Supporting Information

A Tool for the Import of Natural and Unnatural Nucleoside Triphosphates into Bacteria

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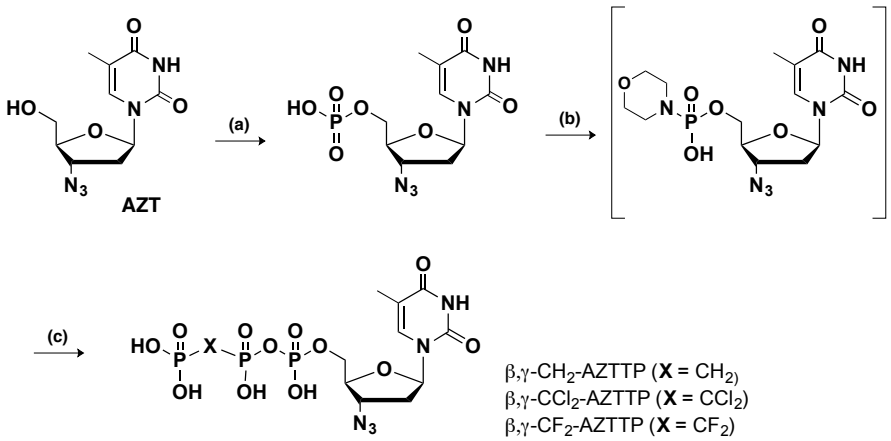


Scheme S1. **Synthesis of β,γ-modified AZT triphosphates**. (a) Proton Sponge, $POCl_3$, $(MeO)_3P$, -15°C, 3hr, (b) Morpholine, DCC, $tBuOH/H_2O$ (50:50), reflux, 5.5hr, (c) $Bu_3N{\bullet}O_3P\text{-}X\text{-}PO_3H$, DMSO, 3d

Methods

Synthetic procedures and compound characterization

Synthesis of AZT monophosphate:

Azidothymidine (AZT) nucleoside (0.1 mmol) and proton sponge (27.9 mg, 0.13 mmol) were placed in a reaction flask along with a magnetic stirbar and dried overnight under vacuum with P_2O_5. The solids were then taken up in freshly distilled trimethyl phosphate (0.47 mL, 4.0 mmol, dried over activated 4 Å molecular sieves) and cooled to -18 °C in a bath of salt and ice. Freshly distilled $POCl_3$ (12.1 μL, 0.13 mmol) was added dropwise, and the reaction was stirred for 3 h between -10 °C and -18 °C. The reaction was quenched by the addition of 0.5

mL 0.5 M TEAB pH 7.5. The crude reaction mixture was purified directly by ion exchange chromatography (DEAE Sephadex A-25; GE Healthcare) with a linear gradient of 0 to 1.2 M TEAB pH 7.5.

General synthesis of β,γ-modified AZT triphosphates:

A solution of AZT monophosphate (35 μmol) and morpholine (9.2 μL, 0.105 μmol) in 50% *t*BuOH/H_2O (1.0 mL) was stirred at room temperature for 15 min before heating to a reflux. A solution of *N,N'*-dicyclohexylcarbodiimide (21.7 mg, 0.105 μmol) in *t*BuOH (0.2 mL) was added dropwise to the refluxing reaction, and the resulting mixture was allowed to reflux an additional 5.5 h, monitoring reaction progress by ^{31}P NMR. Upon cooling to room temperature, solvent was removed *in vacuo* and residue was redissolved in H_2O. Solid side products were removed via filtration through celite, rinsing with H_2O. The combined aqueous solutions were extracted with diethyl ether three times. The resulting aqueous layer was concentrated (Savant SpeedVac concentrator) and dried overnight on high vacuum to yield the AZT morphilidate.

