Supporting Information for:

Development of Covalent Inhibitors for Bacterial Histidine Kinases

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Experimental Methods and Materials

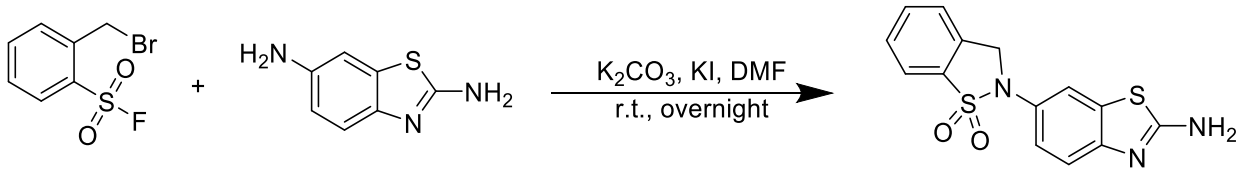
Materials. All chemicals and reagents used in this study were purchased through the following companies. Synthetic reagents: 1,3-benzothiazole-2,6-diamine (Chemcruz, cas# 5407-51-2), 2-amino-6-hydroxybenzothiazole (Sigma-Aldrich, cas# 26278-79-5), 2-amino-6-trifluoromethoxybenzothiazole (Fisher scientific, cas# 1744-22-5), 4-(fluorosulfonyl)benzoyl chloride (Millipore Sigma, cas# 402-55-1), 4-(bromomethyl)benzenesulfonyl fluoride (Millipore Sigma, cas# 76132-88-6), 2-(bromomethyl)benzene-1-sulfonyl fluoride (Enamine, cas# 25300-29-2), 3-bromo-5-((trimethylsilyl)ethynyl)benzenesulfonyl fluoride (Millipore Sigma, cas# 2088829-15-4) ,4-(bromomethyl)-2-((trimethylsilyl)ethynyl)benzenesulfonyl fluoride (Millipore Sigma, cas# 208828-99-1), BODIPY ATP$\boldsymbol{\gamma}$S thioester (Invitrogen, ThermoFisher, cat# A22184), NH125 (Santa Cruz Biotechnology, cas# 268603-08-0), α-cyano-4-hydroxycinnamic acid (Sigma, cas# 28166-41-8), trifluoroacetic acid (Sigma, cas# 76-05-1), formic acid (Sigma, 64-18-6), MS grade water (Sigma, cas# 7732-18-5), MS grade acetonitrile (Sigma, cas# 75-05-8), ammonium bicarbonate (Sigma, cas# 1066-33-7), DTT (dithiothreitol) (BioRad, cas# 27565-41-9), urea (Aldrich, cas# 57-13-6), C3 column (Agilent, 0.3 x 100 mm, 3.5 mm, 300 SB), Proteoextract® Protein Precipitation kit (Sigma-Aldrich), MS grade trypsin (Promega) or chymotrypsin (Promega), ZipTips, Tris(2-carboxyethyl)phosphine hydrochloride (Sigma, cas# 51805-45-9), tris((1-benzyl-4-triazolyl)methyl)amine (Sigma, cas# 510758-28-8), copper II sulfate (Sigma, cas# 7758-98-7), Dde-TAMRA-biotin-azide (Vector Labs, cat# CCT-1367-1) Pierce magnetic streptavidin beads (Thermo Scientific, cat# 88802), Speedbead magnetic neutravidin coated particles (Cytiva, cat 78152104011150), hydrazine hydrate (Acros, cas# 10217-52-4), phenylmethylsulfonyl fluoride (Acros, cas# 329-98-6), (Sigma cas# 86408-36-8) Triton X-100 (Sigma, cas# 9036-19-5), sodium dodecyl sulfate (Sigma, 151-21-3).

LB Media Lennox (Sigma, powder, cat# L3522), agar (Sigma, powder, cat# A1296), plastic 17x100 mm culture tubes (25 pack, VWR, cat# 60818-703), Corning Falcon 100 x 15 mm polystyrene petri dish (20 pack, Fisher scientific, cat# 351029), Costar clear sterile 96 well cell culture plate, flat bottom, tissue culture treated, non-pyrogenic, polystyrene (Corning Incorporated, cat# 3599), ampicillin (Sigma Aldrich, cas# 69-53-4), colistin sulfate (Sigma Aldrich, cas# 1264-72-8), isopropyl-β-D-thiogalactopyranoside (IPTG) (IBI, cas# 367-93-1), bovine serum albumin (Sigma, cas# 9048-46-8), ammonium persulfate (BioRad, cat# 161-0700),

TEMED (BioRad, cat# 161-0800), TrisHCl Stacking (BioRad, cat# 1610799), TrisHCl Resolving (Biorad, cat# 1610798)40% acrylamide (BioRad, cat# 161-0146), 1.5 mm cassettes (ThermoFisher, cat# NC2015), 1.5 mm well comb (ThermoFisher, cat# NC3515), coomassie brilliant blue R-250 staining solution (BioRad, cat#1610436), Pierce Silver Stain Kit (ThermoFisher Scientific, cat# 24612), Sypro Red Protein Gel Stain (Invitrogen, cat# S6653).

Salmonella enterica strains were gifted by Dr. John May, University of Wisconsin – La Crosse (14028, EG9492).

Procedure for the synthesis of ***1a*** from benzylbromide:



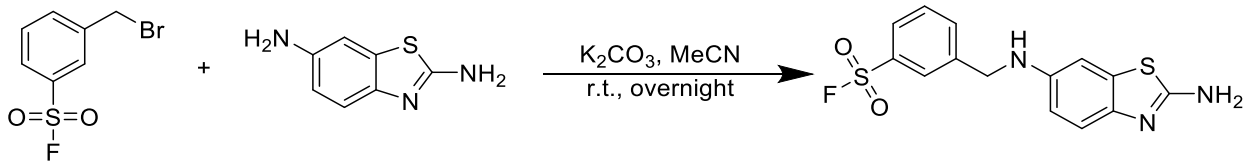
To an oven dried round-bottom flask equipped with a stir bar, 2,6-diaminobenzothiazole (1.1 equiv, 0.02 mmol, 13.7 mg) and potassium carbonate (K_2CO_3, 1.1 equiv, 0.02 mmol, 3.0 mg) were added, sealed with a rubber septum, and vacuum purged with nitrogen cycling (3x). Anhydrous dimethylformamide (DMF, 0.20 mL) was added to the vessel to dissolve the starting material. 3-(bromomethyl)-benzene-1-sulfonyl fluoride (1.0 equiv, 0.080 mmol, 5.0 mg) in the presence of sodium iodide (1.2 equiv, .024 mmol, 3.6 mg) was dissolved in DMF (0.20 mL) as described above and was introduced to the vessel dropwise. The mixture was stirred overnight under nitrogen atmosphere at room temperature. The following day, the reaction was quenched with the addition of water (5 mL) and extracted with 5 mL of ethyl acetate (EtOAc). The aqueous layer was extracted with EtOAc (3x), and the combined organic extracts were washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude material was purified by flash column chromatography (1:4 hexanes:EtOAc) to afford ***1a*** (3.2 mg, 0.010 mmol, 51% yield) as a reddish-brown oil.

Data for compound ***1a:***

^{1}H NMR ($(CD_3)_2SO$, 500 MHz): 7.97 (dd, 1H, J = 8.6, 1.3 Hz, A), 7.81 (s, 1H, J = 2.2 Hz, B), 7.78 (dd, 1H, J = 7.5, 1.0 Hz, C), 7.67-7.69 (m, 2H, G and H), 7.55 (s, 2H, I), 7.40 (d, 1H, J = 8.6 Hz, D), 7.38 (dd, 1H, J = 8.7, 2.2 Hz, E), 5.01 (s, 2H, F).

LCMS *m/z* calcd for $C_{14}H_{12}N_3O_2S_2$ $[M+H]^+$: 318.0365. Found: 318.0.

Procedure for the synthesis of ***1b*** from benzylbromide:



To an oven dried round-bottom flask equipped with a stir bar, 2,6-diaminobenzothiazole (1.1 equiv, 0.083 mmol, 13.7 mg) and potassium carbonate (K_2CO_3, 1.2 equiv, 0.096 mmol, 13 mg) were added, sealed with a rubber septum, and vacuum purged with nitrogen cycling (3x). Anhydrous acetonitrile (MeCN, 2.0 mL) and acetone (0.50 mL) were added to the vessel to dissolve the starting material. 3-(Bromomethyl)-benzene-1-sulfonyl fluoride (1.0 equiv, 0.080 mmol, 20 mg) was dissolved in MeCN (1.0 mL) as described above and was introduced to the vessel dropwise to generate a yellow solution. The resulting mixture was heated to 50-55 °C and stirred overnight under nitrogen atmosphere. The reaction, now a white opaque solution, was cooled to room temperature. The solids were filtered off with a silica plug, washed with 2x ethyl acetate (EtOAc, 2 mL) and the filtrate was concentrated to give a reddish-yellow oil. The crude material was purified by flash column chromatography (100% EtOAc) to afford ***1b*** (17 mg, 0.050 mmol, 64% yield) as a light-yellow crystalline solid.

Data for compound ***1b:***

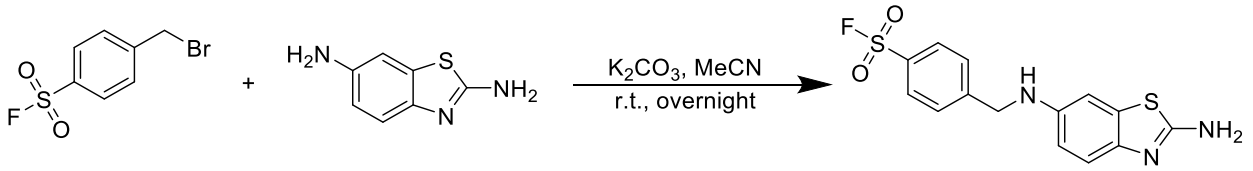
^{1}H NMR (CD_3CN, 500 MHz): δ 8.04 (d, 1H, *J* = 1.9 Hz, a.), 7.91 (dd, 1H, *J* = 8.0, 1.6 Hz, b.), 7.79 (d, 1H, *J* = 7.8 Hz, h.), 7.66 (ddd, 1H, *J* = 8.5, 8.0, 0.9 Hz, i.), 7.18 (d, 1H, *J* = 8.5 Hz, d.), 6.83 (d, 1H, *J* = 2.3 Hz, c.), 6.60 (dd, 1H, *J* = 8.7, 2.5 Hz, e.), 5.55 (br s, 2H, f.), 5.00 (br s, 1H, j.) 4.44 (s, 2H, g.).

^{13}C NMR (CD_3CN, 125 MHz): δ 163.03, 144.60, 143.64, 143.52, 135.00, 133.09, 132.47 (d, $J_{C\text{-}F}$ = 23.8 Hz), 130.15, 126.77, 119.09, 112.62, 103.90, 46.88.

^{19}F{^{1}H}-NMR (CD_3CN, 470.61 MHz): δ 64.5.

LCMS *m/z* calcd for $C_{14}H_{13}FN_3O_2S_2$ $[M+H]^+$: 338.0428. Found: 338.0546.

Procedure for the synthesis of ***1c*** from benzylbromide:



To an oven dried round-bottom flask equipped with a stir bar, 2,6-diaminobenzothiazole (1.1 equiv, 0.083 mmol, 13.7 mg) and potassium carbonate (K_2CO_3, 1.2 equiv, 0.096 mmol, 13 mg) were added, sealed with a rubber septum, and vacuum purged with nitrogen cycling (3x). Anhydrous acetonitrile (MeCN, 2.0 mL) and acetone (0.50 mL) were added to the vessel to dissolve the starting material. 3-(bromomethyl)-benzene-1-sulfonyl fluoride (1.0 equiv, 0.080 mmol, 20 mg) was dissolved in MeCN (1.0 mL) as described above and was introduced to the vessel dropwise to generate a yellow solution. The resulting mixture was heated to 50-55 °C and stirred overnight under nitrogen atmosphere. The reaction, now a white opaque solution, was cooled to room temperature. The solids were filtered off with a silica plug, washed with ethyl acetate (EtOAc, 2x 2 mL) and the filtrate was concentrated to give a yellow oil. The crude material was purified by flash column chromatography (1:4 hexanes:EtOAc) to afford ***1c*** (17 mg, 0.050 mmol, 33%) as a crystalline light-yellow solid.

Data for compound ***1c:***

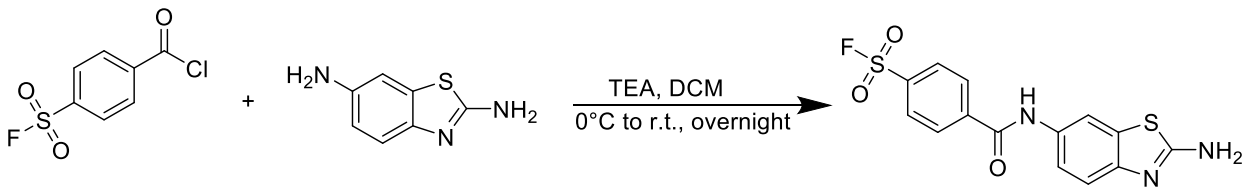
^{1}H NMR (CD_3CN, 500MHz): δ 7.79 (dd, 2H, J = 8.5, 0.5 Hz, a.), 7.68 (dd, 2H, J = 8.5, 0.7 Hz, b.), 7.18 (dd, 1H, J = 8.0, 0.9 Hz, d.), 6.80 (dd, 1H, J = 2.4, 0.5 Hz, c.), 6.61 (dd, 1H, J = 8.5, 2.4 Hz, e.), 5.59 (br s, 2H, f.), 5.55 (br s, 2H, h.), 5.04 (s, 2H, g.).

^{13}C NMR (CD_3CN, 125 MHz): δ 162.93, 150.45, 144.67, 143.66, 133.16, 130.49 (d, $J_{C\text{-}F}$ = 23.8 Hz), 128.55, 119.11, 112.56, 103.81, 47.21.

^{19}F{^{1}H}-NMR (CD_3CN, 470.61 MHz): δ 64.5.

LCMS *m/z* calcd for $C_{14}H_{13}FN_3O_2S_2$ $[M+H]^+$: 338.0428. Found: 338.0546.

<u>Procedure for the synthesis of ***2a*** from acid chlorides:</u>



To an oven dried round-bottom flask equipped with a stir bar, 2,6-diaminobenzothiazole (1.3 equiv, 0.280 mmol, 46.4 mg) was added, sealed with a rubber septum, and vacuum purged

with nitrogen cycling (3x). Anhydrous dichloromethane (DCM, 1 mL) was added to the vessel to dissolve the starting material at room temperature. The solution was cooled in an ice bath prior to the dropwise addition of triethylamine (TEA, 1.5 equiv, 0.292 mmol, 0.041 mL) resulting in a yellow solution. 4-(Sulfonylfluoride)benzoyl chloride (1.0 equiv, 0.225 mmol, 50.0 mg) was dissolved in DCM (1.0 mL) as described above, this solution was introduced to the benzothiazole containing vessel dropwise on ice. The mixture remained yellow and was stirred overnight under nitrogen atmosphere at room temperature. The reaction was quenched with the addition of water (5 mL) and extracted with EtOAc. The aqueous layer was extracted with EtOAc (3x), and the combined organic extracts were washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The reaction was purified by flash column chromatography (0-100% EtOAc/hexanes) to afford ***2a*** (53 mg, 0.151 mmol, 67%) as a bright yellow crystalline solid.

Data for compound ***2a***:

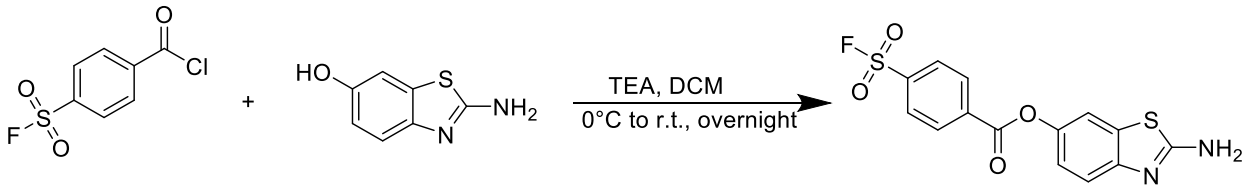
^{1}H NMR ($(CD_3)_2SO$, 500 MHz): δ 10.60 (s, 1H, g), 8.43 (d, 2H, *J* = 8.8 Hz, a.), 8.25 (d, 2H, *J* = 8.8 Hz, b.), 8.12 (s, 1H, c.), 7.56 (dd, 1H, *J* = 8.7, 2.5 Hz, e.), 7.47 (s, 1H, f.), 7.18 (dd, 1H, *J* = 8.7, 0.3 Hz, d.).

^{13}C NMR ($(CD_3)_2SO$, 125 MHz): δ 170.41, 166.26, 163.36, 149.76, 142.17, 141.36, 133.48, 132.13, 129.50, 128.69, 119.09, 117.46, 113.27.

^{19}F{^{1}H}-NMR ($(CD_3)_2SO$, 470.61 MHz): δ 66.0.

LCMS *m/z* calcd for $C_{14}H_{11}FN_3O_2S_2$ $[M+H]^+$: 352.0220. Found: 352.0264.

