bis(benzo[d]thiazol-2-yl)disulfane (16). Yellowish white solid; 26% yield; mp 182 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.67 (d, J = 7.9 Hz, 1H), 7.38 (t, J = 7.7 Hz, 1H), 7.28 (dd, J = 15.9, 7.9 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.0, 156.4, 136.1, 125.7, 123.8, 123.1, 122.1, 119.4; MS (ESI, *m/z)* calc for $[M+H]^+$ = 333.47, found $[M+H]^+$ = 333.13.

1,2-bis(6-ethoxybenzo[d]thiazol-2-yl)disulfane (17). White solid; 5% yield; mp 132 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.81 (d, J = 9.0 Hz, 1H), 7.21 (d, J = 2.5 Hz, 1H), 7.04 (dd, J = 9.0, 2.5 Hz, 1H), 4.05 (q, J = 7.0 Hz, 2H), 1.43 (t, J = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.1, 157.2, 148.7, 137.8, 123.3, 116.2, 104.7, 64.2, 14.8; MS (ESI, *m/z*) calc for $[M+H]^+$ =

421.58, found $[M+H]^+$ = 421.14. Elemental analysis calc (%) for $C_{18}H_{16}N_2O_2S_4$: C 51.40 H 3.83 N 6.66. Found C 51.34 H 4.19 N 6.39.

1,2-bis(6-methylbenzo[d]thiazol-2-yl)disulfane (18). White solid; 5% yield; mp 180 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (s, 2H), 7.74-7.60 (m, 1H), 7.19 (d, J = 8.1 Hz, 1 H), 2.48 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.5, 156.4, 136.8, 126.9, 122.7, 120.8 (2C), 21.5; MS (ESI, *m/z*) calc for $[M+H]^+$ = 361.53, found $[M+H]^+$ = 361.16. HPLC analysis: retention time = 14.300 min; peak area, 98.01%; eluent CH_3CN/H_2O with 0.1% TFA over 30 min with a flow rate of 1 mL min^{-1} and detection at 260 nm; column temperature, rt.

1,2-bis(6-(trifluoromethyl)benzo[d]thiazol-2-yl)disulfane (19). White solid; 45% yield; mp 152 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.05 (dd, J = 10.6, 4.7 Hz, 2H), 7.72 (dd, J = 8.6, 1.4 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.0, 156.4, 136.1, 127.9, 125.7, 123.8 (q, J = 3.4 Hz), 123.1, 122.1, 119.4; MS (ESI, m/z) calc for $[M+H]^+$ = 469.47, found $[M+H]^+$ = 469.18. Elemental analysis (%) calc (%) for $C_{16}H_6F_6N_2S_4$: C 41.02 H 1.29 N 5.98. Found C 41.05 H 1.71 N 5.84.

2.2. General procedure for the synthesis of allicin[45]: A solution of mCPBA (0.48 mmol) in DCM was added to a solution of diallyl disulfide (0.45 mmol) in 3 mL DCM at 0 °C. The reaction mixture was stirred in ice bath for 4 hours and monitored with TLC and LCMS. Upon completion, reaction mixture was washed two times with sodium bicarbonate and two times with water. The combined aqueous layer was then collected and washed two times with ether. The combined organic layer was dried with sodium sulfate. Solvent was removed under reduced pressure and crude compounds were purified via column chromatography (ethyl acetate:hexane, 1:50).

S-allyl prop-2-ene-1-sulfinothioate. Yellow oil; 15% yield; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.03 – 5.82 (m, 1H), 5.53 – 5.11 (m, 2H), 3.96 – 3.66 (m, 2H); MS (ESI, m/z) calc for $[M+H]^+$ = 163.26, found $[M+H]^+$ = 163.84.

2.3. Synthesis of allyl disulfide via oxidation of mCPBA. Allicin (1 mmol) was slowly added into the thiol (0.5 mmol.) in MeOH at room temperature. The reaction mixture was stirred at room temperature for 0.5 – 1 hour and monitored with TLC and LCMS. Upon completion, the solvent was removed under reduced pressure. The crude products were purified with column chromatography.

