Supporting Information for

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

Amanda L. Garner,[†] Anjali K. Struss,[†] Jessica L. Fullagar,[‡] Arpita Agrawal,[‡] Amira Y. Moreno,[†] Seth M. Cohen,*[‡] and Kim D. Janda*[†]

[†]Departments of Chemistry and Immunology and Microbial Science, The Skaggs Institute for Chemical Biology, and The Worm Institute for Research and Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

[‡]Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093

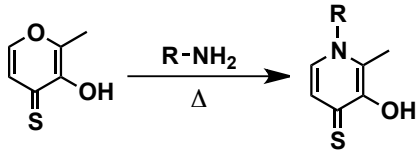
A. General Methods and Materials

General chemistry methods: Reactions were carried out under standard atmospheric conditions. Yields refer to chromatographically and spectroscopically homogenous materials, unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) carried out on silica gel plates using UV-light (254 nm). Flash chromatography separations were performed on Silicycle silica gel (40-63 mesh) or using a CombiFlash® Rf automated chromatography system by Teledyne Isco. All compounds were confirmed to have ≥95% purity by HPLC (254 nm). NMR spectra were recorded on a Bruker or Varian 400 MHz spectrometers and calibrated using a solvent peak as an internal reference. The following abbreviations are used to indicate the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad.

Data measurement and analysis: All fluorescence readings were measured in black 96-well microtiter plates with clear bottoms (Corning® Costar®) on a SpectraMax M2^e Microplate Reader (Molecular Devices). All data was analyzed using GraphPad Prism version 5.0a for Mac OS X (GraphPad Software, www.graphpad.com).

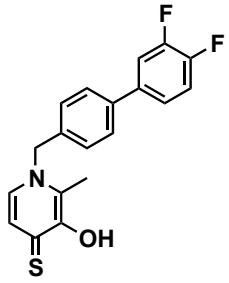
Materials: LasB was purchased from Elastin Products Company and used as received. The LasB pro-fluorescent substrate, Abz-Ala-Gly-Leu-Ala-p-Nitro-Benzyl-Amide (SAG-3905-PI), was purchased from Peptides International and used as received. Molecular biology grade DMF and DMSO were purchased from Sigma Aldrich and used as received. 3,4-HOPTO **7** was synthesized as previously described.[1]

B. General Synthetic Procedure and Characterization and Purity Data for 3,4-HOPTO Analogues 8a and 8c–8g

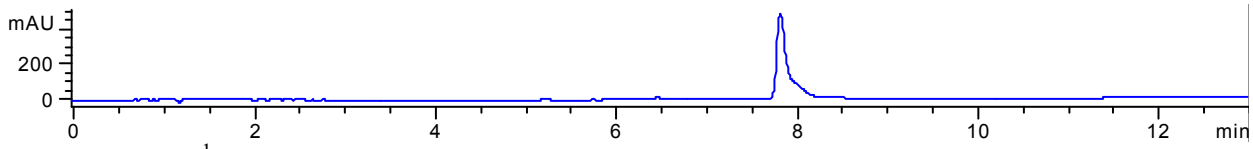


7 8

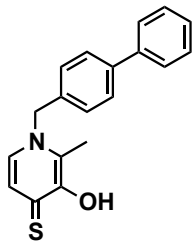
General synthetic procedure for 3,4-HOPTO analogues 8a and 8c–8g. 3-Hydroxy-2-methyl-4*H*-pyran-4-thione (thiomaltol, **7**) (1.0 equiv) and amine (2.0 equiv) were added to a round-bottomed flask and dissolved in toluene (0.8 M). The resulting mixture was heated to 110–115 °C to boil off all solvent. After ~5 min of additional heating, the contents were cooled to 25 °C and the dark residue was dissolved in a minimal amount of EtOAc. Compounds **8a** and **8c–8g** were then obtained via recrystallization from EtOAc and hexanes or flash chromatography to yield yellow powders.



