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3-Hydroxy-1-alkyl-2-methylpyridine-4(1*H*)-thiones: Inhibition of the *Pseudomonas aeruginosa* Virulence Factor LasB

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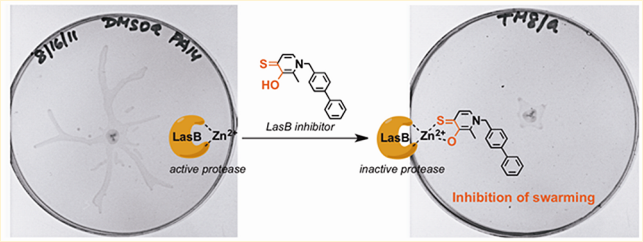
Supporting Information

ABSTRACT: Bacterial resistance coupled to our current arsenal of antibiotics presents us with a growing threat to public health, thus warranting the exploration of alternative antibacterial strategies. In particular, the targeting of virulence factors has been regarded as a "second generation" antibiotic approach. In *Pseudomonas aeruginosa*, a Zn^{2+} metalloprotease virulence factor, LasB or *P. aeruginosa* elastase, has been implicated in the development of *P. aeruginosa*-related keratitis, pneumonia, and burn infection. Moreover, the enzyme also plays a critical role in swarming and biofilm formation, both of which are processes that have been linked to antibiotic resistance. To further validate the importance of LasB in *P. aeruginosa* infection, we describe our efforts toward the discovery of nonpeptidic small molecule inhibitors of LasB. Using identified compounds, we have confirmed the role that LasB plays in *P. aeruginosa* swarming and demonstrate the potential for LasB-targeted small molecules in studying antimicrobial-resistant *P. aeruginosa* phenotypes.



KEYWORDS: *antibiotics, metalloprotease inhibitors, virulence factors, hydroxypyridinthiones*

The emergence of antibiotic resistance to nearly all antimicrobial agents on the market today is a great threat to public health, requiring the development of alternative antibacterial strategies. Of these tactics, the targeting of virulence factors has been regarded as a "second generation" antibiotic approach.[1−3] Pathogenic bacteria produce virulence factors such as adhesion molecules, secretion systems, and other toxic factors, including proteases to enhance their ability to cause disease and damage the host's tissues.[2,3] Importantly, because virulence factors do not alter viability, agents targeting such factors should impose weaker selective pressure for the development of resistance.[2,3] Moreover, as many virulence factors are unique to a specific organism, virulence-targeting drugs are unlikely to impact the host's commensal flora.[2,3] The efficacy of exploring such targets remains to be seen; however, inhibitors targeting toxin function and delivery, the regulation of virulence expression, and adhesion have been reported with promising in vivo anti-infective properties.[2,3]

Pseudomonas aeruginosa is a nosocomial, opportunistic Gram-negative bacteria that is the predominant cause of pneumonia in ventilated patients and lung infection in patients with cystic fibrosis.[4] In addition to respiratory tract infections, the pathogen also causes blood, ear, skin, eye, urinary, and gastrointestinal tract infections among others and is responsible for 10−15% of hospital-acquired infections worldwide.[4,5] To promote these clinical manifestations, *P. aeruginosa* produces a number of surface and secreted virulence factors that facilitate bacterial attachment, colonization and invasion, and tissue damage and cytokine production, respectively.[4,5] Cell-associated virulence factors include flagella, pili, lectins, alginate, and lipopolysaccharide, while extracellular virulence factors include proteases, hemolysins, cytotoxin, pyocyanin, siderophores, and exotoxin A.[5] This arsenal of virulence factors ensures that *P. aeruginosa* infections are both invasive and toxigenic.[5] Thus, because of growing antibiotic resistance in *P. aeruginosa* including the identification of many multidrug resistant strains,[4] new approaches for tackling such infections are warranted.

