Supporting Information

Inhibition of efflux pumps aids small-molecule probe-based fluorescence labeling and imaging in the Gram-negative bacterium *Escherichia coli*

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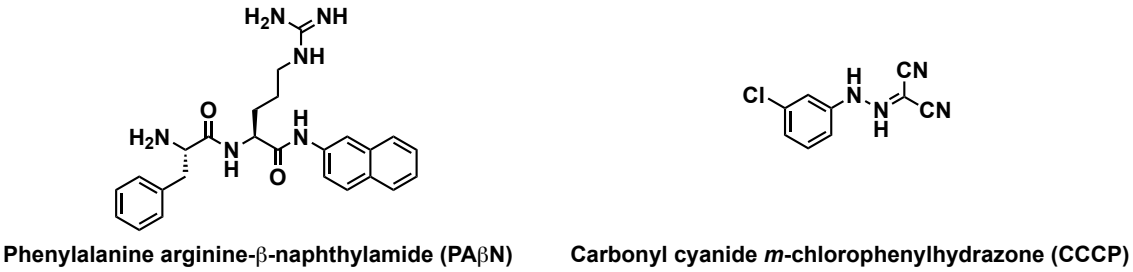


Figure S1. Structures of phenylalanine arginine-β-naphthylamide (PAβN) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).

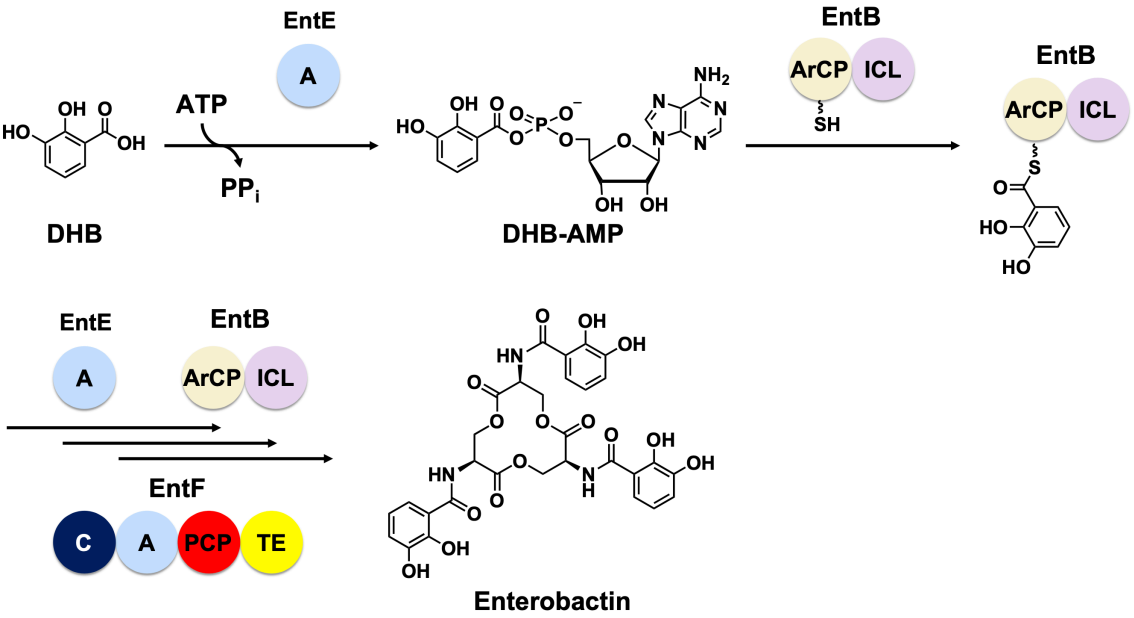


Figure S2. Nonribosomal peptide synthesis of enterobactin. The modules consist of adenylation (A), aryl carrier protein (ArCP), isochorismate lyase (ICL), condensation (C), peptidyl carrier protein (PCP), and thioesterase (TE).

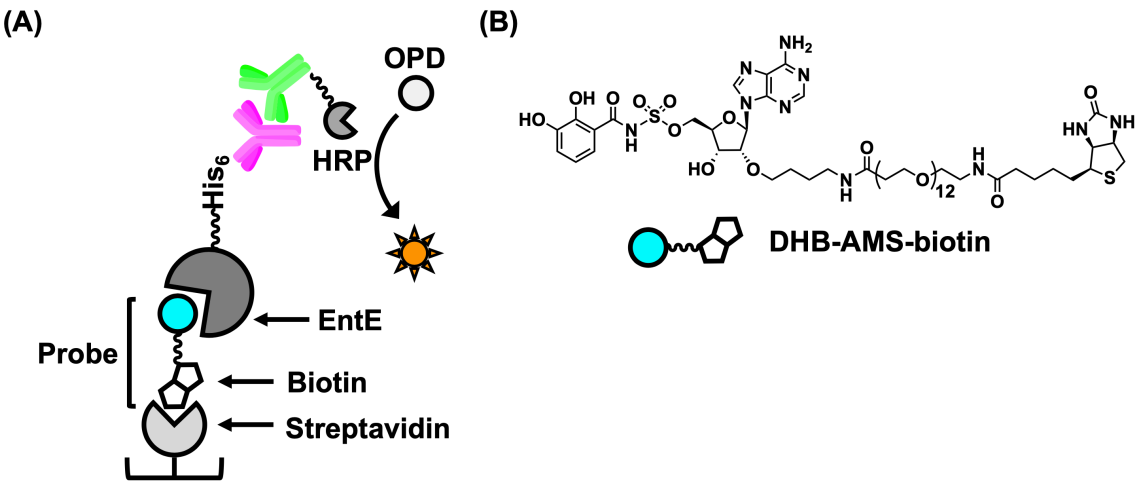


Figure S3. (A) Enzyme-linked immunosorbent assay (ELISA) for the binding of adenylation domains of nonribosomal peptide synthetases.[1,2,3] (B) A structure of the chemical probe described in this ELISA protocol.[1,2,3]