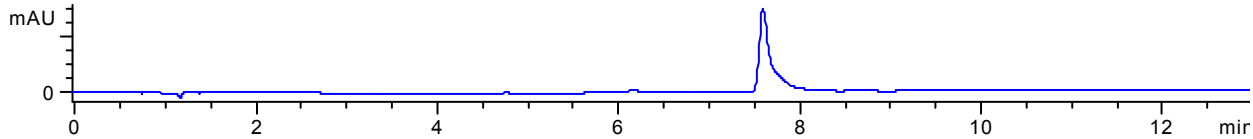
8a



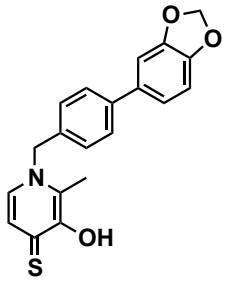
Compound **8a**. ^{1}H NMR (400 MHz, DMSO, 25 °C) δ: 2.34 (s, 3H), 5.51 (s, 2H), 7.23 (d, J = 8.1 Hz, 2H), 7.42 (d, J = 6.6 Hz, 1H), 7.46 (dd, J = 8.4, 6.3 Hz, 2H), 7.73 (d, J = 8.2 Hz, 2H), 7.78 (t, J = 5.9 Hz, 1H), 7.88 (d, J = 6.7 Hz, 1H), 8.76 (s, 1H). ^{13}C NMR (200 MHz, DMSO, 25 °C) δ: 13.25, 57.90, 116.32, 116.50, 118.56, 118.73, 124.07, 124.17, 125.59, 127.86, 128.10, 128.95, 134.25, 135.93, 138.34, 153.36, 170.34; HRMS (ESI-TOF) *m/z* calcd for $C_{19}H_{15}F_2NOS$ $[M+H]^+$ 343.08424, found 344.0914.



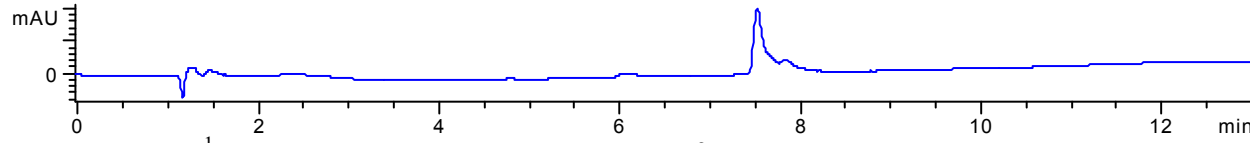
8b



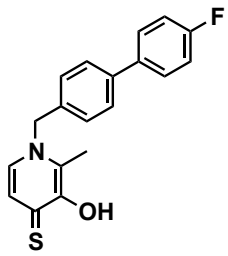
Compound **8b**. See reference [2] for microwave synthesis and compound characterization.



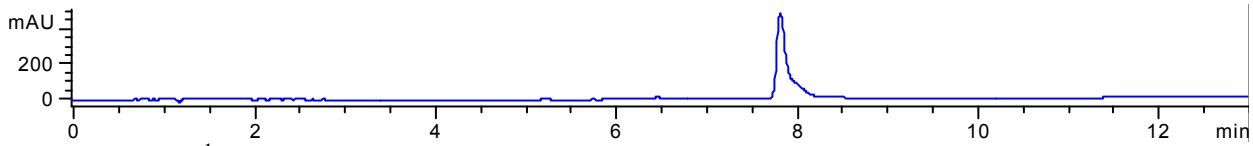
8c



Compound **8c**. ^{1}H NMR (400 MHz, DMSO, 25 °C) δ: 2.35 (s, 3H), 5.48 (s, 2H), 6.05 (s, 2H), 6.99 (d, J = 8.1 Hz, 1H), 7.13 (dd, J = 8.1, 1.6 Hz, 1H), 7.18 (d, J = 8.1 Hz, 2H), 7.24 (d, J = 1.4 Hz, 1H), 7.41 (d, J = 6.6 Hz, 1H), 7.63 (d, J = 8.2 Hz, 2H), 7.87 (d, J = 6.7 Hz, 1H), 8.76 (s, 1H). ^{13}C NMR (200 MHz, DMSO, 25 °C) δ: 13.26, 57.96, 101.86, 107.74, 109.37, 120.96, 125.56, 127.71, 127.74, 128.97, 134.22, 134.30, 134.77, 140.31, 147.69, 148.67, 153.34, 170.24; HRMS (ESI-TOF) m/z calcd for $C_{20}H_{17}NO_3S$ $[M+H]^+$ 351.09291, found 352.1000.