<u>Procedure for the synthesis of ***2b*** from acid chlorides:</u>



To an oven dried round-bottom flask equipped with a stir bar, 2-amino-benzothiazole-6-ol (1.4 equiv, 0.28 mmol, 46.6 mg) was added, sealed with a rubber septum, and vacuum purged with nitrogen cycling (3x). Anhydrous DCM (1 mL) was added to the vessel to dissolve the starting material at room temperature. The solution was cooled in an ice bath prior to the dropwise addition of TEA (1.5 equiv, 0.3 mmol, 0.2 mL) resulting in a yellow solution. 4-(sulfonylfluoride)benzoyl chloride (1.0 equiv, 0.2 mmol, 50.0 mg) was dissolved in DCM (1.0 mL) as described above, this

solution was introduced to the benzothiazole containing vessel dropwise on ice. The mixture remained yellow and was stirred overnight under nitrogen atmosphere at room temperature. The reaction was quenched with the addition of water (5 mL) and extracted with EtOAc. The aqueous layer was extracted with EtOAc (3x), and the combined organic extracts were washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude material was purified by flash column chromatography (1:4 hexanes:EtOAc) to afford ***2b*** (40 mg, 0.114 mmol, 50%) as a white crystalline solid.

Data for ***2b***:

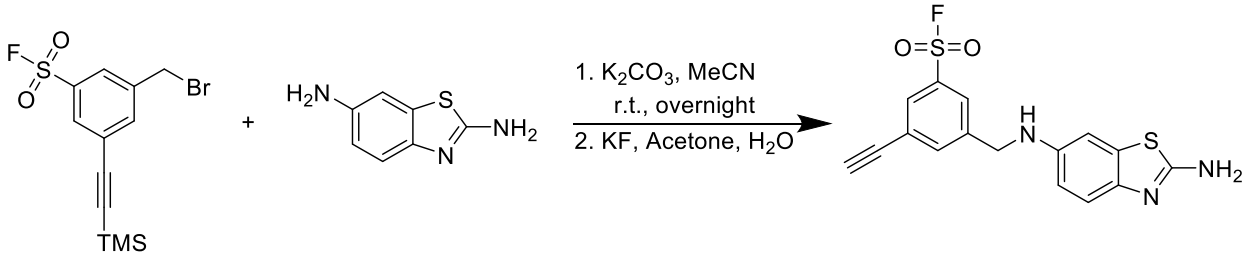
^{1}H NMR (CD_3CN, 500 MHz): δ 8.43 (d, 2H, J = 8.8 Hz, a.), 8.25 (d, 2H, J = 8.8 Hz, b.), 7.56 (dd, 1H, J = 2.5, 0.3 Hz, c.), 7.47 (d, 1H, J = 8.7, 0.3 Hz, d.), 7.18 (dd, 1H, J = 8.7, 2.5 Hz, e.), 6.01 (br s, 2H, f.).

^{13}C NMR (CD_3CN, 125 MHz) δ 167.70, 164.71, 151.97, 146.16, 137.46, 133.21, 132.19, 129.84, 120.39, 119.66, 115.11. (aryl carbon connected to SO_2F is not visible)

^{19}F{^{1}H}-NMR (CD_3CN, 470.61 MHz): δ 64.3

LCMS *m/z* calcd for $C_{14}H_{10}FN_2O_4S_2$ $[M+H]^+$: 353.0061. Found: 353.0289.

Procedure for synthesis of ***P-1*** from benzyl bromides:



To an oven dried round-bottom flask equipped with a stir bar, 2,6-diaminobenzothiazole (1.4 equiv, 0.20 mmol, 33 mg) and K_2CO_3 (1.8 equiv, 0.36 mmol, 50 mg) were added, sealed with a rubber septum, and vacuum purged with nitrogen cycling (3x). Anhydrous MeCN (1 mL) was added to the vessel to dissolve the starting material. 3-(Bromomethyl)-benzene-1-sulfonyl fluoride (1.0 equiv, 0.14 mmol, 50 mg) was dissolved in MeCN (1.0 mL) as described above and was introduced to the vessel dropwise. The reaction was stirred at room temperature overnight under nitrogen atmosphere. The following day the reaction was diluted with 5 mL of EtOAc. The aqueous layer was extracted with EtOAc (3x), and the combined organic extracts were washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude

material subjected to deprotection with potassium fluoride (3 equiv, 0.43 mmol, 25 mg) in the presence of water (3.6 mL) and acetone (1.6 mL) was purified by flash column chromatography (1:4 hexanes:EtOAc) to afford ***P-1*** (39 mg, 0.11 mmol, 75% yield) as a reddish-brown crystalline solid.

Data for compound ***P-1:***

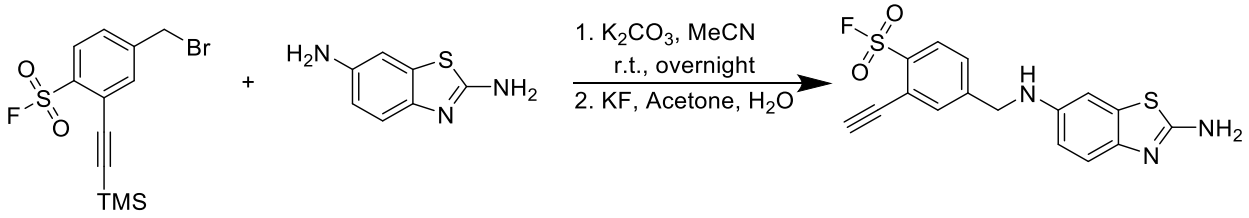
^{1}H NMR (CD_3CN, 500 MHz): δ 8.02 (d, 1H, *J* = 1.3 Hz, a.), 7.99 (d, 1H, *J* = 1.5 Hz, b.), 7.90 (dd, 1H, *J* = 1.5, 0.9 Hz, c.), 7.20 (dd, 1H, *J* = 8.7, 0.5 Hz, d.), 6.81 (dd, 1H, *J* = 2.4, 0.5 Hz, e.), 6.60 (dd, 1H, *J* = 6.2, 2.5 Hz, f.), 5.67 (br s, 2H, g.), 4.42 (s, 2H, h.), 3.59 (s, 1H, j.).

^{13}C NMR (125 MHz, CD_3CN) δ 164.07, 145.60, 145.13, 144.26, 138.71, 134.03, 133.99 (d, $J_{C\text{-}F}$ = 24.5 Hz), 130.73, 127.95, 125.02, 120.01, 113.57, 104.87, 81.94, 81.5, 47.48.

^{19}F{^{1}H}-NMR (CD_3CN, 470.61 MHz): δ 64.3

LCMS *m/z* calcd for $C_{16}H_{13}FN_3O_2S_2$ $[M+H]^+$: 362.0428. Found: 362.0522.

Procedure for synthesis of ***P-2*** from benzyl bromides:



To an oven dried round-bottom flask equipped with a stir bar, 2,6-diaminobenzothiazole (1.2 equiv, 0.069 mmol, 11.3 mg) and Cs_2CO_3 (2.5 equiv, 0.14 mmol, 46.6 mg) were added, sealed with a rubber septum, and vacuum purged with nitrogen cycling (3x). Anhydrous acetone (1.0 mL) and MeCN (0.4 mL) was added to the vessel to dissolve the starting material. 3-(Bromomethyl)-benzene-1-sulfonyl fluoride (1.0 equiv, 0.057 mmol, 20 mg) was dissolved in dry acetone (1.0 mL) as described above and was introduced to the vessel dropwise. The reaction was stirred at room temperature overnight. The following day it was diluted with 5 mL of EtOAc. The aqueous layer was extracted with EtOAc (3x), and the combined organic extracts were washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude material subjected to deprotection with potassium fluoride (3 equiv, 0.43 mmol, 25 mg) in the presence of water (3.6 mL) and acetone (1.6 mL) was purified by flash column chromatography (1:4 hexanes:EtOAc) to afford ***P-2*** (9.5 mg, 0.026 mmol, 46% yield) as a rose-gold colored crystalline solid.

Data for compound ***P-2:***

^{1}H NMR (500 MHz, $(CD_3)_2CO$): δ 8.10 (d, 1H, *J* = 8.6, 0.5 Hz, a.), 7.92 (s, 1H, *J* = 1.9, 0.5 Hz, b.), 7.78 (d, 1H, *J* = 8.7, 1.5 Hz, c.), 7.17 (d, 1H, *J* = 8.7, 0.5 Hz, e.), 6.88 (s, 1H, *J* = 2.5, 0.5 Hz, d.), 6.65 (dd, 1H, *J* = 8.7, 2.4 Hz, f.), 6.31 (br s, 2H, g.), 5.70 (br s, 1H, i.), 4.58 (s, 2H, h.), 4.30 (s, 1H, j.).

^{13}C NMR (125 MHz, $(CD_3)_2CO$): δ 163.77, 151.16, 146.09, 144.39, 135.14, 134.11, 133.26, 131.19, 129.10, 122.58, 119.95, 113.31, 104.56, 88.52, 79.03, 47.82.

^{19}F{^{1}H}-NMR ($(CD_3)_2CO$, 470.61 MHz): δ 59.3.

LCMS m/z calcd for $C_{16}H_{13}FN_3O_2S_2$ $[M+H]^+$: 362.0428. Found: 362.0522.

Cell Culture and Protein Expression.

Protein expression, purification, and subsequent activity and aggregation assays were performed and analyzed as described previously and as follows.[1] Frozen glycerol stock of *E. coli* BL21 overexpression strain containing the pHis‖ HK853 vector[1] was streaked onto a Lennox broth (LB) agar plate containing 100 μg/mL of ampicillin and grown at 37 °C overnight. From the master plate, a singular colony was inoculated into 5 mL of L media containing 100 μg/mL of ampicillin to generate primary cultures and grown at 37 °C overnight with shaking (220 rpm). The primary culture was added to fresh LB-amp media (1L) and grown at 37 °C, with shaking (220 rpm) to an OD_{600} ~0.5-0.6 (Genesys 30 Visible Spectrophotometer). Cells were induced with the addition of 0.22 mM IPTG and grown at 20 °C overnight (~16 h), with shaking (220 rpm). Cells were pelleted at 8,000 x *g* for 30 min at 4 °C and the supernatant discarded.

Protein Purification.

As previously described[2] the pellet was transferred to a glass homogenizer with 20 mL of cold lysis buffer (40 mL) 50 mM Tris, 1 M NaCl, 2 mM β-mercaptoethanol, 5 mM imidazole, 100 mM glycerol, pH 7.6), 1 EDTA free tablet (Roche, cat# 04693159001) and 10 μg/mL DNAse. An additional 10 mL of lysis buffer was used to rinse and collect residual cell pellet from the vessel. Cells were homogenized on ice by hand (12 strokes) and transferred to a 50 mL falcon tube. An additional 10 mL of lysis buffer was used to rinse the glass homogenizer. The falcon tube was submerged in a beaker containing ice and lysed with a Barson wand sonifier (15 s on and 30 s off for 15 min total on time at 30% A). The lysate was centrifuged at 14,000 x *g* for 30 min at 4 °C and clarified with a 0.22 μM PES syringe filter.

The clarified lysate was purified at 4 °C on a nickel affinity (Ni-NTA) column with a gradient of 0-100% buffer B over 20 min and a 2.00 mL/min flow rate. Elution of HK853 was monitored with UV-detection at 215 nm and occurred at approximately 20% buffer B. Fractions containing purified HK853 were collected and concentrated with 30 kDa Ambicon filters at 5,000 x *g* at 4 °C. Concentrated HK853 was washed with storage buffer (50 mM Tris, 500 mM NaCl, 200 mM glycerol, 2 mM β-mercaptoethanol) and centrifuged at 5,000 x *g* and 4 °C for 20 min. Wash steps were repeated 3 times. To assess the protein concentration, 2 µL protein aliquots were measured by Nanodrop at 280 nm (n=3). To avoid freeze-thaw cycles, protein was aliquoted, 5 µL of HK858 into Eppendorf tubes and stored at -80 °C.

1L Buffer A Final Solution:
25 mM Tris-HCl, pH 8, 1 M NaCl, 10% glycerol, 5 mM Imidazole, pH 8, 2 mM DTT. Adjust assembled Buffer A to pH 8. Fill to 1 L with MQ water, and filter through 0.22 µm membrane.

1L Buffer B Final Solution:
25 mM Tris-HCl, pH 8, 1 M NaCl, 10% glycerol, 1 M Imidazole, pH 8, 2 mM DTT. Adjust assembled Buffer B to pH 8. Fill to 1 L with MQ water, and filter 0.22 µm membrane.

Activity Assay.

Stocks of purified HK853 were obtained from storage at -80 °C and thawed on ice. Five-microliters of protein were pipetted into a 30 kDa filter (Ambicon) fitted into a microcentrifuge tube to exchange the protein from protein storage buffer into protein reaction buffer. Four-hundred microliters of chilled protein reaction buffer (kept on ice) were added to the filter and centrifuged at 4 °C, 15,000 x *g* for 5 min. The flowthrough was discarded, and the buffer exchange process was repeated two more times. In a new microcentrifuge tube, the filter was inverted and centrifuged at 15,000 x *g* for 2 min to elute the protein. Protein concentration was measured by the Pierce BCA Protein Assay Kit (ThermoFisher) following the manufactures procedure. BSA serial dilutions were generated in 1x protein reaction buffer as a standard. A working protein stock containing 0.1% (*v/v*) Triton X-100 was prepared by diluting the protein in reaction buffer to a final concentration of 0.50 µM.

Serially diluted inhibitors (final concentration, 0.01 - 1250 µM) were added (1.25 µL) to 23.75 µL of HK853 and incubated at room temperature for 30 min. BODIPY-FL-ATP$\boldsymbol{\gamma}$S (1.25 µL) was added to HK853 (0.46 µM) in the presence of competitors to bring to a final 25 µL volume. The reactions were mixed, incubated at room temperature for 1 h in the dark before quenching with 4x SDS-PAGE sample loading buffer (8.6 µL). Samples (15 µL) were loaded onto a 10% polyacrylamide resolving gel (180 V, on ice).

HK853 activity was elucidated by detected in-gel fluorescence signal and staining the gel with Coomassie Brilliant blue R250 ensured even protein loading. Integrated density values of the fluorescent gel bands were measured on ImageJ and were normalized as "% Inhibition" with respect to a control that contained no inhibitor. Data were plotted in GraphPad Prism version 9.5.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com), with relation to the log of molar inhibitor to determine IC_{50} values (Equation 1). For all activity assays, data were fit to a four-parameter logistic equation,

$$y = Bottom + \frac{(Top - Bottom)}{1 + 10^{((LogIC_{50} - x) * HillSlope)}}$$

where y is the response, Bottom and Top are plateaus in the units of the y-axis, x is the log of the molar concentration of inhibitor, Hillslope is the slope of the curve, and IC_{50} is the concentration of compound required for 50% inhibition of the enzyme (a response halfway between Bottom and Top). All activity experiments were performed in triplicate.

Aggregation Assay.

Stocks of purified HK853 were obtained from storage at -80 °C and thawed on ice. Five-microliters of protein were pipetted into a 30 kDa filter (Ambicon) fitted into a microcentrifuge tube to exchange the protein from protein storage buffer into HEPES buffer (20 mM HEPES in MilliQ H_2O, pH = 7.4). Four-hundred microliters of chilled HEPES buffer (kept on ice) were added to the filter and centrifuged at 4 °C, 15,000 x *g* for 5 min. The flowthrough was discarded, and the buffer exchange process was repeated two more times. In a new microcentrifuge tube, the filter was inverted and centrifuged at 15,000 x *g* for 2 min to elute the protein. Protein concentration was measured by the Pierce BCA Protein Assay Kit (ThermoFisher) following the manufactures procedure. BSA serial dilutions were generated in HEPES buffer. A working protein stock was

prepared by diluting the protein in HEPES buffer to a final concentration of 0.50 μM without Triton X-100 supplementation.

Covalent inhibitors, NH125 (positive control; final concentration, 500 μM), or DMSO (negative control) were added (1.25 μL) to 23.75 μL of HK853 and incubated at room temperature for 30 min. The reactions were quenching with 8.6 μL of 6x Native-PAGE sample loading buffer (40 mM Tris, pH 7.5, 8% glycerol, and 0.08% bromophenol blue (w/v)). Samples (15 μL) were resolved on a 7.5% Native-PAGE gel (7.5% polyacrylamide Tris-glycine resolving gels) with 1x native running buffer (83 mM Tris, pH = 9.4, and 33 mM glycine) on ice, at 180 V for 1.5 h. Native gels were silver stained with a Pierce Silver Stain Kit (ThermoFisher) according to the manufacturer's procedures and scanned in the Silver Stain channel on a Typhoon gel scanner. Gel scans were loaded onto ImageJ and brightness adjusted uniformly. All aggregation experiments were performed in triplicate.

Whole Protein Mass Spectrometry.

HK853 (5 μM) in reaction buffer premixed with Triton X-100 (0.1% v/v) was incubated with covalent inhibitor ***2a*** at (5.5 μM, 4% DMSO) for 30 min unless otherwise specified. Reaction was considered quenched upon injection (8 μL) onto an Agilent 500 Ion Trap LC/MS with C3 column (Agilent, 0.3 x 100 mm, 3.5 mm, 300 SB) heated to 50 ˚C. Separation was performed with mobile phases A (100% water with 0.1% formic acid) and B (100% acetonitrile with 0.1% formic acid). Measurements in positive ion mode. Data were analyzed using the Agilent MassHunter Protein Deconvolution Software and extracted mass ion counts were plotted. Protein was prepared as previously described.[1]

Site of Labeling.

