Supporting Information

Pseudoknot formation seeds the twister ribozyme cleavage reaction coordinate

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Contents

1. Supplementary Methods

1.1. Preparation of RNA

1.1.1. Solid-phase synthesis of oligoribonucleotides. All oligonucleotides were synthesized on a ABI 392 Nucleic Acid Synthesizer following standard methods: detritylation (130 s) with dichloroacetic acid/1,2-dichloroethane (4/96); coupling (4 x 2.5 s) with phosphoramidites/ acetonitrile (0.1 M x 130 µL) and benzylthiotetrazole/acetonitrile (0.3 M x 360 µL); mild capping (2 x 10 s, Cap A/Cap B = 1/1) with Cap A: phenoxyacetic anhydride in tetrahydrofuran (0.2 M) and Cap B: *N*-methylimidazole (0.2 M), sym-collidine (0.2 M) in tetrahydrofuran; oxidation (20 s) with I_2 (20 mM) in tetrahydrofuran (THF)/pyridine/H_2O (35/10/5). The solutions of amidites and tetrazole, and acetonitrile were dried over activated molecular sieves (4 Å) overnight.

Suppliers: 2′-*O*-[(Triisopropylsilyl)oxy]methyl(2′-*O*-TOM)-protected ribonucleoside phosphoramidites (ChemGenes), 2′-*O*-aminopropyl-cytidine and 2′-*O*-aminopropyl-uridine phosphoramidites (ChemGenes), 2′-deoxyuridine phosphoramidite (ChemGenes), 5-aminoallyl-2′-deoxyuridine phosphoramidite (*Berry & Associates, Inc.*), standard polystyrene support (*GE Healthcare*, Custom Primer Support^{TM}, PS 200 (40 µmol g^{-1}) or 5G (300 µmol g^{-1}); 3'-Biotin-lcaa–CPG 500 Å (ChemGenes).

The phosphoramidite reagent used for 5′-phosphate labeling was prepared according to the literature (Horn, T.; Urdea, M. S. *Tetrahedron Lett*. **1986**, *27*, 4705-4708).

1.1.2. Deprotection of oligonucleotides. The solid support was treated with a mixture of CH_3NH_2 in ethanol (33%, 0.75 mL) and CH_3NH_2 in water (40%, 0.75 mL) for 6 to 8 h at 35 °C. The supernatant was removed and the solid support was washed 3x with THF/water (1/1, v/v). The supernatant and the washings were combined and the whole mixture was evaporated to dryness. To remove the 2'-silyl protecting groups, the resulting residue was treated with tetrabutylammonium fluoride trihydrate (TBAF x $3H_2O$) in THF (1 M, 1.5 mL) at 37 °C overnight. The reaction was quenched by the addition of triethylammonium acetate (1 M, pH 7.4, 1.5 mL). The volume of the solution was reduced and the solution was desalted with a size exclusion column (GE Healthcare, HiPrep 26/10 Desalting; 2.6 x 10 cm; Sephadex G25) eluting with H_2O, the collected fraction was evaporated to dryness and dissolved in 1 mL H_2O. Analysis of the crude RNA after deprotection was performed by anion-exchange chromatography on a Dionex DNAPac PA-100 column (4 x 250 mm) at 60 °C. Flow rate: 1 mL min^{-1}, eluant A: 25 mM Tris x HCl (pH 8.0), 6 M urea; eluant B: 25 mM Tris x HCl (pH 8.0), 0.5 M $NaClO_4$, 6 M urea; gradient: 0–60% B in A within 45 min, ultraviolet detection at 260 nm.

1.1.3. Purification of RNA. Crude RNA products were purified on a Dionex DNAPac PA-100 column (9 x 250 mm) at 60 °C with a flow rate of 2 mL min^{-1}. Fractions containing RNA were loaded on a C18 SepPak Plus cartridge (Waters/Millipore), washed with 0.1–0.15 M $(Et_3NH)^+HCO_3^-$, H_2O and eluted with H_2O/CH_3CN (1/1). RNA containing fractions were lyophilized. Analysis of the quality of purified RNA was performed by anion-exchange chromatography with same conditions as for crude RNA; the molecular weight was confirmed by liquid chromatography-electrospray ionization (LC-ESI) mass spectrometry. Yield determination was performed by ultraviolet photometrical analysis of oligonucleotide solutions.

1.1.4. Mass spectrometry of RNA. All experiments were performed on a Finnigan LCQ Advantage MAX ion trap instrumentation connected to an Amersham Ettan micro LC system. RNA sequences were analyzed in the negative-ion mode with a potential of –4 kV applied to the spray needle. LC: Sample (200 pmol RNA dissolved in 30 µL of 20 mM EDTA solution; average injection volume: 30 µL); column (Waters XTerraMS, C18 2.5 µm; 1.0 x 50 mm) at 21 °C; flow rate: 30 µL min^{-1}; eluant A: 8.6 mM TEA, 100 mM 1,1,1,3,3,3-hexafluoroisopropanol in H_2O (pH 8.0); eluant B: methanol; gradient: 0–100% B in A within 30 min; ultraviolet detection at 254 nm.

1.2. Steady-state fluorescence spectroscopy

All experiments were measured on a *Cary Eclipse* spectrometer (Varian, Palo Alto, USA) equipped with a peltier block, a magnetic stirring device.

Using quartz cuvettes, RNA samples were prepared in 0.5 µM concentration in a total volume of 120 µL of buffer (50 mM KMOPS pH 7.5, 100 mM KCl). The samples were heated to 90 °C for 2 min, allowed to cool to room temperature, and held at 20 °C in the peltier controlled sample holder. Then, $MgCl_2$ was pipetted from a stock solution (1 M $MgCl_2$ in water; e.g. 1.2 µL for a final $MgCl_2$ concentration of 10 mM) and manually mixed by pipetting in less than two seconds.

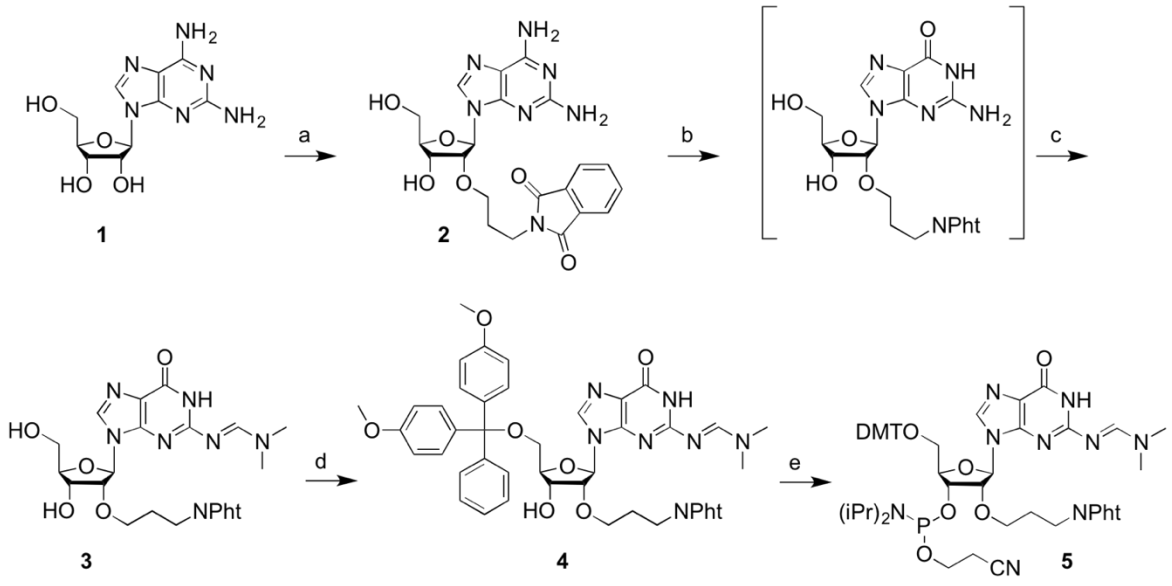
For the LD550/LD650 labeled construct, the fluorescence resonance energy transfer signal was monitored by exciting the donor LD550 at 540 nm and recording the LD650 acceptor emission at 665 nm using the following instrumental parameters: increment of data point collection: 0.2 s; slit widths, 10 nm.

All data processing was performed using Igor Pro software (WaveMetrics Inc.) with the biexponential fitting equation: $y_0 + A_1 \exp(-\text{invTau}_1 * x) + A_2 \exp(-\text{invTau}_2 * x)$

1.3. Synthesis of 2'-*O*-(3-aminopropyl)guanosine phosphoramidite

In analogy to: Griffey, R. H., Monia, B. P., Cummins, L. L., Freier, S., Greig, M. J., Guinosso, C. J., et al. (1996). *Journal of Medicinal Chemistry*, 39(26), 5100–5109.

General. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker DRX 300 MHz or Avance II+ 600 MHz instrument. The chemical shifts are referenced to the residual proton signal of the deuterated solvents: $CDCl_3$ (7.26 ppm), d_6-DMSO (2.49 ppm) for ^{1}H NMR spectra; $CDCl_3$ (77.0 ppm) or d_6-DMSO (39.5 ppm) for ^{13}C NMR spectra. ^{31}P-shifts are relative to external 85% phosphoric acid. ^{1}H- and ^{13}C-assignments were based on COSY and HSQC experiments. MS experiments were performed on a Finnigan LCQ Advantage MAX ion trap instrument. Analytical thin-layer chromatography (TLC) was carried out on Marchery-Nagel Polygram SIL G/UV254 plates. Flash column chromatography was carried out on silica gel 60 (70-230 mesh). All reactions were carried out under argon atmosphere. Chemical reagents and solvents were purchased from commercial suppliers and used without further purification. Organic solvents for reactions were dried overnight over freshly activated molecular sieves (4 Å).

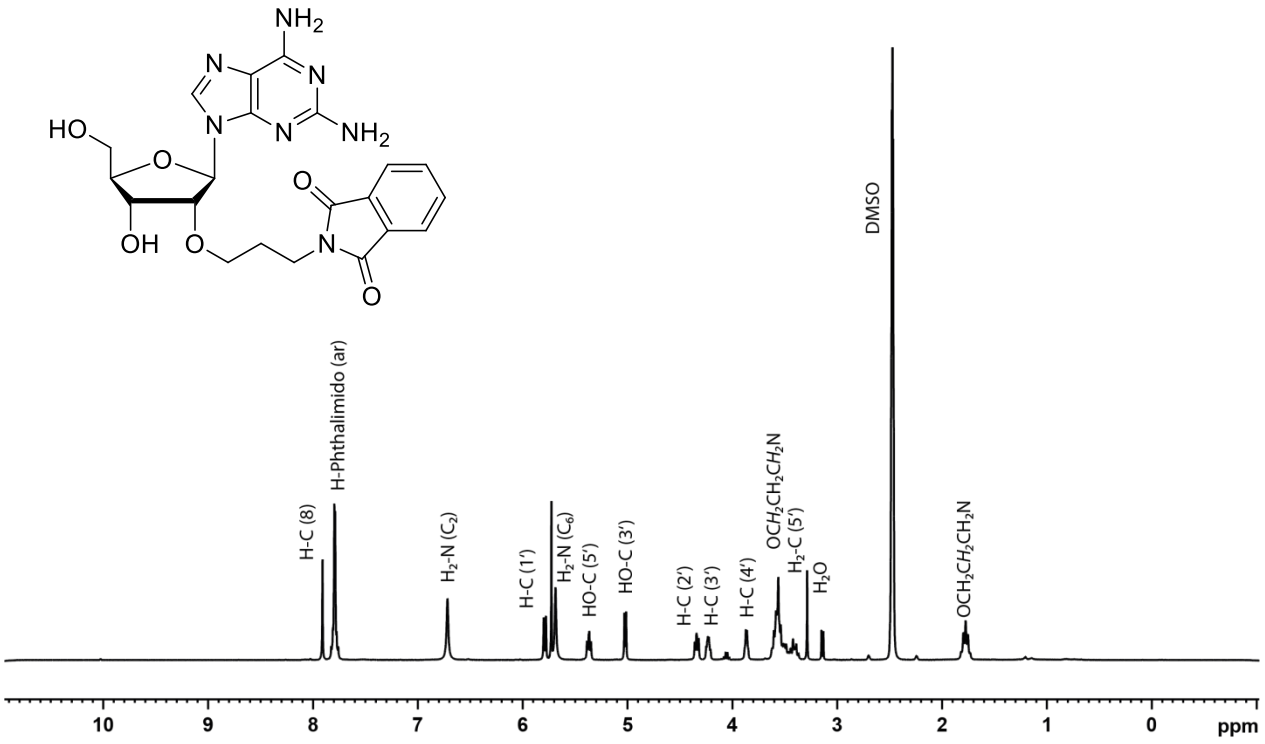


Supporting Scheme S1. Reaction conditions: a. 1.5 equiv NaH, 1.15 equiv 3-bromopropylphthalimide, 0 °C to r.t. overnight, 11% of **2**; b. adenosine deaminase (ADA) from calf intestine (0.44 units per mg of **2**), in DMSO, TRIS-buffer and sodium phosphate buffer, pH 7.4, 37 °C, 5 days; c. 7.5 equiv $(H_3CO)_2CHN(CH_3)_2$, in DMF, r.t., 3.5 h, 32% of **3** (over two steps); d. 1.1 equiv DMT-Cl, 0.1 equiv 4-(dimethylamino)pyridine, in pyridine, overnight, r.t., 53% of **4**; e. 1.5 equiv 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, 10 equiv *N*,*N*-dimethylethylamine, in CH_2Cl_2, 1.5 h, r.t., 41 % of **5**.

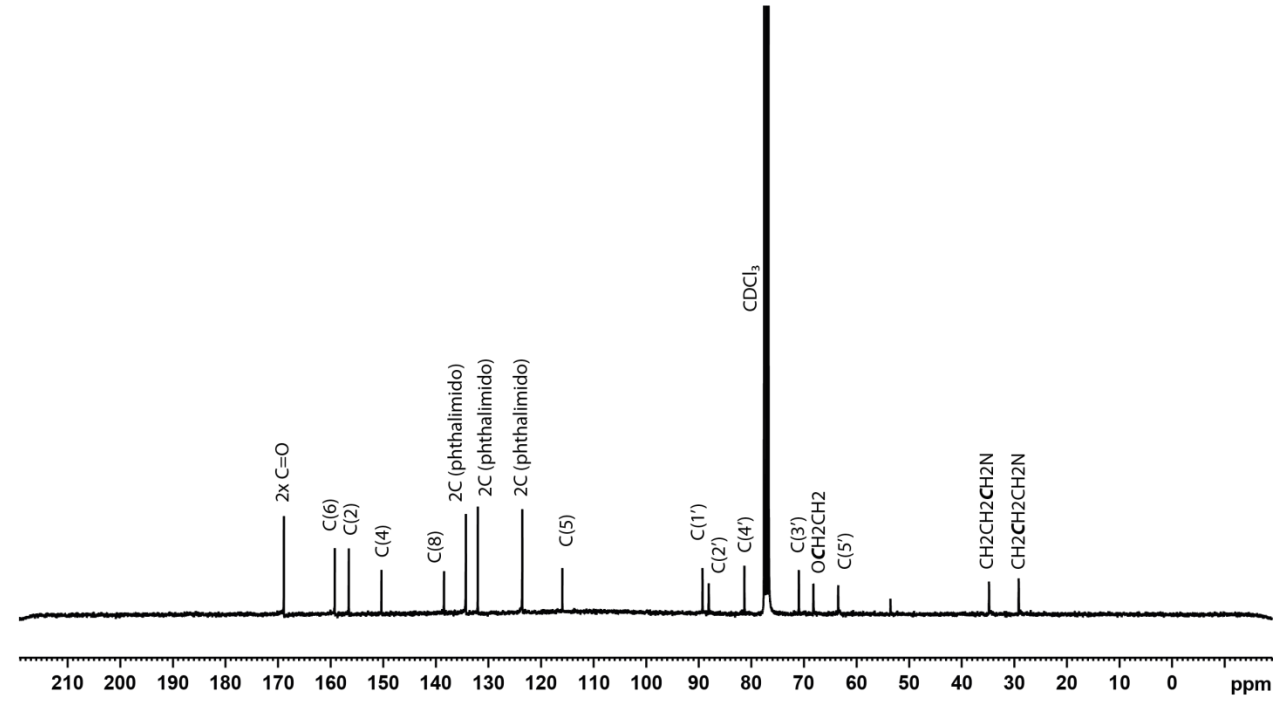
2'-*O*-(*N*-Phthalimidoprop-3-yl)-2-aminoadenosine (2)

2-Aminoadenosine **1** (5.000 g, 17.72 mmol) was dissolved in *N*,*N*-dimethylformamide (137.5 ml) and the solution cooled to 0°C. Sodium hydride (95%; 0.671 g, 26.58 mmol) was added and stirring continued for one hour at 0°C. Then, *N*-(3-bromopropyl)phthalimide (5.464 g, 20.38 mmol) was added and the mixture stirred overnight at room temperature. The reaction was quenched with crushed ice and the solution concentrated to a gum which was partitioned between water and ethyl acetate (4 x 75 ml). The combined organic layers were dried over sodium sulfate, filtered and evaporated under reduced pressure to yield the crude product which was subjected to column chromatographic purification on SiO_2 (CH_2Cl_2/CH_3OH, 95/5). Yield: 0.918 g of compound **2** as greenish foam (11%). TLC (CH_2Cl_2/CH_3OH, 9/1): R_f = 0.52. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.78 (quint, J=6.55 Hz, 2H, -$CH_2CH_2CH_2$-), 3.35-3.53 (m, 2H, CH_2CH_2N), 3.53-3.65 (m, 4H, -$CH_2CH_2CH_2$-, H_2-C(5')), 3.87 (q, J=3.22 Hz, 1H, H-C(4')), 4.20-4.26 (m, 1H, H-C(3')), 4.34 (t, J=5.59 Hz, 1H, H-C(2')), 5.02 (d, J=4.95 Hz, 1H, HO-C(3')), 5.37 (t, J=5.65 Hz, HO-C(5')), 5.69 (s, 2H, NH_2-C(6)), 5.79 (d, J=6.69 Hz, 1H, H-C(1')), 6.72 (s, 2H, NH_2-C(2)), 7.75-7.83 (m, 4H, H-C(phthalimide)), 7.91 (s, 1H, H-C(8)) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.21 ($CH_2CH_2CH_2$); 34.85 (CH_2CH_2N); 63.49 (C(5')); 68.21 (OCH_2CH_2); 70.98 (C(3')); 81.33 (C(4')); 88.11 (C(2')); 89.29 (C(1')); 115.94 (C(5)); 123.57 (2C (phthalimid)); 132.06 (2C (phthalimide)); 134.34 (2C (phthalimide)); 138.49 (C(8)); 150.37 (C(4)); 156.58 (C(2)); 159.26 (C(6)); 168.93 (2C (C=O)) ppm. ESI-MS (m/z): $[M+H]^+$ calculated 470.18, found 470.19.