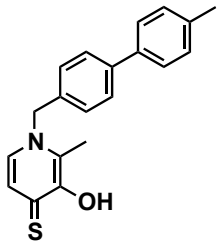


8d

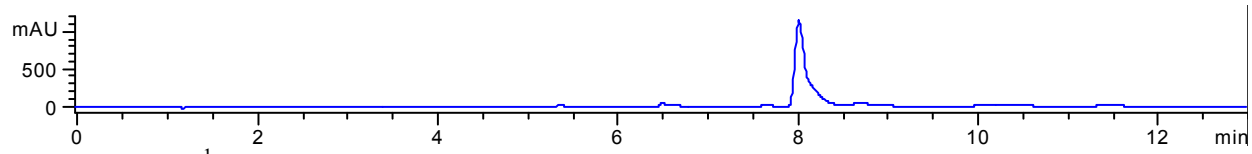


Compound **8d**. ^{1}H NMR (400 MHz, DMSO, 25 °C) δ: 2.34 (s, 3H), 5.51 (s, 2H), 7.23 (d, J = 8.1 Hz, 2H), 7.42 (d, J = 6.6 Hz, 1H), 7.52 (d, J = 4.1 Hz, 2H), 7.72 (d, J = 8.2 Hz, 2H), 7.77 (d, J =

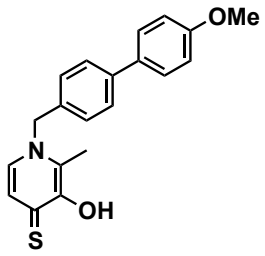
4.8 Hz, 1H), 7.88 (d, J = 8.3 Hz, 2H). ^{13}C NMR (200 MHz, DMSO, 25 °C) δ: 13.25, 54.76, 103.48, 112.40, 135.09, 137.49, 139.99, 142.97, 143.59, 140.31, 147.69, 148.69, 153.65, 170.24, 172.11; HRMS (ESI-TOF) *m/z* calcd for $C_{19}H_{16}FNOS$ $[M+H]^+$ 325.09366, found 326.0938.



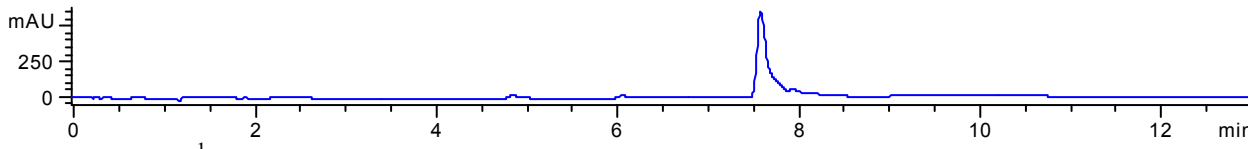
8e



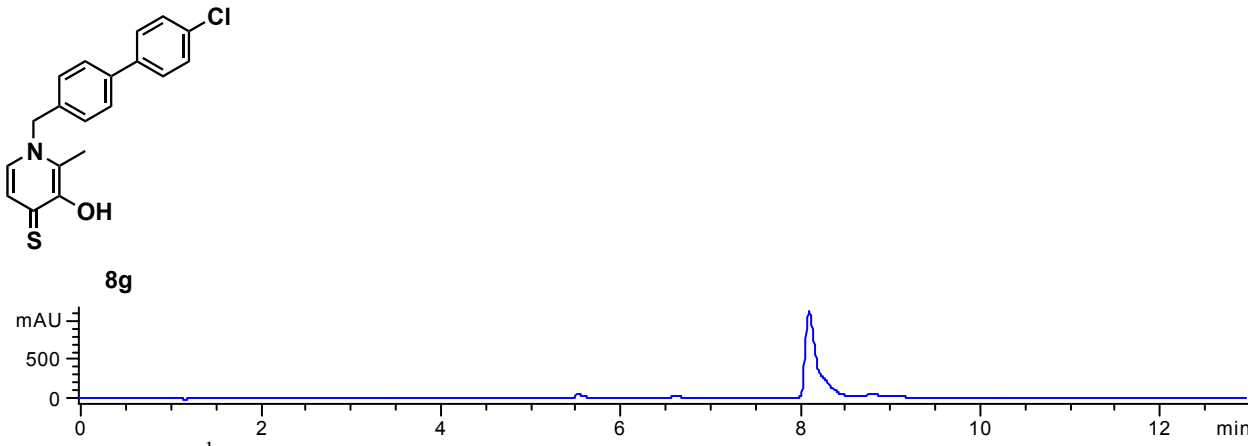
Compound **8e**. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ: 7.60 (m, 4H), 7.48 (d, 2H, J = 7.0 Hz), 7.23 (m, 2H), 7.08 (d, 2H, J = 7.0 Hz), 5.25 (s, 2H), 2.48 (s, 3H), 2.42 (s, 3H); HRMS (ESI-TOF) *m/z* calcd for $C_{20}H_{19}NOS$ $[M+H]^+$ 322.1260, found 322.1254.