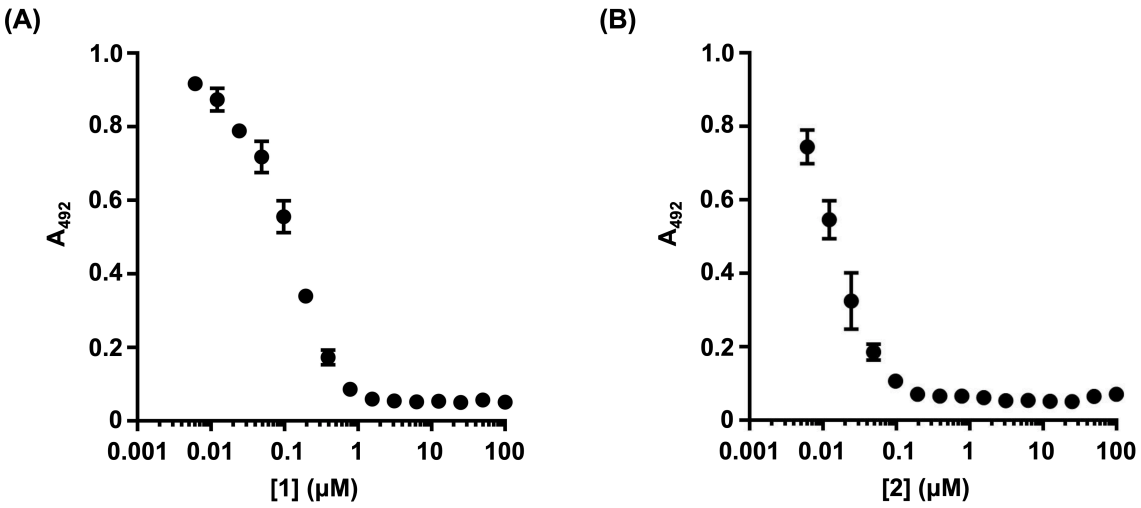


Figure S4. Binding of EntE with (A) probe 1 and (B) probe 2 by competitive ELISA with DHB-AMS-biotin. Streptavidin-coated wells treated with DHB-AMS-biotin (1.25 μg/mL) were incubated with EntE (0.875 μg/mL, 14.8 nM) in either the absence or presence of probes **1** (6.1 nM−100 μM) and **2** (6.1 nM−100 μM).

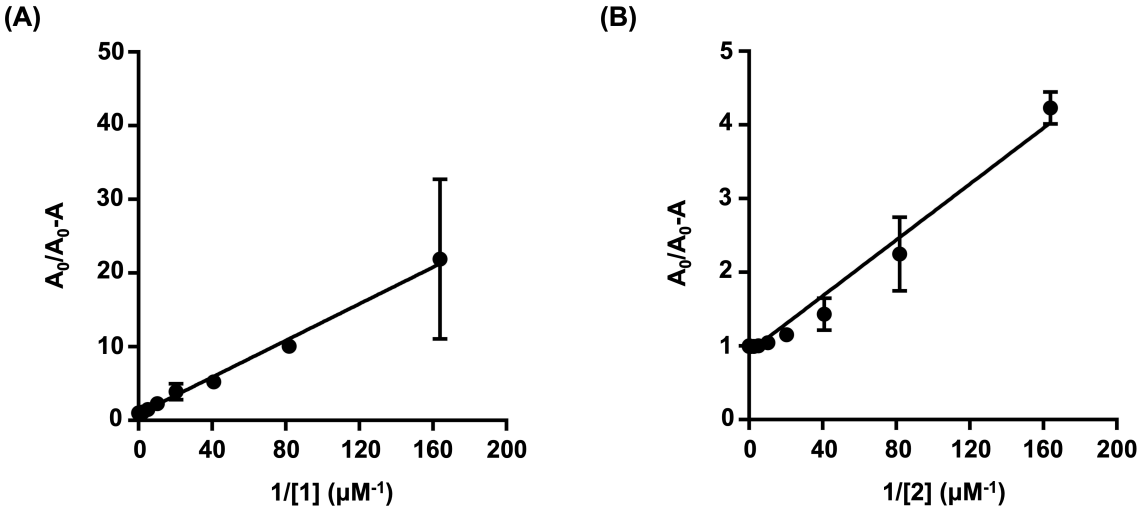


Figure S5. Klotz plots to determine K_d values of EntE with (A) probe 1 and (B) probe 2. The results were analyzed by a Klotz plot (a double reciprocal plot of the inhibition concentration vs the ratio of inhibitor-bound enzyme to the total added enzyme) and the slope of the line yielding the K_d for the probe. All assays were performed in duplicates.

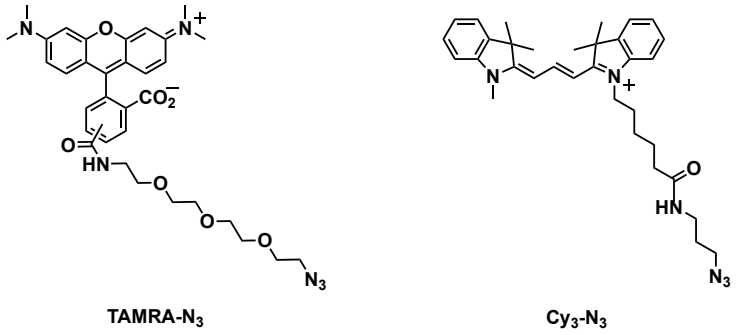


Figure S6. Structures of TAMRA-azide and Cy_3-azide.

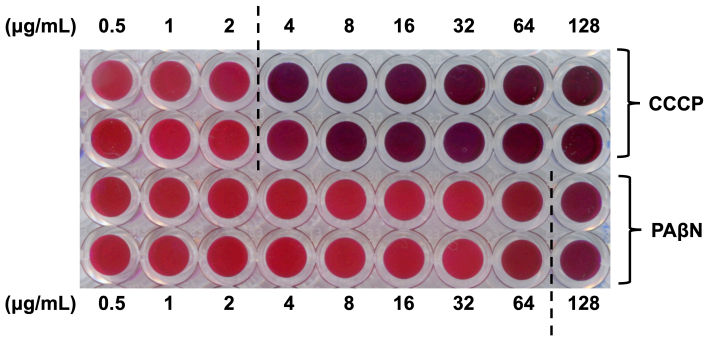


Figure S7. Determination of minimum inhibitory concentration (MIC) determination of arginine-β-naphthylamide (PAβN) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) for *E. coli* BL21 (DE3). Bacterial growth was evaluated using the indicator resazurin, a purple dye, which is reduced to the pink compound resorufin by metabolically live bacteria. The dash lines indicate MIC values in μg/mL.

Table 1. K_d values for the EntE A-domain.[a]

Enzyme	Compound	K_d
EntE	**1**	100 ± 15 nM
	2	19 ± 2 nM

[a]The K_d values were determined at room temperature in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, and 0.0025% NP-40. All assays were performed in duplicates.

