Supporting Information

Selective Inhibitors of Bacterial Phosphopantothenoylcysteine Synthetase

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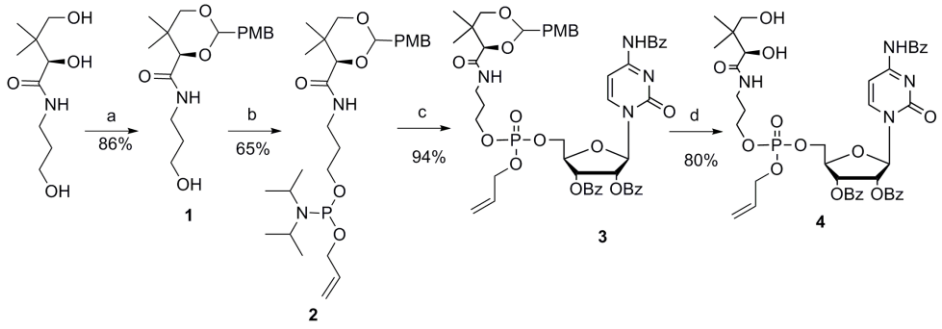
Table of Contents

Experimental

General Methods: All chemicals were used as purchased from Acros, Fisher, Fluka, Sigma-Aldrich, or Specialty Chemicals Ltd. and used without further purification unless otherwise noted. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were recorded on a Bruker Avance DRX 500MHz spectrometer or Bruker Avance DPX 300MHz spectrometer. ^{1}H and ^{13}C assignments are reported in ppm from an internal standard of TMS (0.0 ppm), and phosphorous assignments are reported relative to an external standard of 85% H_3PO_4 (0.0 ppm). Proton spectral data are reported as follows: chemical shift, multiplicity (ovlp = overlapping, s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad), coupling constant in Hz, and integration. All high resolution mass spectra were acquired from the Mass Spectrometry facility in the Chemistry Department at The University of Michigan using either positive-ion or negative-ion mode ESI-MS. Thin layer chromatography was performed using Analtech GHLF 250 micron silica gel TLC plates. All flash chromatography was performed using grade 60 Å 230-400 mesh silica purchased from Fisher.



Reagents and Conditions: a) p-anisaldehyde dimethyl acetal, CSA, DMF b) Allyl-O-P[N(i-Pr_2)]$_2$, 5-(ethylthio)-1H-tetrazole, DCM, c) 1) 5-(ethylthio)-1H-tetrazole,CH_3CN 2) CSO,0^oC d) 80% AcOH

Scheme 1: Synthesis of phosphodiester mimic.

(4*R*)-*N*-(3-hydroxypropyl)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamide (1) D-panthenol (1.0 g, 5 mmol) was rendered anhydrous by evaporation from ethanol stock (5 mL) followed by evaporation from toluene (2 x 5 mL) and dissolved in anhydrous DMF (20 mL). Camphor sulfonic acid (CSA) (0.0116 g, 0.05 mmol) was added and stirred at room temperature for 15 min. *p*-Methoxybenzaldehyde dimethyl acetal (2.55 mL, 15 mmol) was added and the reaction was stirred at room temperature for 24 h. The solvents were removed *in vacuo* and then the resulting syrup was purified over silica (100 mL) eluting with 10% EtOAc in hexanes (300 mL), 25% EtOAc in hexanes (300 mL), and 50% EtOAc in hexanes yielding a white crystalline solid (1.4 g, 86%). Mixture of diastereomers (55%/45%), ^{1}H NMR (DMSO-d_6 major diastereomer): δ 7.55 (s, 1H), 7.44 (d, *J* = 7.05 Hz, 2H), 6.93 (d, *J* = 7.15 Hz, 2H), 5.50 (s, 1H), 4.52 (t, *J* = 4.55 Hz, 1H), 4.08 (s, 1H), 3.75 (s, 3H), 3.61 (q, *J* = 9.74 Hz, 2H), 3.41 (d, *J* = 5.40 Hz, 2H), 3.28-3.06 (m, 2H), 1.56 (t, *J* = 5.75 Hz, 2H), 0.98 (s, 3H), 0.96 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 168.63, 160.01, 130.98, 128.24, 113.79, 100.88, 83.76, 77.87, 59.39, 55.59, 36.34, 32.99, 32.62, 22.05, 19.59. HR-ESI-MS: calcd for $[M+Na]^+$, 346.1625; found 346.1622.

Allyl 3-((4*R*)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamido)propyl diisopropyl-phosphoramidite (2) The protected alcohol (3.1 g, 9.59 mmol) and 5- (ethylthiol)-1H-tetrazole (0.836 g, 6.42 mmol) were dissolved in anhydrous DCM. Allyl- *N*,*N*,*N*,*N*-tetraisopropylphosphoramidite (5.2 mL, 16.3 mmol) was added dropwise to the solution over a period of 5 minutes. The reaction was allowed to stir at room temperature for 6 hours, at which time solvents were removed *in vacuo.* The syrup was then purified over silica (150 mL) eluting with 30% ethyl acetate in hexanes (450 mL), 50% ethyl acetate in hexanes (450 mL), and 100% ethyl acetate (450 mL). Product eluted in 30% ethyl acetate and was obtained as colorless oil (3.2 g, 65%). ^{1}H NMR (DMSO-d_6): δ 7.47-7.42 (ovlp, d,t, 3H), 6.93 (d, *J* = 8.75 Hz, 2H), 5.91-5.78 (m, 2H), 5.53 (s, 1H), 5.26 (d, *J* = 17.15 Hz, 1H), 5.10 (d, *J* = 10.20 Hz, 1H), 4.09 (s, 3H), 3.67-3.61 (m, 2H), 3.55-3.47 (m, 4H), 3.27-3.25 (m, 2H), 3.15-3.12 (m, 2H), 1.70 (t, *J* = 6.65 Hz, 2H), 1.19 (s, 6H), 1.10 (s, 6H), 1.03 (s, 3H), 0.95 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 168.76, 160.00, 134.39, 130.95, 128.31, 116.85, 113.73, 100.97, 83.84, 77.88, 63.55, 55.58, 46.53, 45.73, 35.27, 32.98, 30.52, 22.75, 22.02, 19.60. ^{31}P NMR (DMSO-d_6): δ 145.82 (s, 1P). HR-ESI-MS: calcd for $[M+Na]^+$, 533.2751; found 533.2764.

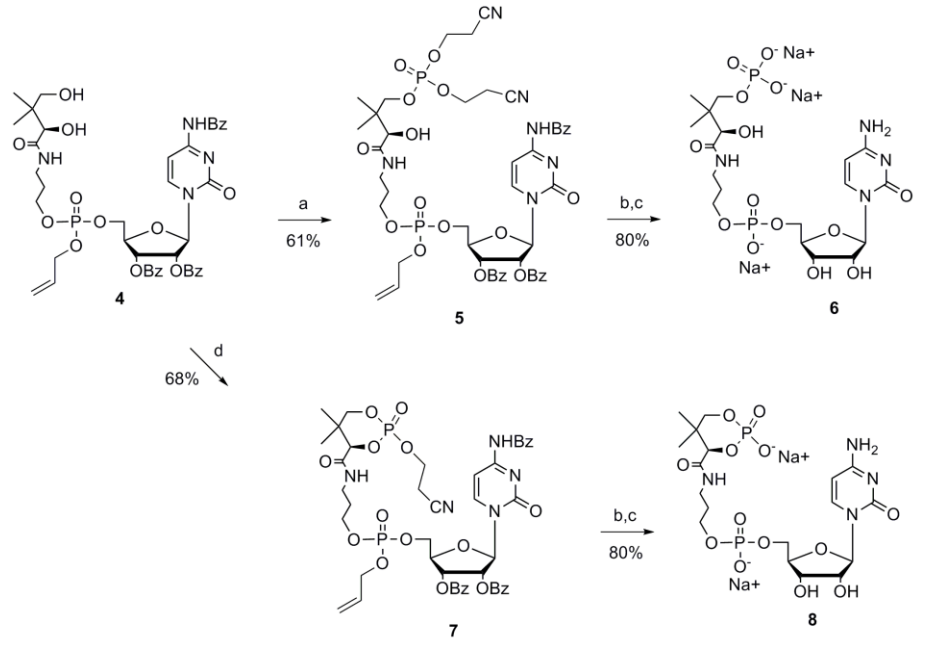
(2*R*,3*R*,4*R*,5*R*)-2-((((allyloxy)(3-((4*R*)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamido)propoxy)phosphoryl)oxy)methyl)-5-(4-benzamido-2-oxopyrimidin-1(2*H*)-yl)tetrahydrofuran-3,4-diyl dibenzoate (3) The tribenzoyl cytidine **17** (858 mg, 1.45 mmol) and the *p*-