8f



Compound **8f**. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ: 7.57 (d, 2H, J = 7.0 Hz), 7.55 (d, 2H, J = 7.0 Hz), 7.48 (d, 1H, J = 6.8 Hz), 7.23 (d, 1H, J = 6.8 Hz), 7.08 (d, 2H, J = 7.0 Hz), 6.96 (d, 2H, J = 7.0 Hz), 5.25 (s, 2H), 3.81 (s, 3H), 2.42 (s, 3H); HRMS (ESI-TOF) *m/z* calcd for $C_{20}H_{19}NO_2S$ $[M+H]^+$ 338.1209, found 338.1214.



Compound **8g**. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ: 7.58 (m, 3H), 7.50 (d, 2H, J = 7.0 Hz), 7.39 (d, 2H, J = 7.0 Hz), 7.19 (d, 1H, J = 6.8 Hz), 7.05 (d, 2H, J = 7.0 Hz), 5.25 (s, 2H), 2.46 (s, 3H); HRMS (ESI-TOF) *m/z* calcd for $C_{19}H_{16}ClNOS$ $[M+H]^+$ 342.0714, found 342.0711.

C. Fluorescence Assay for LasB Activity

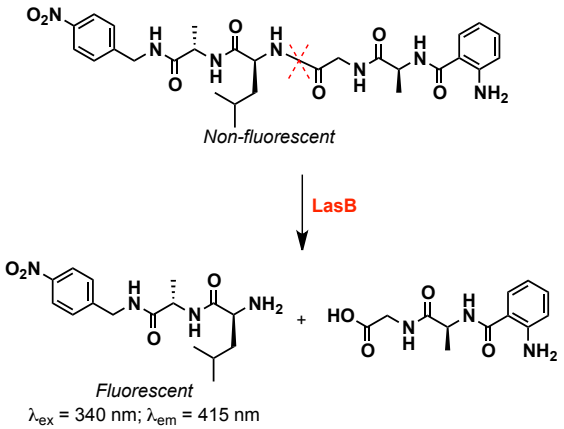


Figure S1. Fluorescence assay for LasB activity. This assay was adapted from that previously reported.[3]

Assay Buffers:

A: 50 mM Tris-HCl, 2.5 mM $CaCl_2$ (pH 7)

To prepare: 25 mL Tris-HCl (1 M solution), 475 mL H_2O, 139 mg $CaCl_2$

B: 50 mM Tris-HCl, 2.5 mM $CaCl_2$, 1% DMF (pH 7)

To prepare: 25 mL Tris-HCl (1 M solution), 475 mL H_2O, 139 mg $CaCl_2$, 5 mL DMF (molecular biology grade)

Assay Stock Solutions:

LasB: 0.1 mg/mL in Buffer A

LasB substrate: 5 mM in DMF (molecular biology grade)

To prepare: 3 mg (MW: 583.64 g/mol), 1 mL DMF

Inhibitor Stock Solutions:

10 mM, 5 mM, 2.5 mM, 500 μM, 250 μM, 50 μM, 25 μM in DMSO

Note: 2 μL of each stock used in the assay

Stock	Assay Final Concentration (μM)
10 mM	200
5 mM	100
2.5 mM	50
500 μM	10
250 μM	5
50 μM	1
25 μM	0.5

Assay Protocol:

1. To 96-well microtiter plate (Corning®Costar®; black with clear bottom), add 91 μL **Buffer B**, 2 μL of each **inhibitor stock** and 2 μL **LasB** solution (2 mg/mL final) to each well (in this order)

   Notes:
   i. Positive control: 2 μL DMSO in place of inhibitor
   ii. Negative control: 4 μL DMSO in place of LasB and inhibitor stock
2. Incubate plate at 37 °C for 30 min
3. While incubating, turn on and set up fluorescence plate reader: λ_{ex} = 340 nm, λ_{em} = 415 nm, 37°C, read on kinetic setting over 30 min
4. After 30 min incubation at 37 °C, add 5 μL **LasB substrate** solution (250 mM final) to each well
5. Transfer plate to fluorescence plate reader and measure fluorescence
   Note: For an inhibitor, *decreased* fluorescence signal will be observed in comparison to the positive control