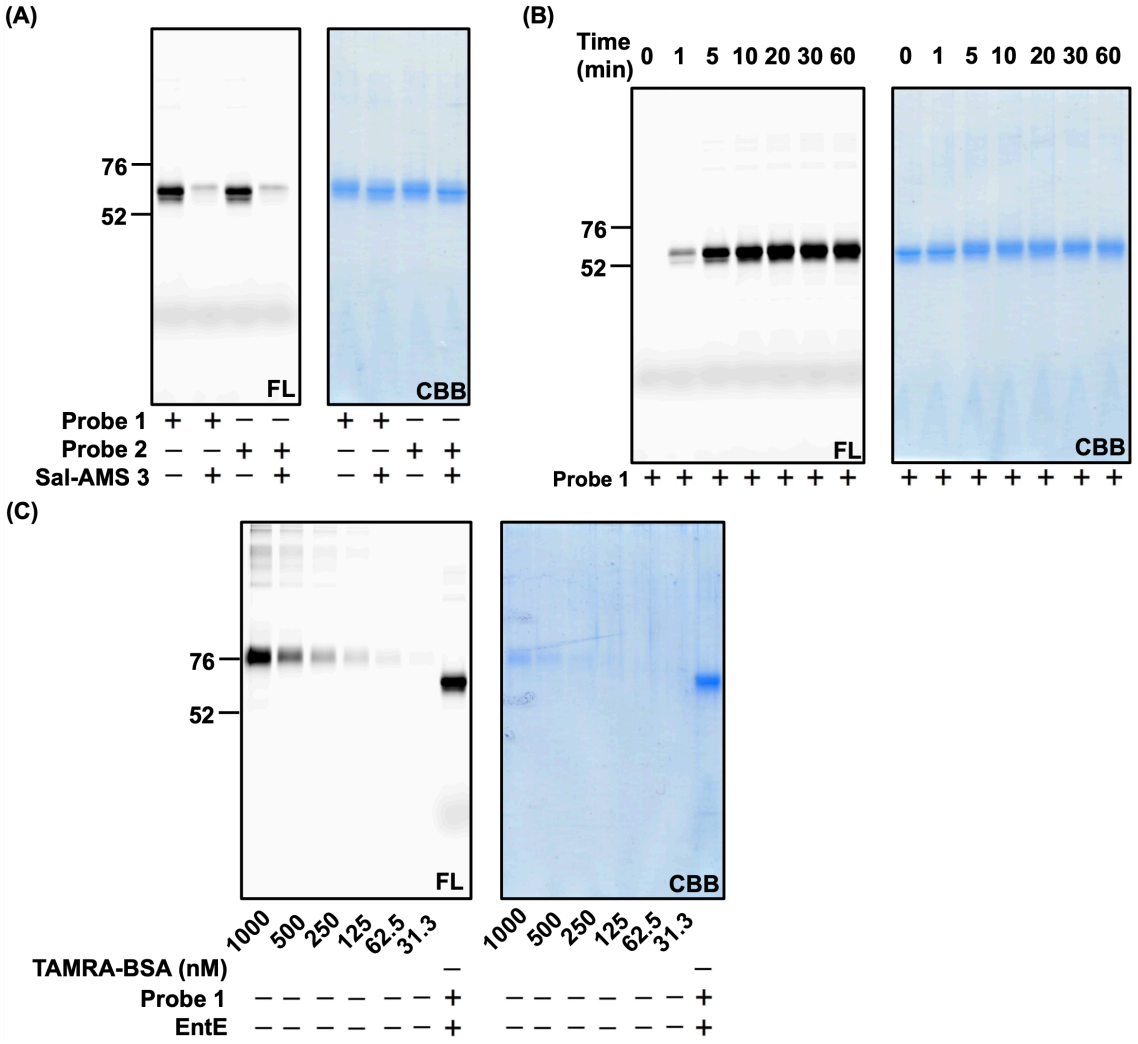


Figure S8. Full images of SDS-PAGE gels from Figure 2. Labeling of recombinant EntE both in purified EntE and within proteomes with probes **1** and **2**. (A) Labeling of EntE and inhibition studies with excess inhibitor **3**. EntE (1 μM) was preincubated in either the absence or presence of 100 μM of Sal-AMS **3** and treated with 1 μM of probes **1** and **2**. (B) UV irradiation time course studies of the labeling of EntE with probe **1**. SDS-PAGE analysis denoting the labeling of EntE (1 μM) with probe **1** (1 μM). (C) Measurement of the labeling efficacy of probe **1**. Fluorescent SDS-PAGE gel of a BSA-TAMRA conjugate, a standard for fluorescent intensity and labeling of EntE (1 μM) with probe **1** (1 μM). (D) Labeling of *E. coli* BL21 (DE3) lysates prepared from cells expressing EntE with probes **1** and **2**. *E. coli* lysates (1 mg/mL) from EntE-expressing cells were incubated with probes **1** and **2** (1 μM) in either the absence or presence of Sal-AMS **3** (100 μM). An arrowhead points to the expressed EntE. The gels were visualized by in-gel fluorescence (FL) and Coomassie brilliant blue (CBB).

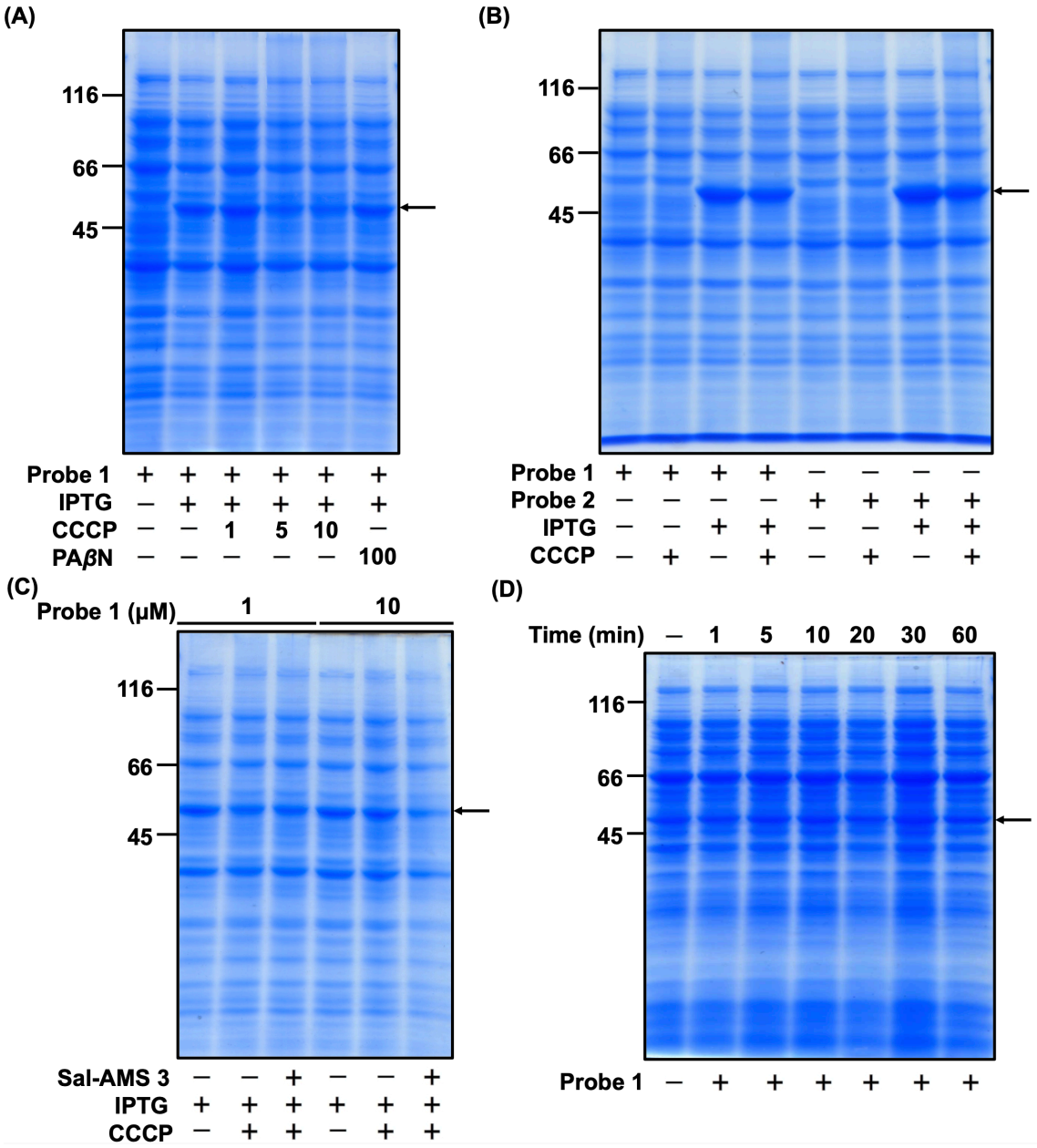
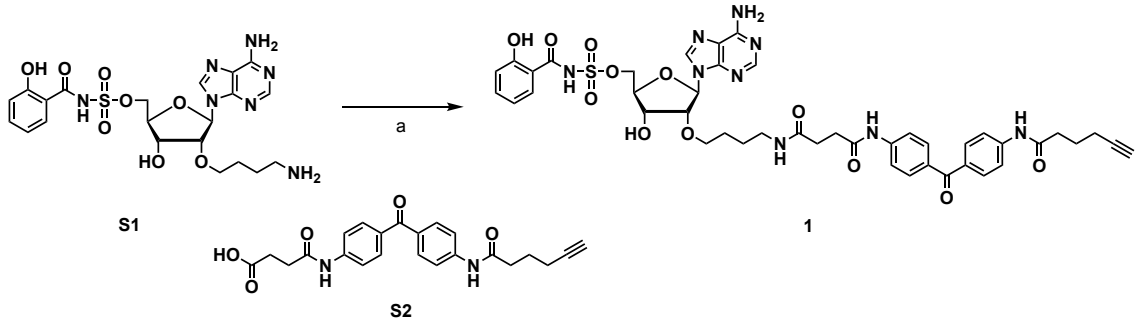