One *P. aeruginosa* virulence factor that has been shown to be highly toxic to the host[6] is a Zn^{2+} metalloprotease, LasB or *P. aeruginosa* elastase,[7] which is secreted by the bacteria in a quorum sensing-dependent fashion.[8] In particular, LasB has been demonstrated to cause tissue damage and persistent inflammation, degrade plasma proteins, and promote invasion and colonization among other pathologies.[6] To do so, the enzyme degrades a wide variety of substrates including elastin, fibrin, immunoglobulins, complement factors, and cytokines.[6] Importantly, infection with a *P. aeruginosa* strain producing a

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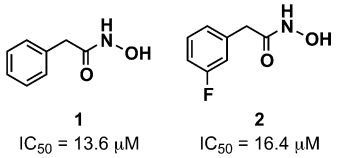
mutant, inactive elastase was found to be less virulent than the parent strain in a chronic lung infection model in rats.[9] Additionally, with respect to the bacteria itself, LasB has been linked to biofilm formation[10] and swarming,[11] further aiding in its elusion from the host's immune system. Accordingly, LasB has been shown to play a role in the development of *P. aeruginosa*-related keratitis, pneumonia, and burn infection.[6]

Our laboratories have invested much effort in exploring the modulation of metalloproteases for human health, such as identifying small molecule inhibitors of the botulinum neurotoxins,[12−18] anthrax lethal factor,[19−22] and matrix metalloproteinases.[21,22] To do so, we have designed and developed nonpeptidic small molecules containing a variety of metal-chelating warheads as active site inhibitors. Because only peptide-based LasB inhibitors have been previously reported[23−26] coupled with the potential therapeutic significance of this *P. aeruginosa* virulence factor, we were interested in expanding the scope of our metalloprotease-targeting design strategies toward the goal of identifying potent small molecule LasB antagonists. Herein, we describe our efforts to discover the first nonpeptidic small molecule inhibitors of LasB and validate the role of this virulence factor in swarming, a multicellular bacterial behavior recently linked to antimicrobial resistant phenotypes.[27]

To initiate our screening efforts, we examined a 323-member small molecule hydroxamic acid library, which was constructed using solid-phase organic synthesis and originally used to identify inhibitors of the botulinum neurotoxin A protease.[14] All compounds were analyzed for LasB inhibition using a previously reported fluorescence assay based on a LasB-cleavable fluorescence resonance energy transfer (FRET) peptide substrate (see the Supporting Information for assay details).[23] Library members were initially examined at 50 μM, and from our screening efforts, eight hits showing 100% inhibition at this concentration were identified following triplicate experiments (see the Supporting Information). Of these hit compounds, only two demonstrated dose-dependent LasB antagonism, hydroxamic acids **1** and **2** (Figure 1), with

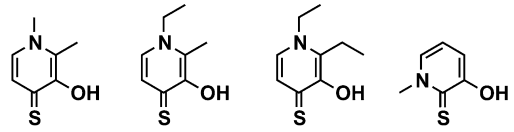


1 IC_{50} = 13.6 μM **2** IC_{50} = 16.4 μM

Figure 1. Hydroxamic acid LasB inhibitor hits with IC_{50} values.

measured IC_{50} values of 13.6 and 16.4 μM, respectively. Despite the in vitro activity of these compounds, unfortunately, no antagonism in bacterial LasB assays was observed. One possible explanation for the lack of bacterial cell activity is enzymatic discharging of the hydroxamate moiety, as this chelating motif is known to be unstable.[28]

Because of the potential instability and off-target effects of the hydroxamate group,[28] we were eager to explore additional Zn^{2+} chelating motifs that may be more stable under biological conditions. Recently, a library of metal chelating fragments (CFL-1.1) was designed by the Cohen laboratory.[21,22] The library consists of 96 fragment chelators, each containing 2−4 donor atoms for metal binding. Some representative library members include picolinic acids, hydroxyquinolones, pyrimidines, and hydroxypyrones in addition to other well-known metal-binding units such as sulfonamides and hydroxamic acids. Members of this fragment library were screened at 1 mM for inhibition of LasB, and 11 fragment hits were identified (see the Supporting Information). Of these compounds, four showed dose-dependent inhibition of LasB with IC_{50} values ranging from 80 to 400 μM (compounds **3**−**6**, Figure 2). Interestingly, all hits contained an O,S atom donor set and have previously been identified from this library as inhibitors of other Zn^{2+} metalloproteases.[19−22]

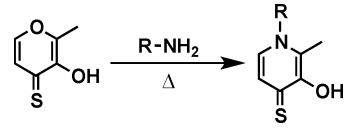


	3	**4**	**5**	**6**
IC_{50} (μM):	80.6 ± 0.05	88.5 ± 0.01	392 ± 0.92	220 ± 0.10

Figure 2. Hit compounds from CFL-1.1 with IC_{50} values for LasB inhibition.