D. Supplemental Screening Results and Library Structures

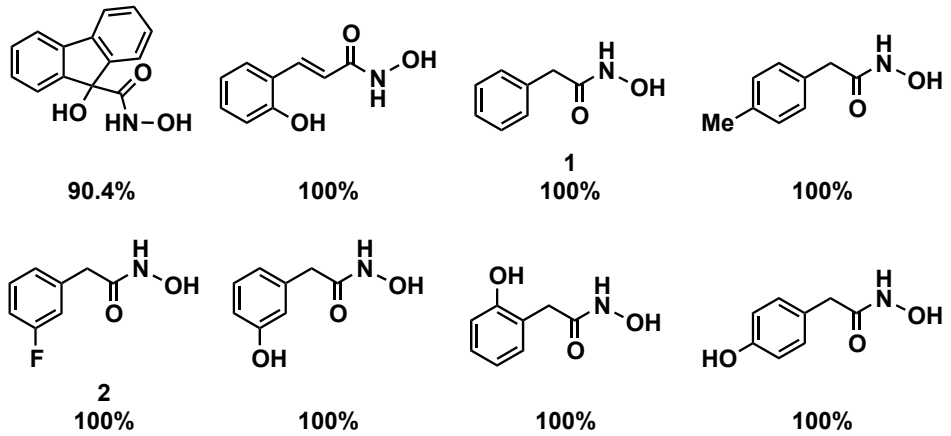


Figure S2. Hits from the hydroxamic acid small molecule library. Percentages indicated are percent inhibition at 50 μM (triplicate experiments). Only compounds **1** and **2** showed dose-dependent LasB antagonism.

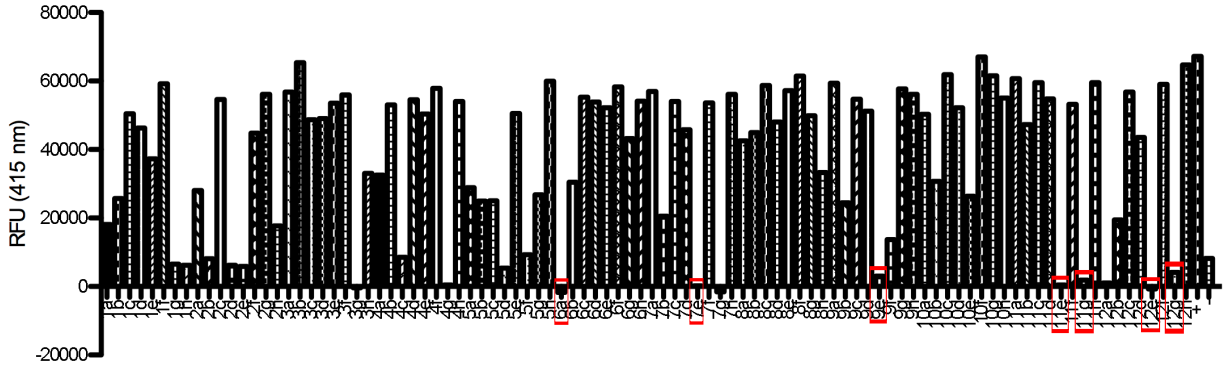


Figure S3. LasB inhibition from 96-member fragment chelator library. Hits receiving secondary screening are indicated in red. RFU = relative fluorescence units at 415 nm.