HK853 (10 μM, 95 μL of 10.4 μM in reaction buffer) was incubated with specified inhibitor or DMSO (5%) in 1x reaction buffer for 90 min. Samples were precipitated to remove detergent and excess probe (Proteoextract® Protein Precipitation kit, Sigma-Aldrich). Samples were re-suspended in 25 μL of solution containing 8 M urea, 0.5 M ammonium bicarbonate, pH 8.0 and 4 mM DTT in ultrapure water and briefly vortexed and centrifuged followed by incubation at 37 ºC for 45 min. Samples were cooled for 5 min at room temperature, then 25 μL of 20 mM

iodoacetamide in MS grade water was added. Samples were briefly vortexed and centrifuged then incubated at room temp for 30 min in the dark. MS grade water (50 µL) was added to dilute urea below concentrations that would inhibit trypsin. For specified samples, 1.6 µL of 0.5 mg/mL mass spec grade trypsin (Promega) or chymotrypsin (Promega) in 50 mM acetic acid were added and incubated for 10-16 h at 37 ºC in a water bath. After incubation, samples were frozen at -80 ºC, speed vacuumed to dryness. Dried samples were reconstituted with 13 µL of 5:95, ACN:H_2O, 0.1% TFA, vortexed for 45 s and centrifuged at 4000 x *g* for 2 min. If pH was >3, 0.3 µL of 10% TFA was added followed by vortexing and centrifuging as above. The pH was adjusted pH to ≤3. ZipTips (10 µL) were used for sample clean-up. Tips were hydrated with 10 µL of 80:20, ACN:H_2O, 0.1% TFA twice followed by of 2x 0.1% TFA in H_2O (10 µL). The sample (10 µL) was loaded slowly to avoid introducing air into the packing material and maximize peptide binding and was aspirated and drawn up again ~5x to ensure full loading. The tip was washed with 10 µL of 0.1% TFA in H_2O five times and the waste expelled. The peptides were eluted by aspiration and expulsion of 1.3 µL of 40:60, ACN: H_2O, 0.1% TFA three times with the epi tube on ice (use caution not to fully release the pipettor plunger). For MALDI-TOF analysis, 0.7 µL of matrix solution (saturated α-cyano-4-hydroxycinnamic acid CCA in H_2O) was mixed with 1 µL of sample and the sample spotted on a MTP 384 Target Plate (Bruker) and analyzed by MALDI-TOF (Applied Biosystems-Sciex 5800 MALDI-TOF/TOF Mass Spectrometer, reflectron positive mode, 1000 Hz, 2000 shots).

Standard Click Reaction.

Per 50 µL of alkyne-containing protein sample, the following was added in order: 0.5 µL of azide (5 mM in DMSO), 0.4 µL of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 50 mM in water), 0.2 µL tris((1-benzyl-4-triazolyl)methyl)amine (TBTA, 1 mM in DMSO), and 1.3 µL copper sulfate ($CuSO_4$, 50 mM in water) and briefly vortexed. Fluorescent click reaction mixtures were placed on a rotator in the dark for 30 min before quenching with 4x SDS loading buffer or precipitation (Proteoextract® Protein Precipitation kit, Sigma-Aldrich). Biotin click reaction mixtures were placed on a rotator for 2 h before precipitation (Proteoextract® Protein Precipitation kit, Sigma-Aldrich) to remove excess biotin.

Pure HK853 Pull-down.

HK853 (5 µM) was incubated in 750 µL Tris reaction buffer (0.1% triton) with 35 µM ***P-1*** or equivalent DMSO (3 µL) on a rotator for 1 h. To probe-treated samples, the following reagents were added in order: 24 µL of Dde-TAMRA-biotin-azide (CCT-1367-1); 5 mM in DMSO), 10 µL of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 100 mM in water), 10 µL tris((1-benzyl-4-triazolyl)methyl)amine (TBTA, 10 mM in DMSO), and 10 µL copper sulfate ($CuSO_4$, 10 mM in water), and briefly vortexed. The reaction mixtures were placed on a rotator in the dark for 1 h. To quench the click reaction and remove excess reagents, protein precipitation solution (Proteoextract® Protein Precipitation kit, Sigma-Aldrich) was added according to manufacture instructions and incubated overnight at -20 °C. Precipitated proteins were isolated and washed according to the manufacture instructions and resolubilized in 100 µL of 1xPBS with 0.1% SDS. Precipitated proteins were isolated and washed according to the manufacture instructions and resolubilized in 400 µL of 1xPBS with 0.1% SDS. Speedbead magnetic neutravidin coated particles (Cytiva, cat 78152104011150) were prepared according to manufacture instructions and resolubilized in 1x PBS to a final concentration of 5 mg/mL. Resolubilized proteins (100 µL) were split into 4-eppie tubes containing 100 µL of magnetic beads for a final bead concentration of 1mg/mL and incubated for 1 h in the dark, rotating at RT. The supernatant was removed, unbound proteins precipitated (as above) and saved for later analysis. Beads with bound proteins were washed with 1x PBS with 0.1% SDS at least 3 times. Beads were treated with 2% hydrazine (50 µL) and incubated for 1 h, rotating in the dark at RT. The supernatant was removed and bound proteins precipitated overnight at -20 °C (Proteoextract® Protein Precipitation kit, Sigma-Aldrich) to remove excess hydrazine. Protein pellets were resolubilized with 50 µL of 1X PBS with 0.1%SDS. Bound and unbound protein samples (7 µL) were combined with 3 µL of 4x SDS-PAGE loading buffer. Samples (8 µL) were loaded onto a 12% tris acrylamide gel and run at 180 V for 1 h in the dark on ice. Gel was removed from the cassette, washed 3x with MilliQ water, and scanned using the TAMRA channel on a Typhoon gel scanner. Gel was stained with Sypro Red Protein Gel Stain (45 min) according to manufacturer instruction, washed with 7.5% *(v/v)* acetic acid for 1 min and scanned using the Sypro Red channel on a Typhoon gel scanner.

Overexpressed HK853 in Lysate Pull-down with *P-1*.

Single colonies of *E. coli* expressing HK853 from LB-amp (100 µg/mL) agar plates were grown overnight in LB-amp (100 µg/mL) broth (Lennox – Sigma Aldrich; 5 mL at 37 ˚C and shaking at 220 RPM). Overnights were diluted 1:100 in fresh media(5mL) and grown to an OD_{600} of 0.6. Cultures were induced for 30 minutes with the addition of 1mM IPTG (5 µL). The culture was centrifuged to pellet (6000 x *g*), supernatant was removed, and cell pellets washed twice with 1 mL PBS. Cells were lysed in 1 mL of PBS with 1mM PMSF (8 cycles, 5 sec, 30 sec rest, 40% amp) with probe sonicator on ice. Protein concentration was adjusted to 5 mg/mL with chilled PBS and 200 µL were aliquoted into eppie tubes on ice. Lysates were treated with ***P-1*** (50 µM) or DMSO (0.8%) and incubated at room temp on a rotator for 1 h. For every 50 µL of alkyne-containing protein sample, the following were added in order: 0.5 µL of Dde-TAMRA-biotin-azide (CCT-1367-1); 5 mM in DMSO), 0.4 µL of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 50 mM in water), 0.2 µL tris((1-benzyl-4-triazolyl)methyl)amine (TBTA, 1 mM in DMSO), and 1.3 µL copper sulfate ($CuSO_4$, 50 mM in water), and briefly vortexed. The reaction mixtures were placed on a rotator in the dark for 1 h. To quench the click reaction and remove excess reagents, protein precipitation solution (Proteoextract® Protein Precipitation kit, Sigma-Aldrich) was added according to manufacture instructions and incubated overnight at -20 °C. Precipitated proteins were isolated and washed according to the manufacture instructions and resolubilized in 100 µL of 1xPBS with 0.1% SDS. Pierce Streptavidin Magnetic Beads (ThermoFisher, cat 88817) were prepared according to manufacture instructions and resolubilized in 1x PBS to a final concentration of 10 mg/mL. Magnetic beads (20 µL) were added to samples for a final concentration of 1 mg/mL and incubated for 1 h in the dark, rotating at RT. The supernatant was removed, unbound proteins precipitated and saved for later analysis. Beads with bound proteins were washed with 1x PBS with 0.1% SDS at least 3 times. Beads were resolubilized in 100 µL of 1xPBS with 0.1% SDS and treated with 2% hydrazine (50 µL) and incubated for 1 h, rotating in the dark at RT. The supernatant was removed and bound proteins precipitated overnight at -20 °C (Proteoextract® Protein Precipitation kit, Sigma-Aldrich) to remove excess hydrazine. Protein pellets were resolubilized with 1X SDS-PAGE loading buffer (50 µL). Samples (10 µL) were loaded onto a 12% tris acrylamide gel and run at 180 V for 1 h in the dark on ice. Gel was removed from the cassette, washed 3x with MilliQ water, and scanned using the TAMRA channel on a Typhoon gel scanner. Gel was stained with Sypro Red Protein Gel Stain (45 min) according to manufacturer

instruction, washed with 7.5% *(v/v)* acetic acid for 1 min and scanned using the Sypro Red channel on a Typhoon gel scanner.

Growth Curves.

Salmonella enterica was struck onto LB agar plates. Primary plates were incubated at 37 °C overnight. Overnight liquid cultures were generated the following day by inoculating a single colony into 1 mL of LB media and incubated at 37 °C with shaking (220 rpm), overnight (~16 h). On the day of the experiment, 2.5 mM inhibitor stocks were generated in DMSO. Eighty microliters of LB medium supplemented with 4% (*v/v*) DMSO were added to a 96-well plate (Corning Incorporated). Covalent inhibitors or LB media (20 µL) were dispensed into wells and mixed by pipetting (total volume 100 µL). Cells were normalized by diluting overnight cultures into fresh LB media (without DMSO) to an OD_{600} of 0.1. One hundred microliters of culture were added to wells containing covalent inhibitors (final concentration, 250 µM) or LB media supplemented with DMSO (final concentration 2.5%) for a final volume of 200 µL. Plate edges were sealed with tape to minimize media evaporation. The tape was punctured with a sterile needle to ensure aerobic conditions throughout the experiment. OD_{600} measurements were taken (in 10-min intervals) on a Tecan Spark Plate Reader with continuous orbital shaking (5 ms) at 37 °C for ~24 h. All measurements were performed in biological triplicate, and the specific growth rate (µ) was calculated for the average of each sample by the following equation:

$$\mu_{max} = \frac{[(log_{10}N - log_{10}N_0) \times 2.303]}{t - t_0}$$

Where N is number of cells at any time (t) and the starting number of cells (N_0) occurs at the beginning timepoint (t_0). Total growth was assessed using GraphPad Prism version 9.5.0 for windows by quantifying the area under curve (AUC) for each condition. Brown-Forsythe one-way ANOVA followed by Dunnett's multiple comparisons test were performed for both specific growth rates and AUC's using GraphPad Prism version 9.5.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com), and compared to the DMSO ($\alpha = 0.05$).

Minimum Inhibitory Concentration Assay.

Salmonella enterica was struck onto LB agar plates. Primary plates were incubated at 37 °C overnight. Overnight liquid cultures were generated the following day by inoculating a single colony into 1 mL of LB media and incubated at 37 °C with shaking (220 rpm), overnight (~16 h).

On the day of the experiment, 4x working stocks (800 µL) of colistin or inhibitors were generated in sterile MilliQ water or LB media supplemented with 4% (*v/v*) DMSO, respectively. One hundred microliters 4x treatment stocks or LB with DMSO (final concentration 2.5%) were added to column 1 wells of a 96-well plate (Corning Incorporated). One hundred microliters of LB media were dispensed into all remaining wells. Column 12 served as a growth control. Treatments were serially diluted by transferring 100 µL from column 1 and mixed with media in column 2. Discarding and changing pipette tips between each dilution. The dilution process was repeated to column 11 and the remaining 100 µL of media containing treatment were discarded. One hundred microliters of normalized cells were added to all wells containing treatments or DMSO and mixed by pipetting for a final volume of 200 µL. Plate edges were sealed with tape to minimize media evaporation. The tape was punctured with a razor blade to ensure aerobic conditions throughout the experiment and incubated at 37 °C for ~16 hrs. After incubation, MIC values were determined visually and recorded as the lowest inhibitor concentration with minimal growth of bacteria in the well. The reported MIC value is the consensus of the replicates, not the average. All covalent inhibitors were present at a 250 µM final concentration. All minimum inhibitory concentration assays were performed in six biological replicates on different days.

Checkerboard Assay.

Salmonella enterica was struck onto LB agar plates. Primary plates were incubated at 37 °C overnight. Overnight liquid cultures were generated the following day by inoculating a single colony into 1 mL of LB media and incubated at 37 °C with shaking (220 rpm), overnight (~16H). On the day of the experiment, 8x and 4x working stocks (0.300 mL and 1.12 mL, respectively) of colistin were generated in sterile MilliQ water. Similarly, 4x working stocks (1.12 mL) of inhibitors were generated in LB media supplemented with 4% (*v/v*) DMSO. One hundred microliters LB media were added to all wells of a 96-well plate (Corning Incorporated). One 96 well plate per replicate of colistin and inhibitor combination is needed. One hundred microliters of 4x colistin working stock were dispensed into wells A 1 to 11. One-hundred microliters of 8x colistin working stock were added to well A 12. Colistin was diluted with a multi-channel pipette by transferring 100 µL from wells A 1-12 to G 1-12, discarding the 100 µL after row G. Column 1 to 11 wells served as internal colistin MIC control. To the wells of column 12, 100 µL from the 4x covalent inhibitor working stock were added and serially diluted by transferring 100 µL to wells

of column 11. Dilution was repeated until column 2 and the media was discarded. Row H 2 to 12 wells served as the internal MIC control for the inhibitor and well H1 served as an internal DMSO growth control. *S. enterica* strains of interest were diluted from overnight cultures 1:100 into fresh LB media. One hundred microliters of diluted cells were added to all wells containing treatments or DMSO and mixed by pipetting for a final volume of 200 µL. Plate edges were sealed with tape to minimize media evaporation. The tape was punctured with a razor blade to ensure aerobic conditions throughout the experiment and incubated at 37 °C for ~16 h. After incubation, decreases in MIC values were determined visually and recorded as the concentration combination of colistin and inhibitor of the corresponding well, with minimal bacterial growth. The reported FIC value was calculated by using the following equation:

$$FIC\ Index = FIC_A + FIC_B = \frac{A}{MIC_A} + \frac{B}{MIC_B}$$

All checkerboard assays were performed in biological triplicate on different days.

Modified Checkerboard Assay.

Salmonella enterica was struck onto LB agar plates. Primary plates were incubated at 37 °C overnight. Overnight liquid cultures were generated the following day by inoculating a single colony into 1 mL of LB media and incubated at 37 °C with shaking (220 rpm), overnight (~16 h). On the day of the experiment, 8x and 4x working stocks (0.300 mL and 1.12 mL, respectively) of colistin were generated in sterile MilliQ water. Similarly, 4x working stocks (1.12 mL) of inhibitors were generated in LB media supplemented with 4% (*v/v*) DMSO. To generate colistin internal MIC control, 100 µL of LB media were added to all wells in columns 1 and 2 of a 96-well plate (Corning Incorporated). One hundred microliters of 4x colistin working stock were dispensed into wells H 1 - 2 and diluted two-fold with a multichannel pipette to wells A 1 – 2 and mixed thoroughly between transfers. Residual media (100 µL) was discarded after wells A 1 - 2. To generate wells with colistin and inhibitors combinations, 50 µL of LB media were added to the remaining wells (columns 3 – 12). From here, 50 µL of 8x colistin working stock were dispensed into wells H 3-10. Colistin was serially diluted 2-fold with a multi-channel pipette by transferring 50 µL from wells H 3-10 to A 3-10 with mixing. Residual media (50 µL) was discarded after row A. From 4x covalent inhibitor working stocks, 50 µL were added to all wells (H to A 3-10) and mixed with a pipette discarding tips in between the addition of inhibitors. *S. enterica* strains of interest were diluted from overnight cultures 1:100 into fresh LB media. One hundred microliters

of diluted cells were added to all wells containing treatments or DMSO and mixed by pipetting for a final volume of 200 µL. Plate edges were sealed with tape to minimize media evaporation. The tape was punctured with a razor blade to ensure aerobic conditions throughout the experiment and incubated at 37 °C for ~16 h. After incubation, decreases in MIC values were determined visually and recorded as a fold change relative to the internal MIC colistin control. Columns 11 and 12 were designated for internal MIC controls for inhibitors and DMSO growth controls. Inhibitors were present at a final concentration of 250 µM. All checkerboard assays were performed in biological and technical triplicate on different days.

Swarming Assay with Modified Fastidious Anaerobe Broth (FAB) Media.

Fastidious Anaerobe Broth media agar plates were made fresh the day of the experiment. Note: plates should be used within 24 h for consistent results. To generate solution A, the following reagents were combined in 200 mL of MilliQ H_2O, 1.8 g sodium phosphate dibasic heptahydrate (Na_2HPO_4 x $7H_2O$ -Sigma Aldrich), 600 mg of potassium phosphate monobasic (KH_2PO_4 -Fischer), 600 mg sodium chloride (NaCl -Fischer), 900 mg Bacto agar (BD), and 200 mg Casamino acids (BD). The solution was autoclaved at 121 °C for 22 min and allowed to cool to 60 °C.

