Development of a Selective Activity-Based Probe for Adenylating Enzymes: Profiling MbtA Involved in Siderophore Biosynthesis from *Mycobacterium tuberculosis*

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SUPPLEMENTAL METHODS

Synthesis of Probe 6

Chemistry. All commercial reagents (Sigma-Aldrich, Fisher, Fluka, Quanta Biochem, Strem) were used as provided. Sulfamoyl chloride was prepared by the method of Heacock without recrystallization.(*1*) An anhydrous solvent dispensing system (J. C. Meyer) using 2 packed columns of neutral alumina was used for drying THF, DMF and DCM and the solvents were dispensed under Argon. Anhydrous DMA (Sigma-Aldrich) was used as provided. All reactions were performed under an inert atmosphere of dry Argon in oven-dried (150 °C) glassware. Flash chromatography was performed on an ISCO Combiflash Companion® purification system with prepacked silica gel cartridges and the indicated solvent system. ^{1}H NMR and ^{13}C NMR experiments were recorded on a Varian 600 MHz spectrometer or a Bruker 850MHz spectrometer with a 5 mm TCI Z-gradient cryoprobe. Proton chemical shifts are reported in ppm from an internal standard of residual chloroform (7.26 ppm), methanol (3.31 ppm), or dimethyl sulfoxide (2.50 ppm). Carbon chemical shifts are reported using an internal standard of residual chloroform (77.0 ppm), methanol (49.1 ppm), or dimethyl sulfoxide (39.5 ppm). Proton chemical data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad), coupling constant, and integration. High-resolution mass spectra were acquired on an Agilent TOF II TOF/MS instrument equipped with either an APCI or ESI interface.

5-(Triisopropylsilyl)pent-4-ynoic acid (7). The title compound was prepared as described.(*2*) ^{1}H NMR, ^{13}C NMR, and HRMS data agree with reported values.

***N*-[4-(4-Aminobenzoyl)phenyl]-5-(triisopropylsilyl)pent-4-ynamide (9).** To a stirring solution of **7** (470 mg, 1.8 mmol, 1.0 equiv) in DMF (18.5 mL) at 23 °C was added 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT) (1.1 g, 3.7 mmol, 2.0 equiv) and triethylamine (520 μL, 3.7 mmol, 2.0 equiv). After 10 min, **8** (590 mg, 2.8 mmol, 1.5 equiv) was added as a solid and the mixture stirred at 23 °C for 72 h. The reaction was diluted with H_2O (400 mL) and extracted with $CHCl_3$ (3 × 400 mL). The combined organic extracts were washed with saturated aqueous NaCl (400 mL), dried ($MgSO_4$), and concentrated to an orange oil. Purification by flash chromatography (step gradient $CHCl_3$ to 30% EtOAc/$CHCl_3$ to 50% EtOAc/$CHCl_3$) yielded the title compound (570 mg, 71%) as an orange solid: R_f = 0.38 (30% EtOAc/$CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.02 (s, 18H)1.03 (s, 3H), 2.63 (t, *J* = 6.6 Hz, 2H), 2.69 (t, *J* = 6.6 Hz, 2H), 6.68 (d, *J* = 6.6 Hz, 2H), 7.61 (d, *J* = 8.4 Hz, 2H), 7.67 (d, *J* = 7.2 Hz, 2H), 7.72 (d, *J* = 7.8 Hz, 2H), 8.02 (br s, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 11.3, 16.5, 18.8, 37.3, 82.5, 106.9, 114.0, 119.1, 127.9, 131.2, 133.0, 134.5, 141.1, 150.8, 170.1, 194.6; HRMS (ESI+): calculated for $C_{27}H_{37}N_2O_2Si$ $[M + H]^+$ 449.2619, found 449.2646 (error 6.0 ppm).