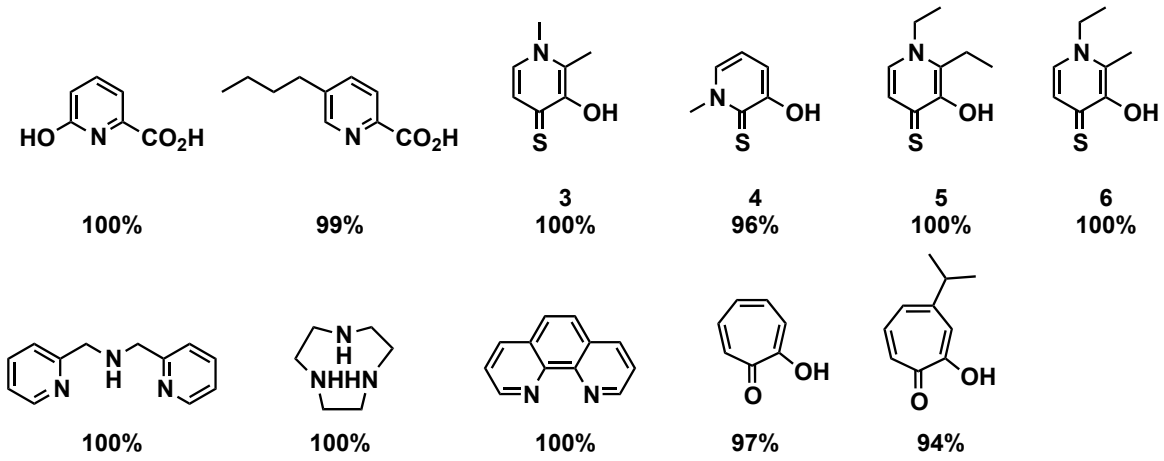


Figure S4. Hits from the fragment chelator library. Percentages indicated are percent inhibition at 1 mM (triplicate experiments). Only compounds **3**–**6** showed dose-dependent LasB antagonism.

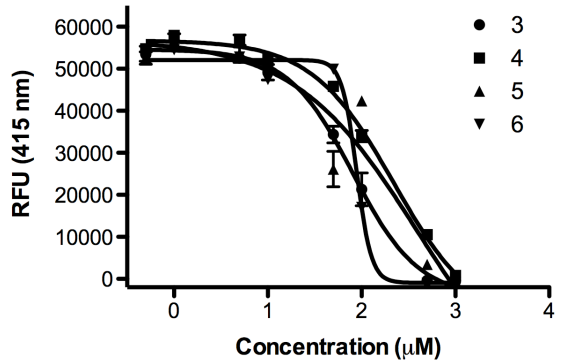


Figure S5. Inhibition curves for compounds **3**–**6**. IC_{50} values were determined from triplicate measurements. RFU = relative fluorescence units at 415 nm.

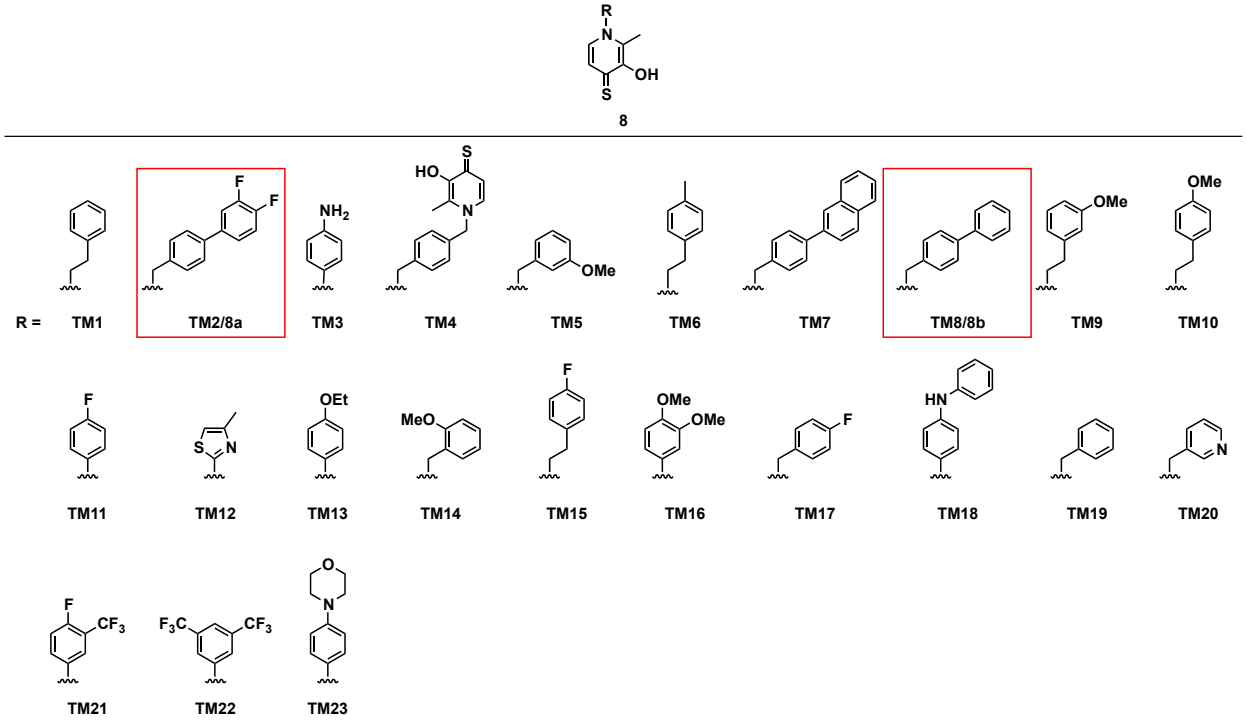


Figure S6. 3,4-HOPTO sub-library structures. Compounds **8a** and **8b** are indicated in red.