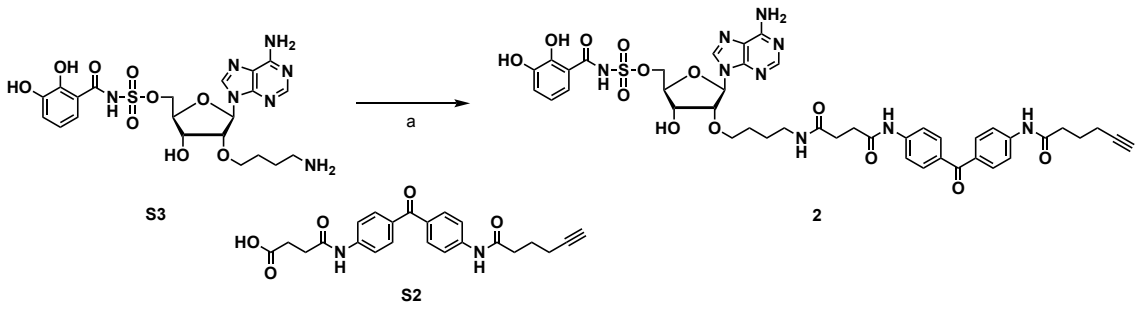
Figure S9. Full images of SDS-PAGE gels from Figure 3. *In vivo* labeling applications of probes **1** and **2**. (A) Effect of the efflux pump inhibitors on the *in vivo* labeling of EntE by probes **1** and **2**. The *E. coli* cells expressing EntE were incubated with CCCP (1-10 μM) and PAβN (100 μM) and subsequently treated with probe **1** (10 μM). (B) Comparison of the *in vivo* labeling properties of EntE by probes **1** and **2** in the presence of CCCP. The *E. coli* cells expressing EntE were incubated with CCCP (5 μM) and probes **1** and **2** (10 μM). (C) *In vivo* labeling of EntE by probe **1** in the presence of Sal-AMS **3**. The *E. coli* cells expressing EntE were treated with CCCP (5 μM) and Sal-AMS **3** (100 μM) before the addition of probe **1** (10 μM). (D) Time-dependent cell

permeability of probe **1**. The *E. coli* cells expressing EntE were incubated with probe **1** (10 μM) in the presence of CCCP (5 μM) for the times indicated before UV irradiation. An arrowhead points to the expressing EntE. The gels were visualized by in-gel fluorescence (FL).

Chemical Synthetic Procedures



Scheme S1. Synthetic route to probe 1. *Reagents and conditions*: [a] **S2**, EDC·HCl, HOBt, Et_3N, DMF, rt, 79% (**1**).



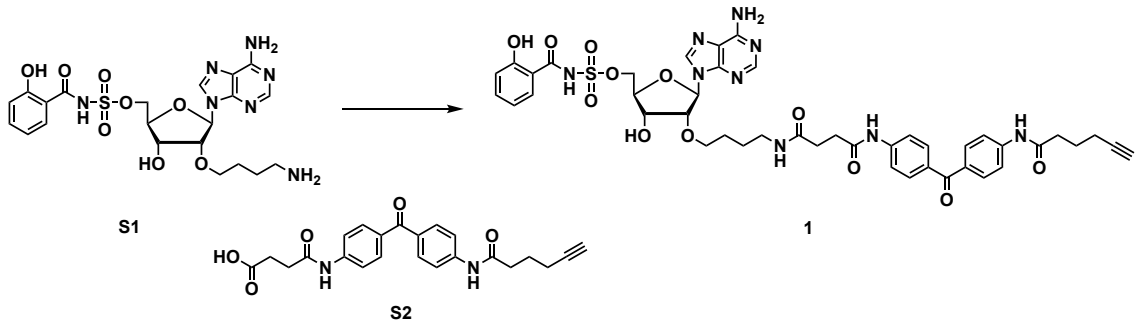
Scheme S2. Synthetic route to probe 2. *Reagents and conditions*: [a] **S2**, EDC·HCl, HOBt, Et_3N, DMF, rt, 39% (**2**).

General Synthetic Methods. All commercial reagents were used as provided unless otherwise indicated. Compounds **S1**, **S2**, and **S3** are known compounds.[1,4] These compounds were prepared according to published literature procedures. All reactions were carried out under an atmosphere of nitrogen in dry solvents and constant magnetic stirring unless otherwise noted. ^{1}H-NMR spectra were recorded at 500 MHz. ^{13}C-NMR spectra were recorded at 125 MHz on JEOL NMR spectrometers and standardized to the NMR solvent signal as reported by Gottlieb.[5] Multiplicities are given as s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet using integration and coupling constant in Hertz. TLC analysis was performed using Silica Gel 60 F254 plates (Merck) and visualization was accomplished with ultraviolet light (λ = 254 nm) and/or the appropriate stain [phosphomolybdic acid, iodine, ninhydrin, and potassium permanganate]. Silica gel chromatography was carried out with SiliaFlash F60 230-400 mesh (Silicycle), according to the method of Still.[6] Mass spectral data were obtained using a LCMS-IT-

TOF mass spectrometer (Shimadzu).