methoxybenzylidene panthenol phosphoramidite **2** (1.26 g, 2.47 mmol) were dissolved in toluene (2 x 5 mL) and evaporated, then dissolved in anhydrous acetonitrile (10 mL) along with 3 Å molecular sieves (0.5 g). Concurrently, in a separate flask 5-ethylthiol-1*H*-tetrazole (566 mg, 4.35 mmol) was dissolved in anhydrous acetonitrile (3 mL) and both flasks were stirred at room temperature for 1 hour. The content of the tetrazole and acetonitrile mixture (~ 3.5 mL) was then added dropwise over 10 minutes to the first flask and the reaction was stirred at room temperature for 4 hours. The phosphite was then oxidized *in situ* upon the addition of (1*R*)-(-)-(8,8-dichloro-10-camphor-sulfonyl) oxaziridine (CSO) (736 mg, 2.47 mmol) in ethyl acetate (3 mL) dropwise over 5 minutes and then allowed to stir for 2 hours. The reaction was quenched upon the addition of dimethyl sulfide (0.2 mL), the reaction was filtered, and then solvents were removed *in vacuo*. The reaction was purified over silica (50 mL) eluting with 25% ethyl acetate in hexanes (150 mL), 50% ethyl acetate in hexanes (150 mL), 75% ethyl acetate in hexanes (150 mL) with the product eluting as white crystalline solid (1.43 g, 94%). ^{1}H NMR (DMSO-d_6): δ 11.41 (s, 1H), 8.31 (d, *J* = 7.12, 1H), 8.03 (d, *J* = 7.25 Hz, 2H), 7.93 (d, *J* = 7.55, 2H), 7.87 (d, *J* = 7.45 Hz, 2H), 7.67-7.64 (m, 4H), 7.55-7.38 (m, 9H), 6.91 (d, *J* = 7.60, Hz, 2H), 6.25 (s, 1H), 5.93 (s, 3H), 5.82 (t, *J* = 6.08 Hz, 1H), 5.50 (s, 1H), 5.36-5.31 (m, 1H), 5.20 (t, *J* = 9.45 Hz, 1H), 4.69 (s, 1H), 4.35 (s, 3H), 4.50-4.42 (m, 1H), 4.40-4.35 (m, 1H), 3.75 (s, 3H), 3.64-3.58 (m, 2H), 1.80-1.72 (m, 2H), 1.01 (s, 3H), 0.92 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 168.82, 167.88, 165.02, 164.36, 160.02, 154.78, 147.35, 134.42, 134.35, 133.40, 133.35, 130.99, 130.93, 129.81, 129.22, 129.00, 128.93, 128.23, 118.37, 113.74, 100.97, 97.28, 91.45, 83.83, 80.63, 77.91, 74.17, 70.95, 68.19, 66.65, 66.17, 55.57, 35.17, 32.96, 30.46, 21.99, 19.57. ^{31}P NMR (DMSO-d_6): δ -0.96 (s, 1P). HR-ESI-MS: calcd for $[M+Na]^+$, 1003.3138; found 1003.3148.

(2*R*,3*R*,4*R*,5*R*)-2-((((allyloxy)(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propoxy)phosphoryl) oxy)methyl)-5-(4-benzamido-2-oxopyrimidin-1(2*H*)-yl)tetrahydrofuran-3,4-diyl dibenzoate (4) The acetal **3** (710 mg, 0.72 mmol) was dissolved in 80% acetic acid (8 mL) and was stirred at room temperature for 20 hours. The solvents were removed *in vacuo* and the syrup was partitioned between DCM and water. The water layer was washed with DCM (2 x 20 mL) and then the organic extracts were dried (Na_2SO_4) and evaporated *in vacuo*. The syrup was purified over silica (50 mL) eluting with 50% ethyl acetate in hexanes (150 mL), 75% ethyl acetate in hexanes (150 mL), and 100% ethyl acetate with the product obtained as white crystalline solid (610 mg, 95%). ^{1}H NMR (DMSO-d_6): δ 11.41 (s, 1H), 8.32 (d, *J* = 7.40 Hz, 1H), 8.03 (d, *J* = 7.41 Hz, 2H), 7.94 (d, *J* = 7.11 Hz, 2H), 7.94 (d, *J* = 7.12 Hz, 2H), 7.86 (d, *J* = 7.15 Hz, 2H), 7.55-7.44 (m, 9H), 6.25 (s, 1H), 5.96-5.90 (m, 2H), 5.83 (t, *J* = 6.35 Hz, 1H), 5.38-5.33 (m, 2H), 5.23-5.19 (m, 1H), 4.70 (s, 1H), 4.56-4.53 (m, 3H), 4.47-4.39 (ovlp, m, 2H) 4.06-4.01 (m, 2H), 3.71 (d, *J* = 5.55 Hz, 1H), 3.20-3.11 (m, 4H), 1.80-1.74 (m, 2H), 0.80 (s, 3H), 0.78 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 173.56, 167.89, 165.09, 165.01, 164.39, 154.81, 147.43, 134.42, 134.36, 133.38, 133.35, 129.82, 129.22, 129.00, 128.93, 128.28, 118.39, 113.73, 97.25, 83.72, 80.67, 75.56, 74.17, 70.95. 68.49, 68.21, 66.67, 66.10, 35.05, 30.51, 21.44, 20.80. ^{31}P NMR (DMSO-d_6): δ -0.99 (s, 1P). HR-ESI-MS: calcd for $[M+Na]^+$, 885.2719; found 885.2733.

(2*R*,3*R*,4*R*,5*R*)-2-((((allyloxy)(3-((*R*)-4-((bis(2-cyanoethoxy)phosphoryl)oxy)-2-hydroxy-3,3-dimethylbutanamido)propoxy)phosphoryl)oxy)methyl)-5-(4-benzamido-2-oxopyrimidin-1(2*H*)-yl)tetrahydrofuran-3,4-diyl dibenzoate (5) The diol **4** (75 mg, 0.087 mmol), *O*,*O*-bis(cyanoethyl)-*N*-diisopropylamine phosphoramidite (36 mg, 0.131 mmol), and 3 Å molecular sieves were dissolved in anhydrous pyridine (0.5 mL) and cooled to -20°C. Pyridinium HCl (15 mg, 0.131 mmol) was dissolved in anhydrous pyridine (1 mL) and then added dropwise to the reaction mixture. The reaction was

allowed to stir at -20°C for 2 h. At this point CSO (39 mg, 0.131 mmol) in DCM (1 mL) was added to the reaction and allowed to stir for 1 h. Solvents were removed *in vacuo* and resulting syrup was purified over silica (5 mL) eluting with 50% EtOAc in hexanes (25 mL), 75% EtOAc in hexanes (25 mL), 100% EtOAc (25 mL), and 10% MeOH in EtOAc (25 mL) to yield 55.6 mg of white crystalline solid (61%). ^{1}H NMR (DMSO-d_6): δ 11.40 (s, 1H), 8.31 (d, *J* = 7.90 Hz, 1H), 8.21-8.19 (m, 1H), 8.03 (d, *J* = 7.45 Hz, 2H), 7.93 (d, *J* = 7.90 Hz, 2H), 7.87 (d, *J* = 7.50 Hz, 2H), 7.69-7.66 (m, 4H), 7.55-7.44 (ovlp, m, 6H), 6.25 (s, 1H), 5.97-5.91 (m, 2H), 5.83 (t, *J* = 6.55, 1H), 5.77 (s, 1H), 5.71 (d, *J* = 5.50 Hz, 1H), 5.35 (dd, *J* = 6.20, 15.90 Hz, 2H), 5.21 (t, *J* = 9.15 Hz, 1H), 4.70 (br,s, 1H), 4.54 (s, 2H), 4.44-4.41 (m, 1H), 4.41-4.39 (m, 1H), 4.25-4.18 (m, 4H), 3.95 (m, 1H), 3.85 (m, 1H), 3.75 (m, 1H), 3.70 (d, *J* = 5.6 Hz, 1H), 3.60 (t, *J* = 6.6 Hz, 1H), 2.92 (t, *J* = 5.2 Hz, 4H),1.84-1.78 (m, 2H), 1.03 (s, 3H), 0.96 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 173.12, 167.45, 164.64, 164.57, 163.94, 154.36, 147.02, 133.99, 133.93, 132.98, 132.89, 129.38, 128.78, 128.57, 128.49, 127.84, 118.47, 117.95, 113.29, 96.83, 91.22, 80.24, 80.14, 75.09, 73.72, 70.50, 68.03, 67.79, 66.24, 65.68, 60.40, 34.60, 30.08, 29.99, 22.24, 21.00, 20.35. HR-ESI-MS: calcd for $[M+Na]^+$, 1071.2913; found 1071.2937.