^{1}H NMR (300 MHz, DMSO-d_6) spectrum of compound **2**:



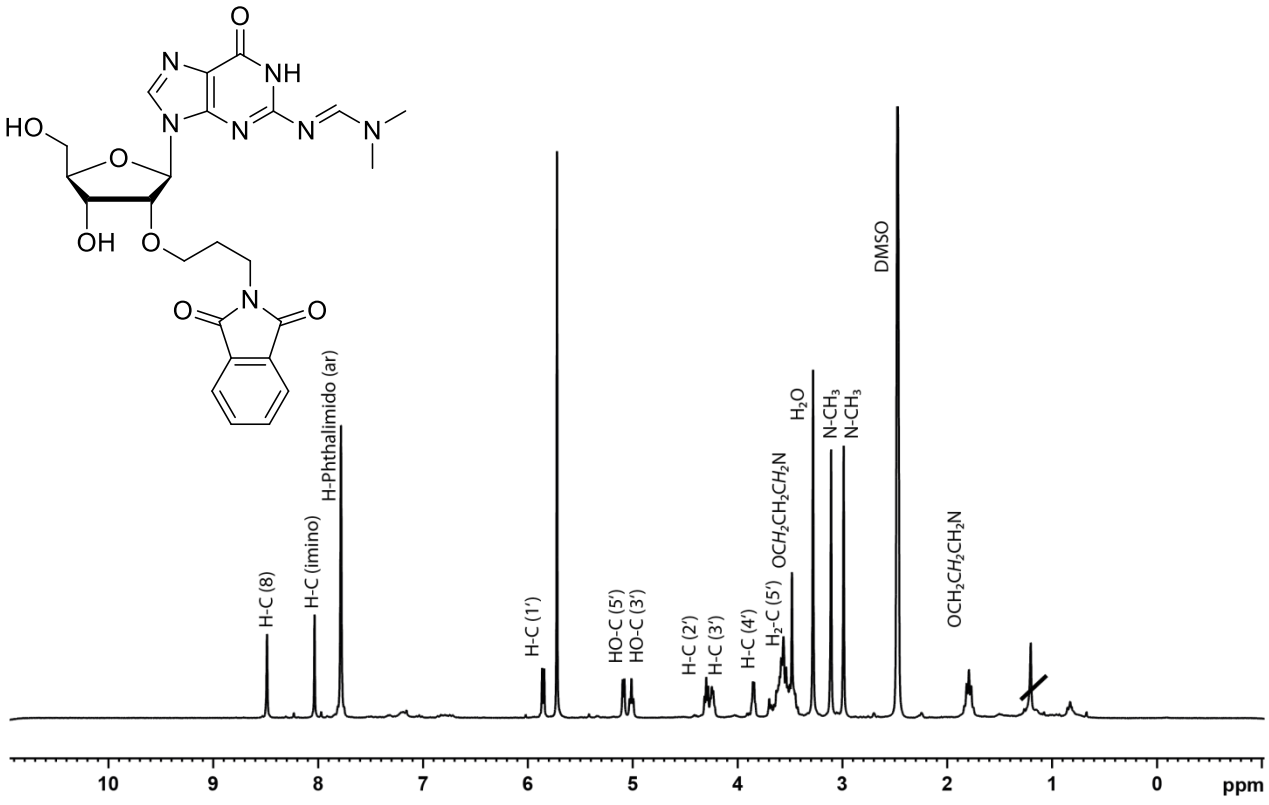
^{13}C NMR (75 MHz, $CDCl_3$) spectrum of compound **2**:



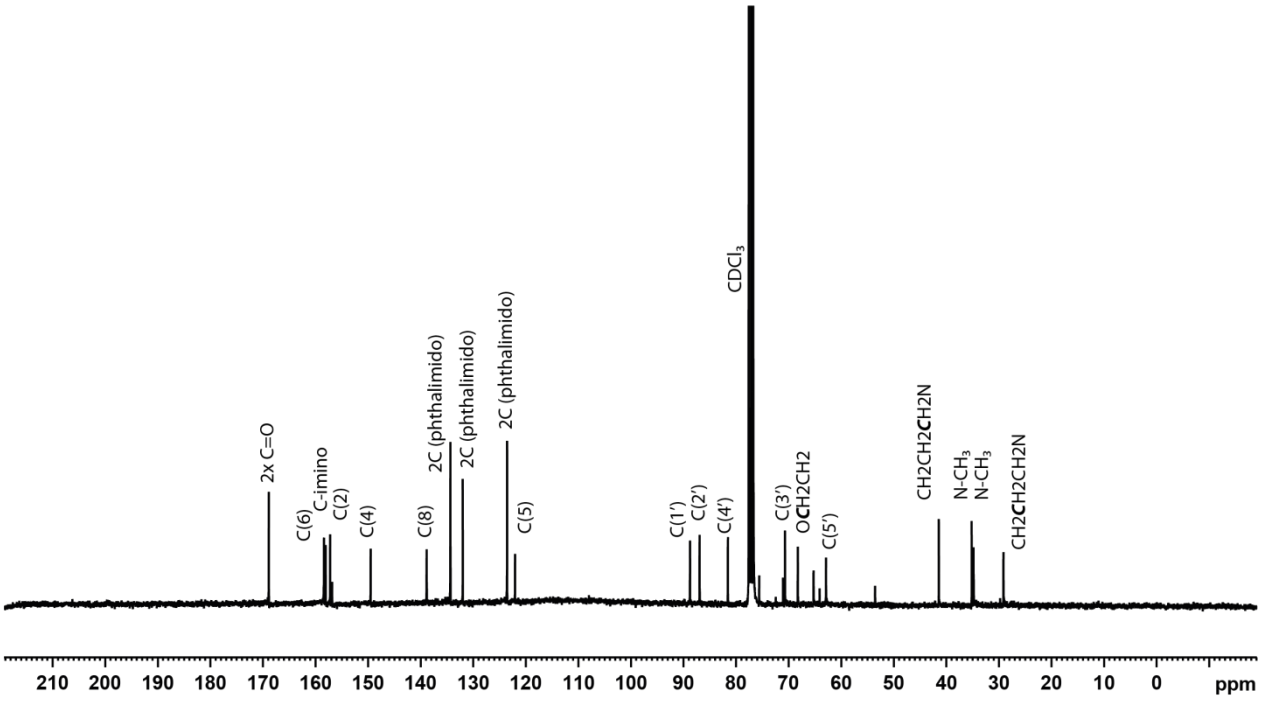
N^2-Dimethylaminomethylidene-2'-*O*-(*N*-phthalimidoprop-3-yl)guanosine (3)

Compound **2** (950 mg, 2.02 mmol) was dissolved in a mixture of dimethyl sulfoxide (13.8 ml), 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) buffer (0.05 M; 20 ml, pH 7.4) and sodium phosphate buffer (0.1 M; 1 ml). The solution was treated with 310 units of adenosine deaminase (ADA) from calf intestine and agitated overnight at 37°C. Then, another 110 units of ADA were added and the reaction mixture was stirred for four days at 37°C. The solvents were evaporated and the crude product was dried on high vacuum overnight. The residue was dissolved in *N*,*N*-dimethylformamide (20.1 ml) and treated with *N*,*N*-dimethylformamide dimethyl acetal (2.0 ml, 15.13 mmol). After stirring for 3.5 hours at room temperature, the solvents were evaporated and crude product was subjected to column chromatographic purification on SiO_2 (CH_2Cl_2/CH_3OH, 96/4-92/8). Yield: 340 mg of compound **3** as white foam (32% over two steps). TLC (CH_2Cl_2/CH_3OH, 9/1): R_f = 0.42. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.80 (quint, J=6.66 Hz, 2H, -$CH_2CH_2CH_2$-), 2.99 (s, 3H, N2-CH_3), 3.11 (s,3H, N2-CH_3), 3.46-3.50 (m, 2H, CH_2CH_2N), 3.52-3.61 (m, 4H, -$CH_2CH_2CH_2$-, H_2-C(5')), 3.85 (q, J=3.84 Hz, 1H, H-C(4')), 4.21-4.27 (m, 1H, H-C(3')), 4.30 (t, J=5.57 Hz, 1H, H-C(2')), 5.01 (t, J=5.26 Hz, 1H, HO-C(5')), 5.09 (d, J=4.92 Hz, HO-C(3')), 5.85 (d, J=5.70 Hz, 1H, H-C(1')), 8.04 (s, 1H, N2-CH(imino)), 8.49 (s, 1H, H-C(8)) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.18 ($CH_2CH_2CH_2$); 34.86 (N-CH_3); 35.22 (N-CH_3); 41.44 (CH_2CH_2N); 62.90 (C(5')); 68.26 (OCH_2CH_2); 70.70 (C(3')); 81.57 (C(4')); 86.94 (C(2')); 88.77 (C(1')); 122.05 (C(5)); 123.56 (2C (phthalimide)); 132.03 (2C (phthalimide)); 134.38 (2C (phthalimide)); 138.89 (C(8)); 149.56 (C(4)); 157.26 (C(2)); 158.11 (N=CH-N); 158.44 (C(6)); 168.92 (2C (C=O)) ppm. ESI-MS (m/z): $[M+TEA]^+$ calculated 627.33, found 627.13.

