

Small Molecule Inhibition of Siderophore Biosynthesis in
Mycobacterium tuberculosis* and *Yersinia pestis

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Supplementary Methods

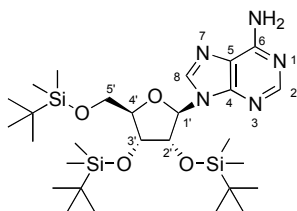
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I. Synthetic Materials and Methods

Reagents were obtained from Aldrich Chemical (www.sigma-aldrich.com) or Acros Organics (www.fishersci.com) and used without further purification. Optima grade solvents were obtained from Fisher Scientific (www.fishersci.com), degassed with Ar, and purified on a solvent drying system as described elsewhere¹. Reactions were performed in flame-dried glassware under positive Ar pressure with magnetic stirring. Cold baths were generated as follows: 0 °C, wet ice/water; -78 °C, dry ice/acetone. TLC was performed on 0.25 mm E. Merck silica gel 60 F254 plates and visualized under UV light (254 nm) or by staining with potassium permanganate (KMnO₄) or cerium ammonium molybdenate (CAM). Flash chromatography was performed on E. Merck 230–400 mesh silica gel 60. NMR spectra were recorded on Bruker DRX500

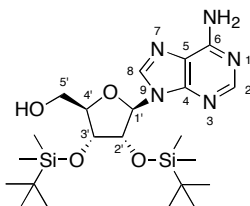
or AMX400 instruments at 24 °C. Chemical shifts are expressed in ppm relative to TMS (^1H , 0 ppm), CDCl_3 (^{13}C , 77.0 ppm), or DMSO-d_6 (^1H , 2.50 ppm; ^{13}C , 39.51 ppm); coupling constants are expressed in Hz. Mass spectra were obtained at the MSKCC Analytical Core Facility on a PE SCIEX API 100 mass spectrometer by electrospray (ESI) ionization.

II. Synthesis of Salicyl-AMS (2) and AMS (8)



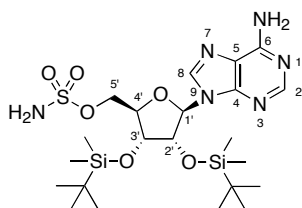
2',3',4'-O,O,O,-Tris(*t*-butyldimethylsilyl)adenosine (9). *tert*-Butyldimethylsilyl chloride (16.3 g, 105 mmol, 3.5 equiv) in anhyd DMF (20 mL) was added to a solution of (–)-adenosine **5** (8.09 g, 30 mmol) and imidazole (18.4 g, 270 mmol, 9.0 equiv) in anhyd DMF (40 mL). The white slurry turned to a colorless solution in 10 min. The resulting clear solution was stirred overnight. The reaction mixture was then diluted with CH_2Cl_2 , washed with 3 × satd aq NaHCO_3 , dried (MgSO_4), filtered, and concentrated. The residue was purified by column chromatography. (elution with 3:1 hexane/EtOAc) to yield **9** as white solid (11.5 g, 63%).

TLC: R_f 0.29 (1:1 hexanes/EtOAc). **$^1\text{H-NMR}$** (400 MHz, CDCl_3): δ 8.33 (s, 1H), 8.15 (s, 1H), 6.02 (d, 1H, $J = 5.2$), 4.69 (t, 1H, $J = 4.9$), 4.32 (t, 1H, $J = 3.8$), 4.12 (m, 1H), 4.03 (dd, 1H, $J = 11.4, 4.4$), 3.79 (dd, 1H, $J = 11.4, 2.8$), 0.95 (s, 9H), 0.93 (s, 9H), 0.79 (s, 9H), 0.13 (s, 3H), 0.12 (s, 3H), 0.10 (s, 3H), 0.09 (s, 3H), -0.04 (s, 3H), -0.23 (s, 3H). **$^{13}\text{C-NMR}$** (125 MHz, CDCl_3): δ 155.7, 153.1, 150.1, 139.8, 120.3, 88.5, 85.7, 75.9, 72.2, 62.7, 26.3, 26.0, 25.6, 18.7, 18.3, 18.1, -4.2 , -4.5 , -4.5 , -4.9 , -5.2 , -5.2 . **ESI-MS** m/z : (pos) 610.3 $[\text{M}+\text{H}]^+$, 632.3 $[\text{M}+\text{Na}]^+$; (neg) 608.4 $[\text{M}-\text{H}]^-$, 644.4 $[\text{M}+\text{Cl}]^-$.