Reagents and Conditions: a) 1) (β-cyanoethyl)$_2$-O-P-N(i-Pr$_2$)$_2$, pyridine HCl, pyridine, -20°C 2) CSO, 0°C b) DBU, TMSCl, DCM c) NH_4OH, 55°C h) d) 1) (β-cyanoethyl)2-O-P-N(i-Pr$_2$)$_2$, 5-(ethylthio)-1H-tetrazole, CH_3CN 2) CSO, 0°C

Scheme 2: Synthesis of phosphodiester mimics

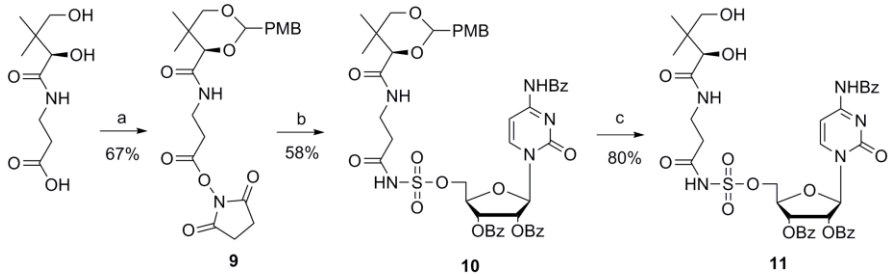
Trisodium (*R*)-4-((3-((((((2*R*,3*S*,4*R*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)oxidophosphoryl)oxy)propyl)amino)-3-hydroxy-2,2-dimethyl-4-oxobutyl phosphate (6) The protected phosphate **4** (56 mg, 0.0537 mmol) was dissolved in anhydrous DCM (1 mL). DBU (0.077 mL, 0.429 mmol) and TMSCl (0.023 mL, 0.215 mmol) were added dropwise to the solution and allowed to stir at rt for 6h. Solvents were removed *in vacuo*, and then the resulting syrup was dissolved in NH_4OH (2 mL). β-mercaptoethanol (0.1 mL) was added and the reaction was stirred at 55°C for 1 h. The reaction was then placed on a C-18 prep sep column and eluted with H_2O. The UV active fractions (fractions 3-5) were collected and manually loaded onto a 15 mL AMGP anion exchange column. The anion exchange column was washed with H_2O (30 mL) and then eluted with a 0-60% gradient of 1M NaCl. The fractions were monitored at 254 nm and the UV active

fractions at 22% 1M NaCl were collected and lyophilized. The powder was then dissolved in H_2O and purified over a 300 mL sephadex size exclusion column. The UV active fractions (16-20) were collected and lyophilized to yield desired trisodium salt as a fluffy white solid (28.5 mg, 80.8%). ^{1}H NMR (D_2O-d_2): δ 7.84 (d, *J* = 7.55 Hz, 1H), 6.00 (d, *J* = 7.65 Hz, 1H), 5.88 (d, *J* = 3.45 Hz, 1H), 4.21-4.16 (m, 2H), 4.16 (s, 1H), 4.09 (d, *J* = 10.10 Hz, 1H), 4.00-3.07 (m, 1H), 3.94 (s, 1H), 3.85-3.81 (m, 2H), 3.68 (dd, *J* = 5.32, 9.85 Hz, 1H), 3.40 (dd, *J* = 4.84, 9.92 Hz, 1H), 3.23 (t, *J* = 6.70 Hz, 2H), 1.78 (t, *J* = 6.52 Hz, 2H), 0.87 (s, 3H), 0.76 (s, 3H). ^{13}C NMR (D_2O-d_2): δ 174.84, 165.99, 157.23, 141.27, 96.35, 89.45, 82.39, 74.54, 74.11, 70.83, 69.14, 64.07, 63.69, 38.23, 35.69, 29.49, 20.99, 18.25. ^{31}P NMR (D_2O-d_2): δ 1.78 (s, 1P), 0.35 (s, 1P). HR-ESI-MS: calcd for $[M+H]^+$, 657.0922; found 657.0936.

Allyl (((2*R*,3*S*,4*R*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl (3-((4*R*)-2-(2-cyanoethoxy)-5,5-dimethyl-2-oxido-1,3,2-dioxaphosphinane-4-carboxamido)propyl) phosphate (7) The diol **3** (190 mg, 0.22 mmol) and 5-(ethylthiol)-1H-tetrazole (86 mg, 0.66 mmol) along with 3 Å molecular sieves were dissolved in anhydrous CH_3CN (5 mL). *O*,*O*-bis(cyanoethyl)-*N*-diisopropylamine phosphoramidite (119 mg, 0.44 mmol) in anhydrous CH_3CN (0.5 mL) was added dropwise and the reaction was stirred at rt for 4 h. CSO (131 mg, 0.44 mmol) in anhydrous CH_3CN (3mL) was added dropwise and allowed to stir for 2 h. The solvents were removed *in vacuo* and the resulting syrup was purified over silica (5 mL) eluting with 50% EtOAc in hexanes (25 mL), 75% EtOAc in hexanes (25 mL), 100% EtOAc (25 mL) to yield 135.6 mg of white crystalline solid (59%). ^{1}H NMR (DMSO-d_6): δ 11.41 (s, 1H), 8.31 (d, *J* = 7.45 Hz, 1H), 8.21-8.19 (m, 1H), 8.03 (d, *J* = 7.60 Hz, 2H), 7.93 (d, *J* = 7.45 Hz, 2H), 7.88 (d, *J* = 7.35 Hz, 2H), 7.69-7.64 (m, 4H), 7.54 (t, *J* = 7.72 Hz, 2H), 7.47 (ovlp,d,t 6H), 6.26 (d, *J* = 2.53 Hz, 1H), 5.97-5.91 (m, 2H), 5.35 (dd, *J* = 5.05, 15.62 Hz, 2H), 4.72-4.70 (m, 1H), 4.61 (s, 1H), 4.55 (br,s, 3H), 4.47-4.45 (m, 1H), 4.41-4.38 (m, 1H), 4.18 (q, *J* = 5.27 Hz, 2H), 4.13 (d, *J* = 11.35, 1H), 43.95 (m, 1H), 3.85 (m, 1H), 3.60 (t, *J* = 6.6 Hz, 1H), 2.97 (t, *J* = 5 Hz, 2H), 1.82-1.78 (m, 2H), 1.03 (s, 3H), 0.96 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 167.65, 165.83, 165.69, 164.65, 164.58, 163.83, 154.22, 146.91, 133.99, 133.93, 133.08, 132.97, 132.89, 129.38, 128.78, 128.57, 128.49, 128.03, 118.42, 117.95, 96.87, 91.16, 84.38, 80.20, 78.00, 73.73, 70.50, 68.06, 67.78, 66.25, 65.59, 62.75, 61.78, 34.29, 29.78, 26.47, 19.98, 19.14, 19.03, 17.64, 15.02. ^{31}P NMR (DMSO-d_6): δ -1.01 (s, 1P), -9.44 (s, 1P). HR-ESI-MS: calcd for $[M+Na]^+$, 1000.2542; found 1000.2576.

