Enzymatic Incorporation and Utilization of an Emissive Fluorescent 6-Aza Uridine
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S1. Synthesis

S1.1 General

Tris(tetrabutylammonium)hydrogen pyrophosphate, phosphoryl chloride, trimethyl phosphate, 1,8-bis(dimethylamino)naphthalene (proton sponge), and anhydrous DMF were purchased from Sigma-Aldrich. All other solvents were purchased from Fisher Scientific. All reagents and solvents were used without further purification.

S1.2 Synthesis of triphosphate 4

Previously published procedure was used with minor changes, as described below.1,2 Tris(tetrabutylammonium) hydrogen pyrophosphate (0.91 g, 1.0 mmol) in a 10 mL round-bottom flask, and 1 (66 mg, 0.20 mmol) in a 25 mL round-bottom flask, were separately coevaporated with anhydrous pyridine and dried (3 × 1mL). Trimethyl phosphate (2 mL) was added to 1 and cooled in an ice bath to 0 °C. Phosphoryl chloride (46 μL, 0.5 mmol) was added slowly, and the reaction was stirred for 2 h at 0 °C. The coevaporated tris(tetrabutylammonium) hydrogen pyrophosphate was dissolved in anhydrous DMF (2 mL) and added to the reaction mixture with 1. Then proton sponge (216 mg, 0.1 mmol) was added and the reaction was kept stirring at 0 °C for 40 min. To the reaction mixture was added 1 M triethylammonium bicarbonate buffer (TEAB) (6 mL), and the mixture was stirred briefly. The mixture was then transferred to a separatory funnel and washed with ethyl acetate (10 mL). The organic layer was then back-extracted with 1 M TEAB (5 mL). The aqueous layers were combined and concentrated under reduced pressure at room temperature to afford an oily yellow residue. The residue was dissolved in 0.05 M ammonium bicarbonate buffer (10 mL) and loaded onto a DEAE Sephadex A25 anion-exchange column kept in a cold room at 4 °C. The column was eluted using a gradient mixer with 0.01–1.0 M of ammonium bicarbonate buffer. A fraction collector was used to collect 260 fractions that were about 8 mL (220 drops). The fractions containing the triphosphate were evaporated under reduced pressure at 10 °C, and the residue was then lyophilized. The triphosphate was further purified by HPLC (Phenomenex Synergi Fusion-RP 80A C18 column, 4 μm, 250 × 10 nm, 5–20% acetonitrile in 50 mM TEAA buffer, pH 6.0, 30 min). Appropriate fractions were lyophilized and treated with 3% sodium perchlorate in acetone and then with Chelex 100 for 30 minutes with shaking, then filtrated and dried to afford nucleotide 4 (29 mg, 20%). 1H NMR (400 MHz, D2O) δ 8.08 – 7.94 (m, 1H), 7.69 – 7.57 (m, 1H), 7.26 – 7.14 (m, 1H), 6.25 – 6.15 (m, 1H), 4.71–4.63 (m, 1H), 4.69 – 4.52 (m, 1H), 4.38 – 4.20 (m, 2H), 4.18 – 4.02 (m, 1H) and triethylammonium (CH3(CH2)3NH+) counter ion (1equiv.) 3.17 (q, J = 8.0 Hz, 6H), 1.25 (t, J = 6.0 Hz, 9H); 13C NMR (125 MHz, D2O) δ 159.18, 151.91, 139.35, 133.88, 129.84, 129.66, 127.91, 89.49, 82.64, 72.95, 70.47, 66.17 and triethylammonium (CH3(CH2)3NH+) counter ion (1equiv.) 46.44, 8.08; 31P NMR (202 MHz, D2O) δ −8.26 (d, J = 19.9 Hz, Pγ), −11.06 (d, J = 19.2 Hz, Pα), −22.34 (t, J = 19.5 Hz, Pβ); HR ESI-MS (negative ion mode)[C13H16N3O15P3S]− calculated 565.9442, found 565.9438.
S1.3 $^1$H-NMR, $^{13}$C-NMR and $^{31}$P-NMR spectra of triphosphate 4

$^1$H-NMR of 5-(thiophen2-yl)-6-azauridine triphosphate (4)