To identify 3,4-HOPTO [3-hydroxy-1-alkyl-2-methylpyridine-4(1*H*)-thione; **3**−**5**] derivatives with improved in vitro potency against LasB, a sublibrary of compounds based on fragments **3** and **4** (see the Supporting Information for structures), was screened.[21] 3,4-HOPTO analogues **8** were constructed via a one-step condensation of a primary amine with thiomaltol **7** (Scheme 1)[21] and initially tested for

Scheme 1. Synthesis of 3,4-HOPTO Analogues



7 **8**

inhibition at 100 μM. From this sublibrary, two compounds were discovered with ∼30-fold improvement in potency: analogues **8a** and **8b** exhibited measured IC_{50} values of 3.34 and 2.73 μM, respectively (Table 1; see the Supporting Information for the activity of other sublibrary members). Structure−activity relationship studies were then conducted on **8b** by varying the substituents of the biphenyl ring; however, no compounds with enhanced LasB inhibition were identified (Table 1). On the basis of initial docking studies, we hypothesize that the diminished activity of compounds **8c**−**g** may be due to the restrictive geometry of the 3,4-HOPTO−Zn^{2+} complex, which forces the biphenyl side chain into solvent space as the active site of LasB is quite open similar to that of thermolysin (data not shown).[23]

With the in vitro activity of 3,4-HOPTO analogues **8a** and **8b** confirmed, we were then anxious to examine their activity in a bacterial phenotypic assay, particularly since hydroxamates **1** and **2** displayed no secreted LasB inhibition. In *P. aeruginosa*, LasB has been demonstrated to be required for both swarming and biofilm formation, and *lasB* mutants were shown to exhibit strong swarming defects and a ∼50% decrease in biofilm formation.[11] Swarming and biofilm formation are both social phenomena that require differentiated cells and are regulated by cell-to-cell communication, in particular, quorum sensing.[27] Of significance, both bacterial cell states have been found to be highly resistant to antibiotics, and it has recently been hypothesized that antimicrobial resistance is a general feature

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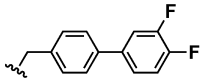
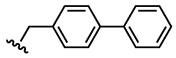
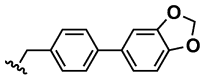
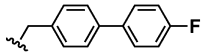
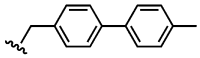
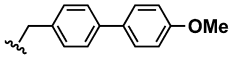
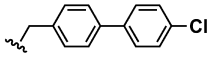
Table 1. Structures and IC_{50} Values for 3,4-HOPTO Analogue Hits

Compound	R	IC_{50} (μM)
8a		3.34 ± 0.03
8b		2.73 ± 0.03
8c		3.58 ± 0.05
8d		4.65 ± 0.17
8e		6.72 ± 0.01
8f		8.58 ± 0.01
8g		5.27 ± 0.01

of bacterial multicellularity.[27] As the study of swarming motility is believed to be a useful model to investigate antibiotic resistance in biofilms,[27] particularly due to reproducibility issues that often go unreported in biofilm assays, we chose to investigate swarming inhibition in the presence of compounds **8a** and **8b**.

To examine the effect of compounds **8a** and **8b** on swarming, *P. aeruginosa* strain PA14 was grown on swarm agar plates containing 25 μM **8a** or **8b** (~95% viability at this concentration; see the Supporting Information) for 16 h at 30 °C. As Figure 3 shows, both **8a** and **8b** almost completely abolished swarming at this concentration in comparison to a DMSO-treated control sample that exhibited a dendritic swarming phenotype typical of *P. aeruginosa*. We highlight this result as **8a** and **8b** are the first targeted compounds to show swarming antagonism. Previously, only general virulence factor expression[29,30] and bacterial adhesion inhibitors[31] have been examined in swarming assays. Similar to biofilm formation, swarming is also of significance pathologically, as the environment required for swarming motility is similar to that covering epithelial surfaces and the hyperabundant mucous found the in lungs of cystic fibrosis patients.[11] Thus, we are hopeful that these compounds will be useful in further understanding the role that LasB plays not only in swarming but also the development of antibiotic resistance in *P. aeruginosa* swarm cells and invasion and colonization of tissues by *P. aeruginosa*.[6] Moreover, upon improvement in potency, we are also interested in exploring the applicability of our nonpeptidic small molecule LasB inhibitors in animal models of *P. aeruginosa* infection to study the impact of this metalloprotease in promoting bacterial virulence. Importantly, 3,4-HOPTO derivatives have been previously demonstrated to be nearly nontoxic at concentrations up to 100 μM in mammalian cells.[32,33] We envision that a combination of small molecule LasB inhibitors with traditional antibiotics may represent a new therapy for targeting multidrug resistant *P. aeruginosa*.