Disodium ((2*R*,3*S*,4*R*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl (3-((*R*)-5,5-dimethyl-2,2-dioxido-1,3,2-dioxaphosphinane-4-carboxamido)propyl) phosphate (8) The protected phosphate **7** (15 mg, 0.0155 mmol) was dissolved in anhydrous DCM (1 mL). DBU (0.02 mL, 0.115 mmol) and TMSCl (0.006 mL, 0.057 mmol) were added dropwise to the solution and allowed to stir at rt for 6h. Solvents were removed *in vacuo*, and then the resulting syrup was dissolved in NH_4OH (2 mL). β-mercaptoethanol (0.1 mL) was added and the reaction was stirred at 55°C for 1 h. The reaction was then placed on a C-18 prep sep column and eluted with H_2O. The UV active fractions (fractions 3-5) were collected and manually loaded onto a 15 mL AGMP1 anion exchange column. The anion exchange column was washed with H_2O (30 mL) and then eluted with a 0-60% gradient of 1 M NaCl. The fractions were monitored at 254 nm and the UV active fractions at 18% 1 M NaCl were collected and lyophilized. The powder was then dissolved in H_2O and desalted over a 300 mL sephadex size exclusion column. The UV active fractions (15-19) were collected and lyophilized to yield desired disodium salt as a fluffy white solid (7.6 mg, 81.1%). ^{1}H NMR (D_2O-d_2): δ

7.82 (d, *J* = 7.10 Hz, 1H), 5.99 (d, *J* = 7.70 Hz, 1H), 5.88 (s, 1H) 4.40 (s, 1H) 4.23-4.21 (m, 2H) 4.17-4.15 (m, 1H), 4.07 (d, *J* = 4.15 Hz, 1H) 3.99 (d, *J* = 11.50 Hz, 2H), 3.82 (q, *J* = 6.05 Hz, 2H), 3.63 (dd, *J* = 11.10, 23.15 Hz, 1H), 3.23 (dm, 2H), 1.75 (t, *J* = 6.20 Hz), 0.91 (s, 3H), 0.86 (s, 3H). ^{13}C NMR (D_2O-d_2): δ 170.48, 166.07, 157.62, 141.24, 96.34, 89.39, 82.49, 82.42, 76.38, 74.12, 69.144, 64.11, 63.58, 35.73, 34.54, 29.51, 19.94, 17.38. ^{31}P NMR (D_2O-d_2): δ -0.367 (s, 1P), -4.21 (s, 1P). HR-ESI-MS: calcd for $[M-H]^-$, 571.1206, found 571.1213.



Reagents and Conditions: a) 1) p-anisaldehyde dimethyl acetal, CSA 2) NHS, DCCb) Cs_2CO_3 c) 80% AcOH

Scheme 3: Synthesis of sulfamate diol intermediate

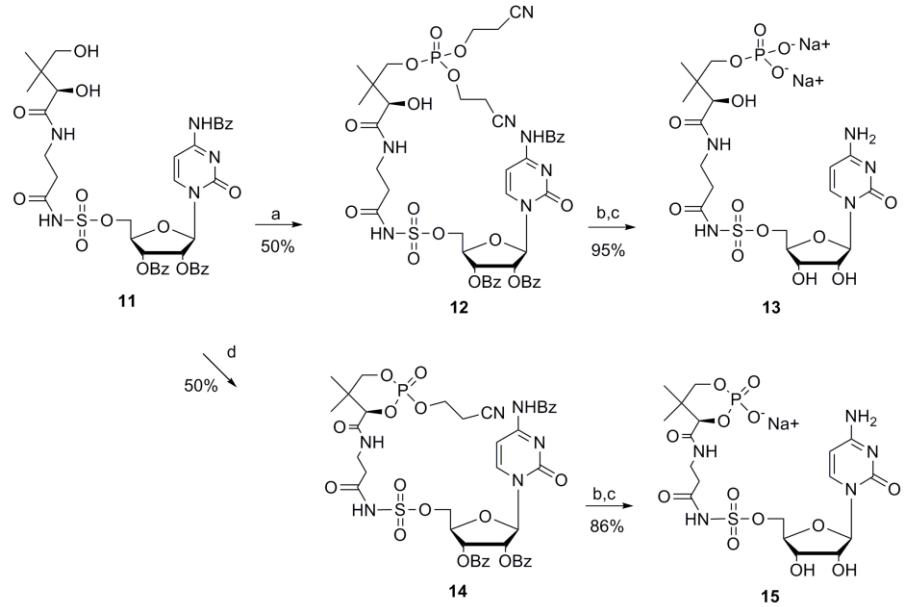
2,5-Dioxopyrrolidin-1-yl-3-((4*R*)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamido)propanoate (9)[1] Pantothenic acid hemicalcium salt (5 g, 20.98 mmol) was dissolved in anhydrous DMF (50 mL). Concentrated H_2SO_4 (0.65 mL, 20.98 mmol) was added dropwise and stirred for 30 min. *p*-Anisaldehyde dimethyl acetal (3.6 mL, 20.98 mmol) and CSA (244 mg, 1.05 mmol) were added and the reaction was stirred for 16 h. Solvents were removed *in vacuo* and the resulting syrup was portioned between EtOAc (500 mL) and H_2O (50 mL). The organic layer was washed with H_2O (2 x 50 mL). The organic layer is then dried (Na_2SO_4) and evaporated. The resulting white solid is then washed with DCM to remove any remaining *p*-anisaldehyde dimethyl acetal to yield the desired product as a white crystalline product (5.1 g, 72%).

The *p*-methoxybenzylidene protected pantothenic acid (750 mg, 2.10 mmol) and *N*-hydroxysuccinimide (242 mg, 2.10 mmol) were dissolved in anhydrous THF (5 mL). A solution of DCC (433 mg, 2.10 mmol) in anhydrous THF (3 mL) was added dropwise and the reaction was stirred for 6h. The reaction mixture was then filtered over celite to remove the white precipitate. The white precipitate was then washed with EtOAc (10 mL). The organic filtrate was then concentrated *in vacuo* to yield the desired product as a glassy clear solid (885 mg, 93%). ^{1}H NMR (DMSO-d_6): δ 7.59 (s, 1H), 7.45 (d, *J* = 8.40 Hz, 2H), 6.94 (d, *J* = 8.80 Hz, 2H), 5.53 (s, 1H), 4.12 (s, 1H), 3.76 (s, 1H), 3.33 (s, 3H), 2.88 (t, *J* = 7.10 Hz, 2H), 2.81 (s, 4H), 2.42-2.40 (m, ovlp, 2H), 1.00 (s, 3H), 0.96 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 170.59, 169.07, 167.84, 160.00, 130.95, 128.27, 113.80, 100.91, 83.63, 77.85, 55.61, 34.30, 33.02, 30.94, 25.90, 21.97, 19.50. HR-ESI-MS: calcd for $[M+Na]^+$, 457.1582; found 457.1589.

5'-*O*-(*N*-(3-((4*R*)-2-(4-Methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamido)propanoyl) sulfamoyl)-2',3'-*O*,*N*^{4}-tribenzoylcytidine (10)[2] NHS ester **9** (442 mg, 0.97 mmol) was dissolved in anhydrous DMF (8 mL). Sulfamoyl cytidine **18** (307 mg, 0.485 mmol) was added and the solution was cooled to 0°C. Cs_2CO_3 (316 mg, 0.97 mmol) was added and stirred at 0°C for 30 min. The ice bath was removed and the reaction was stirred at rt for 16 h. The solvents were removed *in vacuo* and the resulting paste was taken up in EtOAc (50 mL) and filtered. The white precipitate was washed thoroughly with EtOAc (100 mL). The combined filtrate was purified over silica (25 mL) eluting with

75% EtOAc in hexanes (100 mL), 100% EtOAc (100 mL), and 10% MeOH in EtOAc (100 mL) to yield the desired product as a white solid (270 mg, 58%). ^{1}H NMR (DMSO-d_6): δ 11.33 (s, 1H), 8.02 (d, J = 7.20 Hz, 2H), 7.94 (d, J = 7.20 Hz, 2H), 7.83 (d, J = 7.10 Hz, 2H), 7.64 (t, J = 7.55 Hz, 3H), 7.55-7.41 (m, ovlp, 12H), 6.90 (d, J = 8.81 Hz, 2H), 6.30 (d, J = 4.30 Hz, 1H), 5.83 (t, J = 5.15 Hz, 1H), 5.75 (t, J = 5.49 Hz, 1H), 5.48 (s, 1H), 4.69 (m, 1H), 4.47-4.38 (m, 2H), 4.12-3.96 (m, ovlp, 1H), 4.05 (s, 1H), 3.73 (s, 3H), 3.59 (d, J = 2.72 Hz, 1H), 3.29-3.26 (m, 2H), 2.39-2.84 (m, 2H), 0.98 (s, 3H), 0.92 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 167.92, 167.23, 164.49, 164.31, 163.63, 159.39, 154.35, 146.33, 133.80, 133.76, 132.96, 132.68, 130.32, 129.23, 128.67, 128.63, 128.52, 128.40, 128.33, 127.61, 113.21, 100.26, 96.83, 83.08, 80.00, 77.29, 73.94, 71.04, 54.96, 34.48, 32.40, 21.43, 18.91. HR-ESI-MS: calcd for $[M+H]^+$, 954.2863; found 954.2906.