2′,3′-*O*-Isopropylidene-2-{*N*-[4-(4-{*N*-[5-(triisopropylsilyl)pent-4-ynoyl]amino}benzoyl)phenyl]amino}adenosine (11). A solution of $Pd_2(dba)_3$ (0.012 g, 0.011 mmol, 0.10 equiv) and BINAP (0.022 g, 0.035 mmol, 0.15 equiv) in 1,4-dioxane (1.6 mL) that had been pre-mixed for 10 min was added to a Schlenk flask containing **10** (0.100 g, 0.23 mmol, 1.0 equiv). The mixture changed from opaque, dark red to transparent yellow over 10 min. Next, **9** (0.155 g, 0.35 mmol, 1.5 equiv) and Cs_2CO_3

(0.113 g, 0.35 mmol, 1.5 equiv) were added and the resulting mixture was heated at reflux. After 2.5 h, a second portion of $Pd_2(dba)_3$ (0.012 g, 0.011 mmol, 0.10 equiv) and BINAP (0.022 g, 0.035 mmol, 0.15 equiv) in 1,4-dioxane (1.6 mL) was added to the refluxing reaction. After an additional 4 h at reflux, the reaction was cooled to room temperature, filtered, and the filtrate concentrated to a dark brown oil. The oil was redissolved in EtOAc (100 mL), washed with H_2O (3 × 50 mL), dried ($MgSO_4$), filtered, and concentrated to a yellow oil. Purification by three successive flash columns (0–10% MeOH/EtOAc) yielded the title compound (0.074 g, 43%) as a yellow amorphous solid: R_f = 0.47 (10% MeOH/EtOAc); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.03–1.04 (m, 21H), 1.38 (s, 3H), 1.63 (s, 3H), 2.66 (t, *J* = 6.6 Hz, 2H), 2.71 (t, *J* = 6.6 Hz, 2H), 3.81 (d, *J* = 11.4 Hz, 1H), 3.95 (d, *J* = 11.4 Hz, 1H), 4.48 (s, 1H), 5.07 (d, *J* = 6.0 Hz, 1H), 5.34 (t, *J* = 4.8 Hz, 1H), 5.88 (d, *J* = 4.2 Hz, 1H), 5.93 (br s, 1H), 7.29 (br s, 1H), 7.61 (d, *J* = 8.4 Hz, 2H), 7.67 (d, *J* = 7.8 Hz, 2H), 7.71 (d, *J* = 7.8 Hz, 2H), 7.74 (d, *J* = 8.4 Hz, 2H), 8.05 (s, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 11.4, 16.5, 18.8, 25.5, 27.7, 37.3, 63.2, 81.6, 82.6, 83.3, 86.4, 93.0, 106.9, 114.4, 116.1, 118.0, 119.2, 130.9, 131.4, 131.7, 134.0, 138.6, 141.4, 144.2, 150.1, 155.4, 155.9, 170.1, 194.7; HRMS (ESI+): calculated for $C_{40}H_{52}N_7O_6Si$ $[M + H]^+$ 754.3743, found 754.3748 (error 0.7 ppm).

2′,3′-*O*-Isopropylidene-2-[*N*-(4-{4-[*N*-(pent-4-ynoyl)amino]benzoyl}phenyl)amino]adenosine (12). To a solution of **11** (0.044 g, 0.06 mmol, 1.0 equiv) in THF (1 mL) was added a 1.0 M solution of tetrabutylammonium fluoride in THF (0.116 mL, 0.12 mmol, 2.0 equiv) and the mixture stirred at 23 °C for 6 h. The reaction mixture was concentrated to a dark yellow oil and purified directly by flash chromatography (0–10% MeOH/EtOAc) to yield the title compound (0.029 g, 82%)