The tributylammonium salt of disubstituted-methylene bisphosphonate (34.5 μmol) was dissolved in anhydrous DMSO (0.3 mL). Dried AZT morpholidate (0.17 mmol) was dissolved in anhydrous DMSO (0.3 mL), and the resulting solution was added to the reaction vial dropwise. Upon sealing under argon, the reaction was allowed to stir at room temperature for 3 days, monitoring reaction progress by HPLC. The crude reaction mixture was purified by ion exchange chromatography (DEAE Sephadex A-25) with a linear gradient of 0 to 1.2 M TEAB pH 7.5. Upon concentration (SpeedVac), any remaining inorganic phosphonate was removed via further purification by reverse-phase (C18) HPLC (linear gradient of 5% to 35% acetonitrile in 0.1 M TEAB, pH 7.5), and then concentrated to dryness (SpeedVac).

Inhibition based uptake assay

An overnight culture (~3 mL) of the *E. coli* SSO YZ2 (genotype: BL21(DE3) *lacZYA*::P_{lacUV5}*Pt*NTT2(66-575)-T_0 Cm^{R1}) was diluted 100-fold in media supplemented with chloramphenicol (~8 mL) and incubated at 37 °C to an OD_{600} ~ 0.4–0.6 (~1.5 h). For each sample, an aliquot of cells (92.5 μL) was mixed with ATP (50 μM) spiked with [α-^{32}P]-ATP (4 μCi/mL, Perkin Elmer) and an excess of the nucleoside triphosphate being tested for uptake (500 μM) to a final volume of 100 μL, with one sample receiving water in place of additional triphosphate as an ATP-only control. Treated cells were further incubated at 37 °C for 10 min, At this time, the entire culture was collected through a 96-well 0.65 μm glass fiber filter plate (MultiScreen, EMD Millipore) under vacuum and washed with ddH_2O (2 × 100 μL). Filters carrying cells from each sample were exposed overnight to a storage phosphor screen, which was then imaged on a Typhoon 9210 flatbed laser scanner (GE Healthcare). Inhibition of ATP uptake was determined by densitometric analysis of the resulting image using Image Studio Lite (LICOR Biosciences) to quantify radiodensity, normalizing to the ATP-only control and expressing the value as percent

inhibition of ATP uptake. Reported values are the average and standard error of at least three independent determinations.

Radiolabel based uptake assay

An overnight culture of SSO cells (YZ2) was diluted to OD ~ 0.03 in 2×YT supplemented with 50 mM KPi and chloramphenicol (5 μg/mL) and incubated at 37 °C with shaking to an OD600 of ~ 0.3. The culture was then transferred to a 96 well plate in 100 μL aliquots, each supplemented with varying concentrations of the radiolabeled triphosphate of interest. The titration of triphosphate was prepared by mixing unlabeled triphosphate with the corresponding radiolabeled triphosphate, starting at a molar concentration of 1000 μM supplemented to 4 μCi/mL, and decreasing 2-fold in a serial dilution for a total of 8 samples per titration. Upon addition of the triphosphate, initial OD600 measurements were taken using an identical series of cultures in a separate plate that did not receive radiolabeled nucleotide (referred to as the "cold" plate). Both the "hot" plate (containing radiolabeled nucleotides) and the "cold" plate were then incubated at 37 °C for 1 h. At this time, a final OD600 measurement was taken of the "cold" plate, and the cultures in the "hot" plate were collected through a 96-well 0.65 mm glass fiber filter plate (MultiScreen, EMD Millipore) under vacuum and washed with ddH_2O (2 × 100 μL). Filters carrying cells from each sample were exposed overnight to a storage phosphor screen, alongside a calibration curve constructed using a titration of radiolabeled nucleotide blotted onto Whatman filter paper, starting at 0.5 nCi and decreasing 2-fold for a total of 5 calibration points. The storage phosphor screen was then imaged and the resulting radiodensities were quantified as for the inhibition based uptake assay (see above). The radiodensity measurement of each calibration point were plotted against its known molar concentration using the specific activity (determined at the start of the experiment for a given radiolabeled nucleotide of interest) and fit to a linear regression. The equation of this line was used to convert the radiodensity of each filtered culture to a molar concentration of nucleoside triphosphate imported. The rate of uptake was determine by normalizing these values to time and OD600, which was then converted to a per cell rate using the approximation of 1 OD600 = 8×10^8 cells. The rate of uptake was plotted against the molar concentration of triphosphate added to the media, and fit to a curve using the Michaelis-Menton equation to determine V_{max} and K_M.