Trace metal solution was generated by combining the following reagents in sterile MilliQ H_2O, calcium sulfate dihydrate (1 g/L – Sigma Aldrich), ferrous sulfate heptahydrate (1 g/L – Sigma Aldrich), copper sulfate heptahydrate (100 mg/L – Sigma Aldrich), manganese sulfate hydrate (100 mg/L – Sigma Aldrich), zinc sulfate heptahydrate (100 mg/L – Sigma Aldrich), cobalt sulfate pentahydrate (42.5 mg/L – Sigma Aldrich), sodium molybdate dihydrate (50 mg/L – Sigma Aldrich), and boronic acid (25 mg/L – Sigma Aldrich).

To cooled solution A, the following sterile filtered heat sensitive reagents were added, 200 µL (198 g/L) of magnesium chloride heptahydrate ($MgCl_2$ x $7H_2O$ -Fischer), 200 µL (10.5 g/L) of calcium chloride ($CaCl_2$ -Fischer), 2 mL (216 mg/ml) of glucose (Sigma Aldrich), and 40 µL of a trace metal solution. Inhibitors (100 µM final) were added from 25 mM DMSO stocks (0.4% DMSO concentration) to the media and mixed before pouring 20 mL of agar into sterile petri dishes (100 x 15 mm). Plates were dried in laminar flow hood under UV for 45 minutes.

Single colonies of *P. aeruginosa* (PA14) obtained from primary LB agar plates were grown overnight in LB media (Lennox – Sigma Aldrich) to an OD_{600} of 1.2. Cells were pipetted (5 µL) onto the center of freshly made modified FAB agar plates. Spotted culture plates were capped and

allowed to dry in standing incubator at 30 °C for 20 min upright before flipping. Plates were incubated 24 h before imaging on Typhoon FLA 9500 scanner (GE healthcare) using the DY-520XL filter setting. Images were processed and analyzed using ImageJ (NIH) with the oval measurement tool. The area was measured in quadruplicate from furthest points of tendrils. Measurements were averaged and taken as a percentage of the DMSO vehicle control. All experiments were carried out in biological duplicate due to limitations of covalent inhibitor material. Data were compared using a Brown-Forsythe One-way ANOVA followed by Dunnett's multiple comparisons test, which was performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

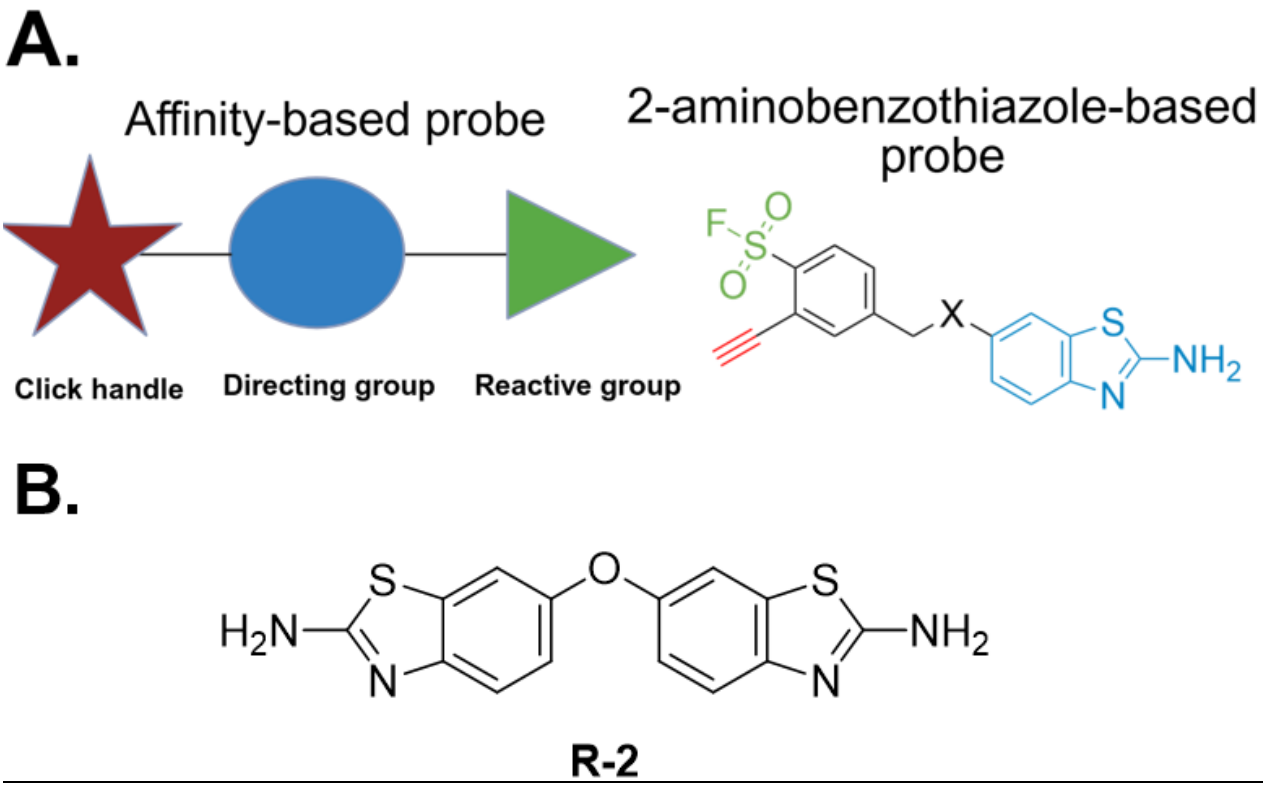


Figure S1. Affinity-based Probe Design and Non-covalent Inhibitor, R-2. A. Affinity-based probes are tripartite structures comprised of a directing group (blue components) for binding to the CA-domain, a reactive group (green components) for covalent modification of amino acid near the binding site, and a bioorthogonal click handle (red components) enabling attachment of fluorophores or affinity tags (e.g., biotin) via copper-mediated azide-alkyne cycloaddition. **B**. The non-covalent inhibitor Rilu-2 (***R-2***) identified through previous high throughput screening, inhibits HK activity *in vitro* and mitigates virulence mechanisms in multiple pathogenic organisms.[1, 3-6]

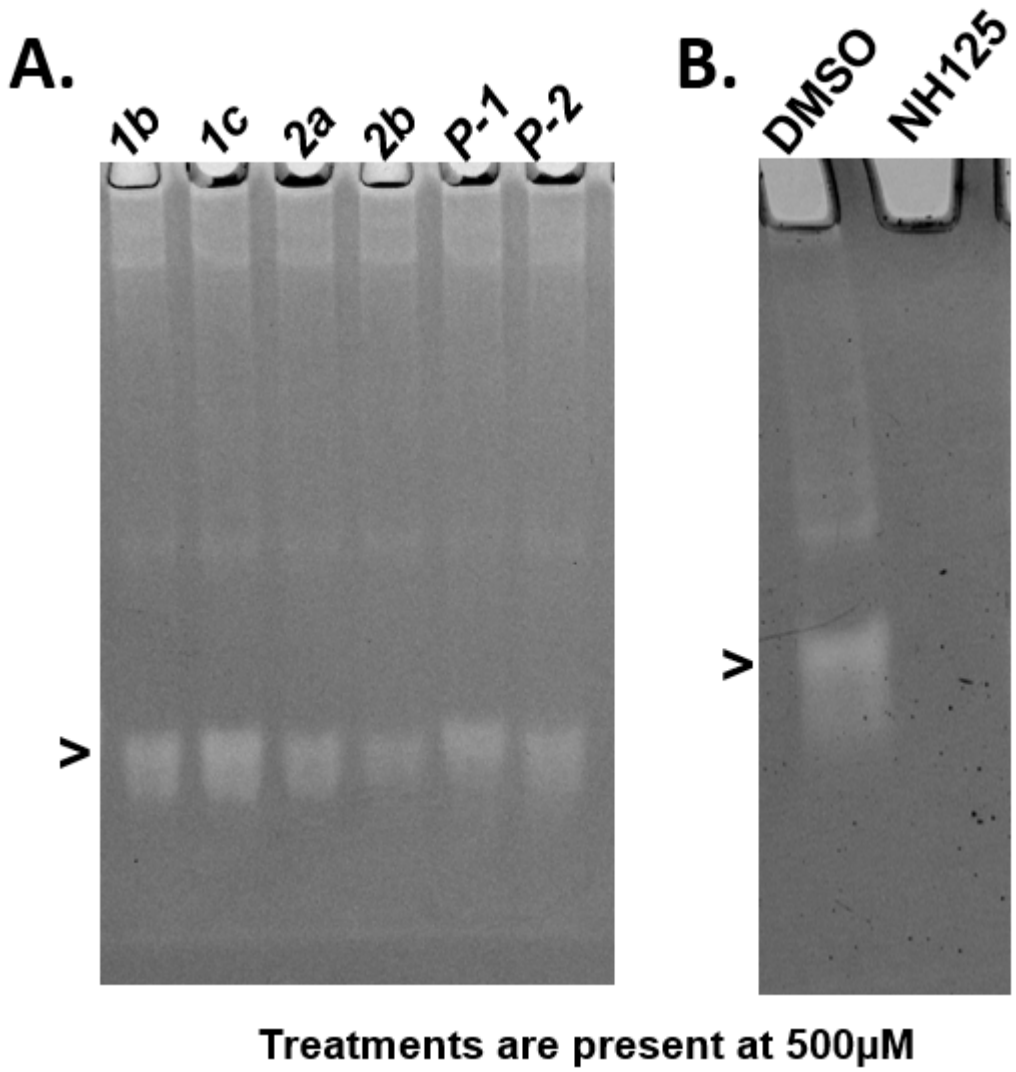


Figure S2. Representative Native-PAGE Gels from *In vitro* Aggregation Assay. HK853 (0.44 µM) was incubated with 0.4% (v/v) DMSO, NH125, or covalent inhibitors (500 µM) for 30 minutes. Reactions were quenched with 6× loading dye and analyzed by non-denaturing native PAGE, followed by silver staining. **Gel A** shows samples treated with covalent inhibitors, while **Gel B** includes DMSO and NH125 controls. Silver staining reveals loss of the HK853 dimer band, indicative of protein aggregation, preventing the protein from migrating into the gel. NH125 was included as a positive control, as it inhibits HK activity by inducing aggregation. None of the test compounds showed notable aggregation effects. Black arrow indicates migration of HK853 in gels.

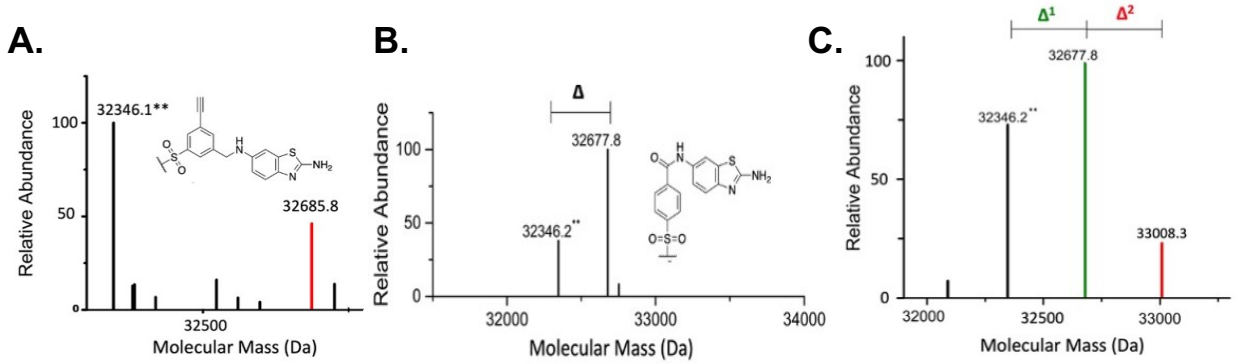


Figure S3. Whole Protein Mass Spectrometry. A. Pure HK853 (5 μM, **expected mass = 32348.61 Da, we measured a species with methionine loss and oxidation, resulting the observed mass) incubated with 1.1 equiv of ***P-1*** in DMSO (4 %) for 30 min. Only one addition of ***P-1*** observed (expected mass for single addition = 341.0 Da ±6.5 Da, observed Δ = 341.7 Da). **B**. Pure HK853 (5 μM) incubated with 1.1 equiv of ***2a*** in DMSO (4%) for 30 min. Unlabeled HK853 **expected mass = 32348.61 Da, we measured a species with methionine loss and oxidation, resulting the observed mass).[7] Only one addition of ***2a*** observed (expected mass for single addition = 332.0 Da (±6.5 Da), observed Δ = 331.6 Da). **C**. Pure HK853 (5 μM) incubated with 3.0 equiv of ***2a*** in DMSO (4 %) for 30 min. Second addition of ***2a*** observed at this higher concentration (expected mass for single addition = 332.0 Da (±6.5 Da), observed Δ = 331.6 Da. Expected mass for double addition = 332.0 Da (±6.6 Da), observed Δ = 662.1 Da). Samples measured in positive ion mode on Agilent 500 Ion Trap LC/MS, data analyzed using the Agilent MassHunter Protein Deconvolution Software and extracted mass ion counts plotted.

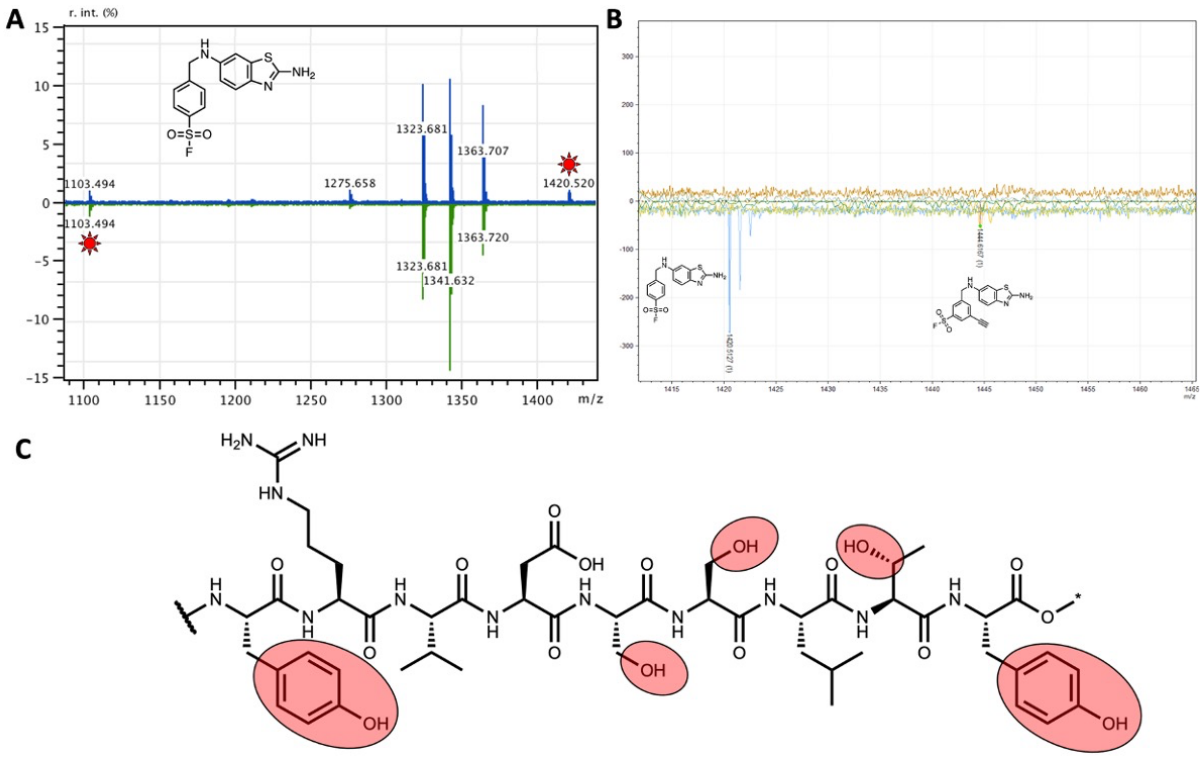


Figure S4. Chymotrypsin Digest Identified Probe Modified Peptide Using MALDI-TOF. A. HK853 (10 µM) incubated with ***1c*** (green, 1.1 equiv) or DMSO (blue, 5%) for 2 h then digested with chymotrypsin. New peak identified in treated sample only, as well as the predicted unmodified peptide peak are noted with a star. Single modification found on one peptide (***1c*** expected mass change: 317.03 ± 0.10 Da, observed mass change: 317.03, predicted peptide sequence: f.YRVDSSLTY.e, possible modified residues: Y, S, T). **B**. Zoom in of spectra of newly detected peptide mass region of HK853 (10 µM) incubated with ***1c*** (blue, 1.1 equiv), ***P-1*** (green, 1.1 equiv), or DMSO (orange, 5%) for 2 h then digested with chymotrypsin. Single modification found on one peptide (***P-1*** expected mass change: 341.03 ± 0.10 Da, observed mass change: 341.11, predicted peptide sequence: f.YRVDSSLTY.e, possible modified residues: Y, S, T). Samples were analyzed with MALDI TOF (Applied Biosystems-Sciex 5800 MALDI-TOF/TOF Mass Spectrometer) and data analysis done using mMass. **C**. Predicted peptide sequence modified by ***1c*** and ***P-1***. Potential residues that are predicted to react with aryl sulfonyl fluorides are highlighted in red.