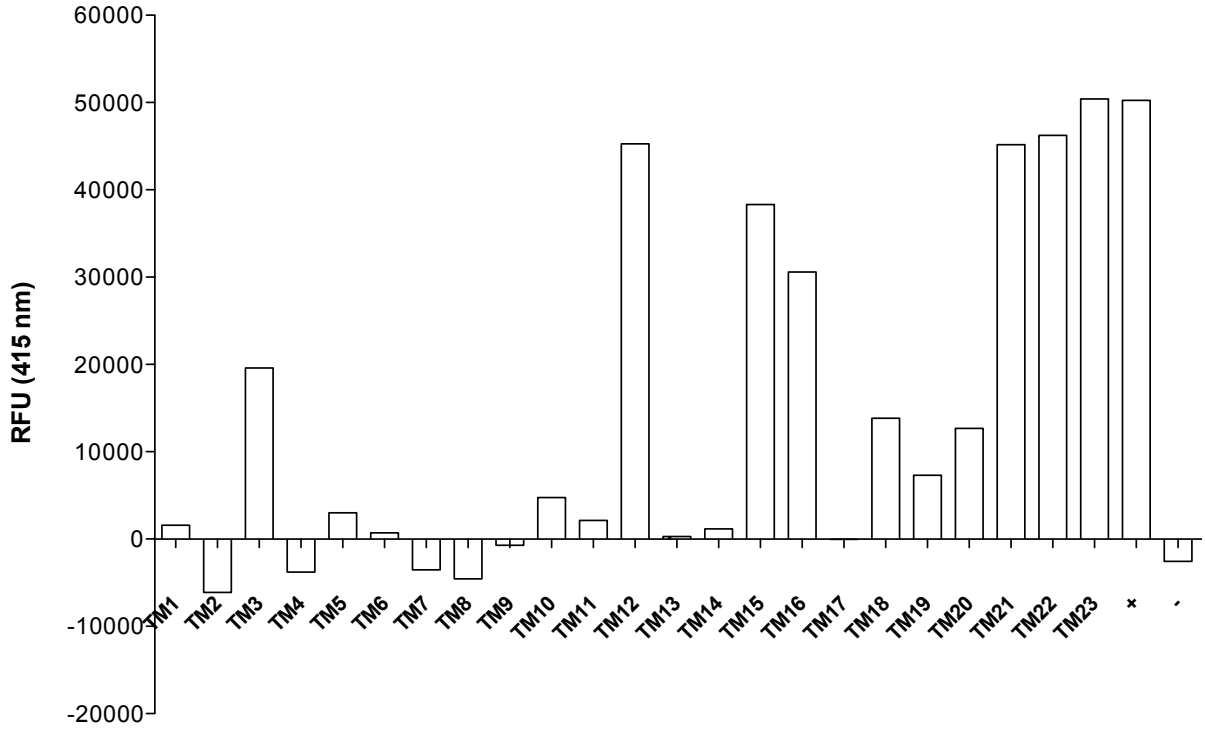


Figure S7. LasB inhibition from 3,4-HOPTO sub-library. TM2 and TM8 are compounds **8a** and **8b**, respectively. RFU = relative fluorescence units at 415 nm.