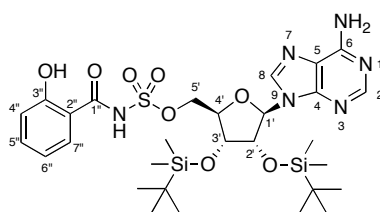
2',3'-O,O-Bis(*t*-butyldimethylsilyl)adenosine (6). A mixture of TFA (1.75 mL) and H_2O (1.75 mL) was added to a cooled ($0\text{ }^\circ\text{C}$) solution of tris(TBS)adenosine **9** (350 mg, 574 μmol) in THF (7 mL). The reaction mixture was stirred at $0\text{ }^\circ\text{C}$. After 5 h, aq NaHCO_3 was added at $0\text{ }^\circ\text{C}$. The aqueous layer was extracted 3 \times with EtOAc, washed with H_2O and brine, dried (MgSO_4), filtered, and concentrated. The residue was purified by flash chromatography (elution with 1:1 hexane/EtOAc) to afford **6** as a white solid (251.6 mg, 88%).

TLC: R_f 0.22 (EtOAc), 0.37 (9:1 CH₂Cl₂/MeOH). **¹H-NMR** (400 MHz, CDCl₃): δ 8.35 (s, 1H), 7.83 (s, 1H), 6.71 (dd, 1H, $J = 12.2, 1.8$), 5.78 (d, 1H, $J = 7.9$), 5.56 (br s, 2H), 5.05 (dd, 1H, $J = 7.9, 4.6$), 4.33 (d, 1H, $J = 4.6$), 4.16 (s, 1H), 3.94 (d, 1H, $J = 13.0$), 3.70 (t, 1H, $J = 12.7$), 0.95 (s, 9H), 0.75 (s, 9H), 0.13 (s, 3H), 0.12 (s, 3H), -0.14 (s, 3H), -0.62 (s, 3H). **¹³C-NMR** (125 MHz, DMSO-*d*₆): δ 156.2, 152.3, 148.9, 140.2, 119.5, 87.6, 87.1, 74.1, 73.0, 61.4, 48.6, 25.7, 25.4, 17.8, 17.5, -4.7, -4.8, -4.9, -5.8. **ESI-MS** m/z : (pos) 496.3 [M+H]⁺, 518.2 [M+Na]⁺; (neg) 530.2 [M+Cl]⁻.



2',3'-O,O-Bis(*t*-butyldimethylsilyl)-5'-O-sulfamoyl-adenosine (7). Bis(tributyltin) oxide (7.43 mL, 14.0 mmol, 3.5 equiv) was added dropwise to a solution of bis(TBS)adenosine **6** (1.98 g, 4.0 mmol) in anhyd benzene (130 mL). The resulting white suspension was refluxed with stirring. After 2 h, the reaction mixture was cooled to 5 °C. Sulfamoyl chloride (2.08 g, 18.0 mmol, 4.5 equiv) in dioxane (65 mL) was added dropwise to the reaction mixture, stirred for an additional 30 min at 5 °C. The solvent was then removed *in vacuo*. The residue was rinsed 3 × with hot (40 °C) hexane. The solid was washed with 1 N NH₃ solution in MeOH. The crude material was purified by column chromatography (elution with 15:1 CH₂Cl₂/MeOH) to yield **7** as white solid (2.2 g, 96%)