^{1}H NMR (300 MHz, DMSO-d_6) spectrum of compound **3**:



^{13}C NMR (75 MHz, $CDCl_3$) spectrum of compound **3**:

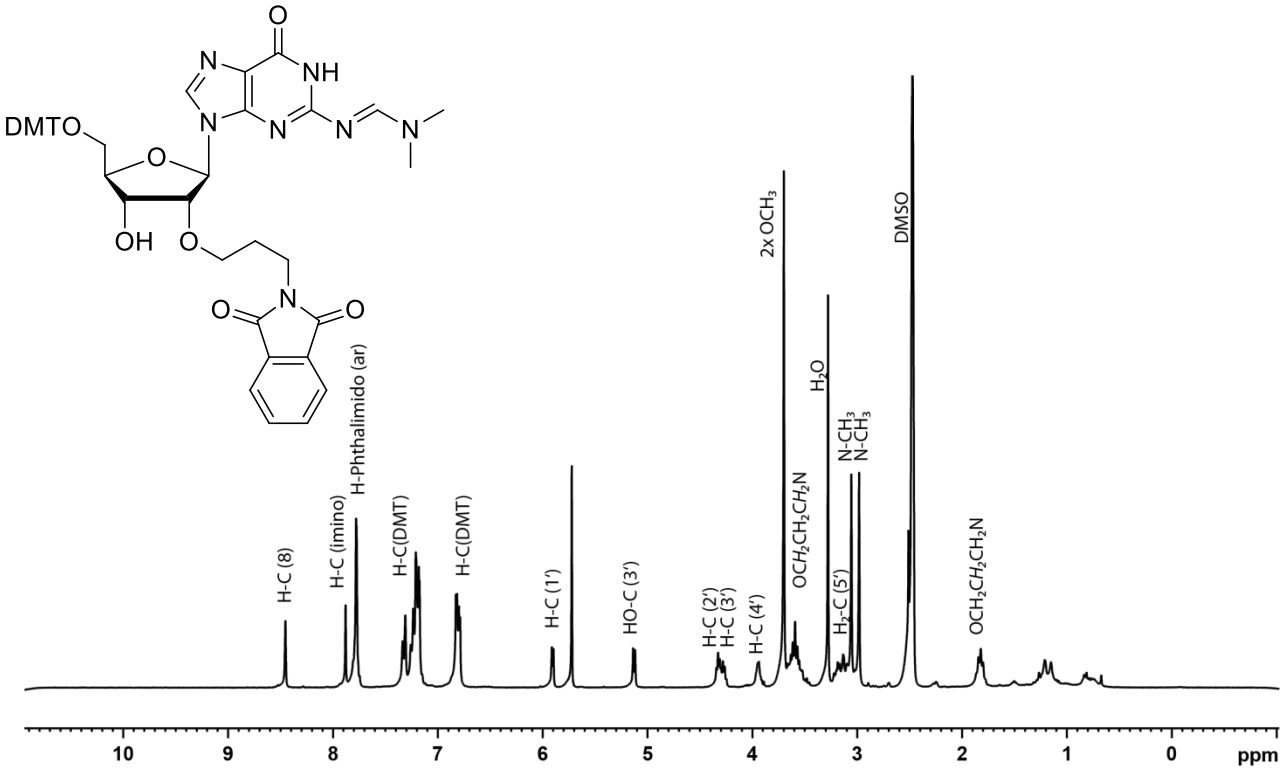


N^2-Dimethylaminomethylidene-5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-(*N*-phthalimidoprop-3-yl)-guanosine (4)

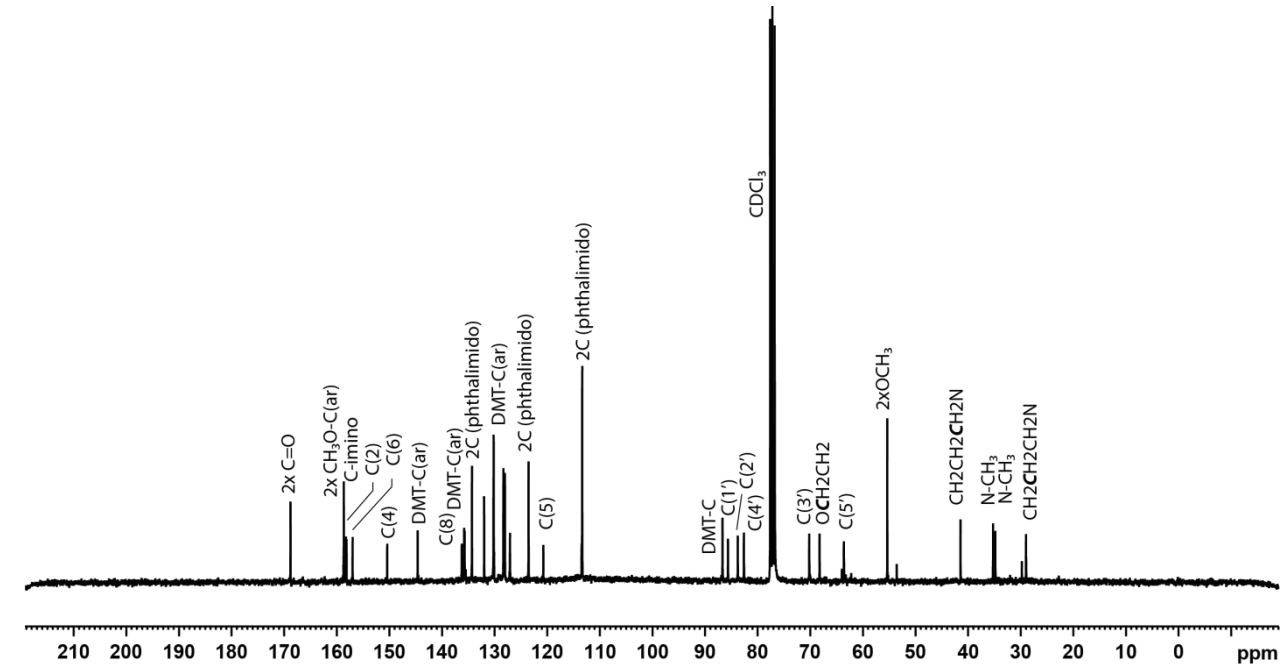
Compound **3** (340 mg, 0.65 mmol) was dried on a high vacuum overnight, then coevaporated with pyridine twice before being dissolved in pyridine (10 ml) and treated with 4-(dimethylamino)pyridine (8 mg, 0.065 mmol). Then, 4,4'-dimethoxytrityl chloride (241 mg, 0.71 mmol) was added in three portions over a period of one hour. After stirring at room temperature overnight, the reaction was quenched with methanol (1.0 ml) and the solvents were evaporated. The residue was dissolved in dichloromethane and washed three times with saturated sodium bicarbonate solution. The organic layers were dried over sodium sulfate, filtered and evaporated under reduced pressure to yield the crude product which was purified by column chromatography on SiO_2 (CH_2Cl_2/CH_3OH, 100/0-96/4). Yield: 285 mg of compound **4** as white foam (53%). TLC (CH_2Cl_2/CH_3OH, 92/8): R_f = 0.50. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.82 (quint, J=6.72 Hz, 2H, -$CH_2CH_2CH_2$-), 2.98 (s, 3H, N2-CH_3), 3.06 (s, 3H, N2-CH_3), 3.12-3.23 (m, 2H, H_2-C(5')), 3.50-3.67 (m, 4H, -$CH_2CH_2CH_2$-), 3.70 (s, 6H, 2xOCH_3), 3.90-3.99 (m, 1H, H-C(4')), 4.24-4.36 (m, 2H, H-C(3'), H-C(2')), 5.13 (d, J=5.61 Hz, 1H, HO-C(3')), 5.91 (d, J=4.50 Hz, 1H, H-C(1')), 6.76-6.86 (m, 4H, H-C(DMT)), 7.16-7.35 (m, 9H, H-C(DMT)), 7.73-7.83 (m, 4H, H-C(phthalimide)), 7.88 (s, 1H, H-C(imino)), 8.46 (s, 1H, H-C(8)) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.03 ($CH_2CH_2CH_2$); 34.87 (N-CH_3); 35.30 (N-CH_3); 41.45 (CH_2CH_2N); 55.37 (2xOCH_3); 63.61 (C(5')); 68.24 (OCH_2CH_2); 70.19 (C(3')); 82.61 (C(4')); 83.78 (C(2')); 85.64 (C(1')); 86.69 (DMT-C); 113.36 (2C (phthalimide)); 120.74 (C(5)); 123.55 (2C (phthalimide)); 127.07, 128.04, 128.13, 128.32, 130.12, 130.24, 132.03 (DMT-C(ar)); 134.35 (2C (phthalimide)); 135.84, 135.73 (DMT-C(ar)); 136.28 (C(8)); 144.66

(DMT-C(ar)); 150.46 (C(4)); 157.03 (C(6)), 158.18 (C(2)); 158.29 (N=CH-N); 158.71 (2xCH_3O-***C***(ar)); 168.83 (2C (C=O)) ppm. ESI-MS (m/z): $[M+TEA]^+$ calculated 929.46, found 929.07.