Fluorescence based uptake assay

An overnight culture of SSO cells (YZ2) was diluted to OD600 ~ 0.03 in 2×YT supplemented with 50 mM KPi and chloramphenicol (5 μg/mL) and incubated at 37 °C with shaking to an OD600 of ~ 0.3. The culture was then transferred to a 96 well plate in 100 μL aliquots, each supplemented with varying concentrations of the triphosphate of interest. The titration of triphosphate started at 125 μM in the media for thCTP, and 1000 μM for thGTP, decreasing 2-fold in a serial dilution for a total of 8 samples per titration. Upon addition of the triphosphate, initial OD600 measurements were taken and the cultures were further incubated at 37 °C for 1 h. A final OD600 measurement was taken and each sample was then transferred to a 1.5 mL microcentrifuge tube and pelleted at 3000×*g* for 10 min. Media was removed, and cell pellets were resuspended in 200 μL 1× PBS. Cells were then

pelleted and resuspended again for a second 200 ×L wash. Cells were pelleted again and resuspended in 70 μL of Lysis Buffer L7 (PureLink, Thermo Fisher Scientific) and incubated at room temperature for 5 minutes before adding 80 μL Precipitation Buffer (PureLink). Samples were gently vortexed and centrifuged at 16000×*g* for 10 min. For each sample, 100 μL of supernatant was transferred to a black, flat-bottom 96-well plate for fluorescence measurements, which were recorded using an Envision microplate reader (Perkin Elmer). For thGTP, fluorescence was measured with λ_{ex}=316 nm and λ_{em}=458 nm. For thCTP, fluorescence was measured with λ_{ex}=320 nm and λ_{em}=429 nm. Fluorescent signals were converted to molar concentrations using an external calibration curve constructed using a protocol identical to that described above, with the exception that nucleoside triphosphates were added just after cell lysis at concentrations starting 1.0 μM and decreasing 2-fold for a total of 8 data points, which were subsequently fit to a linear regression. Uptake values were normalized to time and OD600, which was converted to a per cell rate using the approximation of 1 OD600 = 8×10^8 cells. The rate of uptake was plotted against the concentration of triphosphate added to the media, and fit to a curve using the Michaelis-Menton equation to determine V_{max} and K_M.

LC-MS/MS based uptake assay

An overnight culture of SSO cells (YZ2) and a *Pt*NTT2(-) control were each diluted 100-fold in 2×YT + KPi (50 mM) + chloramphenicol (5 μg/mL), and incubated at 37 °C to an OD600 of ~0.4–0.6. Cultures were then split into 2 sets of 500-μL samples supplemented with the triphosphate of interest at varying concentrations, starting at 1000 μM and decreasing 2-fold to 7.8 μM, for a total of 8 samples per strain. Immediately upon addition of the triphosphate, an initial OD600 reading was taken for each sample, followed by incubation at 37 °C for 1 h. At this time, a final OD600 reading was taken, and samples were rapidly pelleted at 16000 x g for 5 min. Media was removed from each sample, and pellets were carefully washed twice with 500 μL of cold 50 mM KPi in water. Washed pellets were then resuspended in 300 μL of chilled extraction solvent (80:20 MeCN/H_2O with 0.1 M formic acid) and held at 4 °C for 15 minutes. Insoluble material was pelleted at 16000 x g for 5 minutes. Supernatants were collected, and pellets were resuspended in another 200 μL of extraction solvent. Samples were again held at 4 °C for 15 minutes before pelleting and collecting the supernatant. Pellets were then extracted one more time with another 200 μL of extraction solvent. The final 700 μL of collected supernatant per sample was lyophilized to dryness. Dried samples were then resuspended in 45 μL of 1× CutSmart buffer (New England Biolabs) before adding 5 μL of rSAP (NEB, 1000 U/mL) and incubating at 37 °C for 3 h. Digested extracts were then directly analyzed by LC-MS/MS alongside an external calibration curve, monitoring for the mass transition of the corresponding nucleoside. Calibration curves were constructed using pure nucleoside solutions in water (corresponding to the triphosphate of interest) starting from either 10 μM or 1 μM and decreasing 2-fold by serial dilution for a total of 8 samples. The area under the curve (AUC) for the peak in the total ion current (TIC) of each calibration point was plotted against the concentration of analyte, and the resulting plot was fit to a linear regression. Both calibration curves and uptake samples were injected twice, and the areas under the curve for the two injections were averaged for further analysis. Signal from *Pt*NTT2(-) samples were subtracted from their