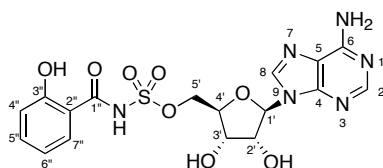
TLC: R_f 0.2 (9:1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$). **$^1\text{H-NMR}$** (400 MHz, DMSO-d_6): δ 8.36 (s, 1H), 8.16 (s, 1H), 7.66 (br s, 2H), 7.33 (br s, 2H), 5.95 (d, 1H, $J = 6.6$), 4.97 (dd, 1H, $J = 6.6$, 4.3), 4.38-4.41 (m, 2H), 4.31 (dd, 1H, $J = 10.8$, 5.8), 4.18 (m, 1H), 0.93 (s, 9H), 0.71 (s, 9H), 0.15 (s, 3H), 0.12 (s, 3H), -0.09 (s, 3H), -0.38 (s, 3H). **$^{13}\text{C-NMR}$** (125 MHz, DMSO-d_6): δ 156.1, 152.7, 149.4, 139.8, 119.3, 87.0, 82.8, 73.7, 72.4, 68.0, 25.7, 25.4, 17.7, 17.5, -4.7 , -4.8 , -4.9 , -5.6 . **ESI-MS** m/z : (pos) 575.3 $[\text{M}+\text{H}]^+$; (neg) 573.2 $[\text{M}-\text{H}]^-$, 609.2 $[\text{M}+\text{Cl}]^-$.



2',3'-O,O-Bis(*t*-butyldimethylsilyl)-5'-O-(*N*-salicylsulfamoyl)adenosine (10). A solution of salicylic acid (66 mg, 477 μmol , 3.0 equiv) and 1,1'-carbonyldiimidazole (93 mg, 575 μmol , 3.6 equiv) in anhyd acetonitrile (4 mL) was stirred at 60 °C for 2 h under argon atmosphere. The reaction mixture was cooled to rt. A mixture of bis(TBS)sulfamoyl adenosine **7** (92 mg, 160 μmol) and DBU (36 μL , 241 μmol , 1.5 equiv) was then added dropwise to the reaction mixture. The resulting yellow solution was again stirred at 60 °C. After an additional 30 min, the reaction mixture was diluted with H_2O . The aqueous layer was extracted 3 \times with EtOAc, washed with 1 N HCl, satd

aq NaHCO₃, and brine, dried (MgSO₄), filtered, and concentrated. The residue was purified by flash column chromatography (elution with 9:1 CH₂Cl₂/MeOH → 5:1 CH₂Cl₂/MeOH) to afford **10** as a white solid (89 mg, 80%).

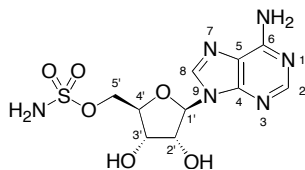
TLC: *R_f* 0.21 (17:3 EtOAc/MeOH). **¹H-NMR** (400 MHz, DMSO-d₆): δ 8.45 (s, 1H), 8.09 (s, 1H), 7.84 (dd, 1H, *J* = 7.8, 1.8), 7.28 (br s, 2H), 7.26 (m, 1H), 6.76-6.71 (m, 2H), 5.95 (d, 1H, *J* = 7.3), 4.92 (dd, 1H, *J* = 4.3, 7.3), 4.36 (d, 1H, *J* = 4.3), 4.29 (dd, 1H, *J* = 4.8, 11), 4.23-4.16 (m, 2H), 0.9 (s, 9H), 0.65 (s, 9H), 0.1 (s, 3H), 0.09 (s, 3H), -0.12 (s, 3H), -0.46 (s, 3H). **¹³C-NMR** (125 MHz, DMSO-d₆): δ 171.1, 160.7, 156.0, 152.6, 149.7, 139.4, 132.6, 129.9, 119.8, 118.9, 117.4, 116.5, 86.2, 83.9, 74.5, 73.2, 67.4, 25.7, 25.4, 17.7, 17.4, -4.8, -4.9, -5.8, -5.9. **ESI-MS** *m/z*: (pos) 695.3 [M+H]⁺, 717.2 [M+Na]⁺; (neg) 693.2 [M-H]⁻.