$^{13}$C-NMR of 5-(thiophen2-yl)-6-azauridine triphosphate (4)
$^{31}$P-NMR of 5-(thiophen-2-yl)-6-azauridine triphosphate (4)
S2. Oligonucleotides synthesis and purification

S2.1 General

All unmodified DNA and RNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. Native NTPs were purchased from Thermo Scientific Dharmacon. Triphosphate 5 (6AzaUTP) was purchased from TriLink BioTechnologies. T7 RNA polymerase (5000 U), T4 Polynucleotide Kinase (PNK) and kinase buffer (2500 U) were purchased from New England BioLabs (NEB). T4 DNA ligase, 50% PEG 4000 and RiboLock (40 U/µl) were purchased from Fermentas Inc. All other solutions and buffers were prepared in our laboratory.

T7 RNA polymerase-mediated in vitro transcription reactions

Transcription reactions with T7 RNA polymerase and the analytically pure triphosphates 4, 5 and 6 were performed to analyze their enzymatic incorporation into short RNA transcripts 7–10. The T7 promoter and template A were annealed, and transcribed in the presence of natural NTPs or with modified UTPs 4, 5, 6 replacing native UTP. An agarose gel electrophoresis revealed full-length 10-mer products (transcripts 7–10). Transcription reaction cocktail contains: annealed template/promoter (500 nM), 1X transcription buffer (pH 9.0), MgCl₂ (16 mM), dithiothreitol DTT (10 mM), RiboLock (1 U/µL), T7 RNA polymerase (0.15 µg/µL), native and modified triphosphates (1 mM). Next, transcription reaction was incubated for 4 h at 37 °C. The precipitated magnesium pyrophosphate was removed by centrifugation. The reaction was concentrated to half of the volume. Then 125 µL of loading buffer was added. The mixture was heated at 75 °C for 3 min, and loaded onto a preparative 20% denaturing polyacrylamide gel. The gel was UV shadowed; appropriate bands were excised, extracted with 0.5 M ammonium acetate, and desalted on a Sep-Pak column. Importantly, when visualizing the gel under UV illumination (365 nm), the product and initiation phase truncated transcripts are highly fluorescent.

T4 polynucleotide kinase (PNK)-mediated phosphorylation

Donor RNA construct for the ligation reaction was purchased from IDT Integrated DNA Technologies and purified before phosphorylation. Phosphorylation reaction cocktail contains: oligonucleotide (0.006 mM), ATP (1 mM), dithiothreitol DTT (5 mM), 10X kinase buffer (0.1 v/v), PNK (0.2 U/µL). After reaction mixture was incubated for 2 h at 37 °C and precipitated from ethanol, an agarose gel electrophoresis revealed quantitative conversion.³

T4 DNA ligase-mediated in vitro ligation reaction

Ligation reaction with T4 DNA ligase and analytically pure donor and acceptor constructs were performed with presence of splint DNA oligonucleotide for better selectivity. The reaction consists of two steps. First, acceptor (10 µM, 1 equiv.), donor (10 µM, 1.5 equiv.) and splint (1.5 equiv.) and Tris-HCl buffer (40 mM, pH 7.8) were mixed and heated at 90 °C for 3 minutes and cooled down slowly until 40 – 50 °C. Second, MgCl₂ (10 mM), dithiothreitol DTT (10 mM), ATP (0.5 mM) 50% PEG 4000 (0.1 V) and T4 DNA ligase (100 U/nmol) were added to the previous reaction mixture and
incubated for 2 h at 37 °C. Then ethanol precipitation was performed and final product was purified by agarose gel electrophoresis.°

**S2.2 T7 RNA polymerase-mediated in vitro transcription reaction**

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**Figure S2.2.** Transcription reaction was carried out with T7 promoter and template B in the presence of natural NTPs and UTP or modified 1. Lanes 1–3: control reaction with all natural NTPs; lanes 4–6: reaction in presence of 1. The reaction was resolved by agarose gel electrophoresis on a denaturing 20% polyacrylamide gel; with UV light at 254 nm (on TLC plate) on the left, and 365 nm on the right.