^{1}H NMR (300 MHz, DMSO-d_6) spectrum of compound **4**:



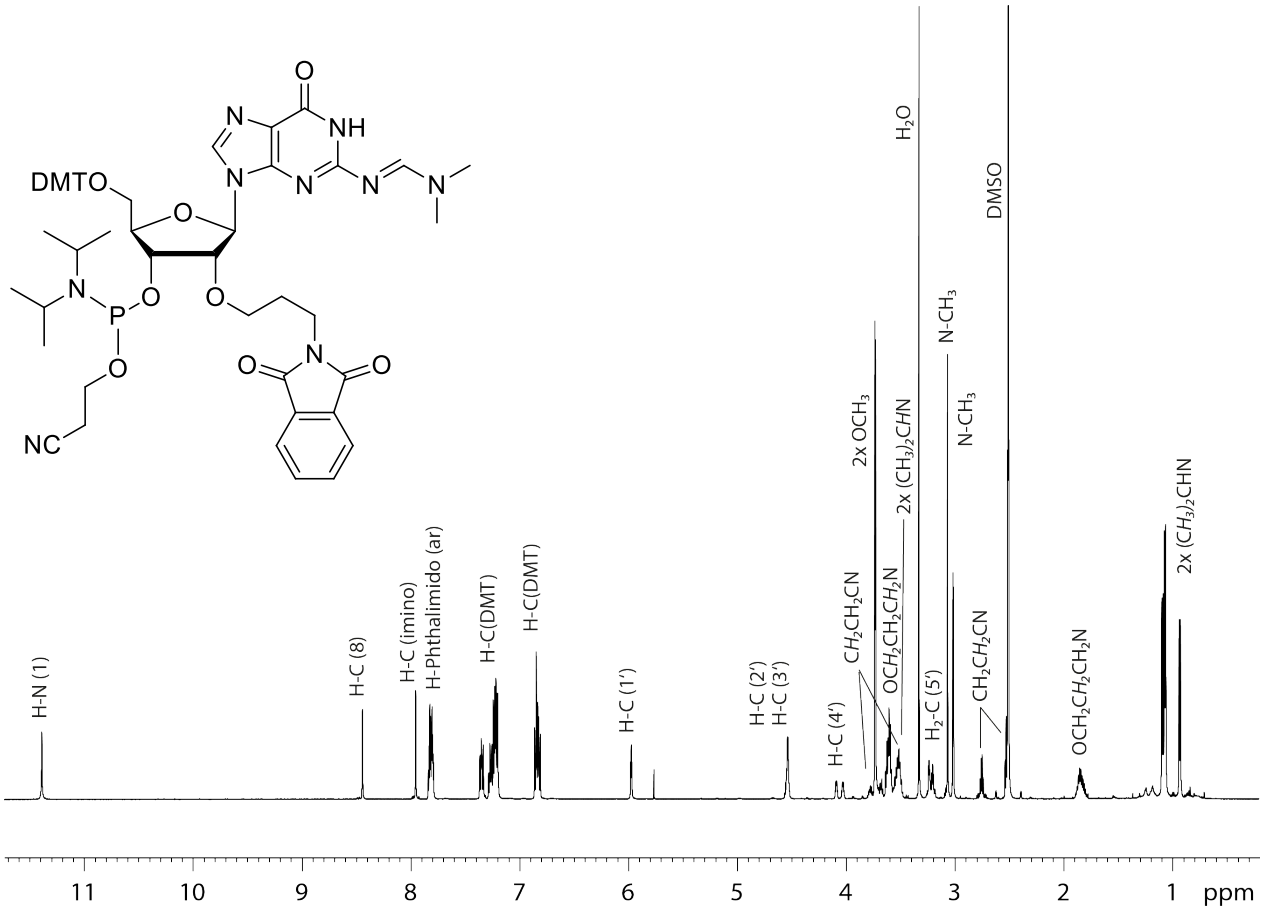
^{13}C NMR (75 MHz, $CDCl_3$) spectrum of compound **4**:



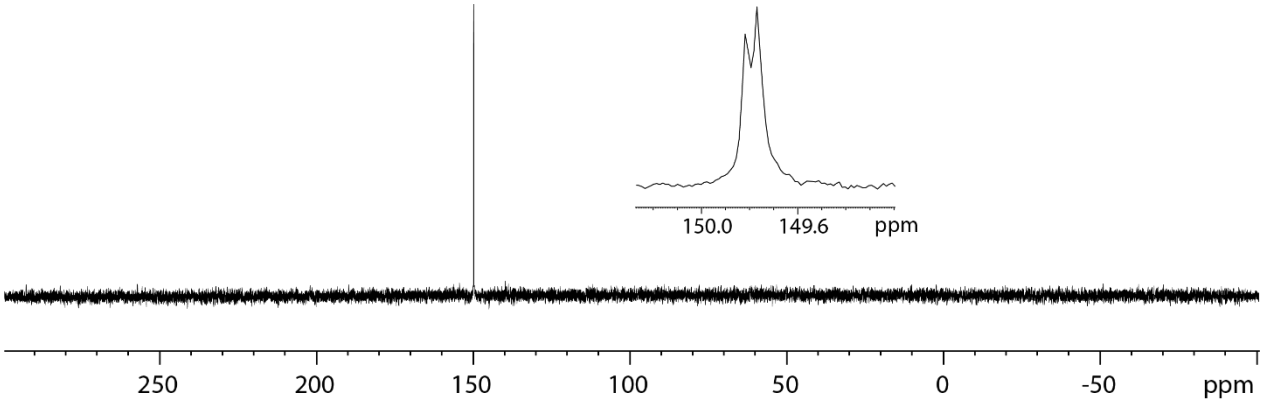
N^2-Dimethylaminomethylidene-5'-*O*-dimethoxytrityl-2'-*O*-(*N*-phthalimidoprop-3-yl)-guanosine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (5)

Compound **4** (285 mg, 340 µmol) was dissolved in dry dichloromethane and treated with *N*,*N*-dimethylethylamine (0.37 ml, 3.4 mmol) under argon atmosphere. After 15 minutes, 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.12 ml, 520 µmol) was added dropwise and the reaction mixture was stirred for 90 minutes, after which it was quenched by the addition of methanol (1.0 ml). The residue was diluted with dichloromethane (10 mL) and washed with saturated sodium bicarbonate solution (10 mL). The organic layer was then dried over Na_2SO_4 and evaporated, and the resulting crude product was purified by flash chromatography on SiO_2 (CH_2Cl_2/CH_3OH, 100/0-96/4). Yield: 145 mg of compound **5** as white foam (41%). TLC (CH_2Cl_2/CH_3OH, 92/8): R_f = 0.50. ^{1}H-NMR (600 MHz, DMSO-d_6): *δ* 0.91-0.95, 1.05-1.11 (m, 12H, N(CH(CH_3)$_2$)$_2$), 1.78-1.90 (m, 2H, $CH_2CH_2CH_2N$), 2.52-2.55, 2.72-2.79 (m, 2H, CH_2CH_2CN), 3.00-3.03 (m, 3H, CH_3N), 3.07 (s, 3H, CH_3N), 3.16-3.26 (m, 2H, 2xH-C(5')), 3.47-3.57 (m, 3H, *H*-CH-CH_2CN, N(C*H*(CH$_3$)$_2$)$_2$), 3.57-3.65 (m, 4H, -$CH_2CH_2CH_2N$), 3.70-3.75 (m, 6H, 2xOCH_3), 3.75-3.81 (m, 1H, *H*-CH-CH_2CN), 4.00-4.13 (m, 1H, H-C(4')), 4.49-4.59 (m, 2H, H-C(2'), H-C(3')), 5.96-6.00 (m, 1H, H-C(1')), 6.78-6.89, 7.19-7.29, 7.31-7.38 (m, 13H, H-C(DMT)), 7.77-7.86 (m, 4H, H-C(phthalimide)), 7.92-7.99 (m, 1H, H-C(imino)), 8.44 (s, 1H, H-C(8)), 11.39 (s, 1H, H-N(1)). ^{31}P (121 MHz, $CDCl_3$): δ 149.82, 149.76 ppm; ESI-MS (m/z): $[M+TEA]^+$ calculated 1129.57, found 1129.15.