corresponding YZ2 samples for background correction, and then converted to molarity using the calibration curve. The resulting uptake values were normalized to time and OD600, which were converted to a per cell rate using the approximation of 1 OD600 = 8×10^8 cells. The rate of uptake was plotted against the concentration of triphosphate added to the media, and fit to a curve using the Michaelis-Menton equation to determine V_{max} and K_M. LC conditions: Buffer A = 10 mM ammonium formate in H_2O; Buffer B = 100% MeOH; flow rate = 0.8 mL/min; run time = 15 min; gradient = 0 – 100% buffer B over 10 min.

Radiolabeling of RNA and DNA in *E. coli*

Strain YZ2, harboring a pUC19 plasmid, was grown for 16 h at 37 °C in 2×YT supplemented with 5 μg/mL chloramphenicol, 50 mM potassium phosphate (pH 7), and 100 μg/mL ampicillin (from here on referred to as media). The culture was diluted to OD600 ~ 0.03 in fresh media and incubated at 37 °C until reaching an OD600 ~ 0.3. At this point the culture was divided into 250 μL aliquots in a 48-well plate, each containing the appropriate triphosphate of interest at a concentration of 500 μM and supplemented with the corresponding [α^{32}P]-labeled analog at 4 μCi/mL (referred to as the "hot" plate). An identical plate of cultures was set up in parallel lacking the radiolabeled nucleotides for monitoring growth (referred to as the "cold" plate). The cultures were then incubated for 1 h at 37 °C before pelleting at 6000 rpm for 5 min. Upon removing the supernatant, cell pellets were frozen at -80 °C.

For RNA extraction, pellets were resuspended in 1.0 mL of Trizol reagent (ThermoFisher), and incubated at room temperature for 2–5 minutes before adding 0.2 mL of chloroform. Samples were shaken vigorously by hand for 15 s and incubated at room temperature for 2–3 minutes before centrifuging at 16000 x g for 15 min. The aqueous upper layer (~0.5 mL) was carefully removed, transferred to a new 1.5 mL microcentrifuge tube, and mixed with 0.5 mL of isopropyl alcohol by inversion. After incubating at room temperature for 10 min, samples were centrifuged at 16000 x g for 15 min. The RNA pellet is usually smeared along the side of the microcentrifuge tube. The supernatant was removed by pipet, and 1.0 mL of ice cold 75% ethanol was added. After carefully inverting each sample, tubes were centrifuged at 16000 x g for 10 min at 4 °C. All traces of ethanol were removed by pipet, and pellets were briefly allowed to air-dry before dissolving in 20 μL of nuclease-free H_2O. Extracted RNA was quantified fluorometrically using a Qubit fluorometer (ThermoFisher, RNA BR Assay Kit). RNA (750 ng) was mixed with 10 μL of 2× RNA Loading Dye (New England Biolabs), adding water to a total volume of 20 μL before loading onto a 6% denaturing PAGE gel (8M urea) and running at 300 V for 30 min. Gels were then dried for 2 h at 80 °C under vacuum prior to imaging. Dried gels were exposed overnight to a storage phosphor screen, which was then imaged on a Typhoon 9210 flatbed laser scanner (GE Healthcare).