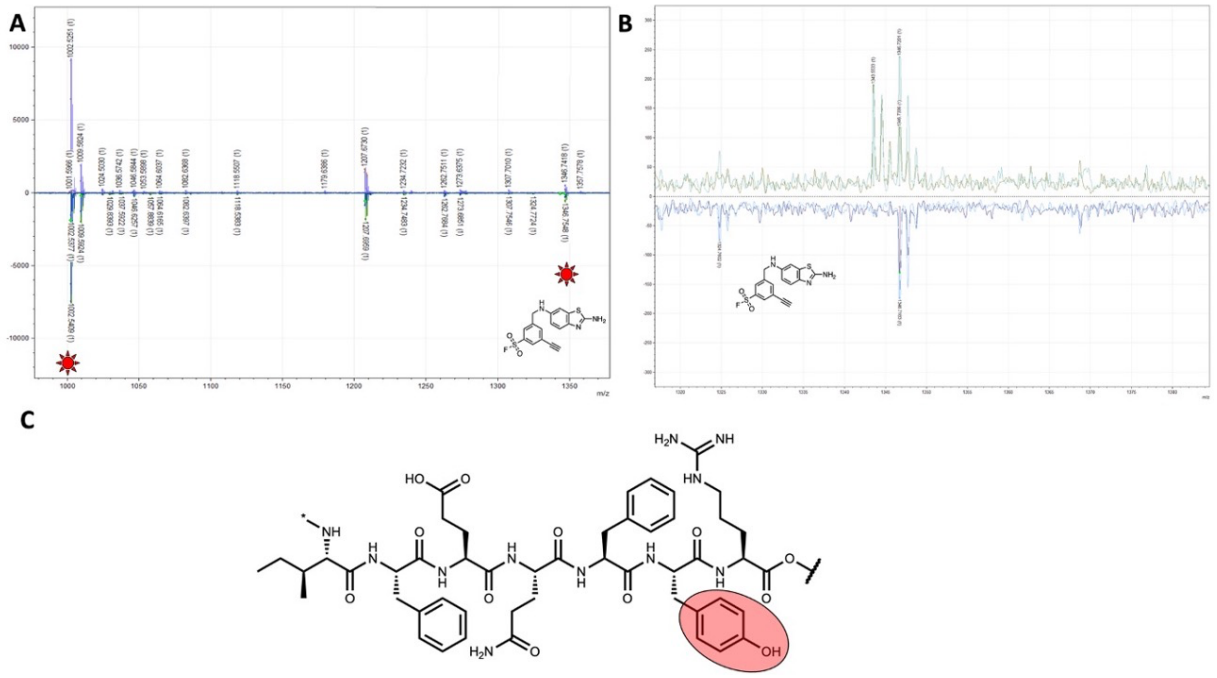


Figure S5. Trypsin Digest Identified Probe Modified Peptide Using MALDI-TOF. A. HK853 (10 μM) incubated with ***P-1*** (green, 1.1 equiv) or DMSO (blue, 5%) for 2 h then digested with trypsin. New peak identified in treated sample, as well as the predicted unmodified peak noted with a star. Single modification found on one peptide (expected mass change: 341.03 ± 0.10 Da, observed mass change: 341.01, predicted peptide sequence: r.IFEQFYR.v, possible modified residues: Y). **B**. Zoom in of spectra of new peptide mass region. Samples were analyzed with MALDI TOF (Applied Biosystems-Sciex 5800 MALDI-TOF/TOF Mass Spectrometer) and data analysis performed using mMass. **C**. Predicted peptide sequence modified by ***P-1***. Residue predicted to react with aryl sulfonyl fluoride is highlighted in red.

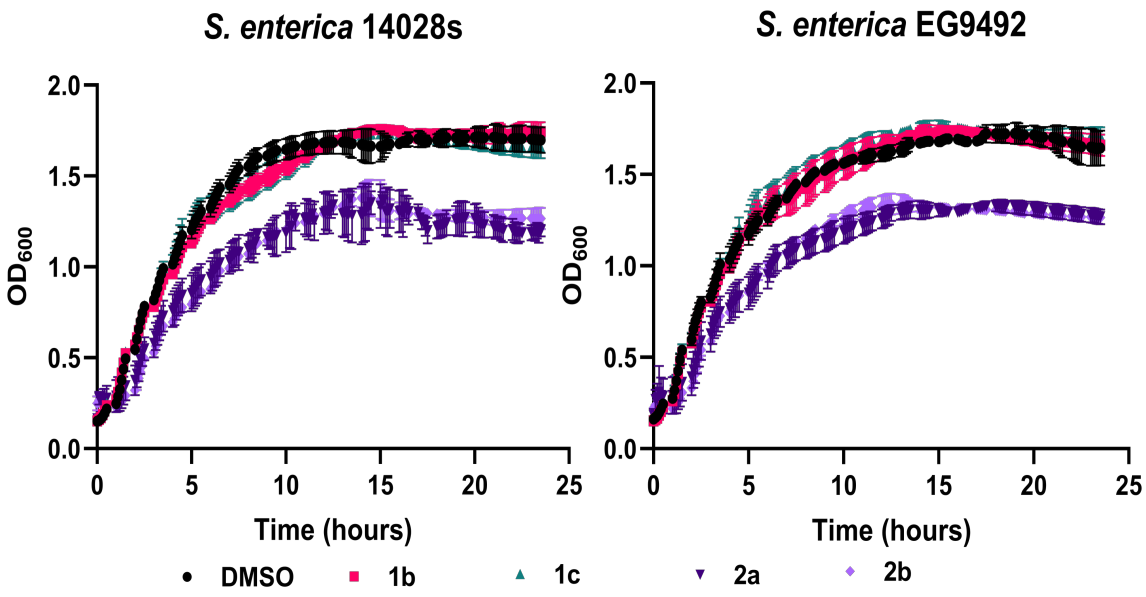


Figure S6. Impacts of Covalent Inhibition on *S. enterica* Growth. *S. enterica* WT and polymyxin-resistant cell growth in the presence of covalent inhibitors (250 μM) or DMSO (2.5% v/v) was monitored for 24 h. Data is plotted as mean ± standard error of three independent experiments.

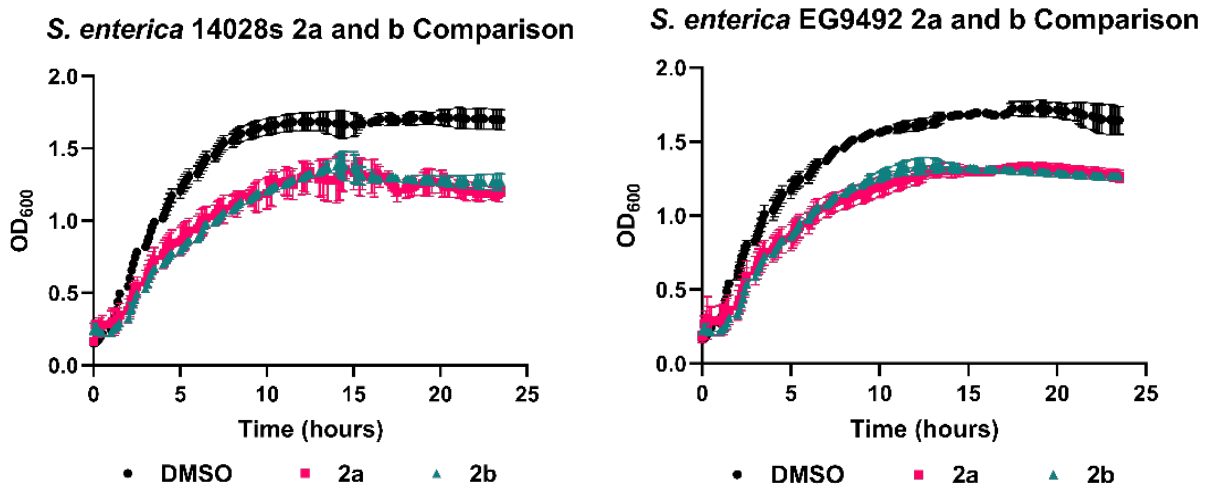


Figure S7. Impacts of Covalent Inhibitors 2a and 2b on *S. enterica* Growth. *S. enterica* WT and polymyxin-resistant cell growth in the presence of covalent inhibitors (250 μM) or DMSO (2.5% v/v) was monitored for 24 h. Growth curves for DMSO control vs. covalent inhibitors ***2a*** and ***2b*** were plotted individually to show differences in optical density measurements over the course of 24 h. Data is plotted as mean ± standard error of three independent experiments.

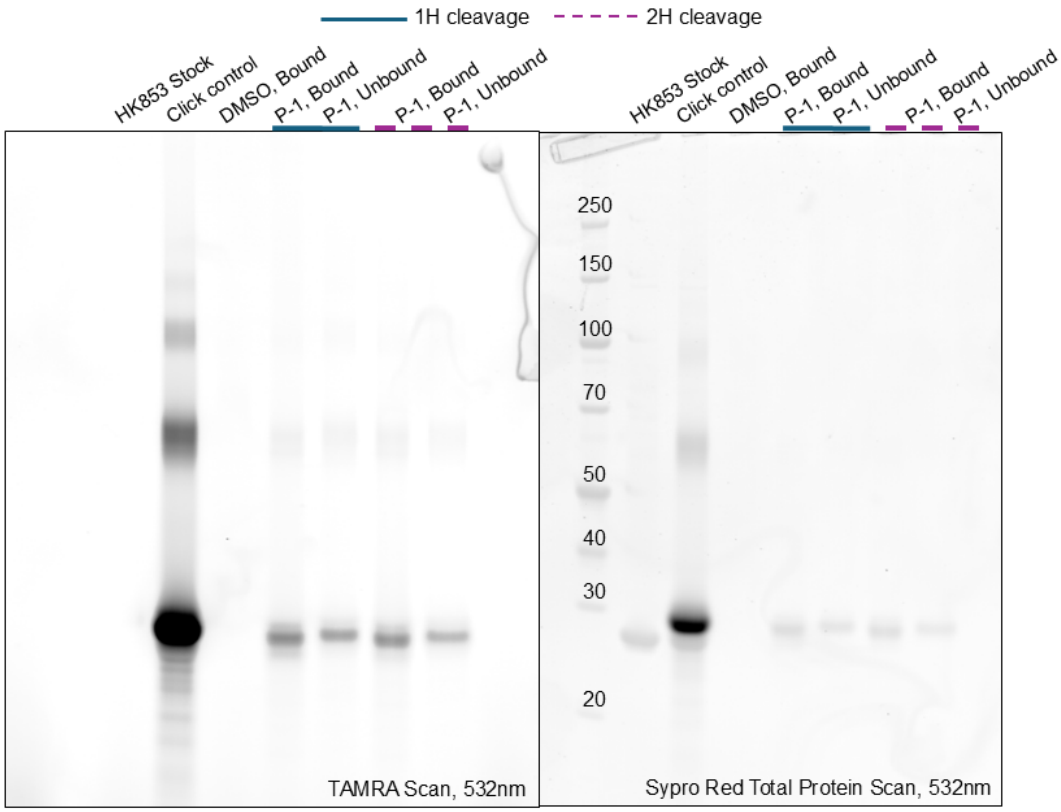


Figure S8. Purified HK853 Pull-down with *P-1*. Purified HK853 was incubated with ***P-1***, or DMSO for 1 h. Following incubation, DDE-TAMRA-Biotin-Azide was reacted with the alkyne handle of ***P-1*** for 2 h. Samples were then incubated with NeutrAvidin beads for 1 h, after which unbound proteins were removed and beads were washed prior to cleavage with 2% hydrazine for 1 h or 2 h, as indicated. DMSO-treated samples show no signal in the TAMRA or total protein scans indicating that ***P-1*** is necessary for protein enrichment. In ***P-1*** treated samples, signal detected as TAMRA fluorescence is observed in both bound and unbound fractions, indicating that a portion of ***P-1***–labeled HK853 remains in solution following bead incubation. Gels were scanned on Typhoon FLA 9500 scanner (GE healthcare) on TAMRA or Sypro Red filter settings. Gels were adjusted for contrast in ImageJ.

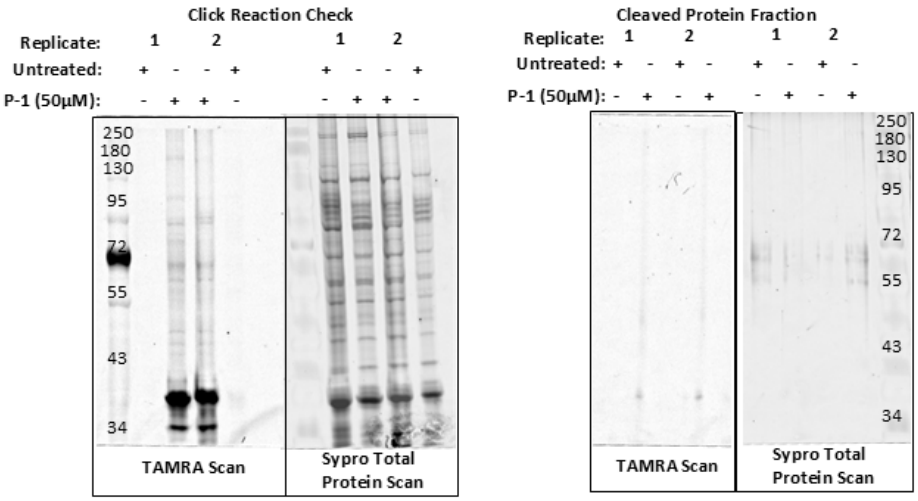


Figure S9. Overexpressed HK853 in Lysate Pull-down with *P-1*. A. *E.coli* that can overexpress HK853 was induced for 1 h with IPTG. Cells were lysed, protein normalized and samples were incubated with ***P-1*** for 1 h. Dde-TAMRA-biotin azide was clicked to the alkyne handle for 2 h, followed by protein precipitation. TAMRA fluorescence is observed exclusively in ***P-1***–treated samples, indicating successful alkyne–azide conjugation, while no fluorescence is detected in DMSO-treated controls. **B**. ***P-1***–labeled lysates were incubated with streptavidin magnetic beads for 1 h. Unbound proteins were removed and bead–bound proteins were released with 2% hydrazine. Only low levels of fluorescence are detected in both the TAMRA and Sypro Red total protein scans, consistent with limited recovery of labeled proteins following bead enrichment from the lysate. Gels were scanned on Typhoon FLA 9500 scanner (GE healthcare) on TAMRA or Sypro Red filter settings. Gels were adjusted for contrast in ImageJ.

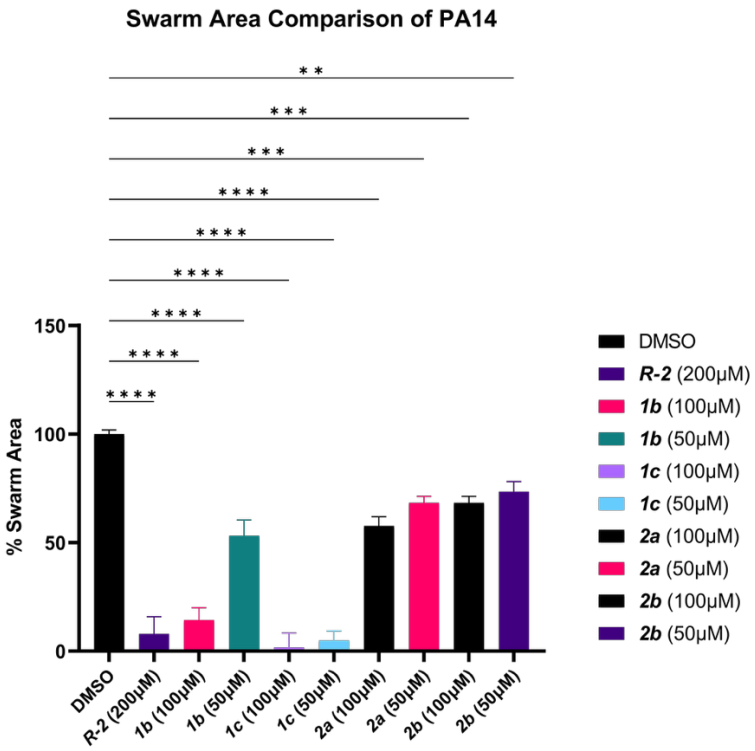


Figure S10. Quantified Swarming Motility Inhibition in *Pseudomonas aeruginosa* PA14. Covalent inhibitors at 50 and 100 μM, ***R-2*** at 200 μM or 0.4% DMSO were incorporated into modified FAB media plates to which PA14 cells from a normalized culture were inoculated and grown for 24 h. Plates were imaged after 24 h and motility diameter was measured using ImageJ with the freehand selection tool. One-Way ANOVA was completed with GraphPad Prism version 9.5.0 comparing each inhibitor treatment to the DMSO control. Statistically significant differences are denoted as follows: *p-value* ≤ 0.01 **, ≤ 0.001 ***, and ≤ 0.0001 ****.