Chemical Synthesis of probe 1 Compound number in bold refers to the structures shown in Schemes S1.

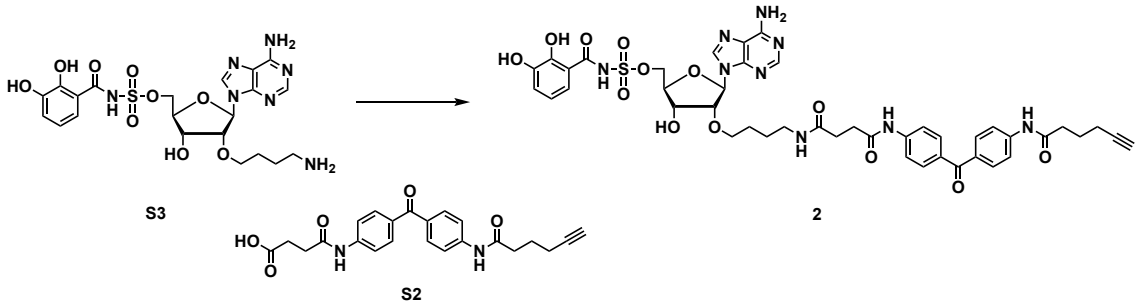
Probe 1



1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (5.8 mg, 0.030 mmol) and 1-hydroxybenzotriazole (4.6 mg, 0.030 mmol) were added to a solution of compound **S1** (16 mg, 0.025 mmol) in DMF (1 mL). The solution was stirred at room temperature for 10 min and **S2** (12 mg, 0.030 mmol) and triethylamine (7 μL, 0.050 mmol) were then added. After 3 h, the reaction mixture was evaporated *in vacuo*. The residue was purified by flash chromatography (8:1 to 5:1 EtOAc/MeOH) to afford probe **1** as a white solid (9 mg, 39%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.54 (s, 1H), 8.16 (s, 1H), 7.95 (dd, J = 7.5, 2.3 Hz, 1H), 7.76–7.69 (m, 8H), 7.23–7.25 (m, 1H), 6.81–6.74 (m, 2H), 6.15 (d, J = 6.3 Hz, 1H), 4.60–4.56 (m, 1H), 4.54–4.50 (m, 1H), 4.44–4.35 (m, 2H), 4.33–4.30 (m, 1H), 3.65–3.59 (m, 1H), 3.53–3.47 (m, 1H), 3.34 (q, J = 6.9 Hz, 12H), 3.12–3.06 (m, 2H), 2.70 (t, J = 6.9 Hz, 2H), 2.58–2.50 (m, 4H), 2.32–2.26 (m, 3H), 1.94–1.86 (m, 2H), 1.55–1.35 (m, 4H), 1.30 (t, J = 6.9 Hz, 18H). ^{13}C NMR (125 MHz, CD_3OD) δ 196.6, 175.2, 174.5, 174.1, 173.3, 162.1, 157.2, 154.0, 150.8, 144.3, 144.2, 141.1, 134.5, 134.1, 133.9, 132.3, 132.2, 131.4, 120.5, 120.13, 120.06, 119.3, 117.9, 87.5, 85.1, 84.1, 83.4, 71.5, 71.3, 70.3, 69.5, 60.0, 47.9, 40.1, 36.7, 33.2, 31.8, 27.8, 26.8, 25.5, 18.6, 9.2, 8.4. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI–): $[M–H]^-$ calcd for $C_{44}H_{46}N_9O_{12}S$, 924.2987; found, 924.2958.

Chemical Synthesis of probe 2 Compound number in bold refers to the structures shown in Schemes S2.

Probe 2



1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (4.2 mg, 0.022 mmol) and 1-hydroxybenzotriazole (3.4 mg, 0.022 mmol) were added to a solution of compound **S3** (12 mg, 0.018 mmol) in DMF (1 mL). The solution was stirred at room temperature for 10 min and **S3** (8.9 mg, 0.022 mmol) and triethylamine (5 μL, 0.036 mmol) were then added. After 3 h, the reaction mixture was evaporated *in vacuo*. The residue was purified by flash chromatography (6:1 to 2:1 $CHCl_3$/MeOH) to afford probe **2** as a white solid (12 mg, 58%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.37 (d, J = 12.6 Hz, 2H), 8.56 (s, 1H), 8.41 (s, 1H), 8.11 (s, 1H), 7.82 (dd, J = 5.7, 5.7 Hz, 2H), 7.78–7.72 (m, 4H), 7.70–7.65 (m, 4H), 7.29–7.25 (m, 3H), 6.80–6.74 (m, 1H), 6.51 (dd, J = 7.5, 7.5 Hz, 1H), 6.01 (d, J = 6.3 Hz, 1H), 5.36 (d, J = 5.2 Hz, 1H), 4.56–4.52 (m, 1H), 4.34–4.31 (m, 1H), 4.24–4.19 (m, 1H), 4.18–4.10 (m, 2H), 3.54–3.50 (m, 1H), 3.45–3.40 (m, 1H), 3.00–2.91 (m, 3H), 2.82 (t, J = 2.3 Hz, 1H), 2.60–2.52 (m, 2H), 2.39–2.33 (m, 2H), 2.24–2.20 (m, 2H), 2.11 (s, 2H), 1.80–1.72 (m, 2H), 1.49–1.38 (m, 2H), 1.36–1.25 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 193.4, 171.3, 170.9, 158.2, 156.0, 152.8, 149.7, 149.5, 145.7, 143.1, 143.0, 139.2, 131.7, 131.6, 130.9, 120.0, 119.9, 118.9, 118.2, 118.1, 117.8, 116.7, 85.2, 84.0, 83.2, 80.7, 71.7, 69.4, 69.3, 67.8, 55.7, 45.4, 43.7, 38.2, 36.9, 35.2, 34.1, 31.9, 30.2, 26.7, 26.6, 25.6, 23.8, 17.4, 15.7. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI–): $[M–H]^-$ calcd for $C_{44}H_{46}N_9O_{13}S$, 940.2936; found, 940.2936.

Chemical Biology Procedures

Culture Procedures: *Escherichia coli* BL21 (DE3) harboring the pET28b-*entE* plasmid was grown overnight at 37 °C and 200 rpm in Lennox Broth (LB) supplemented with kanamycin (50 μg/mL). The overnight culture was added to two LB cultures containing kanamycin (50 μg/mL). The two cultures were grown to an OD_{600} = 0.6−0.8 at 37 °C, after which isopropyl-β-D-thiogalactoside (IPTG) was added to one of the culture at a final concentration of 0.1 mM to one of the cultures. Both cultures were grown for 1 h (for *in vitro* and *in vivo* labeling) and 3 h (for protein preparation) with shaking at 37 °C. For protein preparation, the cultures were centrifuged, and the pellets were frozen at –80 °C.