as a yellow amorphous solid: R_f = 0.20 (10% MeOH/EtOAc); ^{1}H NMR (CD_3OD, 600 MHz) δ 1.39 (s, 3H), 1.61 (s, 3H), 2.30 (t, *J* = 3.0 Hz, 1H), 2.57 (td, *J* = 6.6, 2.4 Hz, 2H), 2.64 (t, *J* = 7.2 Hz, 2H), 3.68 (dd, *J* = 12.0, 5.4 Hz, 1H), 3.72 (dd, *J* = 12.0, 4.2 Hz, 1H), 4.33 (dd, *J* = 7.8, 4.8 Hz, 1H), 5.03 (dd, *J* = 6.0, 3.0 Hz, 1H), 5.43 (dd, *J* = 6.0, 3.0 Hz, 1H), 6.12 (d, *J* = 3.0 Hz, 1H), 7.72–7.74 (m, 6H), 7.90 (d, *J* = 9.0 Hz, 2H), 8.07 (s, 1H); ^{13}C NMR (CD_3OD, 150 MHz) δ 15.5, 25.8, 27.7, 37.1, 63.5, 70.5, 83.1, 83.6, 85.8, 88.6, 92.6, 115.3, 116.1, 118.8, 120.2, 130.9, 132.2, 132.7, 134.9, 139.5, 143.9, 147.3, 151.7, 157.6, 172.7, 196.8 (missing 1 carbon); HRMS (ESI+): calculated for $C_{31}H_{32}N_7O_6$ [M + H]$^+$ 598.2409, found 598.2415 (error 1.0 ppm).

2′,3′-*O*-Isopropylidene-2-[*N*-(4-{4-[*N*-(pent-4-ynoyl)amino]benzoyl}phenyl)amino]-5′-*O*-(sulfamoyl)adenosine (13). To a stirring solution of **12** (0.016 g, 0.025 mmol, 1 equiv) in dimethylacetamide (1.0 mL) at 0 °C was added solid sulfamoyl chloride (0.012 g, 0.10 mmol, 4 equiv). The mixture was warmed to 23 °C over approximately 3 h and then stirred for 17 h at 23 °C. The reaction was diluted with EtOAc (15 mL) and washed successively with millipore H_2O (4 × 15 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated to a yellow oil. Purification by flash chromatography (0–5% MeOH/EtOAc) afforded the title compound (0.0145 g, 80%) as an amorphous off-white solid: R_f = 0.41 (10% MeOH/EtOAc); ^{1}H NMR (CD_3OD, 600 MHz) δ 1.40 (s, 3H), 1.62 (s, 3H), 2.29 (s, 1H), 2.57 (t, *J* = 7.2 Hz, 2H), 2.64 (t, *J* = 7.2 Hz, 2H), 4.21 (dd, *J* = 10.8, 5.4 Hz, 1H), 4.35 (dd, *J* = 10.2, 4.8 Hz, 1H), 4.53 (d, *J* = 2.4 Hz, 1H), 5.16 (d, *J* = 6.0 Hz, 1H), 5.49 (d, *J* = 6.0 Hz, 1H), 6.17 (s, 1H), 7.75–7.76 (m, 6H), 7.91 (d, *J* = 8.4 Hz, 2H), 8.01 (s, 1H); ^{13}C NMR (CD_3OD, 150 MHz) δ 15.5, 25.7, 27.6, 37.1, 70.0, 70.5, 83.2, 83.6, 85.7, 85.9, 92.5, 115.5, 116.1,

119.0, 120.2, 131.0, 132.3, 132.8, 134.9, 139.5, 143.9, 147.3, 151.7, 157.67, 157.72, 172.8, 196.9; HRMS (ESI+): calculated for $C_{31}H_{33}N_8O_8S$ $[M + H]^+$ 677.2137, found 677.2127 (error 1.5 ppm).