Compound Characterization Data

Figure S11. Characterization data for **1a** (sultam derivative):

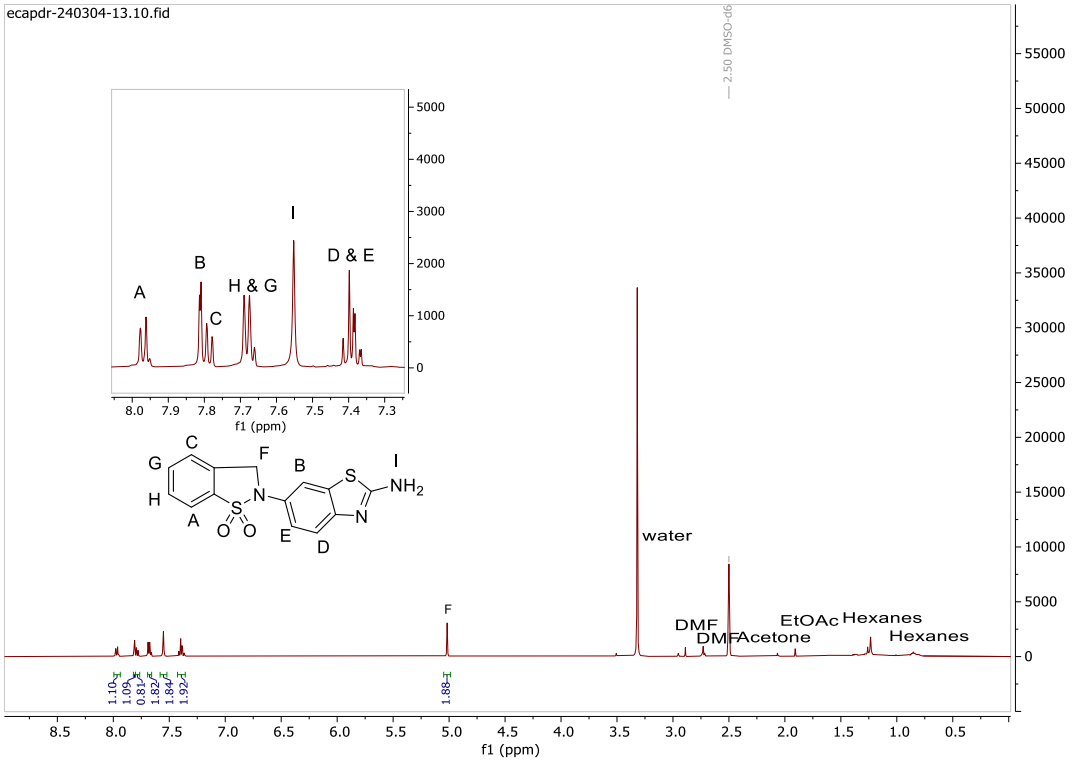
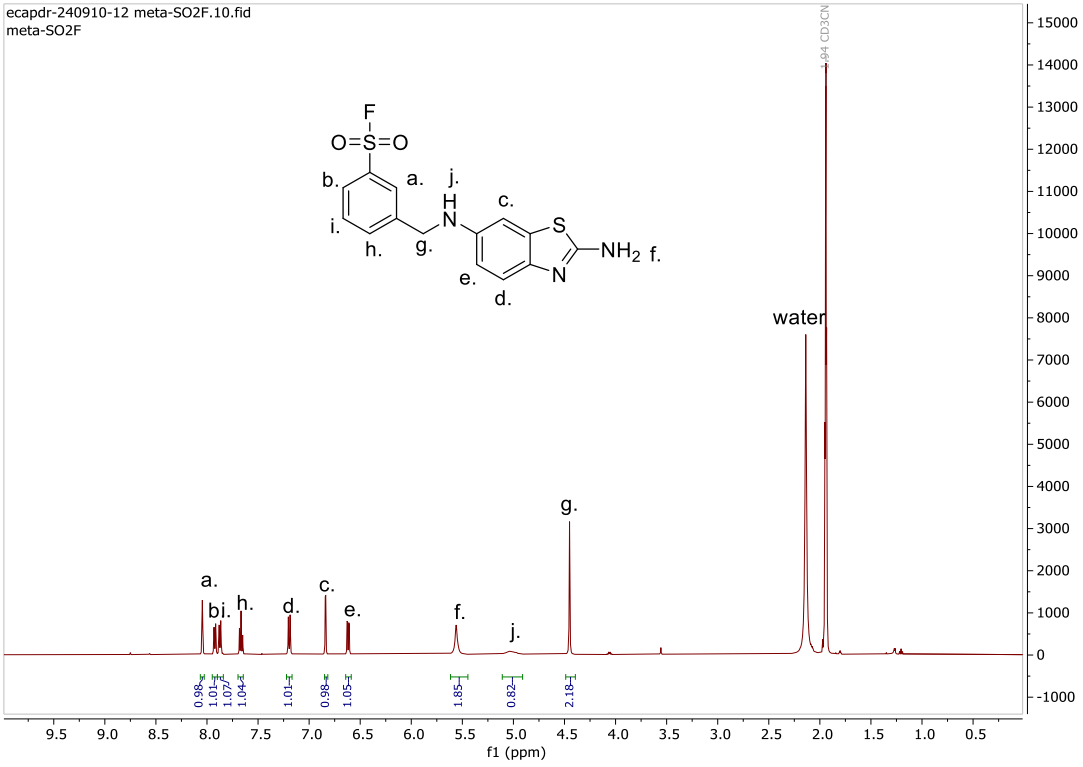
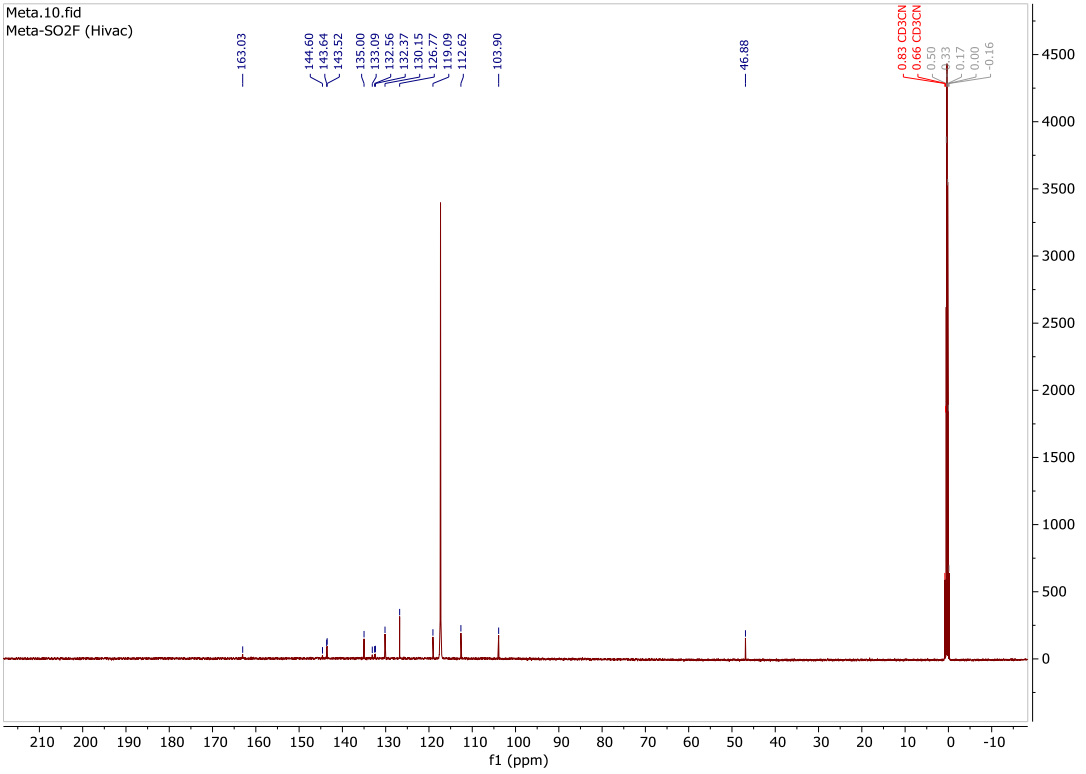


Figure S12. Characterization data for **1b**:


ecapdr-240910-12 meta-SO2F.10.fid
meta-SO2F
F
O=S=O
b.
a.
j.
H
N
c.
i.
h.
g.
S
NH2 f.
N
e.
d.
water
2.94 CD3CN
a.
b.
i.
h.
d.
c.
e.
f.
j.
g.
0.98
1.01
1.07
1.04
1.01
0.98
1.05
1.85
0.82
2.18
f1 (ppm)


Meta.10.fid
Meta-SO2F (Hivac)
163.03
144.60
143.64
143.52
135.00
133.09
132.56
132.37
130.15
126.77
119.09
112.62
103.90
46.88
0.83 CD3CN
0.66 CD3CN
0.50
0.33
0.17
0.00
-0.16
f1 (ppm)

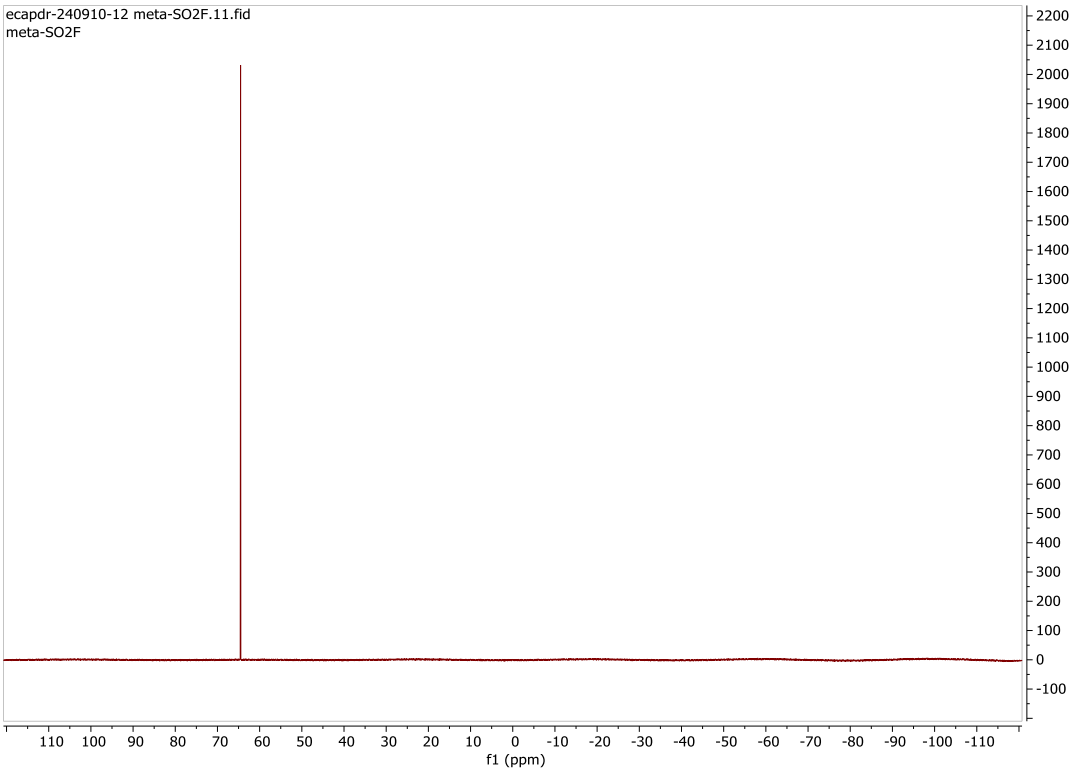
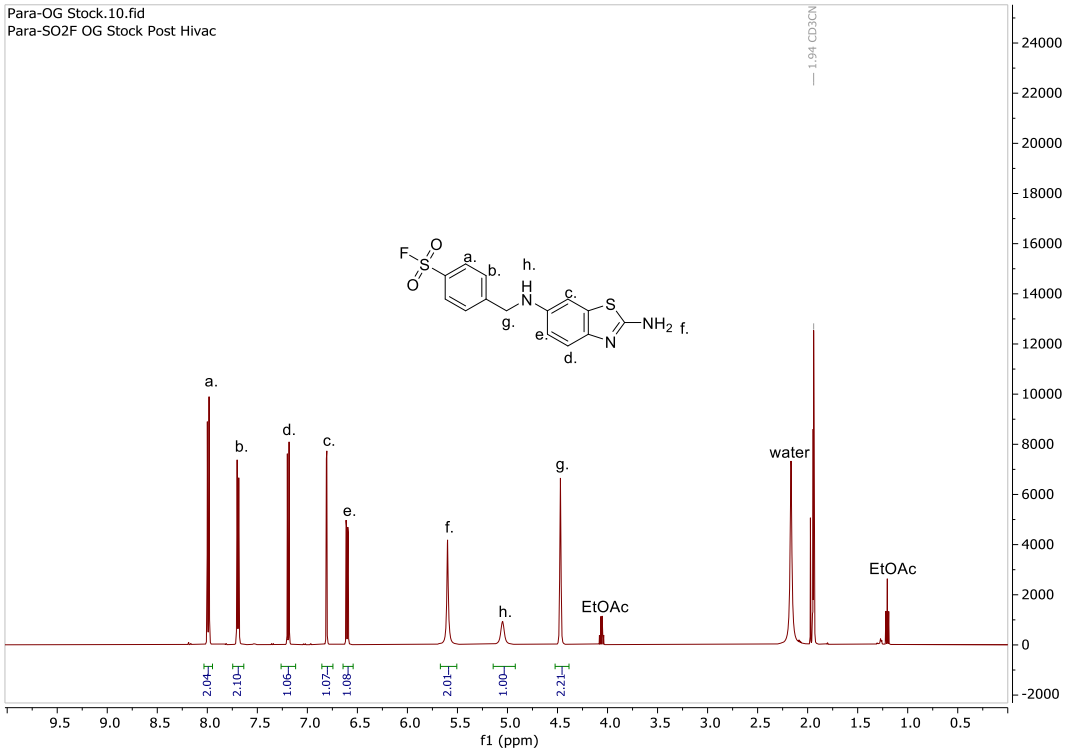


Figure S13. Characterization data for **1c**:



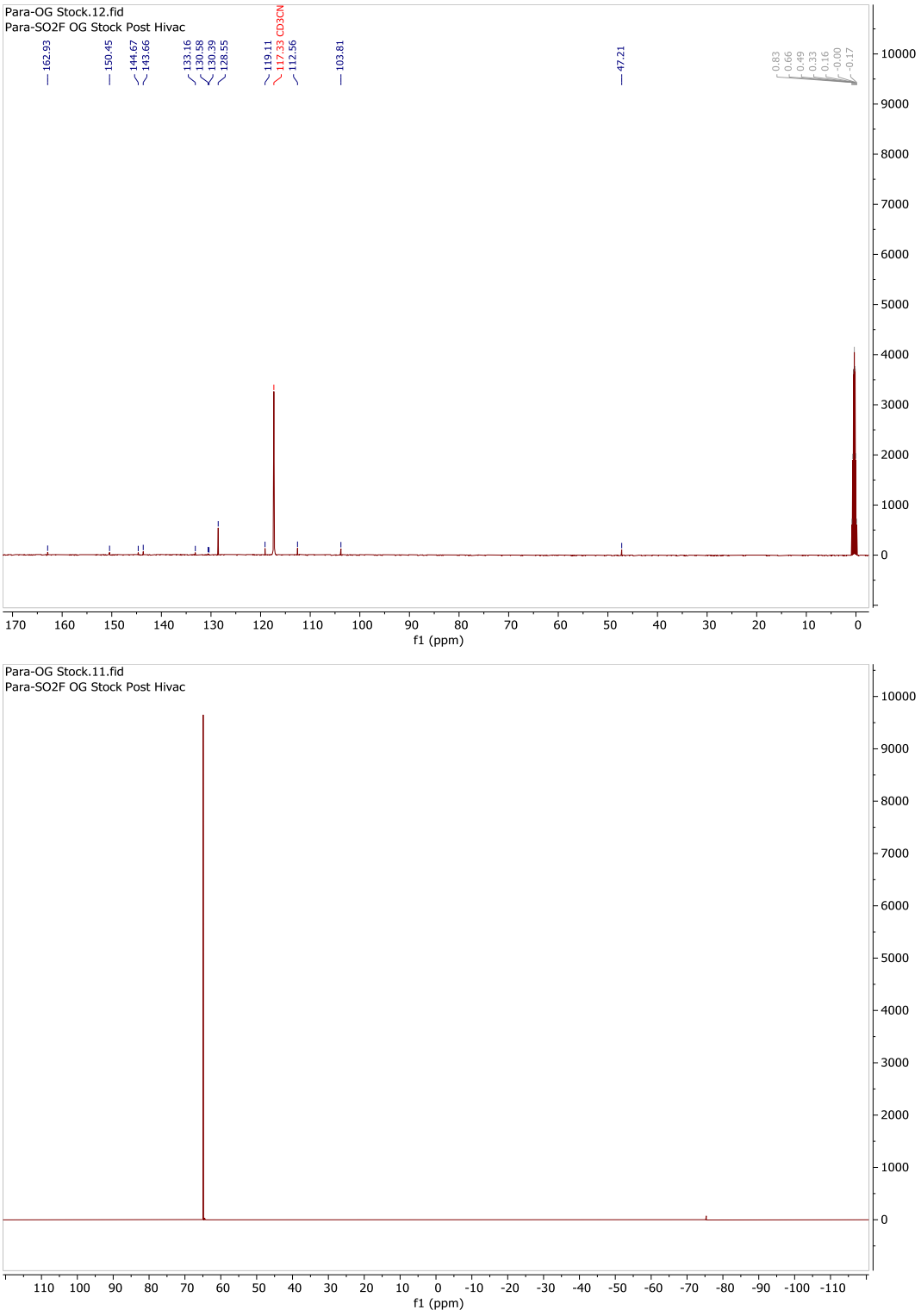
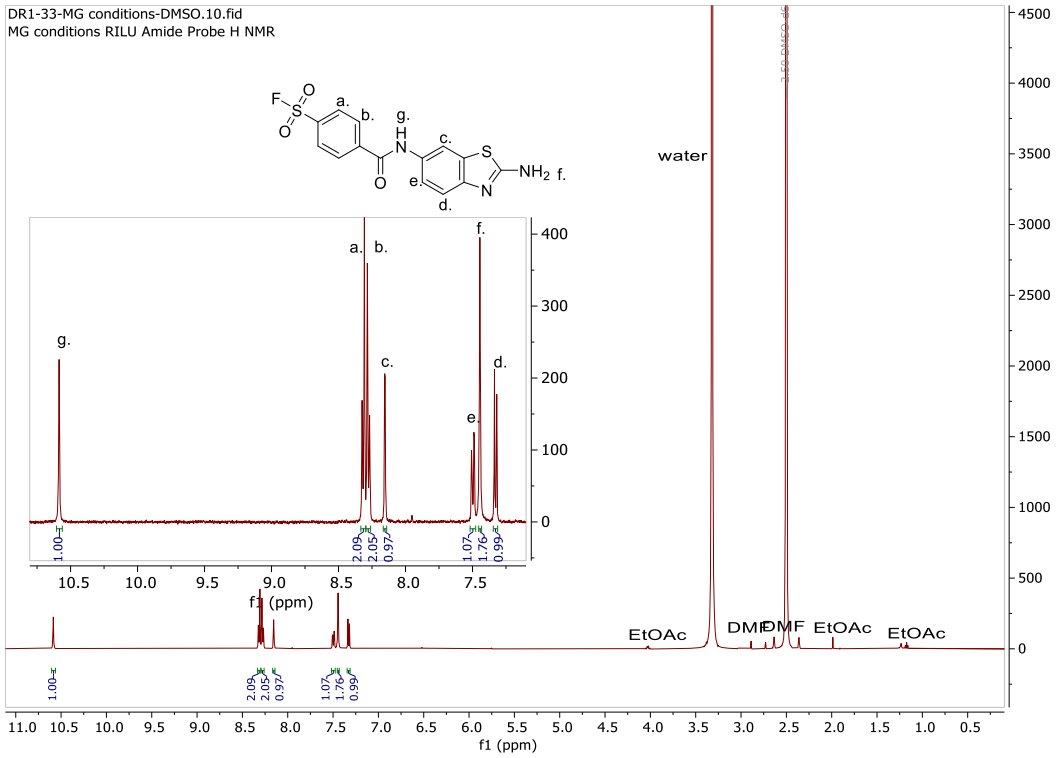
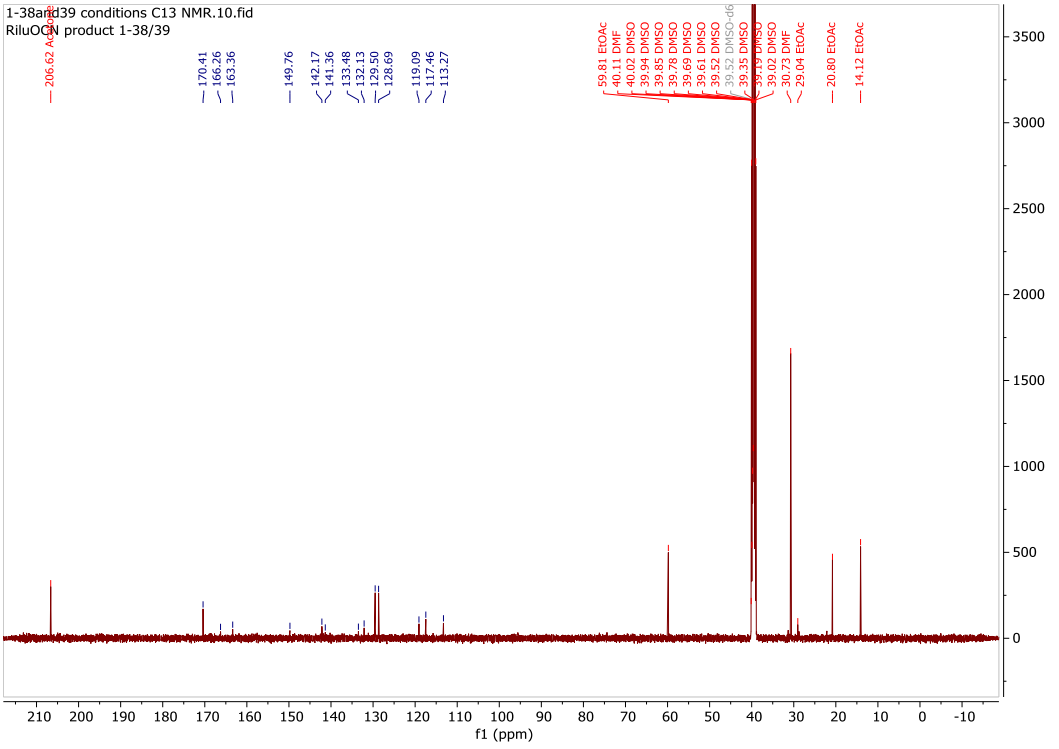


Figure S14. Characterization data for **2a**:


DR1-33-MG conditions-DMSO.10.fid
MG conditions RILU Amide Probe H NMR
water
EtOAc
DMF
DMF
EtOAc
EtOAc
f1 (ppm)


1-38and39 conditions C13 NMR.10.fid
RiluOCN product 1-38/39
f1 (ppm)

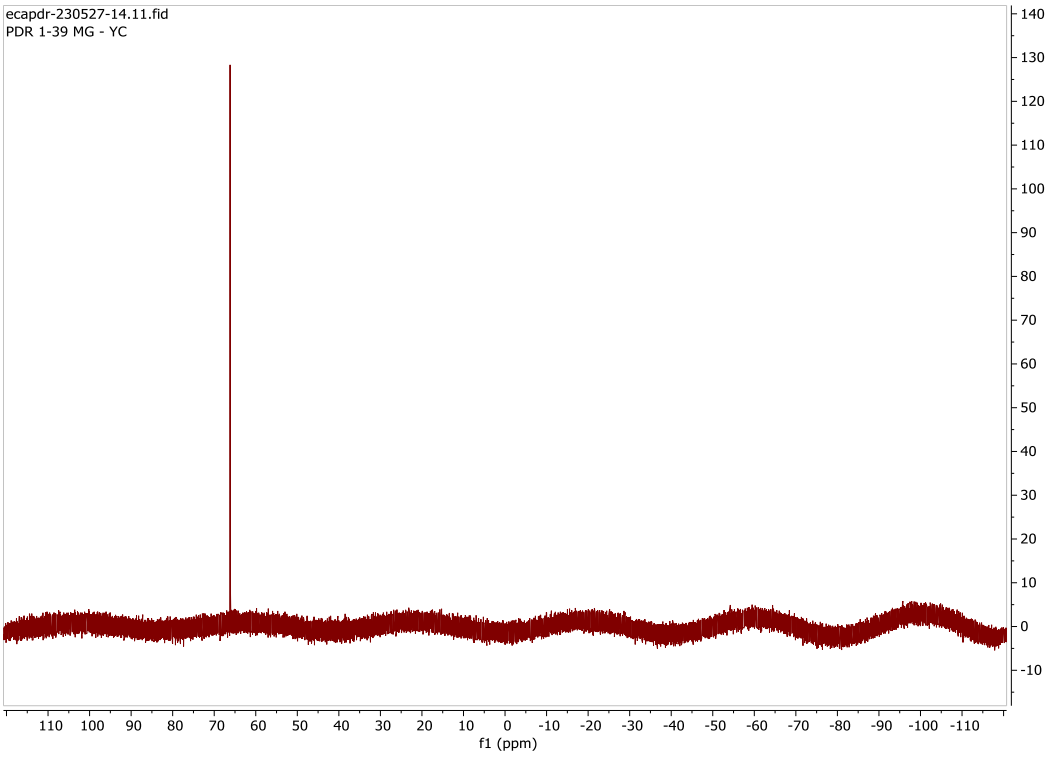
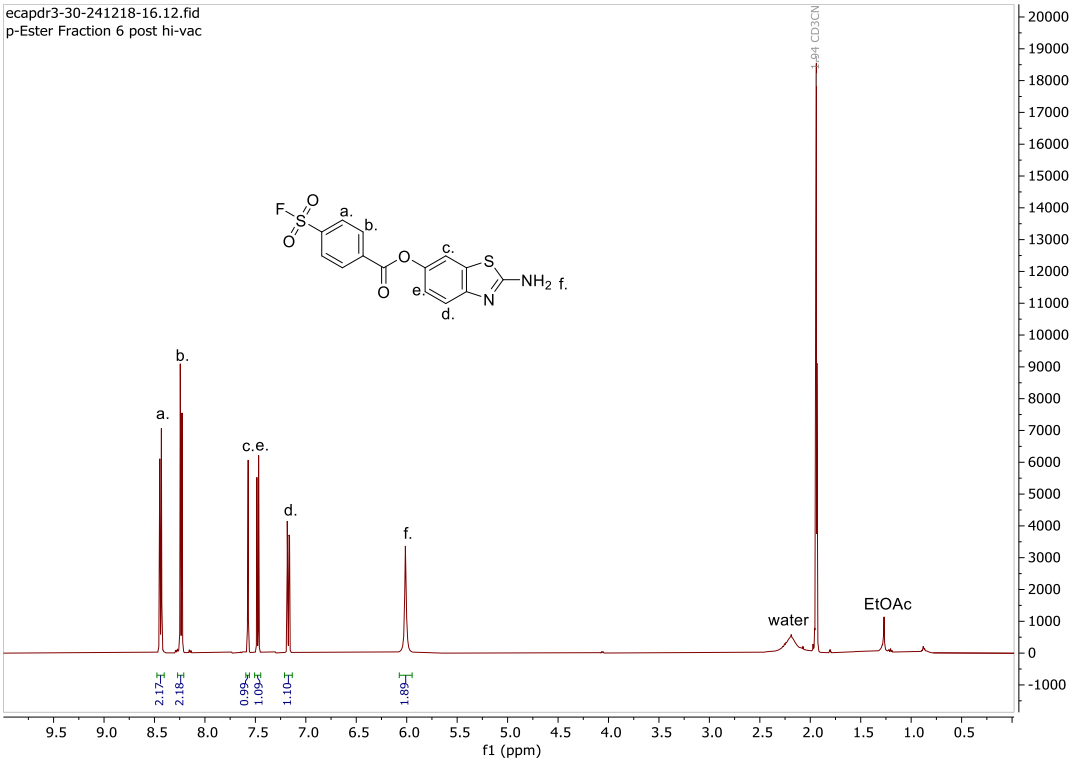


Figure S15. Characterization data for **2b**:



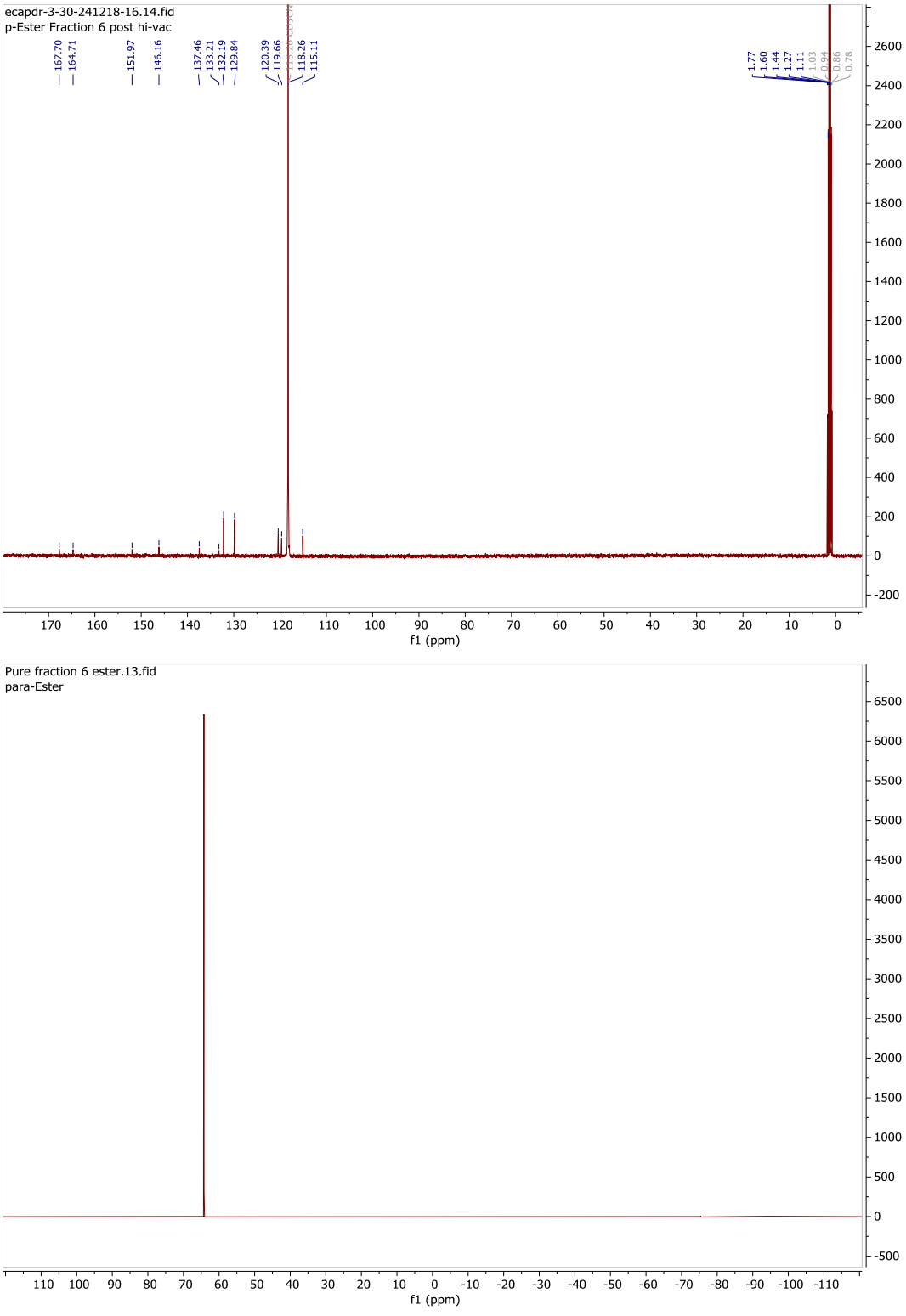
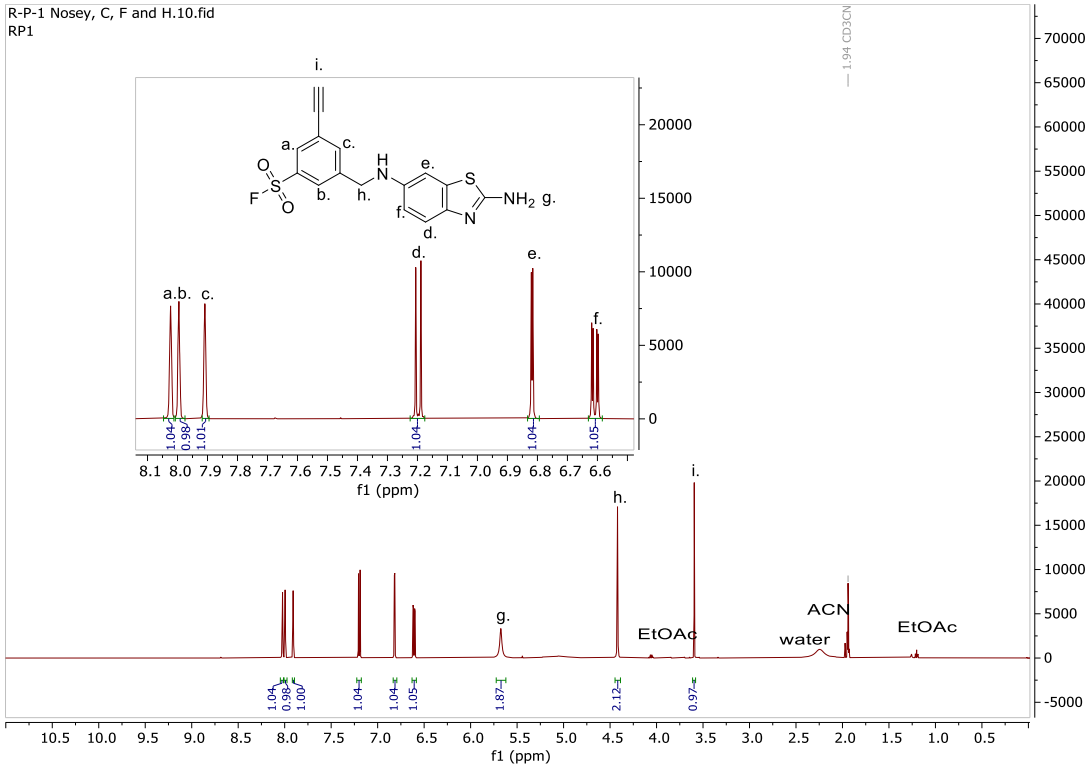
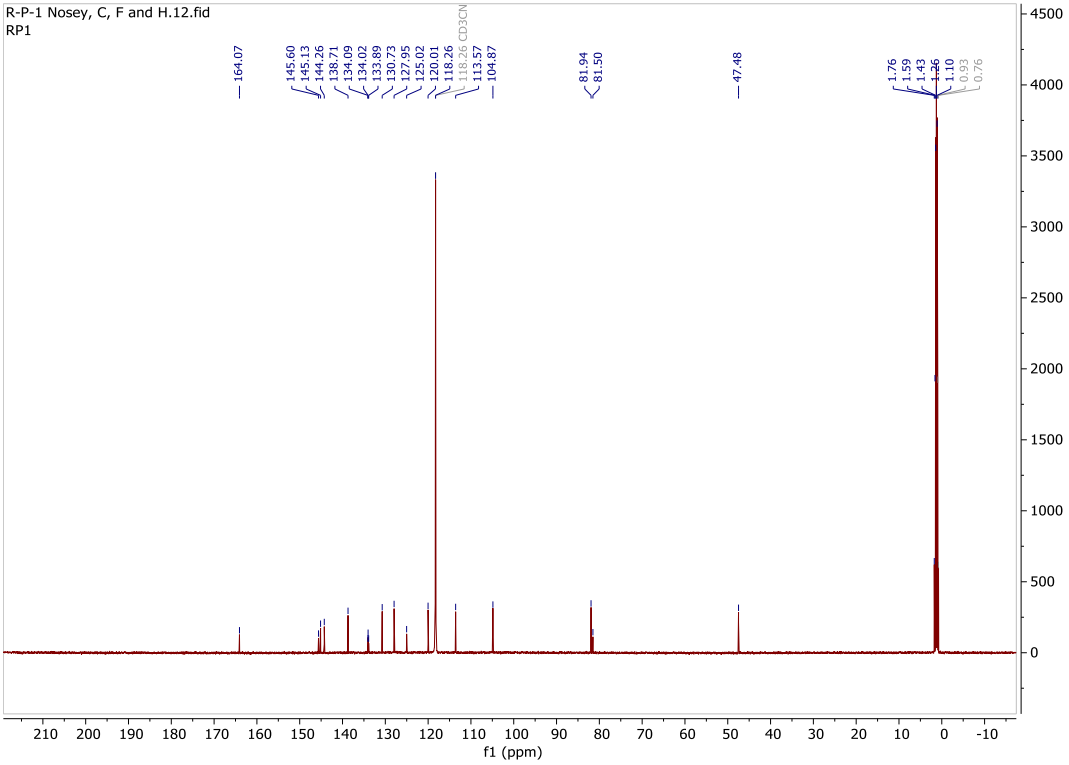