5'-*O*-(*N*-(3-((*R*)-2,4-Dihydroxy-3,3-dimethylbutanamido)propanoyl)sulfamoyl)-2',3'-*O*,*N*^{4}-tribenzoylcytidine (11) The *p*-methoxy benzyl acetal **10** (92.5 mg, 0.097 mmol) was dissolved in 80% AcOH (5 mL) and stirred at rt for 12 h. Solvents were removed *in vacuo* and the resulting syrup was purified over silica (10 mL) eluting with 75% EtOAc in hexanes (25 mL), 100% EtOAc (25 mL), and 10% MeOH in EtOAc (25 mL) to yield the desired product as a white solid (65 mg, 80%). ^{1}H NMR (DMSO-d_6): δ 11.33 (s, 1H), 8.50 (s, 1H), 8.02 (d, J = 7.53 Hz, 2H), 7.95 (d, J = 7.29 Hz, 2H), 7.83 (d, J = 7.65 Hz, 2H), 7.67-7.61 (m, 5H), 7.55-7.41 (m, 8H), 6.32 (s, 1H), 5.84-5.82 (m, 1H), 5.75 (t, J = 5.52 Hz, 1H), 5.35 (d, J = 5.61 Hz, 1H), 4.74-4.70 (m, 1H), 4.44-4.31 (m, 2H), 4.10 (d, J = 5.22 Hz, 1H), 3.67 (d, J = 5.52 Hz, 1H), 3.30-3.26 (m, 2H), 2.30-2.27 (m, 2H), 0.77 (s, 3H), 0.75 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 176.72, 173.03, 165.10, 164.84, 147.23, 147.00, 134.39, 134.34, 133.62, 133.24, 129.82, 129.31, 129.22, 129.00, 128.92, 97.50, 88.70, 81.15, 75.50, 74.77, 72.05, 68.58, 66.71, 66.16, 39.27, 35.75, 21.27, 20.96. HR-ESI-MS: calcd for $[M+Na]^+$, 858.2263; found 858.2286.



Scheme 4: Synthesis of sulfamate mimics

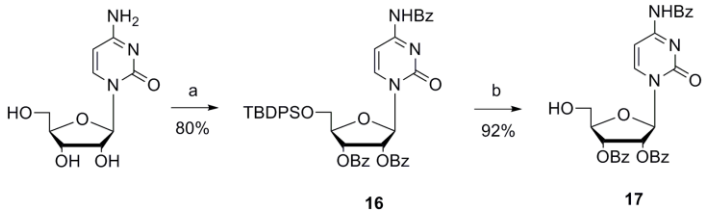
5'-*O*-(*N*-((((*R*)-Bis(2-cyanoethyl) 3-hydroxy-4-(3-oxopropylamino)-2,2-dimethyl-4-oxobutyl) phosphoryl)oxy)sulfamoyl)-2',3'-*O*,N^4-tribenzoylcytidine (12) The diol **11** (65 mg, 0.077 mmol), *O*,*O*-bis(cyanoethyl)-*N*-diisopropylamine phosphoramidite (32 mg, 0.117 mmol), and 3 Å molecular sieves were dissolved in anhydrous pyridine (0.5 mL) and cooled to -20°C. Pyridinium HCl (13.5 mg, 0.117 mmol) was dissolved in anhydrous pyridine (1 mL) and then added dropwise to the reaction mixture. The reaction was allowed to stir at -20°C for 2 h. At this point CSO (35 mg, 0.117 mmol) in DCM (1 mL) was added to the reaction and allowed to stir for 1 h. Solvents were removed *in vacuo* and resulting syrup was purified over silica (5 mL) eluting with 50% EtOAc in hexanes (25 mL), 75% EtOAc in hexanes (25 mL), 100% EtOAc (25 mL), and 10% MeOH in EtOAc (25 mL) to yield 38 mg of white solid (50%). ^{1}H NMR (DMSO-d_6): δ 11.34 (s, 1H), 8.46 (s, 1H), 8.04 (d, *J* = 7.20 Hz, 2H), 7.96 (d, *J* = 7.17 Hz, 2H), 7.86 (d, *J* = 7.14 Hz, 2H), 7.66 (t, *J* = 7.53 Hz, 3H), 7.57-7.43 (m, 8H), 6.34 (d, *J* = 6.30 Hz, 1H), 5.86-5.81 (m, 1H), 5.79-5.70 (ovlp, m, 2H), 4.75 (s, 1H), 4.46-4.40 (m, 2H), 4.28-4.21 (m, 4H), 4.04 (q, *J* = 11.92, 1H), 3.94-3.92 (m, 1H), 3.88-3.86 (m, 1H), 3.29-3.26 (m, 2H), 2.95 (t, *J* = 8.74 Hz, 4H), 2.28-2.23 (m, 2H), 0.89 (s, 3H), 0.85 (s, 3H). ^{31}P NMR (DMSO-d_6): δ -2.13 (s, 1P). HR-ESI-MS: calcd for $[M+Na]^+$, 1044.2458; found 1044.2482.

5'-O-(N-((((*R*)-3-hydroxy-4-(3-oxopropylamino)-2,2-dimethyl-4-oxobutyl)phosphoryl)oxy) sulfamoyl) cytidine disodium salt (13) The protected phosphate **12** (61 mg, 0.0597 mmol) was dissolved in anhydrous DCM (1 mL). DBU (0.086 mL, 0.478 mmol) and TMSCl (0.026 mL, 0.239 mmol) were added dropwise to the solution and allowed to stir at rt for 6h. Solvents were removed *in vacuo*, and then the resulting syrup was dissolved in NH_4OH (2 mL). β-mercaptoethanol (0.1 mL) was added and the reaction was stirred at 55°C for 1 h. The reaction was then placed on a C-18 prep sep column and eluted with H_2O. The UV active fractions (fractions 3-5) were collected and manually loaded onto a 15 mL AGMP1 anion exchange column. The anion exchange column was washed with H_2O (30 mL) and then eluted with a 0-60% gradient of 1M NaCl. The fractions were monitored at 254 nm and the UV active fractions at 58% 1M NaCl were collected and lyophilized. The powder was then dissolved in H_2O and desalted over a 300 mL sephadex size exclusion column. The UV active fractions (15-20) were collected and lyophilized to yield desired trisodium salt as a fluffy white solid (36 mg, 95%). ^{1}H NMR (D_2O-d_2): δ 7.75 (d, *J* = 7.10 Hz, 1H), 5.99 (d, *J* = 7.70 Hz, 1H), 5.86 (s, 1H), 4.31 (d, *J* = 9.60 Hz, 1H), 4.23-4.21 (m, 2H), 4.15 (s, 1H), 3.67-3.64 (m, 1H), 3.37 (t, *J* = 6.80 Hz, 2H), 3.30 (dd, *J* = 4.00, 9.20 Hz, 1H), 2.38 (t, *J* = 6.58 Hz, 2H), 0.88 (s, 3H), 0.72 (s, 3H). ^{13}C NMR (D_2O-d_2): δ 180.70, 174.99, 166.14, 157.66, 141.25, 96.38, 89.51, 81.35, 74.62, 73.99, 70.70, 69.22, 67.57, 38.38, 37.97, 35.70, 21.34, 17.96. ^{31}P NMR (D_2O-d_2): δ 1.42 (s, 1P). HR-ESI-MS: calcd for $[M+Na]^+$, 670.0779; found 670.0780.