For DNA extraction, pUC19 plasmids were isolated using commercial miniprep kits (ZR Plasmid Miniprep Classic, Zymo Research), using half the recommended volumes of solutions P1, P2, and P3, and eluting the purified DNA in 10 μL of nuclease-free H_2O. Each sample was quantified fluorometrically using a Qubit fluorometer (ThermoFisher, dsDNA HS Assay Kit). Plasmids were then digested in 10-μL reactions (15 ng each) using 1 μL SphI-HF (20 U, New England Biolabs), 1 μL NdeI (20U, New England Biolabs), and 1 μL 10× CutSmart buffer

(New England Biolabs), and incubating at 37 °C for 1 h before quenching with 1 µL of 0.5 M EDTA. DNA loading dye was added (2 µL to each reaction of 6× Purple Loading Dye, New England Biolabs), and each sample was run on a 6% acrylamide gel in 1× TBE at 130 V for 45 min. Gels were then dried for 2 h at 80 °C under vacuum prior to imaging. Dried gels were exposed overnight to a storage phosphor screen, which was then imaged as described above.

2'-Fluoro labeling of RNA and DNA in *E. coli*

Strain YZ2, harboring a pUC19 plasmid, was grown for 16 h at 37 °C in 2×YT supplemented with 5 µg/mL chloramphenicol, 50 mM potassium phosphate (pH 7), and 100 µg/mL carbenicillin (from here on referred to as media). The culture was diluted to OD600 ~ 0.03 in fresh media and distributed in 500 µL aliquots into a 48-well plate. The plate was then incubated at 37 °C with shaking (200 RPM) until reaching at OD600 ~ 0.3. At this time, the appropriate nucleoside triphosphate was added at the indicated concentration. Cultures were then incubated for another 1.5 h at 37 °C with shaking before pelleting 230 µL of culture and freezing at -80°C for subsequent RNA extraction. RNA was extracted and purified using the RNA *snap*™ protocol,{Stead, 2012 #75} followed by in-column DNase I treatment of 5 µg of crude RNA (RNA Clean & Concentrator™-5, Zymo Research Corp.) For DNA extraction, 8 identical wells of 500-µL cultures were combined for a total of 4 mL, pelleted, and frozen at -80 °C before isolating pUC19 plasmids using commercial miniprep kits (ZR Plasmid Miniprep Classic, Zymo Research). Isolated nucleic acids were then digested in 60 µL reactions containing 200–500 ng of DNA or RNA, calf intestinal phosphatase (30 U, New England Biolabs), phosphodiesterase I (120 mU, MP Biomedical), and benzonase (250 U, EMD Millipore) in digestion buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, pH 8.0). Each digestion reaction was incubated at 37 °C for 16 h. Digestion reactions were then directly analyzed by LC-MS/MS as described above (see LC-MS/MS based uptake assay). The percent labeling in a given sample was determined by simultaneously quantifying the nucleoside of interest and the corresponding natural nucleoside, and calculating the fraction of labeled nucleoside over the sum of both (e.g. % labeling of 2FdC = [2FdC]/([2FdC]+[C])*100).