Figure S16. Characterization data for **P-1**:


R-P-1 Nosey, C, F and H.10.fid
RP1
f1 (ppm)
EtOAc
water
ACN
EtOAc


R-P-1 Nosey, C, F and H.12.fid
RP1
f1 (ppm)

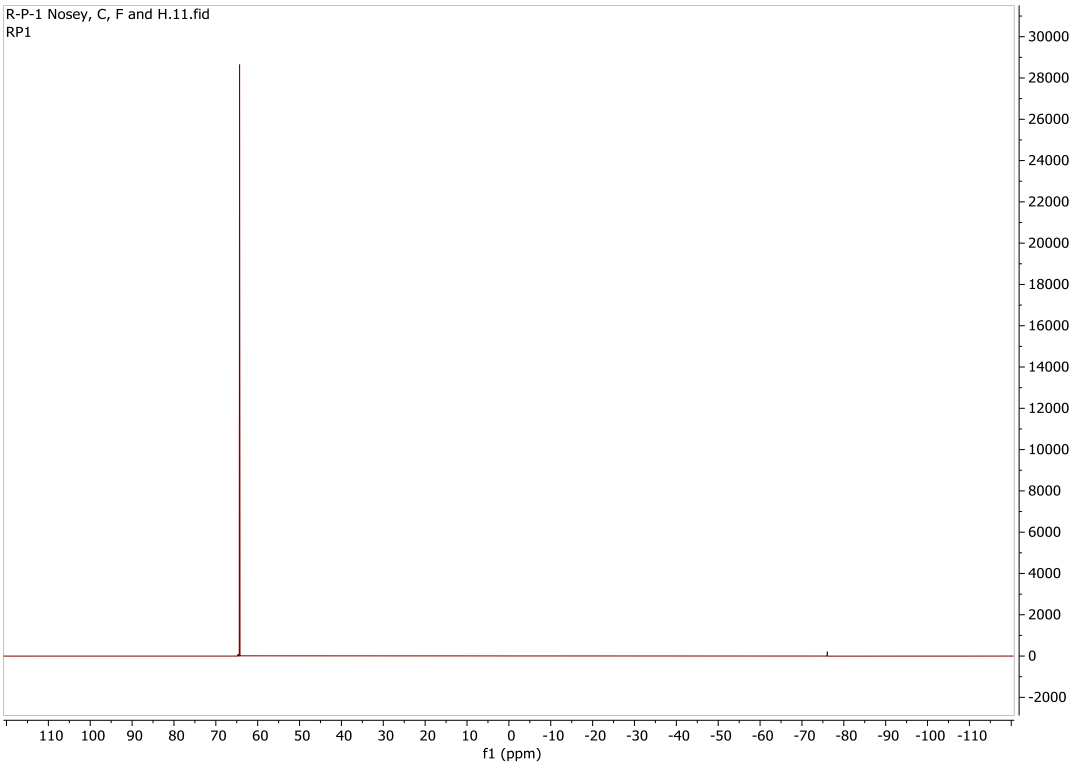
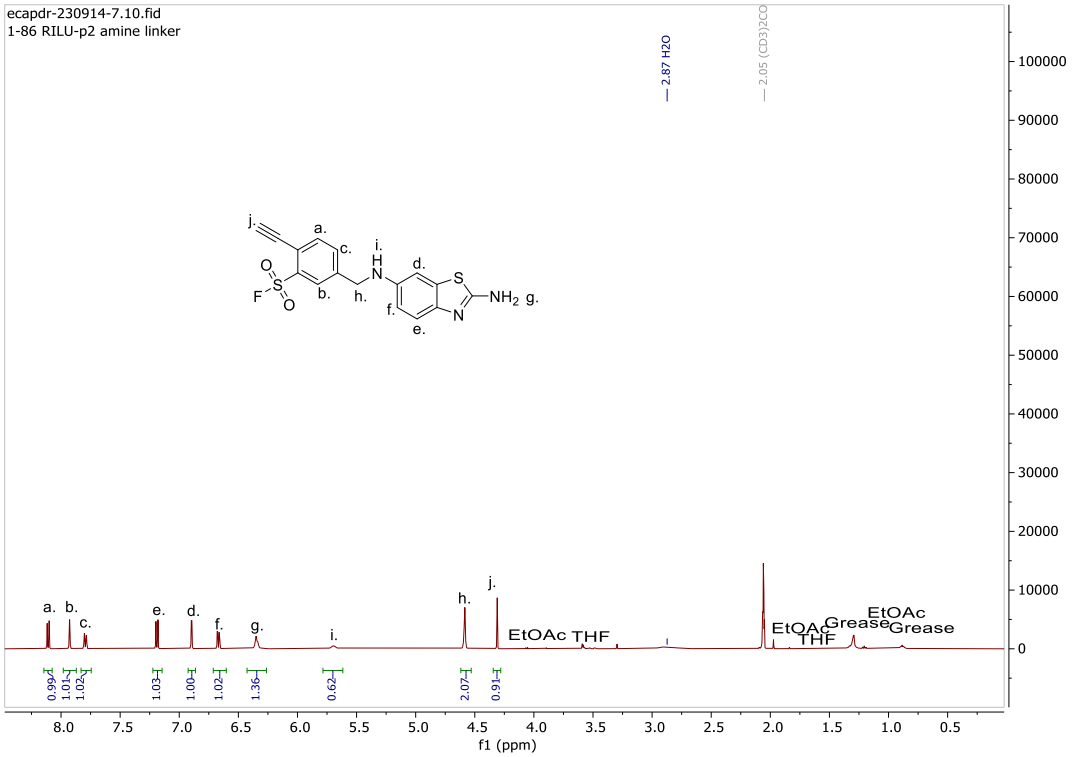
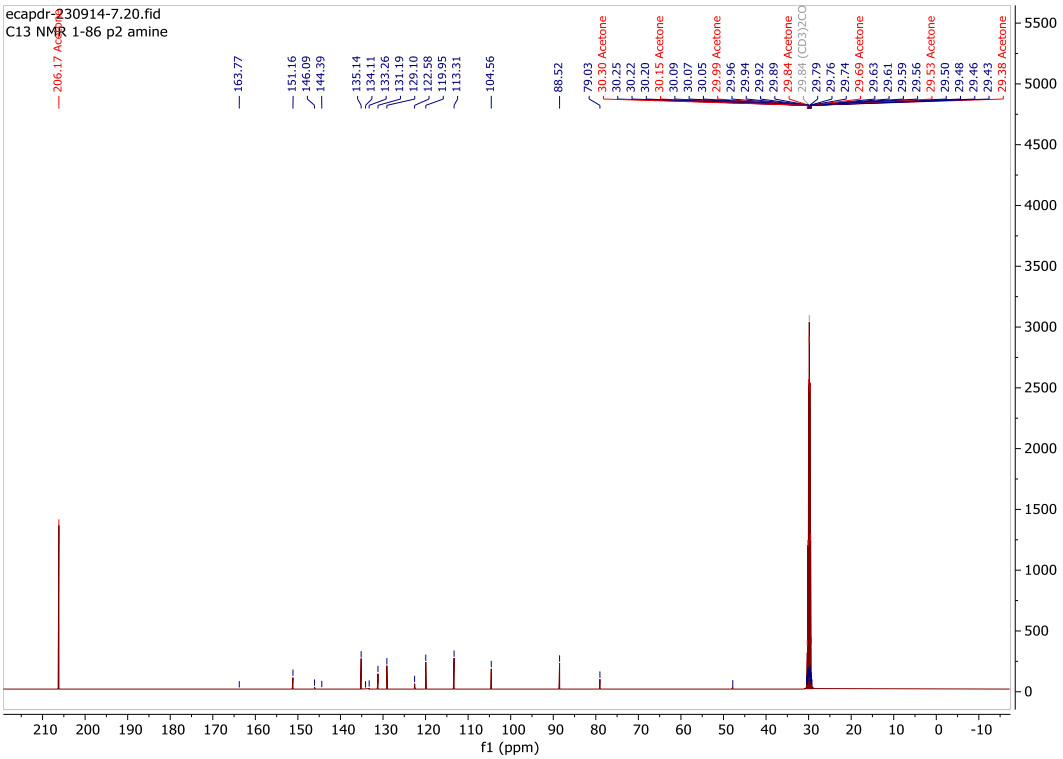
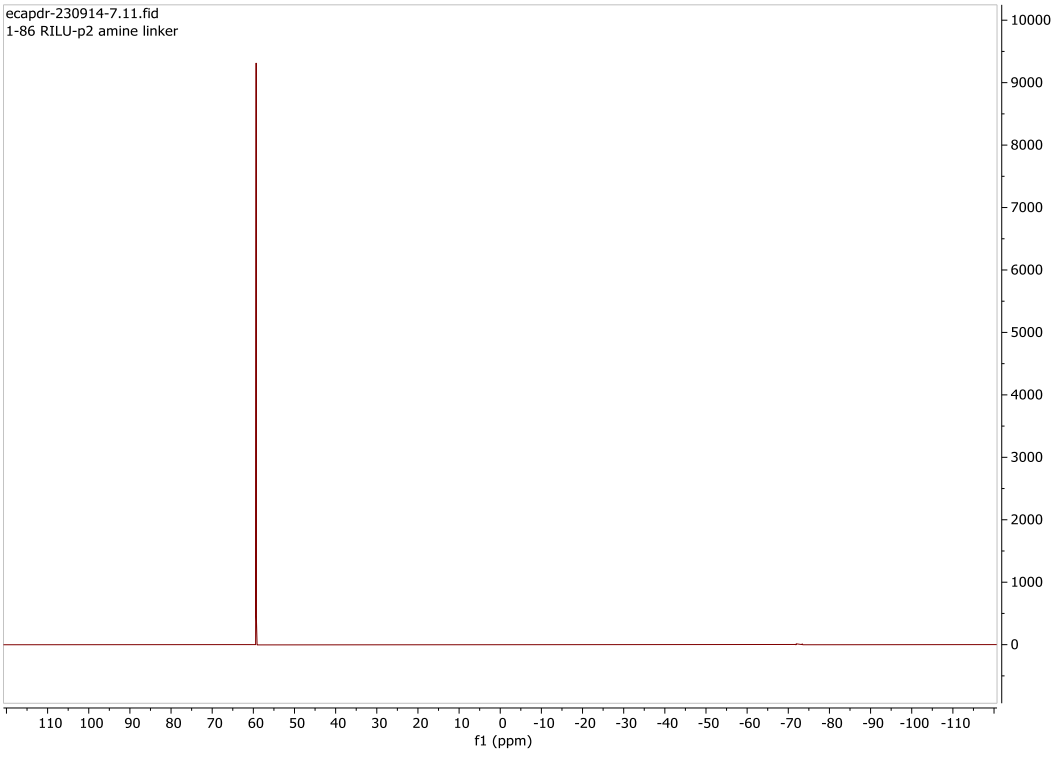


Figure S17. Characterization data for **P-2**:




ecapdr-230914-7.20.fid
C13 NMR 1-86 p2 amine
206.17 Acetone
163.77
151.16
146.09
144.39
135.14
134.11
133.26
131.19
129.10
122.58
119.95
113.31
104.56
88.52
79.03
30.30 Acetone
30.25
30.22
30.20
30.15 Acetone
30.09
30.07
30.05
29.99 Acetone
29.96
29.94
29.92
29.89
29.84 Acetone
29.84 (CD3)2CO
29.79
29.76
29.74
29.69 Acetone
29.63
29.61
29.59
29.56
29.53 Acetone
29.50
29.48
29.46
29.43
29.36 Acetone
f1 (ppm)


ecapdr-230914-7.11.fid
1-86 RILU-p2 amine linker
f1 (ppm)

	MIC (μg/mL)	
Compound	**WT**	**EG9492**
Polymyxin E	**1.2**	**32.0 - 64.0**
R-1	**177**	**177**
R-2	**-**	**> 157**
1b	**>170***	**>170***
1c	**>170***	**>170***
2a	**>176***	**>176***
2b	**>176***	**>176***

Table. S1. Minimum Inhibitory Concentration for Polymyxin E, Non-covalent and Covalent Inhibitors in WT and Polymyxin Resistant *S. enterica*.

The results are from six biological replicates done on different days. DMSO was present at a final concentration of 2.5%. *Denotes the highest concentrations tested due to limited compound solubility in LB media.

Combination[a]	Strain	FIC
R-1	**WT**	**0.30**
R-1	**EG9492**	**0.28**

[a] Compounds were present in combination with polymyxin E.

Table S2. 2-Aminobenzothiazoles Synergize with Polymyxin E.

Serially diluted polymyxin E and inhibitors were combined in a 96-well plate and incubated with WT or polymyxin resistant *S. enterica* for ~16 h. Changes in polymyxin E or inhibitor ***R-1*** minimum inhibitory concentrations (MICs) were compared to combination treatment MICs. The fractional inhibitory concentration (*see checkerboard assay method for equation*) was calculated from six biological replicate experiments done on different days. DMSO was present at a final concentration of 2.5%.

References

1. Fihn, C. A.; Lembke, H. K.; Gaulin, J.; Bouchard, P.; Penningroth, M. R.; Crone, K. K.; Vogt, G. A.; Gilbertsen, A. J.; Ayotte, Y.; Couthino de Oliveira, L.; Serrano-Wu, M. H.; Drouin, N.; Hung, D. T.; Hunter, R. C.; Carlson, E. E., Evaluation of expanded 2-aminobenzothiazole library as inhibitors of a model histidine kinase and virulence suppressors in *Pseudomonas aeruginosa*. *Bioorg. Chem.* **2024,** *153*, 107840.
2. Lembke, H. K.; Carlson, E. E., Activity-based ATP analog probes for bacterial histidine kinases. *Methods Enzymol* **2022,** *664*, 59-84.
3. Goswami, M.; Espinasse, A.; Carlson, E. E., Disarming the virulence arsenal of Pseudomonas aeruginosa by blocking two-component system signaling. *Chem Sci* **2018,** *9* (37), 7332-7337.
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6. Wilke, K. E.; Francis, S.; Carlson, E. E., Inactivation of multiple bacterial histidine kinases by targeting the ATP-binding domain. *ACS Chem Biol* **2015,** *10* (1), 328-35.
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