5'-O-(N-Salicylsulfamoyl)adenosine (Salicyl-AMS, 2). TBAF (1.0 M in THF, 100 μL, 0.1 mmol, 2.5 equiv) was added dropwise to a solution of bis(TBS)salicylsulfamoyl-adenosine **10** (28 mg, 0.04 mmol) in anhyd THF (1.6 mL). The reaction mixture was stirred at rt for 30 min. The solvent was then evaporated. The residue was

purified by flash column chromatography (elution with 5:1 EtOAc/MeOH) to afford **2** as a white solid (14.8 mg, 79%).

TLC: R_f 0.33 (5:1 EtOAc/MeOH). **$^1\text{H-NMR}$** (400 MHz, DMSO- d_6): δ 8.40 (s, 1H), 8.12 (s, 1H), 7.81 (dd, 1H, $J = 7.7, 1.4$), 7.28-7.24 (m, 3H), 6.75 (d, 1H, $J = 7.7$), 6.74 (t, 1H, $J = 7.7$), 5.92 (d, 1H, $J = 6.4$), 5.49 (d, 1H, $J = 6.1$), 5.35 (d, 1H, $J = 4.8$), 4.62 (dd, 1H, $J = 6.4, 5.8$), 4.25-4.08 (m, 4H). **$^{13}\text{C-NMR}$** (125 MHz, DMSO- d_6): δ 171.1, 160.7, 156.0, 152.6, 149.6, 139.3, 132.6, 129.9, 119.9, 118.9, 117.5, 116.6, 86.9, 82.5, 73.5, 70.8, 68.1. **ESI-MS** m/z : (pos) 467 $[\text{M}+\text{H}]^+$, 489 $[\text{M}+\text{Na}]^+$; (neg) 465 $[\text{M}-\text{H}]^-$.



5'-O-Sulfamoyladenine (AMS, **8).** TBAF (1.0 M in THF, 250 μL , 0.25 mmol, 2.5 equiv) was added dropwise to a solution of bis(TBS)sulfamoyladenine **7** (57.4 mg, 0.1 mmol) in anhyd THF (4.0 mL). The reaction mixture was stirred at rt for 30 min. The solvent was then evaporated. The residue was purified by column chromatography (elution with 5:1 EtOAc/MeOH) to afford **8** as a white solid (31 mg, 89%).

TLC: R_f 0.18 (6:1 EtOAc/MeOH). **$^1\text{H-NMR}$** (500 MHz, DMSO- d_6): δ 8.30 (s, 1H), 8.15 (s, 1H), 7.60 (br s, 2H), 7.31 (br s, 2H), 5.93 (d, 1H, $J = 5.3$), 5.62 (d, 1H, $J = 6.0$),

5.44 (d, 1H, $J = 5.4$), 4.63 (app q, 1H, $J = 5.4$), 4.29 (dd, 1H, $J = 10.7, 3.8$), 4.23 (app q, 1H, $J = 4.9$), 4.19 (dd, 1H, $J = 10.6, J = 6.0$), 4.14 (m, 1H). $^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6): δ 156.1, 152.7, 149.4, 139.4, 119.1, 87.5, 81.5, 73.0, 70.3, 68.7. **ESI-MS** m/z: (pos) 347.1 $[\text{M}+\text{H}]^+$, 368.9 $[\text{M}+\text{Na}]^+$; (neg) 344.9 $[\text{M}-\text{H}]^-$, 380.9 $[\text{M}+\text{Cl}]^-$.