5'-*O*-[*N*-(2-Hydroxybenzoyl)sulfamoyl]-2-[*N*-(4-{4-[*N*-(pent-4-ynoyl)amino]benzoyl}phenyl)amino]adenosine Triethylammonium Salt (6). To a solution of **13** (14.5 mg, 0.02 mmol, 1.0 equiv) in DMF (1.0 mL) was added **14**[24] (9.0 mg, 0.03 mmol, 1.5 equiv) and Cs_2CO_3 (21 mg, 0.06 mmol, 3.0 equiv) and the reaction stirred for 20 h at 23 °C. The reaction mixture was filtered and the filtrate was concentrated *in vacuo* to a colorless residue. A solution of 80% aqueous TFA (1.0 mL) was added the crude residue and the mixture was stirred at 23 ° C for 2 h, then concentrated under reduced pressure to remove all traces of TFA. Purification by preparative HPLC using a Phenomenex Gemini 10 μm C18 110Å (250 × 21.2 mm) column at a flow rate of 30 mL/min and a gradient of 5–80% MeCN in 20 mM aqueous triethylammonium bicarbonate over 30 min, followed by 80% MeCN for 5 min. The retention time of the product was 16.7 minutes (k' = 4.9) and the appropriate fractions were pooled and lyophilized to afford the title compound (5.0 mg, 27%) as light yellow solid: ^{1}H NMR (CD_3OD, 600 MHz) δ 1.20 (t, J = 7.2 Hz, 9H), 2.30 (s, 1H), 2.57 (t, J = 7.2 Hz, 2H), 2.64 (t, J = 7.2 Hz, 2H), 2.94 (d, J = 3.0 Hz, 6H), 4.31 (d, J = 2.4 Hz, 1H), 4.37 (d, J = 11.4 Hz, 1H), 4.42–4.45 (br m, 1H), 4.47 (dd, J = 11.4, 1.8 Hz, 1H), 4.82–4.83 (m, 1H), 6.06 (d, J = 6.0 Hz, 1H), 6.75–6.79 (m, 2H), 7.27 (t, J = 8.4 Hz, 1H), 7.75–7.77 (m, 6H), 7.93–7.96 (m, 3H), 8.26 (s, 1H); ^{13}C NMR (CD_3OD, 213 MHz) δ 8.7, 15.7, 37.2, 47.8, 69.9, 70.6, 72.9, 75.9, 83.7, 84.7, 89.4, 115.9, 118.1, 118.6, 119.5, 120.3, 120.9, 130.8, 131.7, 132.3, 133.0, 134.6, 135.1, 139.5, 143.9, 147.7, 152.8, 157.6, 157.8, 162.3, 172.9, 175.3,

197.1; HRMS (ESI–): calculated for $C_{35}H_{31}N_8O_{10}S$ $[M - H]^-$ 755.1889, found 755.1916 (error 3.6 ppm).

***Mycobacterium tuberculosis* H37Rv MIC assay.** The broth microdilution assay was used to determine minimum inhibitory concentrations (MIC) against *Mtb* H37Rv.(*3*) Assays were performed in quadruplicated in GAST media with and without Fe^{3+} using probe **6** from a DMSO stock solution. Control wells contained either an equal volume of DMSO or isoniazid as a positive control. Cells were grown to an OD_{650} of 0.2, diluted 1000-fold in either GAST or GAST/Fe media. 50 μL of the cell suspension (cell density of 10^4 cells/well) was added to each well in a 96-well plate containing **6** in a two-fold dilution series across the wells. Plates were incubated at 37 °C and growth was monitored at 7 and 14 days. The MIC was determined as the lowest concentration of compound that resulted in complete inhibition of growth.

% Labeling Efficiency. In order to determine the efficiency of photolabeling with probe **2**, a BSA-TAMRA conjugate was first synthesized. BSA (100 mg, 1.5 μmol) was dissolved in 10 mM NaH_2PO_4, pH 9.0 buffer (1.0 mL). Propargyl isothiocyanate (2.9 μL, 30 μmol, Sigma) and DMSO (100 μL) were added to the BSA solution, and this mixture was rotated at room temperature for 16 h. Excess propargyl isothiocyanate was removed using a Nap-5 column (GE Healthcare) with 10 mM HEPES, pH 8.0 as the elution buffer to provide BSA-alkyne. For conjugation of TAMRA onto BSA, BSA alkyne (8.0 nmol) was reacted with TAMRA-N_3 (100 nmol), TCEP (1.0 μmol), TBTA ligand (100 nmol) and $CuSO_4$ in a final reaction volume of 1.0 mL in phosphate buffered saline (PBS). After 1 h at rt, the reaction was diluted to 2.5 mL with PBS, and excess TAMRA-N_3 was removed using a PD-10 columng (GE Healthcare) with PBS as the elution buffer. The