Protein Expression and Purification: Recombinant EntE was expressed and purified as described previously.[1]

Preparation of *E. coli* Cellular Lysates for Proteomic Studies: Pellets were resuspended in 20 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, 0.05% NP-40, and protease inhibitor cocktail (EDTA free) (100×) (Nacalai Tesque, Inc.) and sonicated. The cell lysates were centrifuged for 20 min at 15,000 rpm, and the pellets were discarded. The total protein concentration was measured by the Bradford assay using BSA as a standard.[7]

Labeling of Purified EntE by Probes 1 and 2: Standard conditions for reactions between probes **1** and **2** and recombinant protein, respectively, were as follows: recombinant EntE (1 μM) was treated with probes **1** or **2** (1 μM from a 100 μM stock in DMSO) in assay buffer [20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, and 0.0025% NP-40]. Inhibition studies were performed by pre-incubation of EntE (1 μM) with Sal-AMS **3** (100 μM from a 10 mM stock in DMSO) for 10 min at room temperature. In all the experiments, the total DMSO concentration was maintained at 2.2%. After 10 min at room temperature, the samples were irradiated at 365 nm for 30 min on ice. To initiate the click reaction, TAMRA-azide, tris(2-carboxyethyl)phosphine) (TCEP), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA), and $CuSO_4$ were added to obtain final concentrations of 100 μM, 1 mM, 100 μM, and 1 mM, respectively. After 1 h at room temperature, 5× SDS-loading buffer (strong reducing) was added, and the samples were heated at 95 °C for 5 min. Samples were separated by 1D SDS-PAGE, and fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

Ultraviolet Photolysis Time Studies: Recombinant EntE (1 μM) was treated with probe **1** (1 μM from a 100 μM stock in DMSO; final DMSO concentration of 2.2%) in assay buffer, respectively. After 10 min at room temperature, the samples were irradiated at 365 nm for the indicated time (0–60 min) on ice, reacted with TAMRA-azide, and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

Measurements of EntE Labeling with Probe 1: The probe **1**-recombinant EntE reactions were performed by incubating recombinant EntE (1 μM) with probe **1** (1 μM from a 100 μM stock solution in DMSO; final DMSO concentration of 1.1%) for 10 min at room temperature in assay buffer. The sample was then irradiated at 365 nm for 30 min on ice, reacted with TAMRA-azide, and subjected to SDS-PAGE. The concentration of TAMRA-conjugated BSA (Invitrogen) was determined using the extinction coefficient of TAMRA (ε_{560} = 65,000 M^{-1} cm^{-1}). Samples (5 μL) were separated by 1D SDS-PAGE and fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager. Protein labeling by probe **1** was quantified by measuring the integrated band intensities using ImageJ.

Labeling of *E. coli* Cellular Lysates Expressing EntE by Probes 1 and 2: *E. coli* lysates (1.0 mg/mL) were treated with probes **1** or **2** (1 μM from a 100 μM stock in DMSO) in 20 mM Tris

(pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, 0.05% NP-40, and a protease inhibitor cocktail. For inhibition studies, *E. coli* lysates (1.0 mg/mL) were preincubated with Sal-AMS **3** (100 μM from a 10 mM stock in DMSO) for 10 min at room temperature. In all the experiments, the total DMSO concentration was maintained at 2.2%. After 10 min at room temperature, the samples were irradiated at 365 nm for 30 min on ice and reacted with TAMRA-azide for 1 h at room temperature. Reactions were treated with 5× SDS-loading buffer and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

Determination of the Minimum Inhibitory Concentration (MIC): *E. coli* BL21 (DE3) harboring the pET28b-*entE* plasmid was cultured overnight at 37 °C in a cation-adjusted Mueller Hinton Broth-II (CA-MHB-II) with supplemented with kanamycin (50 μg/mL) and adjusted to obtain turbidity comparable to 0.5 McFarland standards before MIC determination. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and phenylalanine arginine-β-naphthylamide (PAβN) were assayed for antibiotic activity using the broth microdilution method in a 96-well microtiter plate. Prior to the MIC test, CCCP and PAβN were diluted in DMSO. The MIC was then determined at concentrations ranging from 0.5 μg mL^{-1} to 128 μg mL^{-1} toward CCCP and PAβN. The test compound was added to sterile CA-MHB-II in a microtiter plate before the bacterial suspension, prepared as described above, was added. Inoculated and uninoculated wells of compound-free broth were also included (the first controls the adequacy of the broth to support the growth of the organism, and the second is a check of sterility). The assay was performed in duplicates. For image acquisition (Figure S6), resazurin was used as an indicator to support visualization using a method similar to that of Sarker *et al*.[8] A 10-μL sample of sterile aqueous resazurin solution (0.7% w/v) was added to the wells. After further incubation for 6 h at 37 °C, the plates were then photographed.

***In Vivo* Labeling of EntE with Probe 1 in the Presence of CCCP or PAβN:** Cultures (1 mL) were harvested and treated with CCCP (1 μM, 5 μM, 10 μM from 1 mM, 5 mM, 10 mM stock in DMSO) or PAβN (100 μM from a 100 mM stock in DMSO). Controls were treated with DMSO (vehicle). After 10 min at room temperature, the bacterial cells were treated with probe **1** or **2** (10 μM from a 10 mM stock in DMSO) for 10 min at room temperature. In all the experiments, the total DMSO concentration was maintained at 0.1%. The bacterial cells were harvested, washed twice with 20 mM Tris (pH 8.0) (500 μL) containing CCCP or PAβN, resuspended in 100 μL of 20 mM Tris (pH 8.0) (500 μL) containing CCCP or PAβN, and transferred into a 96-well plate. The plate was placed on ice and exposed to 365 nm light for 30 min. The cells were then harvested by centrifugation and stored in a freezer until further use. Cell lysates were prepared by treating cell pellets with 20 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, 0.05% NP-40, and protease inhibitor cocktail (EDTA free) (100×) (Nacalai Tesque, Inc.) as described above. Lysates (1.0 mg mL^{-1}) were then reacted with TAMRA-N_3 for 1 h at room temperature and separated by

gel electrophoresis. Fluorescence gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

***In Vivo* Labeling of EntE with Probes 1 and 2 in the Presence of CCCP:** Cultures (1 mL) were harvested and treated with CCCP (5 μM from 5 mM stock in DMSO). Controls were treated with DMSO (vehicle). After 10 min at room temperature, the bacterial cells were treated with probe **1** or **2** (10 μM from a 10 mM stock in DMSO) for 10 min at room temperature. In all the experiments, the total DMSO concentration was maintained at 0.1%. The bacterial cells were harvested, washed once with 1 mL of 20 mM Tris (pH 8.0) (1 mM) containing CCCP (5 μM from 5 mM stock in DMSO), resuspended in 100 μL of 20 mM Tris (pH 8.0) (100 μL) containing CCCP (5 μM from 5 mM stock in DMSO), and transferred into a 96-well plate. The plate was placed on ice and exposed to 365 nm light for 30 min. The cells were harvested by centrifugation and stored in a freezer until further use. Cell lysates were prepared by treating cell pellets with 20 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, 0.05% NP-40, and protease inhibitor cocktail (EDTA free) (100×) (Nacalai Tesque, Inc.) as described above. Lysates (1.0 mg mL^{-1}) were then reacted with TAMRA-N_3 for 1 h at room temperature and separated by gel electrophoresis. Fluorescence gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