III. Protein Production

MbtA and YbtE were expressed in *E. coli* BL21(DE3) as IPTG-inducible N-terminally His₆Smt3-tagged proteins using plasmids pSmt3MbtA and pSmt3YbtE, respectively. Plasmid pSmt3MbtA was constructed by inserting the MbtA coding segment as a *Bam*HI–*Hind*III fragment into pSMT3². The MbtA fragment was PCR amplified with primers JfmbtaF (5'-aaggaggatccatgccaccgaaggcggcag-3') and JfmbtaR (5'-ttgacaagcttcaatggcagcgcctgggtcg-3') from plasmid pMBTA³. Plasmid pSmt3YbtE was constructed by cloning the YbtE coding region as a *Bam*HI–*Hind*III fragment into pSMT3. This fragment was PCR amplified with primers JfybteF (5'-aaaggggatccatgaattcttcttgaatc-3') and JfybteR (5'-ggtttaagcttattgggcagaatggcgataac-3') from genomic DNA. Cloning was carried out using standard methods⁴. For protein production, the strains expressing the tagged proteins were cultivated in LB broth⁴ (5 L) containing kanamycin (30 $\mu\text{g}/\text{ml}$) with orbital shaking (250 rpm) at 37 °C. Cultures ($\text{OD}_{600} = 0.6$) were induced with 0.5 mM IPTG for 4 h before the cells were harvested and resuspended in lysis buffer (75 ml, 50 mM Tris·HCl pH 8, 0.5 M NaCl, 20% sucrose, 1 mM BME, 1 mM PMSF, 10 mM imidazole, 0.1% IGEPAL). Resuspended cells were disrupted using a French pressure cell and cellular debris was

removed from the lysates by ultracentrifugation. The tagged proteins were purified by nickel column chromatography using Ni-NTA Superflow resin according to the manufacturer's instructions (Qiagen). Purified proteins were dialyzed against a solution of 0.2 M NaCl, 25 mM Tris-HCl (pH 8.0), 2 mM DTT, and 5% glycerol. Tagged MbtA and YbtE were treated with SUMO protease (Invitrogen) to remove the tag as reported⁵. Tag-free MbtA and YbtE were purified by gel filtration using Superdex 200 resin according to the manufacturer's instructions (Amersham Biosciences). Protein samples were concentrated (≤ 10 mg/ml) and stored at -80 °C. Recombinant C-terminally His₆-tagged PchD (PchD-H6), C-terminally His₆-tagged *Y. pestis* ArCP domain (ypArCP-H6), and phosphopantetheinyl transferase Sfp were purified as reported⁶⁻⁸.

IV. Adenylation assay and data analysis

Adenylation was measured with an ATP-[³²P]-pyrophosphate (PPi) exchange assay as reported^{3,6,8}. Reactions (100 μ l) were initiated by addition of the domain salicylation enzyme. Exchange reactions used to obtain dose-response curves (**Figs. 2a–c**) each contained: 75 mM Tris-HCl (pH 8.8); 10 mM MgCl₂; 2 mM DTT; 5% glycerol; 1 mM sodium [³²P]-PPi (5 Ci/mol, PerkinElmer); 10 mM ATP ($\approx 60 \times K_m^{\text{ATP}}$); 250, 500, and 140 μ M salicylate ($\approx 50 \times K_m^{\text{Sal}}$) in reactions with MbtA, YbtE, and PchD, respectively; 20 nM domain salicylation enzyme; and inhibitor added in DMSO (1% of reaction volume) at the concentrations indicated. Reactions were incubated at 37 °C for 30 min for MbtA and for 15 min for YbtE and PchD. Data analysis was done using

methods for TBIs as described⁹. The dose-response data sets were fitted to the

$$\text{Morrison equation, } \frac{v_i}{v_c} = 1 - \frac{([E] + [I] + K_i^{\text{app}}) - \sqrt{([E] + [I] + K_i^{\text{app}})^2 - 4[E][I]}}{2[E]}, \text{ Eq. (1),}$$