2-(allyldisulfanyl)benzothiazole (2). Yellow oil; 90% yield; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.86 (d, J = 7.9 Hz, 1H), 7.82 – 7.72 (m, 1H), 7.48 – 7.37 (m, 1H), 7.36 – 7.24 (m, 1H), 5.88 (ddt, J = 17.2, 9.9, 7.4 Hz, 1H), 5.20 (dd, J = 12.7, 11.8 Hz, 2H), 3.57 (d, J = 7.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.7, 155.1, 135.8, 131.5, 126.3, 124.61, 122.2, 121.2, 120.5, 42.31; MS (ESI, *m/z)* calc for $[M+H]^+$ = 240.37, found $[M+H]^+$ = 240.95. Elemental analysis calc (%) for $C_{10}H_9NS_3$: C 50.18 H 3.79 N 5.85. Found C 50.24 H 4.06 N 5.67.

2-(allyldisulfanyl)-5-methoxybenzooxazole (20). Yellow sticky oil; 31% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34 (dd, J = 16.2, 8.9 Hz, 1H), 7.14 (dd, J = 15.9, 2.5 Hz, 1H), 6.99 – 6.77 (m, 1H), 5.97 – 5.66 (m, 1H), 5.34 – 5.00 (m, 2H), 3.99 (dd, J = 7.4, 0.9 Hz, 1H), 3.84 (s, 3H), 3.62 (d, J = 7.4 Hz, 1H); ^{13}C NMR (75.6 MHz, $CDCl_3$): δ 165.3, 157.5, 147.1, 142.7, 131.5, 120.1, 113.1, 110.4, 102.6, 55.9, 42.1; MS (ESI, *m/z)* calc for $[M+H]^+$ = 254.33, found $[M+H]^+$ = 254.06. Elemental analysis calc (%) for $C_{11}H_{11}NO_2S_2$: C 52.15 H 4.38 N 5.53. Found C 52.15 H 4.63 N 5.50.

2-(allyldisulfanyl)-6-chlorobenzooxazole (21). Yellow oil; 40% yield; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.87 – 7.81 (m, 1H), 7.44 (ddd, J = 8.3, 7.2, 1.4 Hz, 1H), 7.34 (ddd, J = 8.4, 7.5, 1.3

Hz, 1H), 5.89 (ddt, *J* = 17.2, 9.9, 7.4 Hz, 1H), 5.27 – 5.18 (m, 2H), 3.61 – 3.57 (m, 2H); ^{13}C NMR (75.6 MHz, $CDCl_3$): δ 165.3, 150.9, 143.0, 131.4, 124.9, 120.3, 119.3, 110.9, 42.1; MS (ESI, *m/z)* calc for $[M+H]^+$ = 258.75, found $[M+H]^+$ = 258.00. Elemental analysis calc (%) for $C_{10}H_8ClNOS_2$: C 46.60 H 3.13 N 5.43. Found C 46.70 H 3.14 N 5.45.

1-allyl-2-benzyldisulfane (22). Colourless oil; 30% yield; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.39 – 7.22 (m, 5H), 5.75 (ddt, *J* = 17.3, 10.0, 7.4 Hz, 1H), 5.14 – 5.02 (m, 2H), 3.91 (s, 2H), 3.03 (d, *J* = 7.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 137.45, 133.31, 129.43 (2C), 128.57 (2C), 127.54, 118.53, 43.69, 41.88.