***In Vivo* Labeling of EntE with Probe 1 in the Presence of Sal-AMS 3 and CCCP:** Cultures (1 mL) were harvested and treated with CCCP (5 μM from 5 mM stock in DMSO) and/or Sal-AMS (10 μM from 10 mM stock in DMSO). Controls were treated with DMSO (vehicle). After 10 min at room temperature, the bacterial cells were treated with probe **1** or **2** (1 μM, 10 μM from 1 mM, 10 mM stock in DMSO) for 10 min at room temperature. In all the experiments, the total DMSO concentration was maintained at 0.3%. The bacterial cells were harvested, washed once with 1 mL of 20 mM Tris (pH 8.0) (1 mM) containing CCCP (5 μM from 5 mM stock in DMSO), resuspended in 100 μL of 20 mM Tris (pH 8.0) (100 μL) containing CCCP (5 μM from 5 mM stock in DMSO), and transferred into a 96-well plate. The plate was placed on ice and exposed to 365 nm light for 30 min. The cells were harvested by centrifugation and stored in a freezer until further use. Lysates (1.0 mg mL^{-1}) were then reacted with TAMRA-N_3 for 1 h at room temperature and separated by gel electrophoresis. Fluorescence gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

Time-dependent Cell Permeability of Probe 1: Cultures (1 mL × 7) were treated with CCCP (5 μM from a 5 mM stock in DMSO) for 10 min at room temperature. The samples were incubated with probe **1** (10 μM from a 10 mM stock in DMSO) for the indicated times (1, 5, 10, 20, 30, and 60 min) at room temperature. In all reactions, the DMSO concentration was maintained at 0.2%. The bacterial cells were harvested, washed once with PBS (1 mL) containing CCCP (5 μM), and resuspended in 100 μL of PBS containing CCCP (5 μM), followed by UV irradiation (365 nm) for 10 min on ice. The cells were harvested by centrifugation and stored in a freezer until further

use. Lysates were prepared by treating cell pellets with 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, 0.05% NP-40, and protease inhibitor cocktail (EDTA free) (100×) (Nacalai Tesque, Inc.), and sonicated. Lysates (1.0 mg mL^{-1}) were then reacted with TAMRA-N_3 for 1 h at room temperature and separated by gel electrophoresis. Fluorescence gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

***In Vivo* Labeling and Imaging of EntE with Probe 1 in the Presence of CCCP:** Cultures (10 mL × 2) were treated with CCCP (5 μM from a 5 mM stock in DMSO) for 10 min at room temperature. One sample was incubated with probe **1** (10 μM from a 10 mM stock in DMSO) for 60 min at room temperature. Another sample was treated with DMSO (vehicle). In all reactions, the DMSO concentration was maintained at 2.0%. The bacterial cells were harvested, washed once with PBS (10 mL) containing CCCP (5 μM), and resuspended in 1 mL of PBS containing CCCP (5 μM), followed by UV irradiation (365 nm) for 10 min on ice. Cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, washed three times with PBS (200 μL), and permeabilized with 0.3% v/v Triton X-100 for 15 min at room temperature. Additionally, the cells were washed three times with PBS (200 μL). The optimized conditions for in-cell CuAAC were as follows: Cy_3-N_3 (25 μM from a 20 mM Cy_3-N_3 in DMSO, stored at –80 °C), $CuSO_4$ (100 μM from 20 mM $CuSO_4$ in water, stored at 4 °C), tris[(1-hydroxypropyl-1*H*-1,2,3-triazol-4-yl)methylamine (THPTA) (500 μM from 50 mM THPTA in water; stored at 4 °C), aminoguanidine hydrochloride (5 mM from 100 mM aminoguanidine hydrochloride freshly made in water), and sodium ascorbate (5 mM from 100 mM sodium ascorbate freshly prepared in water) for 1 h in PBS at room temperature. Further, 1.25 μM of a 20 mM $CuSO_4$, 2.5 μL of a 50 mM THPTA, and 0.3 μL of 20 mM Cy_3-azide (structure are shown in Figure S5) were mixed and allowed to react for 3 min at room temperature in the dark (i.e., dye premix). Next, 100 mM aminoguanidine hydrochloride (12.5 μL) and 100 mM sodium ascorbate (12.5 μL) were added to 221 μL PBS. The dye premix was then added to the solution. For in-cell labeling, samples were resuspended in 221 μL PBS, and the solutions were added as described above. The tubes were inverted once and incubated in the dark at room temperature for 1 h. Samples were then washed three times with PBS, and subsequently three times in an increasing ethanol series (50%, 80%, and 96%). Cells were resuspended in PBS, transferred onto glass slides, and analyzed microscopically.

For *in vitro* labeling, cell lysates (1 mg/mL) were prepared by treating cell pellets with 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, 0.05% NP-40, and protease inhibitor cocktail (EDTA free) (100×) (Nacalai Tesque Inc.), and sonicated as described above. Lysates (1.0 mg mL^{-1}) were then reacted with TAMRA-N_3 for 1 h at room temperature and separated by gel electrophoresis. Fluorescence gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

Fluorescence Microscopy: We used a MetaMorph system (Universal Imaging Corp., Downingtown, PA, USA) with an Olympus IX81 fluorescence microscope equipped with a UPLSAPO ×100 lens to acquire images. A WIGA filter was used to detect Cy_3.

Competitive ELISA Protocol and Determination of the Dissociation Constant of Enzyme-Inhibitor Equilibrium in Solution using ELISA[2]: Prior to carrying out the competitive ELISA, the optimum enzyme concentration and the incubation time on streptavidin high binding capacity Coated 96-well plates (Thermo Fisher Scientific K.K.) (precoated with 1.25 μg/mL of DHB-AMS-biotin, structure are shown in Figure S2) were determined to establish a reproducible titration curve. In our experiments, the final concentration and the incubation time were typically EntE and 15–20 min.[2] Competitive ELISA experiments were conducted as follows: a 60-μL solution of each inhibitor in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP and 0.0025% NP-40 was serially diluted 2-fold across a 96-well flat-bottom plate (Corning) containing 60 μL of buffer per well. To each well, 60 μL of enzyme solution (final concentration was preadjusted) was added to each well, and the mixture was equilibrated for 1 h at room temperature. Determination of the K_d values of probes **1** and **2** for EntE was conducted as follows. Final reaction concentrations: EntE (0.875 μg/mL, 14.8 nM) and **1** (6.1 nM to 100 μM) or **2** (6.1 nM to 100 μM). Control samples (DMSO alone) were incubated under the same conditions. In all the experiments, the total DMSO concentration was maintained at or below 2.0%. The resulting enzyme-inhibitor solutions (100 μL) from each well were transferred to wells treated with probes and incubated for 15–20 min. After extensive washing with 200 μL of PBST, wells were treated with a solution of 100 μL of an anti-6× His, monoclonal antibody (9C11, Wako Pure Chemical Industries, Ltd.) (1:5000 in PBST) for 1 h at room temperature. After three washes with 200 μL of PBST, a solution of 100 μL of goat anti-mouse-HRP conjugate (Bio-Rad Laboratories, Inc.) (1:5000 in PBST), was incubated for 1 h at room temperature, followed by three washes with 200 μL of PBST, and each well was then treated with 100 μL of 0.4 mg/mL *o*-phenylenediamine (OPD) in 0.05 M phosphate-citrate (pH 5.0) containing 0.4 mg/mL urea hydrogen peroxide at room temperature. The yellow color was allowed to develop for approximately 5 min and the reaction was quenched by the addition of 50 μL of 1 M H_2SO_4. The absorbance at 492 nm was measured using an EnVision Multilabel Reader (PerkinElmer). The results were analyzed using a Klotz plot (a double reciprocal plot of the inhibition concentration vs. the ratio of inhibitor-bound enzyme to the total added enzyme) and the slope of the line yielding the K_d for the inhibitor. All assays were performed in duplicates.