where v_i and v_c are the activities measured in inhibitor-containing reactions and DMSO-containing (1%) controls, respectively. IC_{50} values were calculated with the equation

$$IC_{50} = \frac{1}{2}[E] + K_i^{\text{app}}, \text{ Eq. (2), and using the } K_i^{\text{app}} \text{ derived from the Eq (1) curve fit. In}$$

experiments to determine IC_{50} values at different YbtE concentrations (**Fig. 2d**), each

reaction had the composition noted above except that ATP and salicylate were both at 1

mM, the inhibitor was included in a 0–200 nM range, and YbtE was added at the

concentrations indicated. Reactions were incubated at 37 °C for 15 min. The dose-

response data were fitted to Eq. (1) and the IC_{50} values were calculated with Eq. (2).

In the experiments to determine K_i^{app} values (**Figs. 2e and f**), dose-response curves were

determined with the inhibitor in a 0–150 nM range, YbtE at 20 nM, and either ATP

fixed at 10 mM and salicylate at 1.3, 2.6, 5.2, 10.4, 20.8, 50, and 250 μM , or salicylate

fixed at 1 mM and ATP at 0.04, 0.08, 0.16, 0.32, 0.64, 1.5, and 10 mM. [YbtE K_m^{ATP}

= 172 μM as determined in this study (not shown) and 350 μM as reported elsewhere⁸;

YbtE K_m^{Sal} = 5.4 μM as determined in this study (not shown) and 4.6 μM as reported

elsewhere⁸]. Other components were included as indicated above. Dose-response

data sets were fitted to Eq. (1) to obtain a K_i^{app} for each dose-response curve. For

competitive TBIs, $K_i^{\text{app}} = K_i(1 + \frac{[S]}{K_m})$, thus $K_i^{\text{app}} = K_i$ when $[S] = 0$, and the K_i value

was calculated as the y -intercept of the line fitted to the data⁹ (**Fig. 2e**). For non-

competitive inhibition $K_i^{\text{app}} = K_i$, and the K_i was calculated by averaging the K_i^{app} values⁹ (**Fig. 2f**). All data sets were fitted using KaleidagraphTM software.

V. Domain salicylation assay

The assay was performed in 96-well Nickel Chelate Coated FlashPlate[®] PLUS plates (flash plates) (PerkinElmer). Plate wells have a scintillant coat and a Ni²⁺ coat for His₆-tagged protein binding. YbtE-catalyzed incorporation of the [³H]-salicyl group into well-bound phosphopantetheinylated ypArCP-H6 leads to [³H]-salicyl-ypArCP-H6 formation, which is quantified with a plate counter. To obtain phosphopantetheinylated domain, ypArCP-H6 was co-expressed with Sfp (expressed from plasmid pSU20-Sfp¹⁰), and the purified ypArCP-H6 domain was further incubated with Sfp and coenzyme A for maximum modification as reported¹¹. Domain binding to wells was done as recommended by the plate manufacturer. After binding, wells were loaded with reaction mixtures (30 μ l) containing 75 mM MES (pH 6.5), 1 mM TCEP, 100 μ M ATP, 150 nM [³H]-salicylate (33 Ci/mmol, Vitrox Inc.), and inhibitor (added in DMSO as 1% of the reaction volume) at the concentrations indicated (**Fig. 2g**). Reactions were started by addition of YbtE (20 μ l) at 70 nM. After incubation at 37 °C for 1.5 h, reactions were chased with 300 μ l of PBS containing 1 mM salicylate, the wells were washed with chase solution (3 \times 100 μ l), and [³H]-salicyl-ypArCP-H6 was quantified using a TopCount[®] Microplate counter (Packard BioScience). Dose-response data were fitted to Eq. (1) and the IC₅₀ was calculated with Eq. (2).

VI. References

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