2-(allyldisulfanyl)-6-ethoxybenzo[d]thiazole (23). Yellow oil. 95% yield; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (d, *J* = 8.9 Hz, 1H), 7.27 (m, *J* = 2.9 Hz, 1H), 7.02 (dd, *J* = 9.1, 2.3 Hz, 1H), 5.95 – 5.83 (m, 1H), 5.22 (dd, *J* = 19.8, 13.3 Hz, 2H), 4.08 (dd, *J* = 13.9, 7.0 Hz, 2H), 3.58 (d, *J* = 7.4 Hz, 2H), 1.45 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.7, 156.7, 149.3, 137.3, 131.5, 122.7, 120.3, 115.6, 104.9, 64.2, 42.4, 14.8; MS (ESI, *m/z*) calc for $[M+H]^+$ = 284.42, found $[M+H]^+$ = 284.11. Elemental analysis calc (%) for $C_{12}H_{13}NOS_3$: C 50.85 H 4.62 N 4.94. Found 50.76 H 4.82 N 4.72.

2-(allyldisulfanyl)-6-chlorobenzo[d]thiazole (24). Yellow oil. 40% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.89 – 7.64 (m, 2H), 7.36 (dd, *J* = 8.8, 2.0 Hz, 1H), 5.87 (ddt, *J* = 17.3, 9.9, 7.4 Hz, 1H), 5.34 – 5.05 (m, 2H), 3.57 (dd, *J* = 7.4, 0.6 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.5, 153.6, 136.9, 131.3, 130.6, 127.0, 122.85 (s), 120.7 (2C), 42.3; MS (ESI, *m/z*) calc for $[M+H]^+$ = 274.81, found $[M+H]^+$ = 274.98.

2-(allyldisulfanyl)-6-methylbenzo[d]thiazole (25). Yellow oil. 90% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.65 (d, *J* = 8.1 Hz, 2H), 7.14 (dd, *J* = 7.8, 1.0 Hz, 1H), 5.88 (ddt, *J* = 17.2, 9.9, 7.4 Hz, 1H), 5.30 – 5.12 (m, 2H), 3.58 (t, *J* = 7.3 Hz, 2H), 2.46 (s, 3H); ^{13}C NMR (100 MHz,

$CDCl_3$): δ 172.5, 155.4, 136.4, 132.8, 131.5, 126.1, 122.3, 120.6, 120.4, 42.3, 21.5; MS (ESI, *m/z*) calc for $[M+H]^+$ = 254.40, found $[M+H]^+$ = 254.08. Elemental analysis calc (%) for $C_{11}H_{11}NS_3$: C 52.14 H 4.38 N 5.53. Found C 52.22 H 4.46 N 5.55.

2-(allyldisulfanyl)-6-(trifluoromethyl)benzo[d]thiazole (26). Yellow oil. 97% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (s, 1H), 7.93 (d, J = 8.6 Hz, 1H), 7.67 (dd, J = 8.6, 1.3 Hz, 1H), 5.89 (ddt, J = 17.3, 9.9, 7.4 Hz, 1H), 5.31 – 5.09 (m, 2H), 3.60 (d, J = 7.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.0, 157.1, 135.9, 131.2, 126.9, 126.6, 123.3, 122.4, 120.7, 118.7, 42.3; MS (ESI, m/z) calc for $[M+H]^+$ = 308.37, found [M] = 308.95.

2.4. Synthesis of unsymmetrical sulfide via alkylation. 2-mercaptobenzothiazole (0.5 mmol) and thiol (0.5 mmol) were dissolved in water. NEt_3 (0.6 mmol) or K_2CO_3 (0.75 mmol) were added and reaction mixture was stirred at room temperature. Reaction was monitored with TLC and LCMS. Upon completion, reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Solvent was removed under reduced pressure and crude compounds were purified via column chromatography (ethyl acetate 1% in hexane).

2-(propylthio)benzo[d]thiazole (27). Yellow oil; 36% yield; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.87 (d, J = 8.2 Hz, 1H), 7.748 (d, J = 8.0 Hz, 1H), 7.42 - 7.40 (m, 1H), 7.30-7.27 (m, 1H), 3.33 (t, J = 7.3 Hz, 2H), 1.86 (m, 2H), 1.09 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.0, 153.0, 135.9, 126.2, 124.5, 122.1, 121.1, 36.5, 22.4, 13.1; MS (ESI, *m/z)* calc for $[M+H]^+$ = 210.33, found $[M+H]^+$ = 210.03.