**S2.3 T4 DNA ligase-mediated in vitro ligation reaction**

<table>
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<tr>
<td>Lane</td>
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<td>2</td>
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**Figure S2.3.** Ligation reaction was carried out with T4 DNA ligase in the presence of donor and native acceptor (11) or modified acceptor (12). Lane 1: control reaction with native acceptor (transcript 11); lane 2: reaction with modified acceptor (transcript 12). The reaction was resolved by agarose gel electrophoresis on a denaturing 20% polyacrylamide gel; with UV light at 254 nm (on TLC plate) on the left, and 365 nm on the right.
S3. MALDI-TOF MS measurements

S3.1 Transcripts 7–12

Figure S3.1a. MALDI-TOF MS results for (a) transcript 7 calculated [M] 3413 found 3411, (b) transcript 8 calculated [M] 3497 found 3492, (c) transcript 9 calculated [M] 3414 found 3412, (d) transcript 10 calculated [M] 3496 found 3493.

Figure S3.1b. MALDI-TOF MS results for 11-mer ligase acceptor RNA oligonucleotides (a) transcript 11 calculated [M+K] 3741 found 3742, (b) transcript 12 calculated [M+K] 3825 found 3825.

S3.2 Ligation constructs 13–14

Figure S3.2. MALDI-TOF MS results for (a) ligation products 13 calculated [M] 10175 found 10172, (b) ligation products 14 calculated [M] 10258 found 10254.
S4. Digestion of oligonucleotides

All transcripts 7–12 and ligation products 13–14 (1–2 nmol) were incubated with S1 nuclease in a reaction buffer (Promega) for 2 h at 37 °C. The reaction was further treated with alkaline phosphatase and dephosphorylation buffer (Promega) for 2 h at 37 °C. The ribonucleoside mixture obtained was analyzed by reverse-phase analytical HPLC with an Agilent column eclipse XDB-C18 (5 μm, 4.6 × 150 mm). Mobile phase: 0–5% acetonitrile (0.1% formic acid) in water (0.1% formic acid) over 10 min and then increase 5–35% acetonitrile (0.1% formic acid) in water (0.1% formic acid) 10–17 min; flow rate 1 mL/min. Only for transcript 9 and corresponding standard mixture the conditions were changed. Mobile phase: 0% acetonitrile (0.1% TEAA buffer) in water (0.1% TEAA buffer) over 5 min and then 0–7% acetonitrile (0.1% TEAA buffer) in water (0.1% TEAA buffer) 5–17 min; flow rate 1 mL/min.

S4.1 Transcripts 7–12

Figure S4.1a. HPLC profile of enzymatic digestion (a) mixture of nucleosides used as a standard and digestion results of (b) transcript 7, (c) transcript 11, (d) transcript 8 (e) transcript 12. The ribonucleoside mixture obtained was analyzed by reverse-phase analytical HPLC, using a mobile phase of 0–5% acetonitrile (0.1% formic acid) in water (0.1% formic acid) over 10 min and then increase 5–35% acetonitrile (0.1% formic acid) in water (0.1% formic acid) 10–17 min; flow rate 1 mL/min.
Figure S4.1b. HPLC profile of enzymatic digestion (a) mixture of nucleosides used as a standard and digestion results of (b) transcript 9. The ribonucleoside mixture obtained was analyzed by reverse-phase analytical HPLC, using a mobile phase: 0% acetonitrile (0.1% TEAA buffer) in water (0.1% TEAA buffer) over 5 min and then 0–7% acetonitrile (0.1% TEAA buffer) in water (0.1% TEAA buffer) 5–17 min; flow rate 1 mL/min.

Figure S4.1c. HPLC profile of enzymatic digestion (a) mixture of nucleosides used as a standard and digestion results of (b) transcript 10. The ribonucleoside mixture obtained was analyzed by reverse-phase analytical HPLC, using a mobile phase of 0–5% acetonitrile (0.1% formic acid) in water (0.1% formic acid) over 10 min and then increase 5–35% acetonitrile (0.1% formic acid) in water (0.1% formic acid) 10–17 min; flow rate 1 mL/min.
S.4.2 Ligation construct 13

Figure S4.2. HPLC profile of enzymatic digestion (a) mixture of nucleosides used as a standard and (b) digestion results of ligation product 13. Digestion of 1–2 nmol of transcript was carried out using S1 nuclease for 2 h at 37 °C and followed by dephosphorylation with alkaline phosphatase for 2 h at 37 °C. The ribonucleoside mixture obtained was analyzed by reverse-phase analytical HPLC, using a mobile phase of 0–5% acetonitrile (0.1% formic acid) in water (0.1% formic acid) over 10 min and then increase 5–35% acetonitrile (0.1% formic acid) in water (0.1% formic acid) 10–17 min; flow rate 1 mL/min.