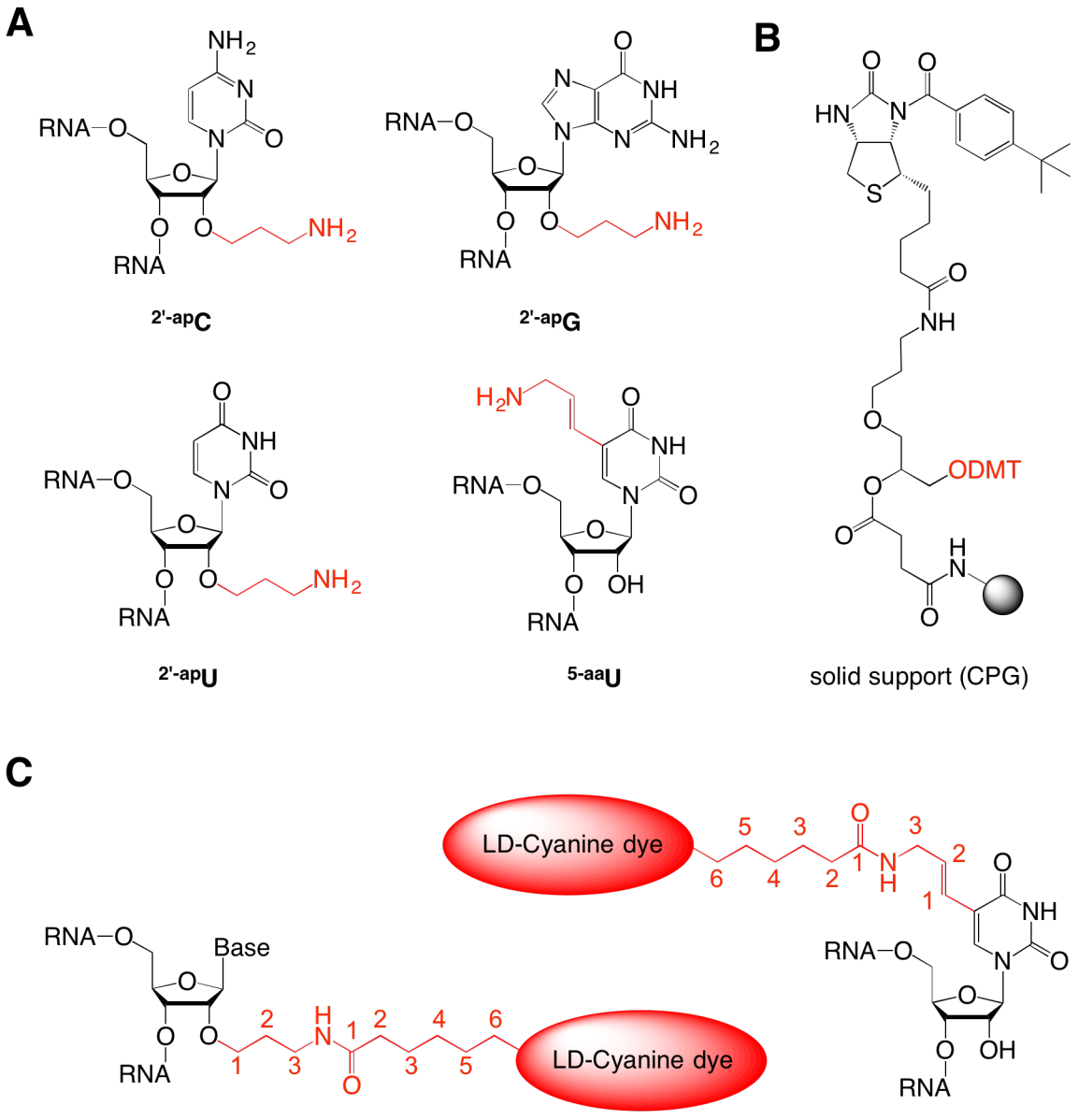
^{1}H NMR (600 MHz, DMSO-d_6) spectrum of compound **5**:



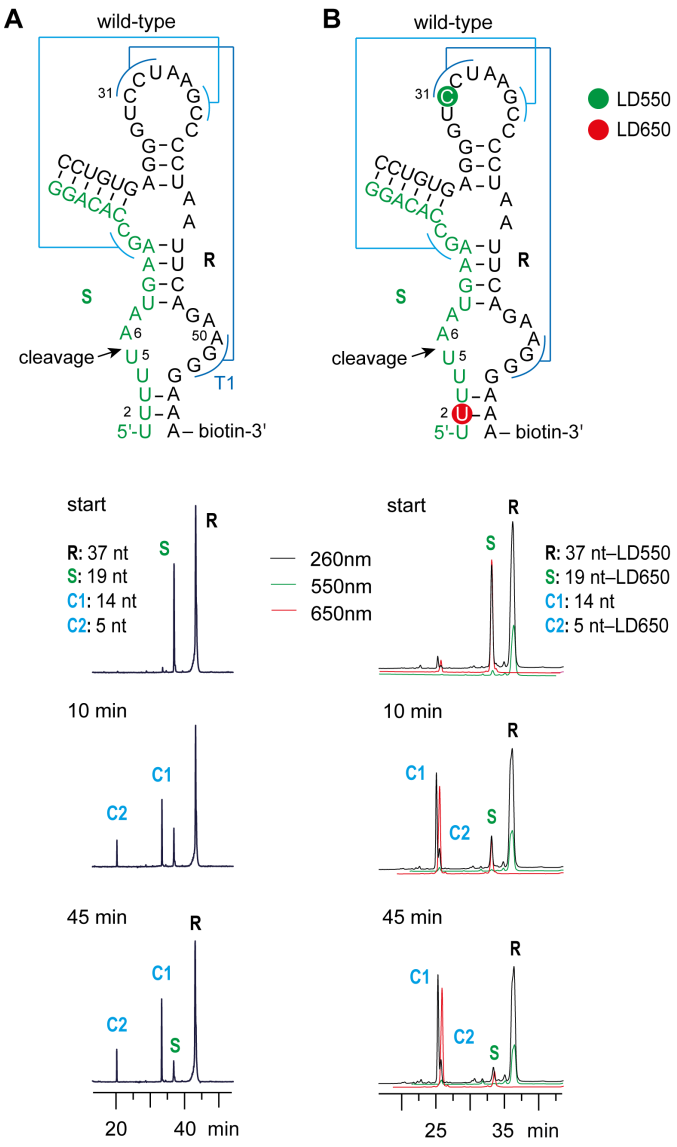
^{31}P (121 MHz, $CDCl_3$) spectrum of compound **5**:



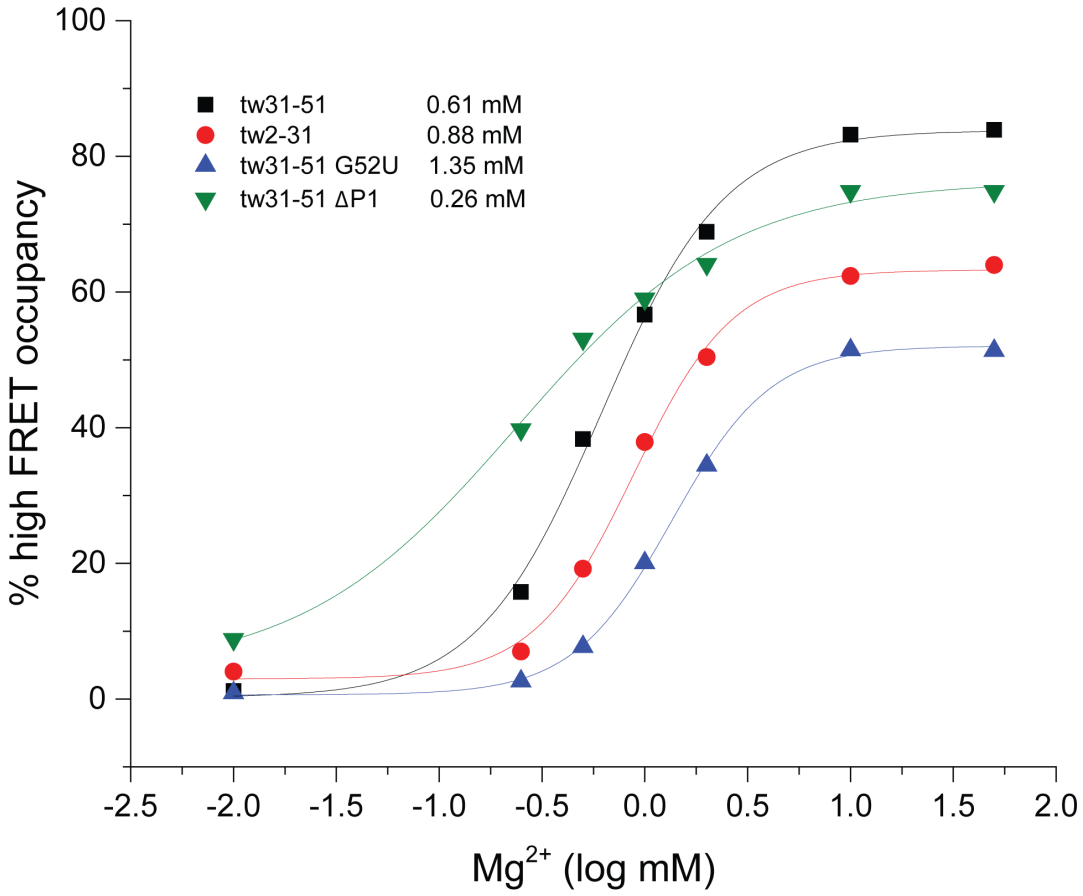
2. Supporting Figures



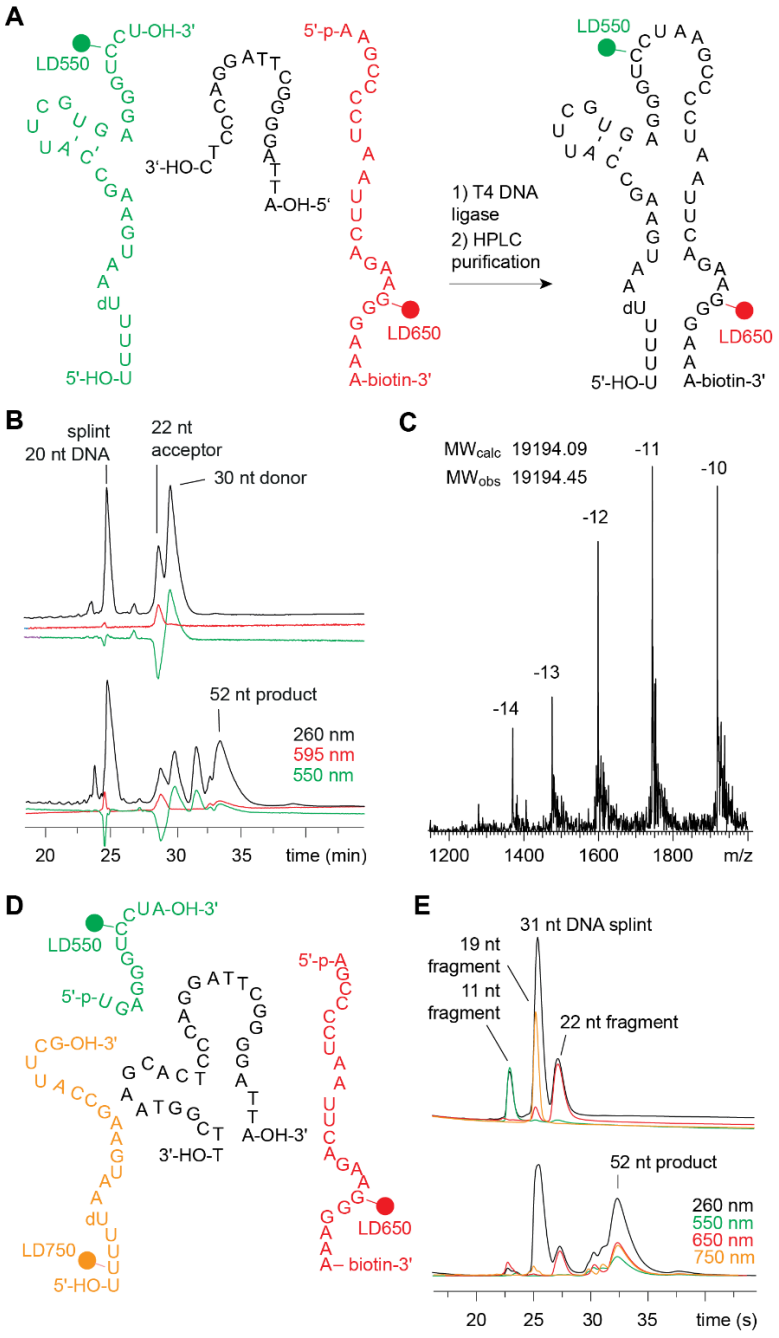
Supporting Figure S1. Chemical structures. (**A**) Aminoalkyl-modified nucleosides used for pre-functionalized RNA. (**B**) Solid support for synthesis of 3'-biotinylated RNA. (**C**) Tether structure and length of fluorophore-labeled RNA.



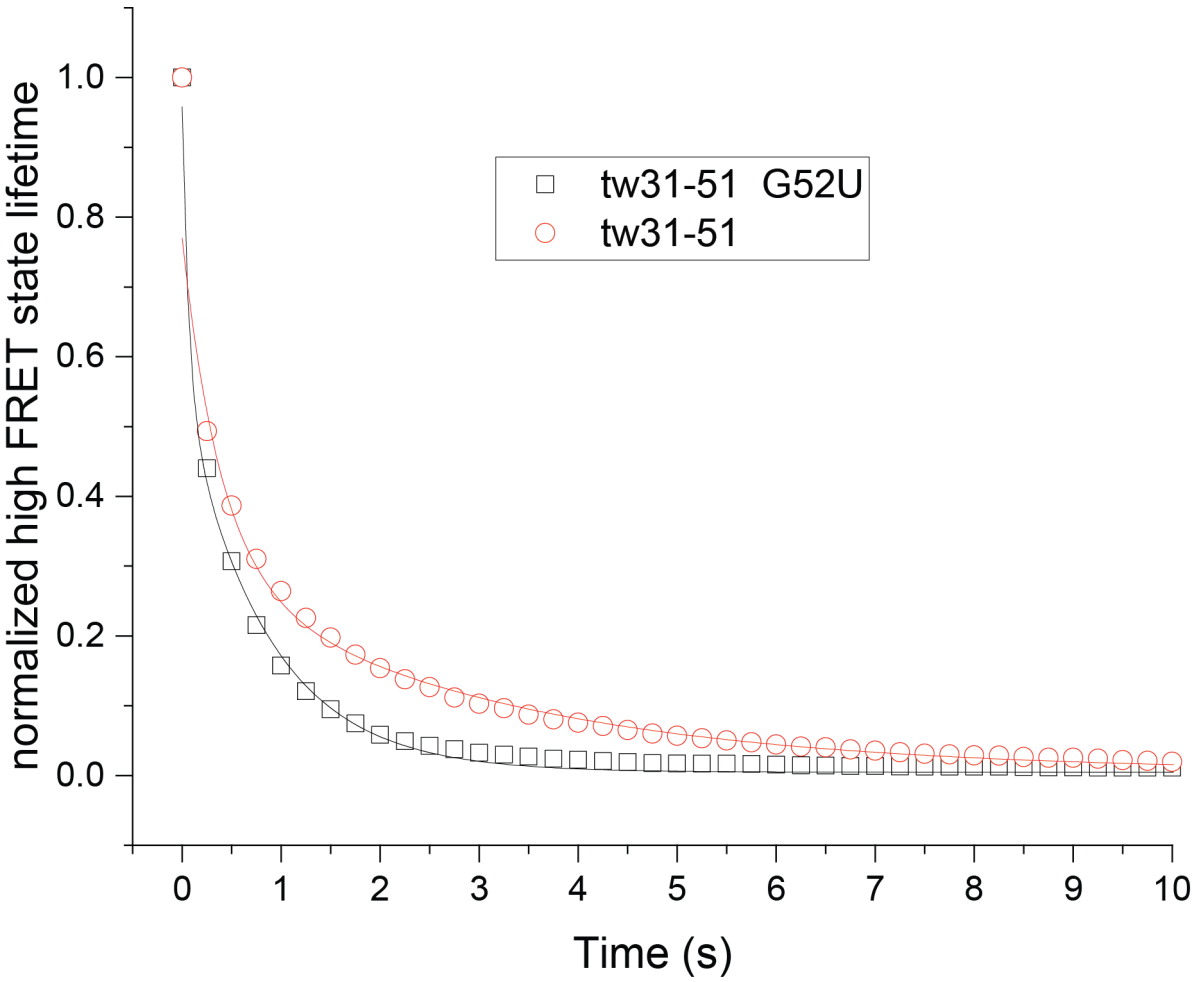
Supporting Figure S2. Comparison of cleavage activities of unmodified (**A**) and fluorophore-modified (**B**) bimolecular twister ribozymes.