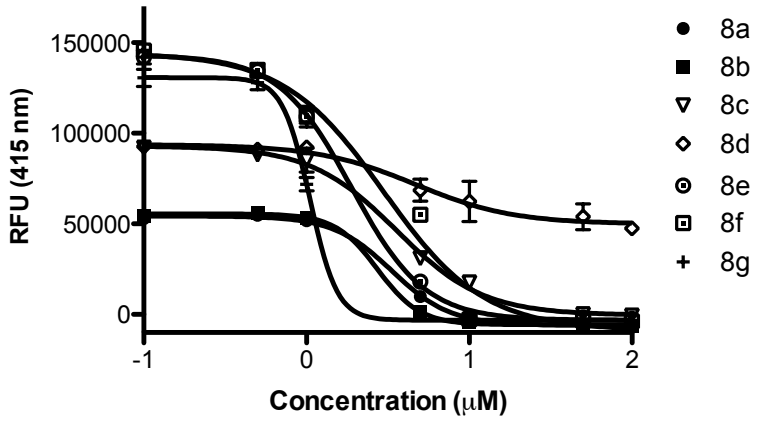


Figure S8. Inhibition curves for compounds **8a–8g**. IC_{50} values were determined from triplicate measurements. RFU = relative fluorescence units at 415 nm. Different enzyme batches were used for compounds **8a–8b**, **8c–8d** and **8e–8g**, accounting for the differential maximum fluorescence readings. Compound **8d** never fully inhibited the enzyme in this assay.

E. Stability of 8a and 8b

The stability of **8a** and **8b** was assessed in PBS (pH 7.4) and Tryptic soy broth. Samples containing 50 μM compound were incubated at 25 °C or 37 °C for 48 h. Aliquots were removed and analyzed using LC-MS at t = 0, 4 h, 8 h, 24 h and 48 h. For all samples, no detectable S-oxidation or decomposition was observed within the 48 h time period.

F. Bacterial Viability with 8a and 8b

Overnight cultures of *P. aeruginosa* strain PA14, prepared in Tryptic soy broth (TSB) at 37 °C (250 rpm), were diluted with fresh media (1:50,000 or 1:1,000) and treated with varying concentrations of **8a** or **8b**. Bacterial viability was then determined by measuring the OD_{600} of the culture in the presence of compound. All concentrations were analyzed in triplicate and the average of the results with 1:1,000 dilution are shown in Table **S1**. Toxicity was observed at concentrations >25 μM (highlighted in yellow in Table **S1**).

Table S1. Viability of PA14 in the presence of **8a** or **8b**.

Concentration (μM)	% Cell Viability	
	8a	**8b**
TSB	100	100
1% DMSO	97	96
0.78125	97	97
1.5625	97	98
3.1250	98	98
6.250	96	97
12.50	96	95
25.00	94	95
50.0	85	88

100.0	80	85
500	59	79

G. *P. aeruginosa* Swarming Assay

A swarming motility assay using *P. aeruginosa* strain PA14 was executed as previously described.[5] Overnight cultures of PA14 strain, prepared in TSB at 37 °C (250 rpm), were washed (3×) with phosphate-buffered saline buffer (pH 7.4). The washed culture was then diluted with the same buffer to an OD_{600} of ~3.0. Swarm agar medium was a modified M9 agar medium and contained: 20 mM NH_4Cl, 12 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$ 2 H_2O, 11 mM dextrose, 0.5% casamino acids (Difco) and Bacto-agar (Difco). The medium was autoclaved and cooled to touch, to which filter-sterilized $MgSO_4$ and $CaCl_2$ $2H_2O$ were added. ~20 mL of swarm agar medium containing compounds **8a** or **8b** (25 μM) was poured into 100 × 25 mm Petri dishes housed in a laminar flow cabinet and dried for 1 h. 5 μL of the bacterial culture (OD_{600} of ~3.0) was spotted onto each plate followed by incubation at 30 °C for 16 h.

H. References

1. Lewis, J. A.; Puerta, D. T.; Cohen, S. M. Metal complexes of the *trans*-influencing ligand thiomaltol. *Inorg. Chem.* **2003**, *42*, 7455.
2. Agrawal, A.; Johnson, S. L.; Jacobsen, J. A.; Miller, M. T.; Chen, L.; Pellecchia, M.; Cohen, S. M. Chelator fragment libraries for targeting metalloproteinases. *ChemMedChem* **2010**, *5*, 195.
3. Nishino, N.; Powers, J. C. *Pseudomonas aeruginosa* elastase: development of a new substrate, inhibitors, and an affinity ligand. *J. Biol. Chem.* **1980**, *255*, 3482.
4. Smith, K. M.; Bu, Y.; Suga, H. Induction and inhibition of *Pseudomonas aeruginosa* quorum sensing by synthetic autoinducers. *Chem. Biol.* **2003**, *10*, 81.
5. Tremblay, J.; Deziel, E. Improving the reproducibility of *Pseudomonas aeruginosa* swarming motility assays. *J. Basic Microbiol.* **2008**, *48*, 509.