concentration of BSA-TAMRA was determined using the extinction coefficient of TAMRA (ε_{540} = 95,000 M^{-1} cm^{-1}). Photo-labeling experiments were performed in a final volume of 25 μL in phosphate buffered saline (PBS). MbtA (final concentration of 1.0 μM) was incubated with ABP **2** (0.25 μL of a 100 μM DMSO stock) to provide a final probe concentration of 1.0 μM. After 10 min at rt, samples were irradiated at 365 nm for 30 min on ice. To initiate the click reaction, TAMRA-N_3, TCEP, TBTA ligand, and $CuSO_4$ were added to provide final concentrations of 100 μM, 1.0 mM, 100 μM, and 1 mM, respectively. After 1 h at rt, 25 μL of 2× SDS gel loading buffer was added and the samples were heated at 95°C for 5 min. Samples (15 μL) were separated by 1D SDS-PAGE (4–20% Tris-HCl Rgels, Biorad) and fluorescent proteins were visualized by in-gel fluorescence scanning using a FMBIO III flatbed scanner. Varying concentrations of BSA-TAMRA were also run on the same gel. Band intensities were measured as band volumes using the software provided with the scanner (Image Analysis, 3.0) and standard curve of band volume versus BSA-TAMRA was constructed and used to determine the % labeling efficiency.

Sal-AMS dose response curve. MbtA (final concentration of 1.0 μM) was preincubated with either DMSO (0.25 μL) or Sal-AMS (**4**) (0.25 μL of 100× stock in DMSO) for 10 min at room temperature. ABP **6** (0.25 μL of a 100 μM DMSO stock) was added to provide a final probe concentration of 1.0 μM. After 10 min at rt, the samples were subjected to UV photolysis, reacted with TAMRA-N_3, and separated by gel electrophoresis as described above. Fluorescent proteins were imaged, and the band intensities were calculated as band volumes using the software provided with the fluorescent scanner (Image Analysis 3.0, Hitachi MiraiBio Group, South San Francisco,

CA). The data were converted to % bound by dividing the band volume at a given [Sal-AMS] by the band volume of the DMSO control. The date were then fit by nonlinear regression analysis to the 'One Site Competion' equation using GraphPad Prism version 4.0c to obtain the IC_{50} value for Sal-AMS.

***E. coli* Cell Lysate Labeling Studies.** *E. coli* cultures harboring the pET-SUMO-MbtA and pGRO7 plasmids(*4*), were grown overnight at 37 °C in LB media (5.0 mL) supplemented with kanamycin (50 μg/mL) and chloramphenicol (25 μg/mL). The overnight culture (100 μL) was added to LB (5.0 mL) containing kanamycin, chloramphenicol and $MgCl_2$ (10 mM). One culture (5.0 mL) also contained arabinose (0.5 mg/mL). After growing both cultures to an OD_{600} = 0.6 at 37 °C, IPTG was added to the culture containing arabinose to a final concentration of 0.4 mM. After shaking at 30 °C for 4 h, the cultures were centrifuged, and the pellet was washed 2× with PBS and frozen at –80 °C overnight. For overexpression of EntE, *E. coli* cultures harboring the pET15b-EntE plasmid(*5*) were grown overnight at 37 °C in LB media (5.0 mL) supplemented with ampicillin (100 μg/mL). The overnight culture (150 μL) was added to two LB (5.0 mL) cultures containing ampicillin. The two cultures were grown to an OD_{600} = 0.6 at 37 °C, after which IPTG was added to a final concentration of 0.5 mM to one of the cultures. Both cultures were grown overnight with shaking at 18 °C. The cultures were centrifuged, and the pellet was washed 2× with PBS and frozen at –80 °C overnight. Pellets were resuspended in PBS (300 μL) and sonicated. The lysate was centrifuged 5 min at 14,000 rpm and the pellet was discarded. The protein concentration was measured by the Bradford Assay (Bio-Rad) using BSA as a standard and cell lysates were diluted to 1.0 mg/mL with PBS. Cell lysate (24.5 μL) was preincubated in a 96-well