5'-*O*-(*N*-(((*R*)-2-(2-cyanoethoxy)-5,5-dimethyl-*N*-(3-oxopropyl)-1,3,2-dioxaphosphinane-4-carboxamide 2-oxy)sulfamoyl)-2',3'-*O*,N^4-tribenzoyl cytidine (14) The diol **11** (115 mg, 0.14 mmol) and 5-(ethylthiol)-1H-tetrazole (54 mg, 0.411 mmol) along with 3 Å molecular sieves were dissolved in anhydrous CH_3CN (5 mL). *O*,*O*-bis(cyanoethyl)-*N*-diisopropylamine phosphoramidite (75 mg, 0.275 mmol) in anhydrous CH_3CN (0.5 mL) was added dropwise and the reaction was stirred at rt for 4 h. CSO (82 mg, 0.275 mmol) in anhydrous CH_3CN (3mL) was added dropwise and allowed to stir for 2 h. The solvents were removed *in vacuo* and the resulting syrup was purified over silica (5 mL) eluting with 50% EtOAc in hexanes (25 mL), 75% EtOAc in hexanes (25 mL), 100% EtOAc (25 mL), and 10% MeOH in EtOAc (25 mL) to yield the desired product as a white solid (68 mg, 52%). ^{1}H NMR (DMSO-d_6): δ 11.34 (s, 1H), 8.55 (s, 1H), 8.20 (s, 1H), 8.03 (d, *J* = 7.25 Hz, 2H), 7.96 (d, *J* = 7.15 Hz, 2H), 7.84

(d, *J* = 8.0 Hz, 2H), 7.69 (t, *J* = 7.35 Hz, 2H), 7.65 (t, *J* = 7.35 Hz, 2H), 7.52 (q, *J* = 8.15 Hz, 4H), 7.45 (t, *J* = 7.90 Hz, 3H), 6.35 (d, *J* = 4.50 Hz, 1H), 5.83 (t, *J* = 5.40 Hz, 1H) 5.76 (ovlp,m, 2H), 4.73 (s, 1H), 4.51 (d, *J* = 7.75 Hz, 1H), 4.37-4.31 (m, 2H), 4.23-4.18 (m, 2H), 3.88 (t, *J* = 4.65 Hz, 1H), 3.18 (m, 1H), 3.15 (q, J = 7.35 Hz, 1H), 2.94 (t, *J* = 4.30Hz, 2H), 2.28-2.23 (m, 2H), 1.02 (s, 3H), 0.95 (s, 3H). ^{31}P NMR (DMSO-d_6): δ -9.46 (s, 1P). HR-ESI-MS: calcd for $[M+Na]^+$, 973.2087; found 973.2098.

Sodium 5'-*O*-(*N*-(((*R*)-5,5-dimethyl-4-((3-oxopropyl)carbamoyl)-1,3,2-dioxaphosphinan-2-olate 2-oxy)sulfamoyl) cytidine (15) The protected phosphate **14** (38 mg, 0.0402 mmol) was dissolved in anhydrous DCM (1 mL). DBU (0.058 mL, 0.322 mmol) and TMSCl (0.015 mL, 0.16 mmol) were added dropwise to the solution and allowed to stir at rt for 6h. Solvents were removed *in vacuo*, and then the resulting syrup was dissolved in NH_4OH (2 mL). β-mercaptoethanol (0.1 mL) was added and the reaction was stirred at 55°C for 1 h. The reaction was then placed on a C-18 prep sep column and eluted with H_2O. The UV active fractions (fractions 3-5) were collected and manually loaded onto a 15 mL AMGP anion exchange column. The anion exchange column was washed with H_2O (30 mL) and then eluted with a 0-60% gradient of 1M NaCl. The fractions were monitored at 254 nm and the UV active fractions at 54% 1M NaCl were collected and lyophilized. The powder was then dissolved in H_2O and purified over a 300 mL sephadex size exclusion column. The UV active fractions (15-20) were collected and lyophilized to yield desired trisodium salt as a fluffy white solid (21 mg, 86%). ^{1}H NMR (D_2O-d_2): δ 8.61 (s, 1H), 7.82 (d, *J* = 7.70 Hz, 1H), 6.03 (d, *J* = 7.70 Hz, 1H), 5.85 (d, *J* = 3.35 Hz, 1H), 4.40 (s, 1H), 4.31 (d, *J* = 10.10 Hz, 1H), 4.23 (s, 2H), 4.21-4.17 (m, 2H), 3.98 (d, *J* = 11.20 Hz, 1H), 3.62 (dd, *J* = 11.42, 22.83 Hz, 1H), 3.45-3.35 (m, 2H), 2.40 (t, *J* = 6.70 Hz, 2H), 0.93 (s, 3H), 0.87 (s, 3H). ^{13}C NMR (D_2O-d_2): δ 180.55, 174.77, 166.12, 160.40, 141.23, 96.36, 89.54, 82.26, 81.30, 74.66, 73.92, 69.23, 67.65, 39.23, 38.31, 37.94, 35.66, 21.44, 17.85. ^{31}P NMR (D_2O-d_2): δ -4.26 (s, 1P). HR-ESI-MS: calcd for $[M-H]^-$, 606.0883; found 606.0901.

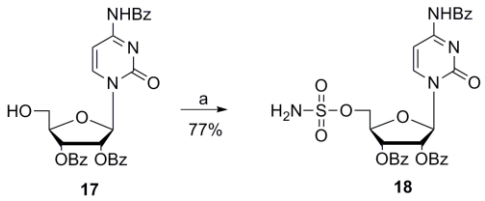


Scheme 5: Synthesis of tribenzoyl cytidine

2'-3'-*O*,N^4-Tribenzoyl-5'-*O*-*tert*-butyldiphenylsilyl cytidine (16)[3] Cytidine (6.0 g, 25 mmol, 1.0 equiv) and imidazole (4.2 g, 63 mmol) were dissolved in DMF (45 mL). *tert*-Butyldiphenylsilyl chloride (7.0 mL, 27 mmol) was added dropwise over 10 min. The reaction was stirred at room temperature for 1 h and then quenched by addition of methanol (10 mL). The solvents were removed *in vacuo* and the resulting syrup was partitioned between water and DCM. The aqueous layer was further washed with DCM (2x) and then the organic extracts were combined and upon standing the product crystallized out within 10 min. The crystals were dried under vacuum and then dissolved in pyridine (40 mL). Benzoic anhydride (56 g, 250 mmol, 10 equiv) was added and the reaction was stirred at room temperature for 2 days. The reaction was quenched with water (20 mL). The solvents were removed *in vacuo* and the resulting syrup was partitioned between water and DCM. The aqueous layer was furthered extracted with DCM (2x), and the combined organic extracts were washed with 5% aqueous HCl, saturated aqueous sodium bicarbonate, and brine solution. The organic layer was then dried over sodium sulfate and then the solvent was removed *in vacuo* and the product was purified over silica (300 mL) eluting

with 10% ethyl acetate in hexanes (900 mL), 25% ethyl acetate in hexanes (900 mL), and 50% ethyl acetate in hexanes (900 mL). The product was obtained as a white solid (15.4 g, 76%). ^{1}H NMR (DMSO-d_6): δ 11.38 (s, 1H), 8.31 (d, J = 6.85 Hz, 1H), 8.02 (d, J = 7.55 Hz, 2H), 7.87-7.80 (m, 3H), 7.68-7.60 (m, 7H), 7.54-7.30 (m, 14H), 6.28 (d, J = 2.30 Hz, 1H), 5.93-5.90 (m, 2H), 4.61 (q, J = 4.52 Hz, 1H), 4.11 (dd, J = 3.25, 11.70 Hz, 1H), 3.97 (dd, J = 4.10, 11.55 Hz, 1H), 1.01 (s, 9H). ^{13}C NMR (DMSO-d_6): δ 168.05, 165.07, 165.02, 164.09, 154.62, 146.22, 135.59, 135.52, 134.41, 134.34, 133.55, 133.31, 132.88, 132.58, 130.54, 129.84, 129.77, 129.24, 129.00, 128.93, 128.50, 128.41, 97.18, 90.56, 82.28, 74.67, 70.82, 63.54, 27.07, 19.23. HR-ESI-MS: calcd for $[M+Na]^+$, 816.2712; found 816.2740.