S5. Thermal denaturation curves for control and modified RNA ligation constructs

Figure S5. Thermal denaturation curves: (a) forward curve (■) for control RNA ligation product 13 and forward curve (■) for modified RNA ligation product 14; (b) forward (■) and reverse (□) curve for modified RNA ligation product 14 (c) forward (■) and reverse (□) curve for modified RNA ligation product 13. Tm of control RNA ligation product 13 is 82.8 ±0.1 °C, Tm of modified RNA ligation product 14 is 79.1 ±0.3 °C.
S6. Photophysical studies of modified A-site construct

S6.1 General

Aqueous samples were prepared with de-ionized water. For all spectroscopic measurements a 1 cm Hellma Analytics quartz cuvette was used. Steady state emission spectra were taken on a PTI luminescence spectrometer with a 1 nm resolution. The sample temperature was kept constant at 20 °C with a Quantum Northwest TLC50 fluorescence cuvette holder in conjunction with a software controllable TC 125 temperature controller.

EC50 values were calculated using OriginPro 8.5 software by fitting a dose response curve (eq 1) to the fractional fluorescence saturation (Fs) plotted against the log of antibiotic (A) concentration.

\[ Fs = F_0 + \left( F_\infty [A]^n / ([EC50]^n + [A]^n) \right) \]  

Fs is the fluorescence intensity at each titration point. F0 and F∞ are the fluorescence intensity in the absence of aminoglycoside or at saturation, respectively, and n is the Hill coefficient or degree of cooperativity associated with binding. All reported standard deviations were calculated using STDEVP in Microsoft Excel.

**Binding of aminoglycosides to a fluorescently modified A-site**

All titrations were performed with working solutions of 1 μM labeled A-site 14 in 20 mM cacodylate buffer (pH 7.0, 100 mM NaCl, 0.5 mM EDTA). The solutions were heated to 75 °C for 5 min and cooled to room temperature over 2 h. Aliquots (1 μL) of increasing concentrations of an aminoglycoside (15–16) were added. Fluorescence spectrum was recorded after each addition until saturation was achieved. A background spectrum (buffer) was subtracted for each sample. Modified A-site 14 was excited at 320 nm (slit width 8 nm), and changes in emission were monitored at 420 nm. For figure 6, each event was fitted separately for the binding of neomycin to the A-site; critical points were fixed at y=0, x=0.1 mM (for the first event) and y=0, x=1 × 10⁻³ mM (for the second event). Errors were calculated as standard deviation from two measurements.

**Displacement of A-site bound 6''-amino-6''-deoxykanamycin-coumarin with unlabelled RNA binders**

All titrations were performed with working solutions of 1 μM labeled A-site 14 in 20 mM cacodylate buffer (pH 7.0, 100 mM NaCl, 0.5 mM EDTA). The solutions were heated to 75 °C for 5 min and cooled to room temperature over 2 h 6''-amino-6''-deoxykanamycin-coumarin was added, to give a working concentration of 0.53 μM, just prior to aminoglycoside titrations (just as described above). For binding studies, 14 was excited 320 nm (slit width 8 nm), and changes in emission upon titration with aminoglycosides were monitored at 420 nm and at 470 nm. For figure 6, each event was fitted separately for the binding of neomycin to the A-site; critical points were fixed at y=0, x=0.1 mM (for the first event) and y=0, x=1 × 10⁻³ mM (for the second event). Errors were calculated as standard deviation from two measurements.
S6.2 Binding of aminoglycosides to a fluorescently modified A-site

S6.2a Tobramycin titration

Figure S6.2 Fluorescence titrations of the A-site construct 14 with tobramycin (16). (a) Emission spectra for titrations of A-site hairpin 14 with tobramycin (16). (b) Curve fit for the titrations of A-site hairpin 14 with tobramycin (16) (fractional saturation vs. antibiotic concentration). Two slopes were separated and fitted individually.

S7. References