plate with either DMSO (0.25 μL) or 500 μM Sal-AMS (**4**) (0.25 μL of a 50 mM DMSO stock) at room temperature for 10 min. ABP **6** (0.25 μL of a 100 μM DMSO stock) was added to provide a final probe concentration of 1.0 μM. After 10 min at room temperature, the reactions were subjected to UV photolysis, conjugation with TAMRA-N_3, separation and visualization as described above.

***M. smegmatis* Cell Lysate Labeling Studies.** All bacterial fermentation was performed in low iron GAST medium (2.0 g/L ammonium chloride, 1.0 g/L L-alanine, 0.3 g.L Bacto Casitone (Difco), 4.0 g/L dibasic potassium phosphate, 2.0 g/L citric acid, 1.2 g/L magnesium chloride hexahydrate, 0.6 g/L potassium sulfate, 0.72 g/L sodium hydroxide, 12.62 g/L glycerol. Lysates were generated by inoculating 4 mL GAST medium supplemented with 1 μM $FeCl_3$ with *Mycobacterium smegmatis* $mc^2$4517. The cultures were grown for 48 h at 37 °C and 100 μL of the starter culture was inoculated into 200 mL GAST medium. After growing for an additional 56 h at 37 °C the cells were harvested by centrifugation and frozen at -80 °C. The cell pellet was thawed in 5 mL PBS and sonicated 5 times 30 s on ice using a Branson Sonifier 250 set to power 4 and 100% duty cycle with 1 min between pulses. The protein concentration of the lysate was measured using the Bradford protein assay and adjusted to 2 mg/mL with PBS for affinity labeling.

Protein labeling was performed at a final volume of 50 μL in a 96 well UV clear plate (corning 3679). Lysate (49 μL) was incubated with 0.5 μL DMSO or 50 mM Sal-AMS in triplicate for 2.5 h on ice followed by 20 min at 25 °C. Affinity probe (0.25 μL of a 20 μM solution) was added to each reaction for a final concentration of 100 nM and allowed to incubate for an additional 10 min. The reaction plate was set in ice under a UV lamp

set to 365 nm for 30 min. After irradiation 7 μL of click reaction master mix (7 mM TCEP, 0.7 mM ligand, 7 mM $CuSO_4$, 0.7 mM biotin azide) was added to each well and allowed to conjugate for 1 h at 25 °C. The proteins were precipitated by the addition of 250 μL ice-cold acetone and place at -80 °C overnight. The precipitated proteins were pelleted at 16,000 × g for 5 min at 4 °C and subsequently washed 2 times with 500 μL ice-cold methanol and allowed to dry for 10 min at 25 °C. The pellets were resuspended in 100 μL 1.2% SDS in PBS in a sonicating water bath for 4 min followed by heating 5 min at 90 °C. The reactions were diluted to 0.4% SDS with PBS and 2.5 μL of washed streptavidin beads (washed 3 × with 50 volumes of PBS) were added to each tube. The beads were incubated with the labeled protein for 2 hours at 25 °C and then spun at 1,400 × g for 3 min at 25 °C (all subsequent washes used the same centrifugation conditions). The beads were washed 1 × 200 μL 0.4% SDS in PBS, 1 × 200 μL PBS, 3 × 200 μL water. Beads were resuspended in 50 μL 6 M urea in PBS and 2.5 μL 200 mM freshly prepared TCEP. The reactions were incubated at 65 °C for 15 min and allowed to slowly cool to 35 °C. The reactions were held at this temperature with gentle agitation for 30 min after the addition of 2.5 μL 400 mM iodoacetamide. The reactions were then diluted with 500 μL PBS and centrifuged 1,400 × g for 3 min and the supernatant was discarded.