2'-3'-*O*,*N*^{4}-Tribenzoyl cytidine (17)[3] The protected cytidine derivative **16** (15.5 g, 19.5 mmol) was dissolved in THF (30 mL). Acetic acid (1.7 mL, 29 mmol) was added followed by tetrabutylammonium fluoride solution (1.0 M, 59 mL, 59 mmol). The reaction was stirred at room temperature for 1 h and then the solvents were removed *in vacuo*. The resulting syrup was partitioned between saturated aqueous sodium bicarbonate and DCM, and the aqueous layer was further extracted using DCM (2x). The combined organic extracts were washed with a brine solution, and dried over sodium sulfate. The product was purified over silica (150 mL) eluting with 50% ethyl acetate in hexanes (450 mL), 75% ethyl acetate in hexanes (450 mL), and 100% ethyl acetate (450 mL) with the product obtained in 100% ethyl acetate as a white solid (9.9 g, 90%). ^{1}H NMR (DMSO-d_6): δ 11.31(1s, 1H), 8.52 (d, J = 7.00 Hz, 1H), 8.02 (d, J = 7.25 Hz, 2H), 7.93 (d, J = 8.05 Hz, 2H), 7.83 (d, J = 7.15, 2H), 7.68-7.58 (m, 3H), 7.53-7.39 (m, 7H), 6.38 (d, J = 4.95 Hz, 1H), 5.85 (t, J = 5.31 Hz, 1H), 5.79 (t, J = 5.64 Hz, 1H), 5.50 (t, J = 5.11 Hz, 1H), 4.53 (q, J = 3.65 Hz, 1H), 3.88 (m, 1H), 3.81 (m, 1H). ^{13}C NMR (DMSO-d_6): δ 167.33, 164.68, 164.43, 163.54, 154.46, 145.65, 133.82, 133.78, 133.00, 132.74, 129.24, 129.23, 128.76, 128.67, 128.45, 128.39, 96.82, 88.38, 83.15, 74.39, 71.44, 60.47. HR-ESI-MS: calcd for $[M+Na]^+$, 578.1534; found 578.1538.



Reagents and Conditions: a) sulfamoyl chloride, DMA

Scheme 6: Synthesis of sulfamoyl tribenzoyl cytidine

Sulfamoyl chloride[4] Chlorosulfonyl isocyanate (1.2 mL, 14.1 mmol) was dissolved in anhydrous DCM (7 mL) and cooled to 0°C. 88% Formic acid (0.65 mL, 14.7 mmol) was added dropwise and stirred at 0°C for 15 min. The reaction was stirred at rt for 45 min and then heated to reflux for 45 min. The reaction mixture was then placed in the -20°C freezer overnight. The next day the mixture was cooled to -48°C and the sulfamoyl chloride crystallized out. The crystals were vacuumed filtered and washed with DCM to yield the desired product (1.6 g, 96%).

2'-3'-*O*,*N*^{4}-Tribenzoyl-5'-*O*-sulfamoyl cytidine (18) Tribenzoyl cytidine **4** (1.54 g, 2.7 mmol) was dissolved in anhydrous DMA (5 mL). A solution of freshly made sulfamoyl chloride (940 mg, 8.11 mmol) dissolved in anhydrous DMA (2 mL) was added dropwise at 0°C. The ice bath was removed and the reaction was stirred at rt for 3 h. The reaction was quenched with H_2O (1 mL) and the solvents were

removed *in vacuo*. The resulting syrup was partitioned between EtOAc (250 mL) and brine (50 mL). The brine layer was washed with EtOAc (2 x 50 mL) and then the organic extracts were combined, dried (Na_2SO_4), and concentrated *in vacuo*. The crude mixture was then purified over silica (50 mL) eluting with 50% EtOAc in hexanes to yield the desired product as a white solid (1.32 g, 77%). ^{1}H NMR (DMSO-d_6): δ 11.41 (s, 1H), 8.29 (d, *J* = 7.50 Hz, 1H), 7.87 (d, *J* = 8.10 Hz, 2H) 8.03 (d, *J* = 8.00 Hz, 2H), 7.95 (d, *J* = 8.20 Hz, 2H), 7.75 (s, 2H), 7.70-7.65 (m, 3H), 7.52-7.40 (m, ovlp, 7H), 6.27 (d, *J* = 3.65 Hz, 1H), 5.91 (t, *J* = 5.05 Hz, 1H) 5.81 (t, *J* = 6.10 Hz, 1H), 4.77 (s, 1H), 4.50-4.44 (m, 1H), 4.43-4.41 (m, 1H). ^{13}C NMR (DMSO-d_6): δ 167.53, 164.57, 164.47, 163.70, 154.19, 146.54, 133.91, 133.87, 133.00, 132.80, 129.30, 128.72, 128.70, 128.47, 128.41, 128.36, 96.93, 90.66, 79.46, 73.64, 70.64, 67.88. HR-ESI-MS: calcd for $[M-H]^-$, 635.1448, found 635.1445.

Purification of *E. faecalis PPCS*: *E. coli* BL21 AI/pUMGD1 was used to express *E. faecalis* PPCS and the enzyme purified by previously published methods.[5]

Purification of C-terminal Hexa-Histidine Tagged Human *PPCS*: *E. coli* BL21 (DE3)/pUMJY120ho was used to express human PPCS and the enzyme purified by previously published methods.[6]

Cloning, Overexpression, and Purification of *E. coli PPCS*: The *coaB* coding region of the *dfp* gene (encoding ser181-arg406 of the *E. coli* CoaBC protein)[7, 8] was PCR amplified using *E. coli* MG1655 genomic DNA as a template, and the primers, coabec1(forward primer), 5' – CGCGCATA TGTCGCCCGTCAACGACCTGAAACATCTG-3' and dfp3 (reverse primer), 5'-GCGCCTCGAGACGTCGATTTTTTTCATCATAACGGG-3'. The forward primer introduces an *NdeI* site (shown underlined) to provide a start codon for the coaB coding region, and the reverse primer creates a *XhoI* site (shown underlined) downstream of the stop codon of the open reading frame. The PCR products were digested with *NdeI* and *XhoI*, and ligated into pET23a(+) (Novagen) cut with *NdeI* and *XhoI*. The resulting plasmid was designated pUMDOT3 and the insert was confirmed by DNA sequencing.

E. coli BL21 AI (Invitrogen) harboring the plasmid pUMDOT3 were grown in 500 mL LB-ampicillin media (5 g of NaCl, 5 g of yeast extract, 10 g of tryptone, and 100 mg of ampicillin per L) at 37°C and 250 rpm to a OD_{600} of 0.6. The cells were then cooled by shaking at 16°C for 10-15 min, induced with 0.07% L-arabinose, and continued to grow at 16°C and 250 rpm for 12-16 hours. The cells were harvested at 6000 x g for 10 minutes at 4°C, washed, and then suspended in 12 ml of 20 mM HEPES pH 8.0. Cells were lysed by French Press and crude cytosol obtained by centrifugation at 20,000 x g for 25 minutes at 4°C.

ecPPCS was purified using a tandem anion exchange column (Source 15Q (GE Healthcare); 20 mL) and cation exchange column (Source 15S (GE Healthcare); 8 mL). The 12 mL of crude cytosol was loaded onto the tandem chromatography columns which had been pre-equilibrated with 20 mM HEPES pH 8.0. The columns were then washed with another 40 mL of equilibration buffer and the anion exchange column was removed. Under these conditions the ecPPCS does not bind to the anion exchange resin, but does bind to the cation exchange resin. The cation exchange column was eluted with a linear gradient of 0-0.4 M NaCl in 20 mM HEPES pH 8.0, with a total gradient volume of 100 mL. ecPPCS eludes as a single peak at 75 mM NaCl and was greater than 98 % pure as determined by SDS-PAGE.

Cloning, Overexpression, and Purification of the C-terminal Hexa-Histidine Tagged *Streptococcus pneumoniae* PPCS. The *coaB* gene was amplified from *S. pneumonia* TIGR4 genomic DNA via PCR, using the forward primer CATATGAAAATTTTAGTTACATC and reverse primer CTCGAGAGAATGATAGGCTTGAATTTTTTC to introduce a *Nde*I site before and *Xho*I site after the gene. The PCR product from the amplification was digested with *Nde*I and *Xho*I, and then ligated into pET23a(+) (Novagen) also digested with *Nde*I and *Xho*I. The desired plasmid was designated pUMJY140h, and the sequence of the inserted *coaB* gene was confirmed by DNA sequencing. Since the reverse primer was designed to exclude the stop codon of the gene, the linker and hexa-histidine tag encoded by the pET23a(+) vector is expressed with the gene to generate the C-terminal hexa-histidine tagged PPCS.