2-(but-3-en-1-ylthio)benzo[d]thiazole (28). Yellow oil; 49% yield; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.91 (d, J = 8.1 Hz, 1H), 7.78 (d, J = 8.0 Hz, 1H), 7.49 – 7.40 (m, 1H), 7.38 – 7.25 (m, 1H), 5.92 (m, J = 16.9, 10.2, 6.7 Hz, 1H), 5.16 (m, J = 13.7, 11.4, 1.4 Hz, 2H), 3.46 (t, J = 7.3 Hz, 2H), 2.68 – 2.58 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.0, 153.2, 135.7, 135.1, 126.1,

124.2, 121.5, 121.0, 117.0, 33.3, 32.8; MS (ESI, *m/z)* calc for $[M+H]^+$ = 222.34, found $[M+H]^+$ = 222.03. Elemental analysis calc (%) for $C_{11}H_{11}NS_2$: C 59.69 H 5.01 N 6.33. Found C 59.97 H 5.24 N 6.17.

2-(allylthio)benzo[d]thiazole (29). Colourless oil; 93% yield; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.90 (d, J = 8.2 Hz, 1H), 7.76 (d, J = 8.0 Hz, 1H), 7.43 (dd, J = 11.2, 4.2 Hz, 1H), 7.29 (dd, J = 16.5, 9.1 Hz, 1H), 6.03 (ddt, J = 16.9, 10.0, 6.9 Hz, 1H), 5.39 (dd, J = 16.9, 1.2 Hz, 1H), 5.21 (d, J = 10.0 Hz, 1H), 4.01 (d, J = 6.9 Hz, 2H); ^{13}C NMR (75.6 MHz, $CDCl_3$): δ 166.6, 153.0, 135.3, 132.4, 126.3, 124.5, 121.64, 121.1, 119.4, 36.5; MS (ESI, m/z) calc for $[M+H]^+$ = 208.31, found $[M+H]^+$ = 208.02. Elemental analysis calc (%) for $C_{10}H_9NS_2$: C 57.94 H 4.38 N 6.76. Found C 58.09 H 4.32 N 6.73.

2.5. Synthesis of heterocyclic thiols with aryl iodides[46] : CuI (0.025 mmol), 1,10-phenanthroline (0.05 mmol), and K_2CO_3 (0.65 mmol) were dissolved in 1.5 mL of DMF. Aryl iodides (0.6 mmol) was slowly added into the solution, followed by heterocyclic thiols (0.5 mmol) in DMF (1.5 mL). The reaction mixture was stirred at 120 °C for 10 h. Reaction was monitored with TLC and LCMS. After completion, it was cooled to room temperature. Reaction mixture was added with water and extracted with ethyl acetate, brine and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure and crude compounds were purified via column chromatography (ethyl acetate 1% in hexane).

2-((4-chlorophenyl)thio)benzo[d]thiazole (30). White solid; 93% yield; mp 59 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.90 (d, J = 8.2 Hz, 1H), 7.74 – 7.62 (m, 3H), 7.49 – 7.38 (m, 3), 7.29 (dd, J = 15.0, 7.7 Hz, 1H); ^{13}C NMR (75.6 MHz, $CDCl_3$): δ 168.7, 153.55, 137.04, 136.45, 135.43, 130.22 (2C), 128.33 (2C), 126.39, 124.65, 122.03, 120.90; MS (ESI, m/z) calc for $[M+H]^+$ =

278.78, found $[M+H]^+ = 278.10$. Elemental analysis calculated (%) for $C_{13}H_8ClNS_2$: C 56.21, H 2.90, N 5.04. Found: C 56.36 H 2.91 N 5.06.

ASSOCIATED CONTENT

Supporting Information. Additional figures and NMR spectra as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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L.Y., C. H. T., T. E. N. and M. G. designed methods and experiments. J. F., Y. M. J., T. H. J. carried out the laboratory experiments, analyzed the data and interpreted the results. M. M. S. S. and S. L. C. co-designed the animal experiments and worked on associated data collection and its interpretation. L. Y. and M. G. discussed analyses, interpretation, and presentation. J. F. and L.Y. wrote the paper. All authors have contributed to, seen and approved the manuscript.