The proteins were digested at 37 °C with gentle agitation after the addition of 10.3 μL trypsin mixture (1.9 M urea, 1.9 mM $CaCl_2$, 9.7 ng/μL trypsin). After trypsin digest the streptavidin beads were removed by centrifugation at 1,400 × g for 3 min. The peptide solution was adjusted to 50 μL with water and then desalted after acidification with TFA using stage tips.(*6*) The peptides loaded onto a 12 cm × 75 μm fused silica pulled-tip capillary column packed in-house with Magic C18AQ, 5 μm, 200 Å resin (Michrom

BioResources, Inc.) using load solvent (98/2/0.01, water/acetonitrile/formic acid) at a flow rate of 800 nL/min. Peptides were eluted from the capillary column using a gradient of 10–40% acetonitrile containing 0.1% formic acid over 40 min at a flow rate of 320 nL/min. The column was housed in a nanospray source coupled directly with a Velos Orbitrap mass spectrometer (ThermoFisher Scientific). The spray voltage was 2.0 kV and the capillary was heated to 260 °C. The mass spectrometer was set to acquire a full scan (360–1800 m/z), and the 6 most intense ions from this full scan were fragmented for MS/MS analysis. Data were acquired using Xcalibur software v2.1.0. MS/MS spectra were searched against *Mycobacterium smegmatis* str. MC2 155 (species ID 246196) protein sequences from NCBI. Sequest v27.0 was used for database searches and the following search parameters were used: fixed modification of carbamidomethyl cysteine; variable modifixation of methionine oxidations; partial trypsin specificity; one missed trypsin cleavage site; 100 ppm precursor tolerance; monoisotopic mass and 0.8 Da product ion tolerance monoisotopic mass. Results were analyzed using Scaffold v3.3.1 (Proteome Software) to provide spectral counts.

SUPPLEMENTAL FIGURES

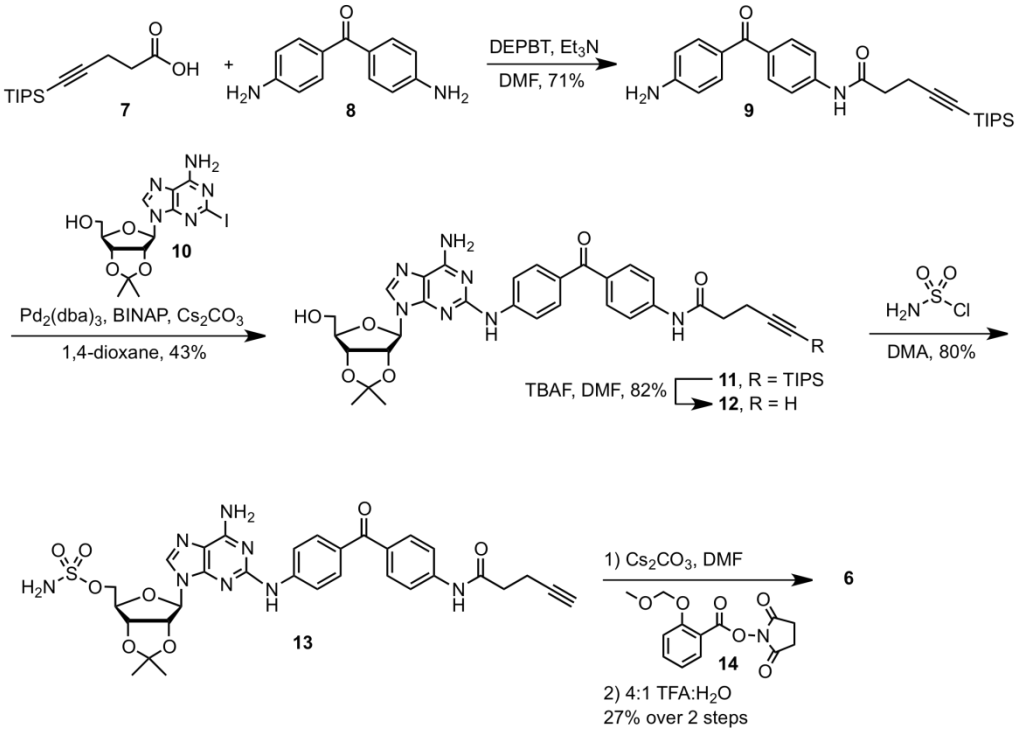

Figure S1. Synthesis of ABP **6**.