E. coli strain BL21 AI, transformed with plasmid pUMJY140h, was incubated in four 1 L flasks containing 250 ml each of LB-ampicillin media at 37ºC and 250 rpm to a OD_{600} of 0.6-0.8. Then, the culture was cooled to 16ºC and induced with L-arabinose (0.065% w/v final concentration). Incubation at 16ºC and 250 rpm shaking was continued for 16 hours. Cells from 1 L of culture were harvested via centrifugation at 6,000 x g, washed with 20 mM HEPES pH 8.0, and resuspended in 60 ml of 20 mM HEPES pH 8.0. The harvested cells were lysed via French Press, and the lysed mixture was centrifuged at 20,000 x g for 30 minutes to spin down the cellular debris as the pellet.

The resulting supernatant was shaken gently with Ni-NTA resin (4 mL per 1 L culture) in a solution of 10 mM imidazole and 20 mM HEPES pH 8.0 for 10 minutes. Then, the mixture was poured into a 20 mL column and the resin was collected in the column. The column was washed with 5 column volumes of 50 mM imidazole, 20 mM HEPES pH 8.0, followed by 5 column volumes of 50 mM imidazole, 500 mM NaCl, 20 mM HEPES pH 8.0. The column was pre-equilibrated prior to elution by flowing through 2 column volumes of 50 mM imidazole, 20 mM HEPES pH 8.0, and then eluted with 250 mM imidazole, 20 mM HEPES pH 8.0 solution. The elution was collected as 1 mL fractions, until proteins were no longer eluted. The fractions containing protein were collected, diluted 4 fold with 20 mM HEPES pH 8.0, and chromotographed on a Mono Q 5/50 GL column pre-equilibrated with 20 mM HEPES pH 8.0 buffer. The column was eluted over a 10 column volume linear gradient of 0 – 0.5 M NaCl buffered with 20 mM HEPES pH 8.0. Approximately 30 mg of spPPCS was purified per liter of culture.

PPCS inhibition assays: The PPCS reaction was observed in the forward reaction via an enzyme coupled assay.[5, 6] The coupled assay measured production of pyrophosphate from PPCS activity via the oxidation of NADH, which could be monitored as a disappearance of absorption at 340 nm. For each mole of pyrophosphate produced, two moles of NADH are oxidized. The commercially available Pyrophosphate Reagent (PR) from Sigma-Aldrich was used as the pyrophosphate detection system and each vial of the PR was initially suspended in 4.5 mL of 100 mM Tris-HCl pH 7.6. Assays were performed on a SpectraMax M5 (Molecular Devices) microplate reader using 96-well half-area plates (Costar UV), with a final assay volume of 100 μL. The pre-incubation mixture consisted of 30 μL PR, 20 μL PPCS (20-400 nM final assay concentration), and 20 μL of varying concentrations of inhibitor in a total volume of 70 μL. These solutions were preincubated at 37°C for 15 minutes. The enzymatic reaction was initiated by addition of the substrates (also pre-incubated at 37°C for 15 minutes) to the assay mixture, to a final concentration of 0.6 mM MgCTP, 1.0 mM L-cysteine, and 0.6 mM PPA. The oxidation of NADH, monitored by a decreasing UV absorbance at 340 nm (ε = 6.22 mM^{-1} cm^{-1}), is monitored over the course of the assay. Assays were run in triplicate with the average IC_{50} being reported. As a control, pre-incubation of the PR (in the absence of PPCS) with the highest

concentrations of inhibitors (1000 nM) showed no inhibition of the coupling enzymes when assayed as above with the addition of magnessium pyrophosphate (0.1 mM).

Mode of Inhibition of Bisubstrate Inhibitor: Assays were performed on a SpectraMax M5 (Molecular Devices) microplate reader using 96-well half-area plates (Costar UV), with a final assay volume of 100 μL. The final assay mixture consisted of 30 μL of PR, 0.86 mM MgCTP, 0.1 mM PPA, 0.3 mM L-cysteine, 10 mM DTT, 100 nM efPPCS, varying concentrations of CTP (0.05-1.0 mM), and varying concentrations of compound **13** (compound **7** in manuscript; 0-1 μM). The assay mixture minus PPA was preincubated at 37°C for 20 minutes in a total volume of 90 μL. The enzymatic reaction was initiated by addition of 10 μl of 1.0 mM PPA (also pre-incubated at 37°C for 20 minutes). The oxidation of NADH, monitored by a decreasing UV absorbance at 340 nm (ε = 6.22 mM^{-1} cm^{-1}), is monitored over the course of the assay (assays ran in duplicate).

The initial velocity data was analyzed and fit via non-linear fitting, and replotted as double reciprocal plots (Figure 1). The plot shows a noncompetitive mode of inhibition (lines intersecting to the left of the y-axis) for compound **13**, indicating that the bisubstrate inhibitors have some affinity for the enzyme substrate complex, as well as to the free enzyme.

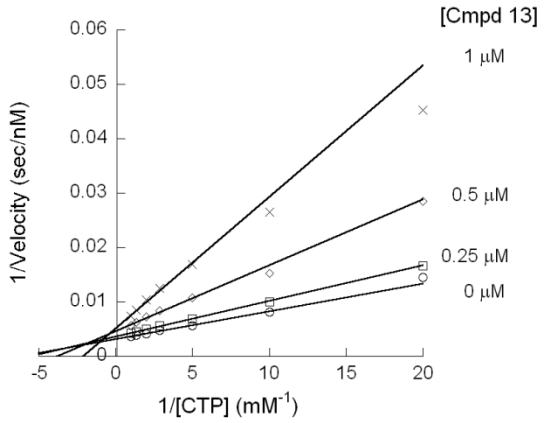


Figure 1. Double reciprocal plot obtained from initial velocity versus [CTP] at various fixed concentrations of compound **13** (compound **7** in manuscript).

The data was then fit to equation 1, which gave a K_i = 0.4 μM and α = 2.9.

$$v = \frac{V_{max}[S]}{[S]\left(1+\frac{[I]}{\alpha K_i}\right)+K_M\left(1+\frac{[I]}{K_i}\right)} \qquad (1)$$

***K_i* Determination**:[9] Assays were performed on a SpectraMax M5 (Molecular Devices) microplate reader using 96-well half-area plates (Costar UV), with a final assay volume of 100 μL. The assay mixture consisted of 30 μL of PR, 0.86 mM MgCTP, 0.86 mM PPA, 1.43 mM L-cysteine, 14 mM DTT, and 19 μL of varying concentrations of inhibitor in a total volume of 70 μL. The assay mixture was preincubated at 37°C for 15 minutes. The enzymatic reaction was initiated by addition of 30 μl of efPPCS (also pre-incubated at 37°C for 15 minutes) to the assay mixture, to a final concentration of 27 nM. The oxidation of NADH, monitored by a decreasing UV absorbance at 340 nm (ε = 6.22 mM^{-1} cm^{-1}), is monitored over the course of the assay (assays ran in triplicate).

The inhibition progress curves displayed a time-dependent decrease in reaction rates indicative of slow-onset, tight-binding inhibition. In order to obtain the apparent first-order rate constant k_{obs}, the inhibition progress curves were fit, using the software KaleidaGraph (Synergy Software, Inc.), to equation 2, where P is the amount of pyrophosphate produced during a period of time t, v_i and v_s are the initial and equilibrium rates, [E] is the concentration of efPPCS in the assay, and [I] is the concentration of inhibitor in the assay.

$$P = v_s t + \frac{(v_i - v_s)(1-\gamma)}{k_{obs}\gamma} ln\left\{\frac{[1-\gamma \exp(k_{obs}t)]}{1-\gamma}\right\} \quad (2)$$

where

$$\gamma = \frac{[E]}{[I]}\left(1 - \frac{v_s}{v_i}\right)^2 \quad (3)$$

Subsequent plotting of the k_{obs}, obtained from the fit of the inhibition progress curves, against inhibitor concentration resulted in data points with a linear relationship (conforming to equation 4). This is indicative of a single-step enzyme inhibition mechanism characterized by slow association and slow dissociation of compound **6** (compound **3** in manuscript).

$$k_{obs} = k_3^{app}[I] + k_4 \quad (4)$$

K_i^{app} was obtained from equation 5. Taking into account the noncompetitive mode of inhibition, K_i^{app} was converted to K_i using equation 6 (α= 2.9).[10]

$$K_i^{app} = k_4/k_3^{app} \quad (5)$$

$$K_i^{app} = \frac{[S]+K_M}{\frac{K_M}{K_i}+\frac{[S]}{\alpha K_i}} \quad (6)$$

Complete Reference 4.[11]

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