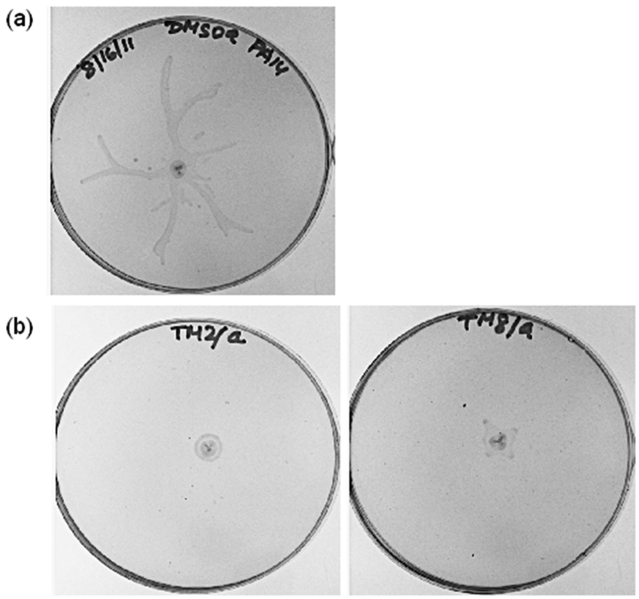


Figure 3. Swarming of *P. aeruginosa* strain PA14 in the presence of 25 μM (a) DMSO (control), (b) **8a** (left), or **8b** (right).

Lastly, we wish to bring to light that although hydroxamic acids **1** and **2** were only ~5-fold less potent than 3,4-HOPTO analogues **8a** and **8b** in the in vitro LasB assay, these compounds showed no swarming inhibition (data not shown). As indicated, it is quite possible that instability and off-target effects may be at play as highlighted by others;[28] however, it remains to be seen if other factors are involved such as bacterial cell metabolism. Initial stability studies in bacterial culture medium indicate the hydrolytic instability of **1** and **2** in comparison to **8a** and **8b**, which showed no detectable S-oxidation or decomposition over a 48 h period (see the Supporting Information). Regardless, compounds **1** and **2** will serve as useful scaffolds for future warhead "hopping" experiments, and these studies will be reported in due course.

In conclusion, we have identified small molecule, Zn^{2+}-chelating inhibitors of LasB with antiswarming activity. These inhibitors were identified via initial screening of a chelator fragment library, further validating this approach for the identification of metalloprotein inhibitors. Although compounds **8a** and **8b** are not the most potent LasB inhibitors to date, they are the first nonpeptidic small molecule antagonists discovered.[26] Moreover, they are the first targeted compounds to exhibit swarming inhibition. On the basis of our preliminary findings described here, LasB-targeted chemical probes should aid in elucidating the role of LasB in *P. aeruginosa*-related infections and its ability to resist antibiotics. Additionally, the discovery and study of such small molecule virulence factor inhibitors will provide additional evidence to discern the impact of targeting bacterial virulence factors as a "second generation" antibiotic approach.

■ ASSOCIATED CONTENT

Supporting Information

Representative synthetic procedure, characterization of compounds **8a** and **8c**–**8g**, LasB fluorescence assay protocol, supplemental screening results, stability and bacterial toxicity of **8a** and **8b**, and swarming details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

P. aeruginosa, *Pseudomonas aeruginosa*; LasB, *P. aeruginosa* elastase; FRET, fluorescence resonance energy transfer; 3,4-HOPTO, 3-hydroxy-1-alkyl-2-methylpyridine-4(1*H*)-thione

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