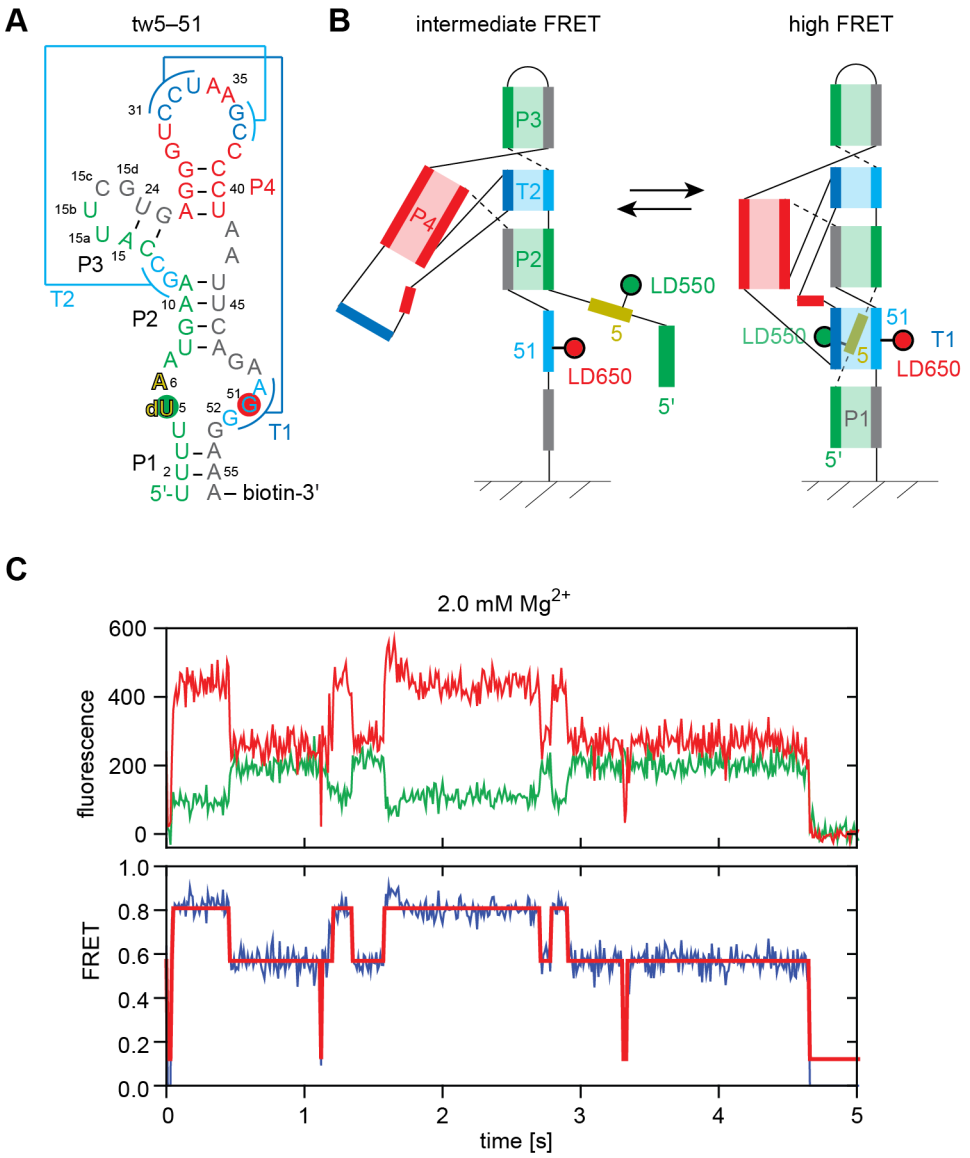
Supporting Figure S3. Apparent Mg^{2+} EC_{50} for folding equilibrium of twister constructs tw2–31, tw31–51, tw31-51 G52U, and tw31-51 ΔP1.



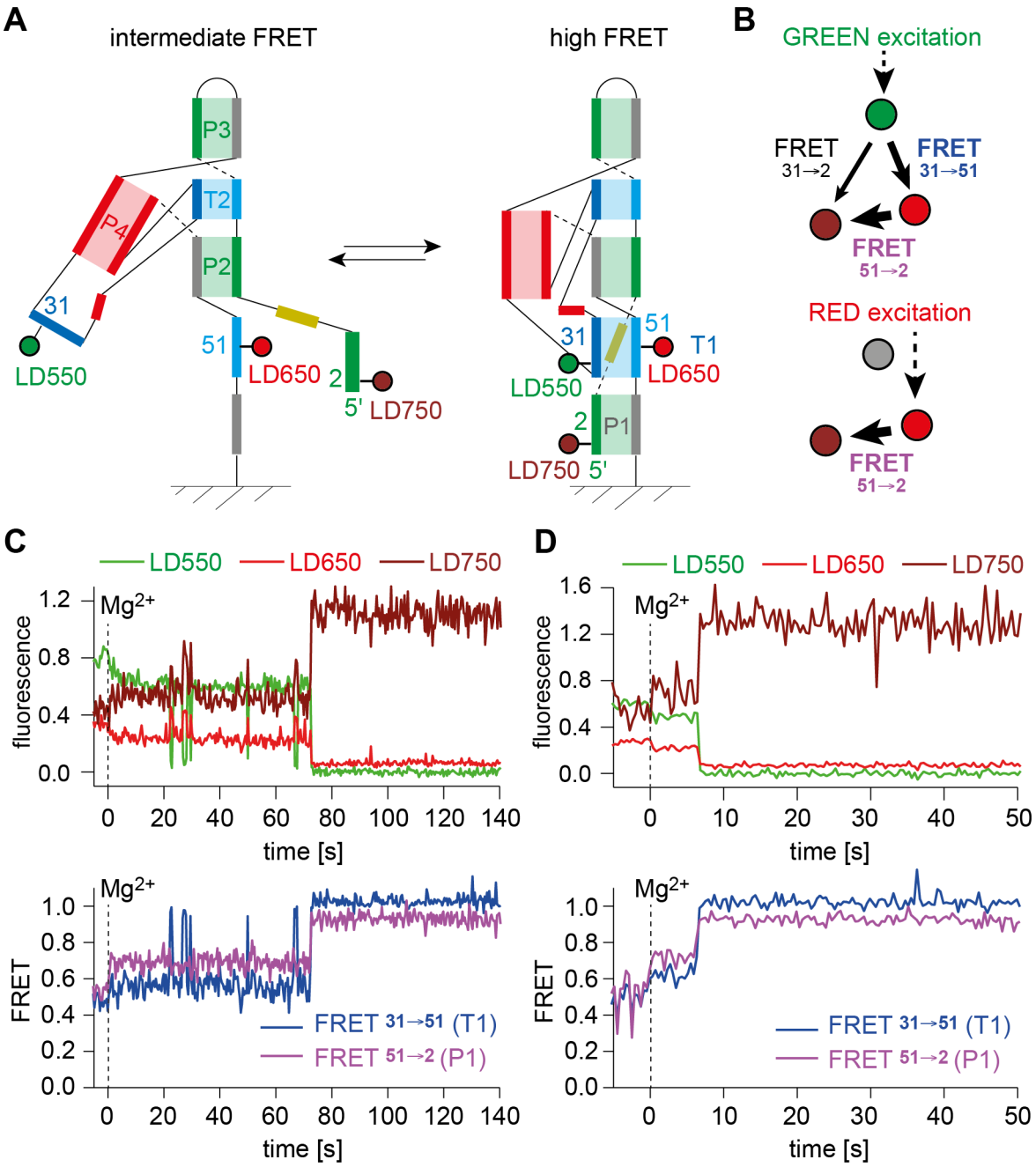
Supporting Figure S4. Preparation of LD550/LD650/LD750 labeled twister variants. (**A**) RNAs and DNA splint sequences for the enzymatic ligation of tw31-51 using T4 DNA ligase. (**B**) Anion exchange (AE) HPLC trace of enzymatic ligation at the start (0 h) and after completion (5 h). The product (retention time = 32.5 min) was purified by AE-HPLC. (**C**) LC-ESI mass spectrum of purified ligation product. (**D**) RNAs and DNA splint sequences for the enzymatic ligation of tw2-31-51 using T4 DNA ligase. (**E**) Anion exchange (AE) HPLC trace of enzymatic ligation at the start (0 h) and after completion (5 h). The product (retention time = 32.5 min) was purified by AE-HPLC. See Supporting Fig. S1 for the chemical structures of modified nucleosides.



Supporting Figure S5. Survival plots of high-FRET states for tw31-51 and G52U tw-31-51. Lifetimes for wild-type and G52U constructs were estimated by fitting each distribution to a double exponential decay function. For the wild-type construct, the population-weighted average of short- (388ms; ~62%) and long-lived (~3 s; ~38%) states was 1.37 s. For the G52U, the population-weighted average of short- (65ms; ~43%) and long-lived (~842 ms; ~62%) states was 0.5 s.



Supporting Figure S6. Twister ribozyme with dU5-G51 labeling pattern. (**A**) Sequence and secondary structure. (**B**) Schematics of anticipated dynamics. (**C**) Representative fluorescence (green, LD550; red, LD650) and FRET (blue) trajectories of twister molecules, where idealization of the data to a two-state Markov chain is shown in red.



Supporting Figure S7. Non-cleavable (dU5) twister ribozyme with U2-C31-G51 labeling pattern. **A**) Schematic of the secondary structure with anticipated dynamics. **B**) Schematics of the alternating laser excitation (ALEX) experiments. **C-D**) Representative fluorescence (green, LD550; red, LD650; dark red, LD750) and FRET (blue, $FRET^{31-51}$; magenta, $FRET^{51-2}$) trajectories, where Mg^{2+} (2 mM final concentration) was delivered at time=0 (vertical dotted line). The two distinct twister molecules represent the two primary subpopulations: those with slow folding and T1 sampling events (panel C), and those with rapid simultaneous T1 and P1 folding with no T1 sampling events (panel D).