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ABBREVIATIONS

GFP, green fluorescent protein; DMSO, dimethyl sulfoxide; DCM, dichloromethane; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; mCPBA, meta-Chloroperoxybenzoic acid; DMF, dimethylformamide.

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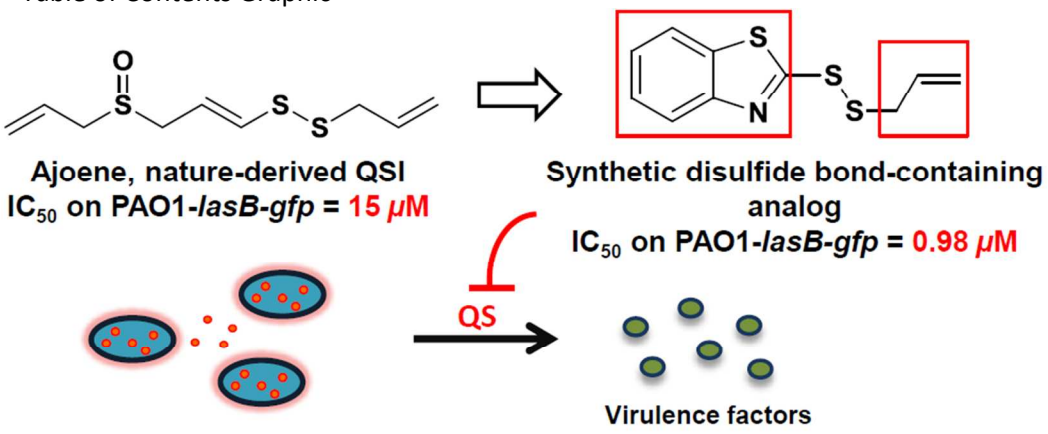
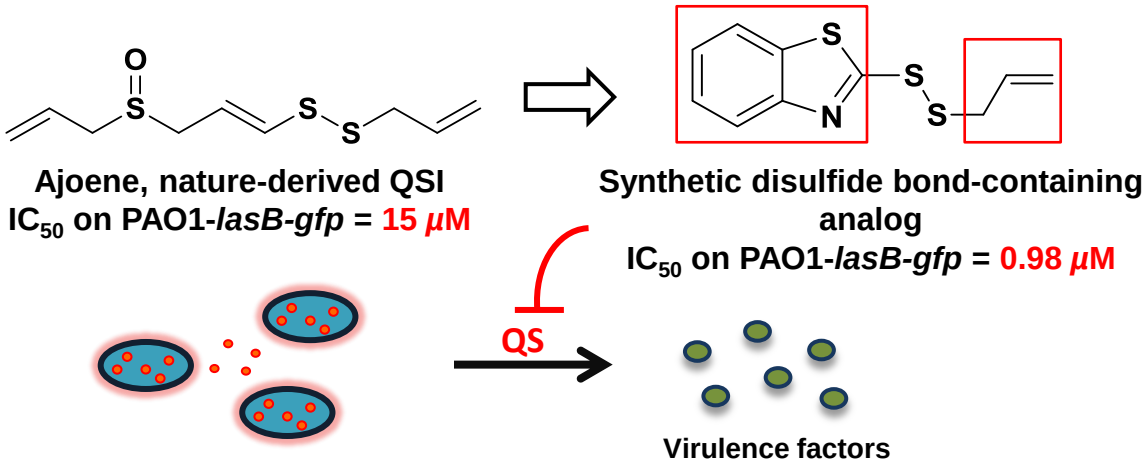
Table of Contents Graphic





Ajoene, nature-derived QSI
IC_{50} on PAO1-*lasB-gfp* = 15 μM

Synthetic disulfide bond-containing analog
IC_{50} on PAO1-*lasB-gfp* = 0.98 μM

Virulence factors