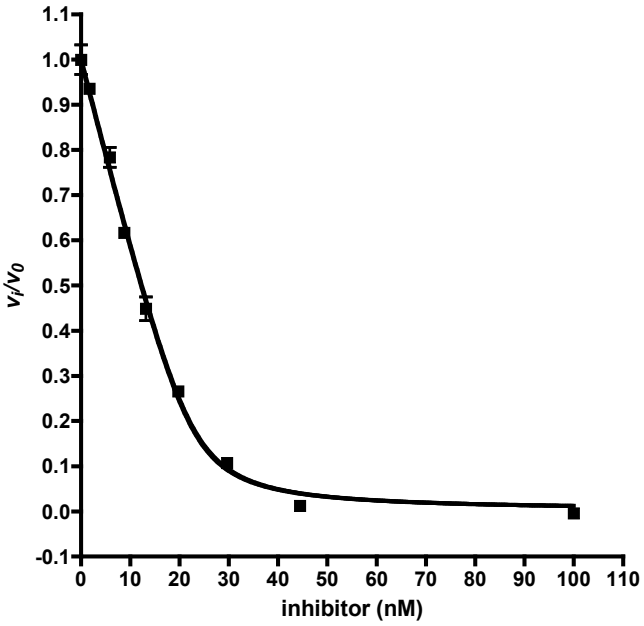

Figure S2. Dose-response of fractional initial velocity of γ-[^{32}P]-ATP formation by MbtA in the presence of varying concentrations of ABP **2**. The curve represents the best nonlinear fit of the data to the Morrison equation. Data points represent the mean with standard error of duplicate experiments.

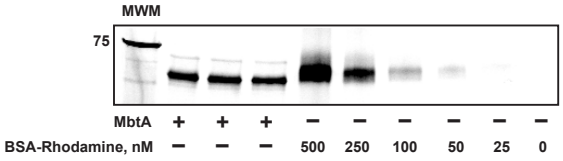

Figure S3. % labeling efficiency of MbtA photoaffinity labeling using ABP **2**.

References.

1. Heacock, D., Forsyth, C. J., Shiba, K., and Musier-Forsyth, K. (1996) Synthesis and aminoacyl-tRNA synthetase inhibitory activity of prolyl adenylate analogs, *Bioorg. Chem. 24*, 273–289.

2. Dunetz, J. R., and Danheiser, R. L. (2005) Synthesis of highly substituted indolines and indoles via intramolecular [4 + 2] cycloaddition of ynamides and conjugated enynes, *J. Am. Chem. Soc. 127*, 5776–5777.

3. De Voss, J. J., Rutter, K., Schroeder, B. G., Su, H., Zhu, Y., and Barry, C. E., 3rd. (2000) The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages, *Proc. Natl. Acad. Sci. USA 97*, 1252–1257.

4. Somu, R. V., Wilson, D. J., Bennett, E. M., Boshoff, H. I., Celia, L., Beck, B. J., Barry, C. E., 3rd, and Aldrich, C. C. (2006) Antitubercular nucleosides that inhibit siderophore biosynthesis: SAR of the glycosyl domain., *J. Med. Chem. 49*, 7623–7635.

5. Drake, E. J., Nicolai, D. A., and Gulick, A. M. (2006) Structure of the EntB multidomain nonribosomal peptide synthetase and functional analysis of its interaction with the EntE adenylation domain, *Chem. Biol. 13*, 409–419.

6. Rappsilber, J., Ishihama, Y., and Mann, M. (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics, *Anal Chem 75*, 663-670.