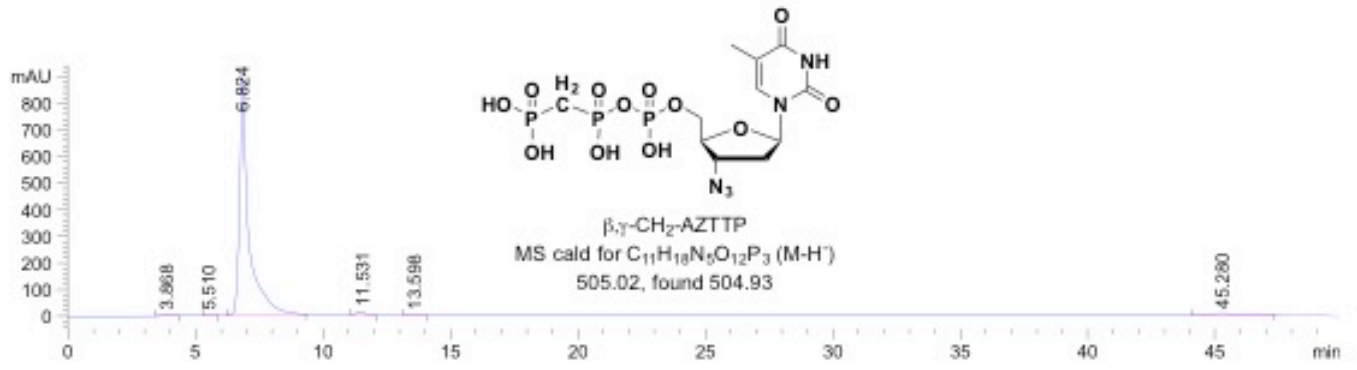


Figure S1. HPLC trace of pure β,γ-CH_2-AZT. Overlayed is the structure, molecular formula, and mass as measured by MALDI

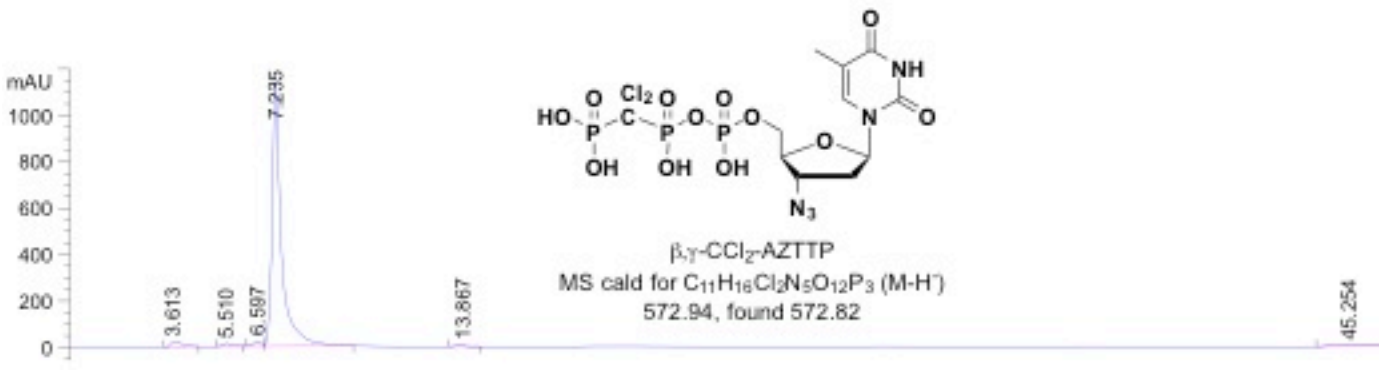


Figure S2. HPLC trace of pure β,γ-CCl_2-AZT. Overlayed is the structure, molecular formula, and mass as measured by MALDI