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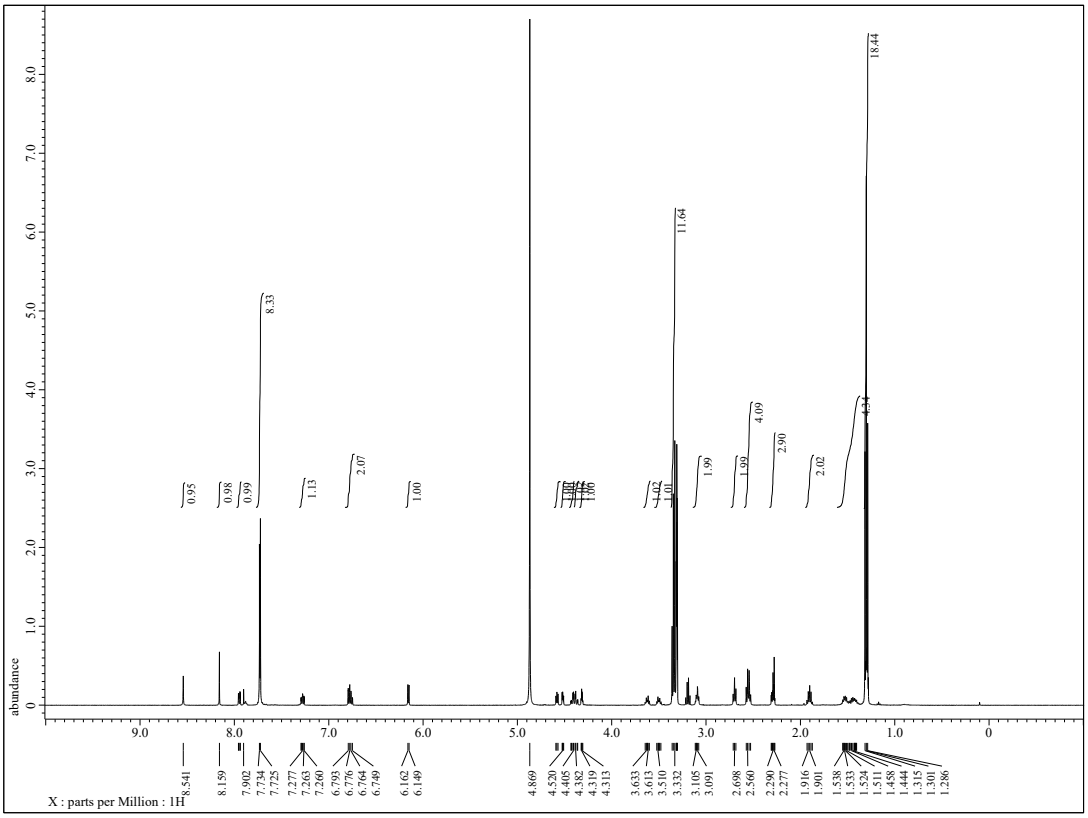
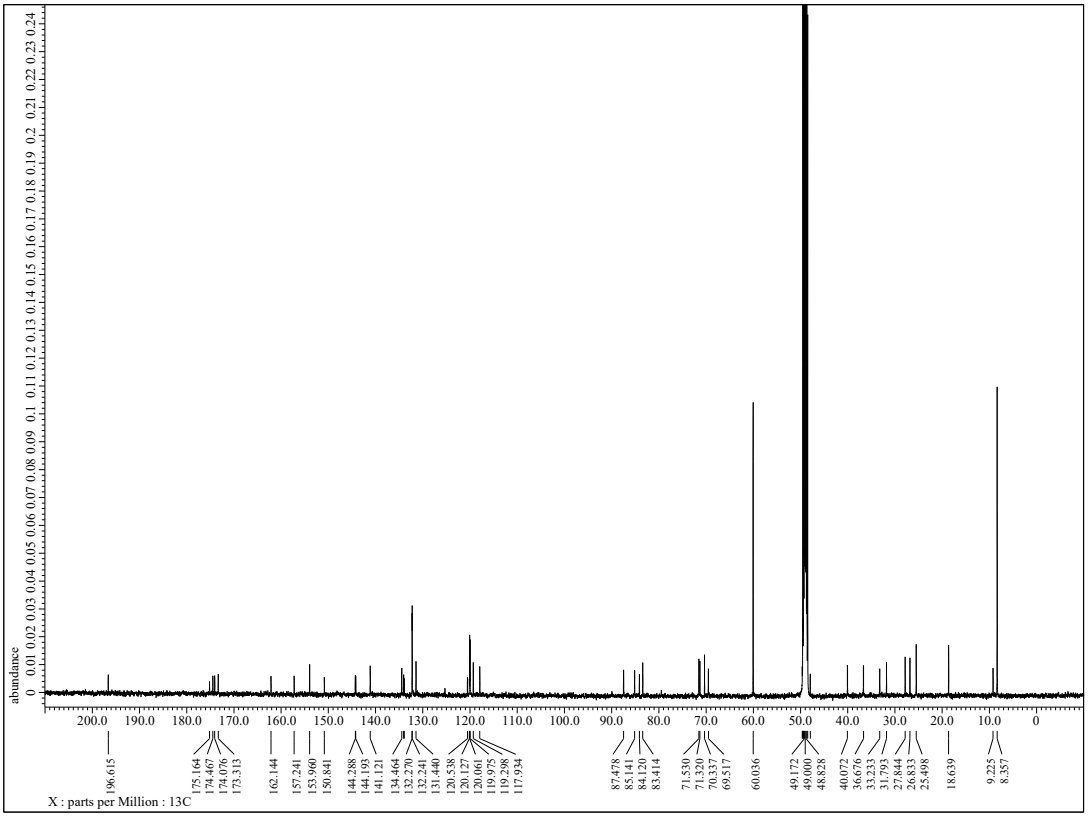
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **1** in CD_3OD





^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **2** in DMSO-d_6