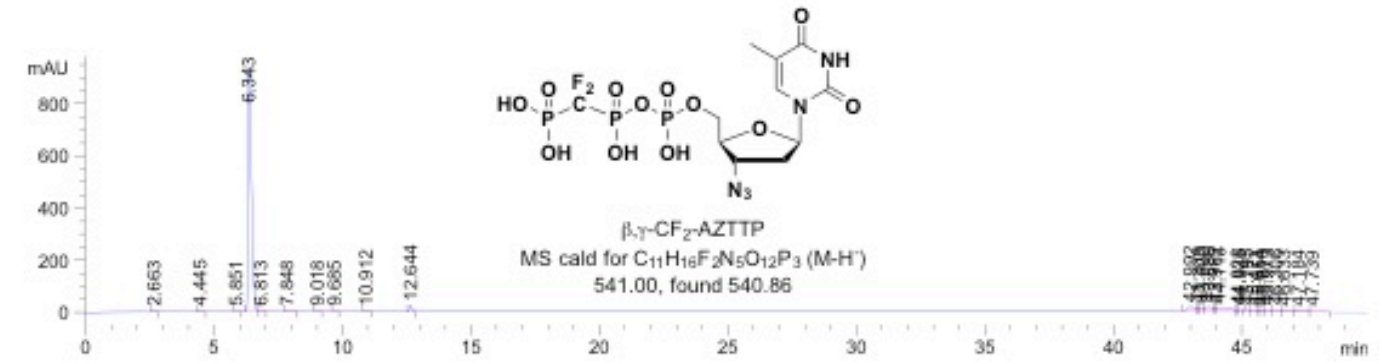


Figure S3. HPLC trace of pure β,γ-CF_2-AZT. Overlayed is the structure, molecular formula, and mass as measured by MALDI

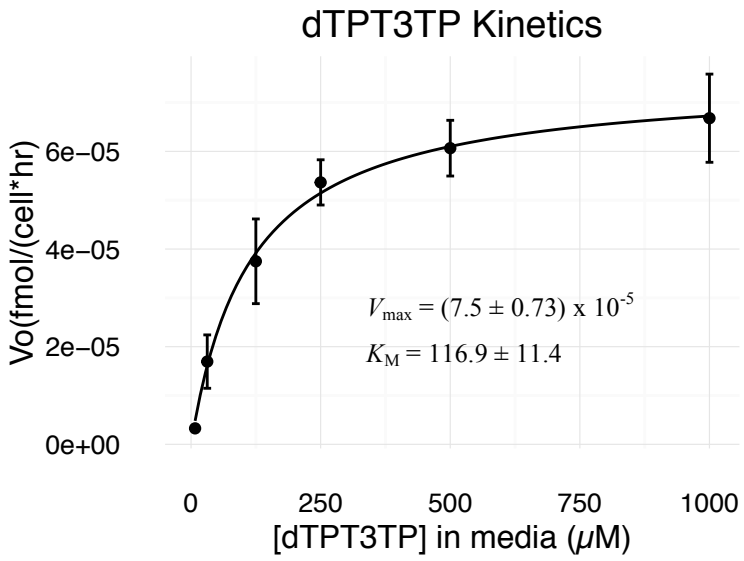


Figure S4. Representative Michaelis-Menten plot. [d**TPT3**TP] refers to the concentration of d**TPT3** triphosphate supplemented in the media. Vo is the initial velocity measured for a given [d**TPT3**TP].

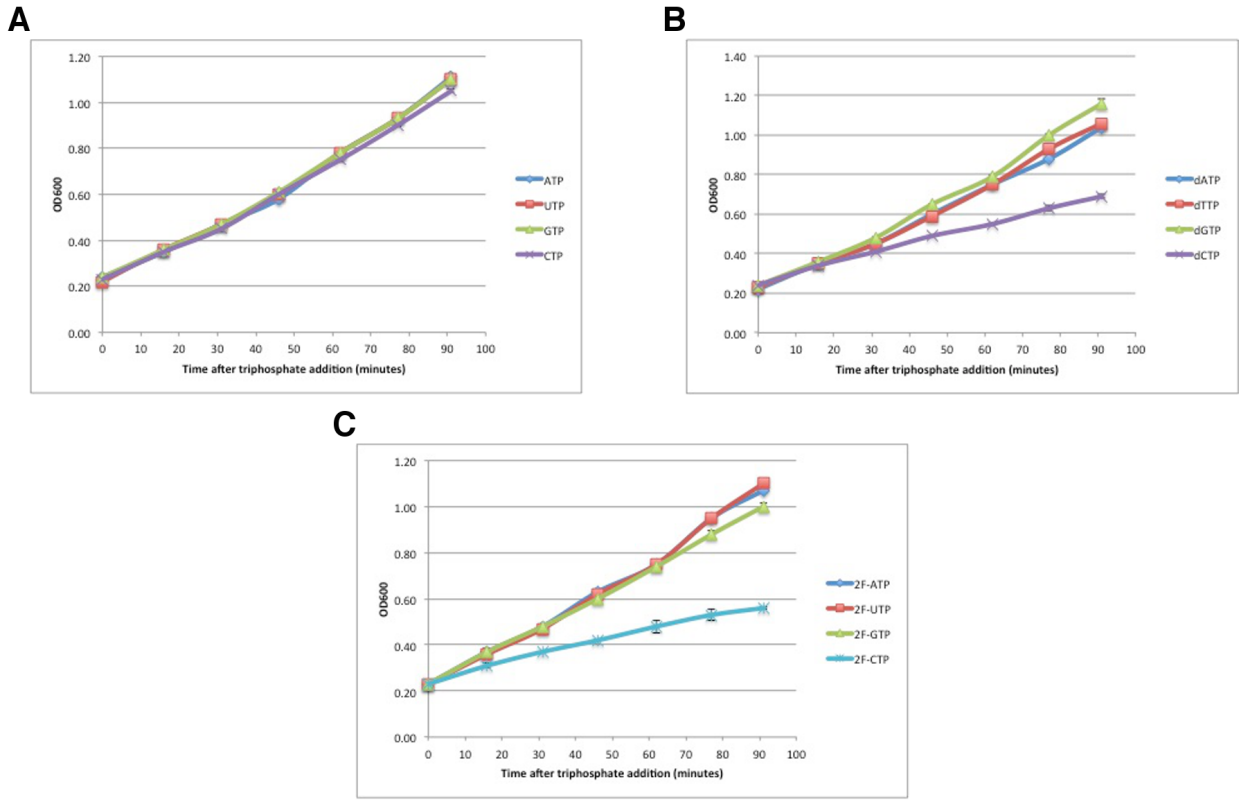
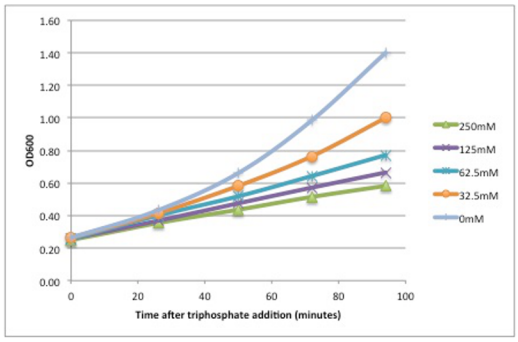


Figure S5. Growth curves of YZ2 after treatment with NTPs **(A)**, dNTPs **(B)**, or 2F NTPs **(C).**



B

[2F CTP] added (μM)	% labeling of C
250	7.15
125	6.69
62.5	5.47
31.25	2.44
0	0.00

Figure S6. Growth curve **(A)** and % labeling **(B)** after treatment of YZ2 cells with a titration of 2'F CTP.

Table S1. Rates of uptake for various additional nucleoside triphosphates by *Pt*NTT2. [a] Assayed by fluorescence. [b] Assayed by radioactivity. [c] Not determined.

Triphosphate	V_{max} (fmol/(cell*hr))	K_M (μM)	V_{max}/ K_M
thCTP[a]	**1.93 (±0.24) × 10^{-3}**	**17.8 (±1.1)**	**1.09 (± 0.09) × 10^{-4}**
thGTP[a]	**4.91 (±0.56) × 10^{-4}**	**92.2 (±9.7)**	**5.34 (± 0.31) × 10^{-6}**
5-IdCTP[b]	**-**[c]	**-**[c]	**1.38 (± 0.11) × 10^{-7}**

Table S2. Specific MS/MS transition monitored for each analog analyzed by LC-MS/MS.

Analog	MS/MS transition
dA	***m/z* 252.31 - 136.33**
3'dA	***m/z* 252.27 - 136.33**
ddA	***m/z* 236.27 - 136.31**
2'F dA	***m/z* 270.43 - 136.32**
tA	**m/z 238.34 - 136.35**
c^3dA	**m/z 251.40 - 118.26**
c^7dA	**m/z 251.33 - 136.33**
d**NaM**	**m/z 292.50 - 197.49**
d**TPT3**	**m/z 284.38 - 168.36**