Supplementary Data

Interbase FRET in RNA – From A to Z

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Synthesis and characterization of the tC_nitro monomer and its intermediates

Reagents and conditions: a) t-Bu2Si(OTf)2, DMF, 0 °C, 1 h; then imidazole, 0 °C, 30 min; then TBS-Cl, RT, 12 h. b) NH2NH2, EtOH, RT, 18 h. c) CuI, Cs2CO3, DMSO, 60 °C, 24 h. d) PyBOP, DBU, MeCN, 0 °C, 1 h; then RT, 4 h. e) HF, Py, DCM, 0 °C --> RT, 6 h. f) DMTr-Cl, Py, 0 °C, 30 min; then RT, 4 h. g) CEP-Cl, DIPEA, THF, RT, 20 h.

Synthesis experimental details
All reactions were performed under a nitrogen atmosphere unless otherwise noted. Reagents were purchased from various chemical vendors and either used as received or purified according to standard techniques. All solvents used for reactions were HPLC-grade and purchased dry. Reactions were monitored by TLC on silica gel plates analyzed under UV (254 nm), and by UPLC-MS (ESI/UV), using a Waters Acquity system equipped with either an Acquity UPLC HSS C18 column (1.8 µm, length 50 mm, ID 2.1 mm) running a gradient of water-MeCN (95:5) to water-MeCN (5:95), with the water eluent containing 1% formic acid (pH 3) or an Acquity UPLC BEH C18 column (1.7µm, length 50 mm, ID 2.1 mm) running a gradient of water-MeCN (95:5) to water-MeCN (5:95), with the water eluent containing 1% ammonium hydroxide (pH 10). Flash chromatography was performed by automated column chromatography using pre-packed silica columns. 1H, 13C and 31P NMR spectra were recorded on a Bruker system (500 or 600 MHz) equipped with a CryoProbe. All shifts are recorded in ppm relative to the deuterated solvent: CDCl3 (7.26 ppm for 1H and 77.16 ppm for 13C) or CD3CN (1.94 ppm for 1H and 118.26 ppm for 13C). 31P NMR spectra were referenced to external 85% orthophosphoric acid (0.00 ppm).
2'-O-[tert-butyl(dimethyl)silyl]-3',5'-O-[di-tert-butylsilylidene]-5-iodouridine (1)
Adapted from literature.[1] 5-Iodouridine (15.3 g, 40.6 mmol) was charged in an oven-dried RBF and co-evaporated with pyridine (2 × 15 mL) and toluene (15 mL). DMF (324 mL) was added and the RM was cooled to 0 °C. tBu2Si(OTf)2 (13.9 mL, 41.4 mmol) was added to the RM with a syringe pump over 20 minutes. After a total of 1 hour, imidazole (13.8 g, 203 mmol) was added and the RM was left to stir at 0 °C for additional 30 minutes. TBDMS-Cl (15.3 g, 101 mmol) was added and the RM was stirred at RT for 12 hours. The reaction mixture was diluted with water (700 mL) and extracted with EtOAc (800 mL). The aqueous phase was extracted with EtOAc (500 mL), and the combined organic phases were concentrated to circa 200 mL. The organic phase was washed with water (2 × 200 mL) and brine (200 mL). The organic phase was dried over Na2SO4, filtered and concentrated. The residue was loaded to Celite and purified by flash column chromatography (330 g SNAP column, EtOAc in heptane, 0-30%) to afford 21.3 g (84%) of the title compound as a white solid.

δH NMR (500 MHz, CDCl3) δ = 8.60 (s, 1H), 7.66 (s, 1H), 5.64 (s, 1H), 4.53 (dd, J = 9.3, 5.2 Hz, 1H), 4.27 (d, J = 4.6 Hz, 1H), 4.17 (dt, J = 10.2, 5.2 Hz, 1H), 3.98 (dd, J = 10.5, 9.5 Hz, 1H), 3.85 (dd, J = 9.7, 4.6 Hz, 1H), 1.06 (s, 9H), 1.03 (s, 9H), 0.93 (s, 9H), 0.18 (s, 3H), 0.14 (s, 3H). 13C{1H} NMR (126 MHz, CDCl3) δ = 159.7, 149.3, 144.1, 94.1, 76.1, 75.5, 74.8, 68.2, 67.7, 27.6, 27.1, 26.0, 23.0, 20.5, 18.4, -4.1, -4.9. HRMS (ESI-TOF) m/z calcd for C23H42IN2O6Si2 [M + H]+: 625.1626, found: 625.1678.

2-amino-5-nitrobenzene-1-thiol (2)[2]
Special safety measures for handling hydrazine: BARRIER© chemical resistant gloves were used in combination with nitrile gloves, enhanced ventilation in fume hood.

6-nitrobenzothiazole (25.0 g, 133 mmol) was dissolved in EtOH (99.5%, 250 mL). Hydrazine hydrate (64% hydrazine, 61 mL, 805 mmol) was added, and the RM was evacuated and refilled with nitrogen 3 times. The RM was stirred at RT for 18 hours, after which it was diluted with aqueous HCl (1 M, 1 L). The mixture was extracted with CH₂Cl₂ (3 × 400 mL). The combined organic phases were washed with brine (2 × 300 mL), dried on a phase separator and concentrated, affording 18.5 g (84%) of the title compound as an orange solid. Used in the next step without further purification.

δH NMR (500 MHz, CDCl3) δ = 8.34 (dd, J = 2.6, 0.9 Hz, 1H), 8.02 (dd, J = 8.9, 2.6 Hz, 1H), 6.71 (d, J = 8.9 Hz, 1H), 4.93 (s, 2H), 3.00 (s, 1H). 13C{1H} NMR (126 MHz, CDCl3) δ = 153.4, 139.0, 132.0, 126.0, 113.4, 110.9. LRMS (ESI-TOF): m/z calcd for C32H32N2O8S2 [M + H]+: 625.1626, found: 625.1678.

5-[2-amino-5-nitrophenylsulfanyl]-2'-O-[tert-butyl(dimethyl)silyl]-3',5'-O-[di-tert-butylsilylidene]uridine (3)
Compound 1 (12.5 g, 20.0 mmol) was charged into an oven-dried RBF, followed by the addition of CuI (1.43 g, 7.48 mmol), Cs2CO3 (7.24 g, 22.2 mmol), and 2 (5.7 g, 33.4 mmol). DMSO (100 mL) was added, and the RM was stirred at 60 °C for 24 hours. The RM was cooled to RT and diluted with water (400 mL) and EtOAc (400 mL) resulting in a black homogenous mixture. The mixture was vacuum filtered through a pad of Celite, whereupon a black wax was collected on top of the Celite pad. The filtrate, now partitioned into 2 phases, was collected. The wax was thoroughly washed with EtOAc (3 × 80 mL) and the combined washes were added to the filtrate. The combined fractions were partitioned. The aqueous phase was washed with EtOAc (2 × 150 mL). The combined organic fractions were washed with water (2 × 500 mL) and brine (2 × 200mL). The organic phase was dried over MgSO4, filtered and concentrated. The residue was loaded onto Celite and purified by flash column chromatography (330 g SNAP column, EtOAc in heptane: 7-30%) to provide 10.86 g (81%) of the title compound as a yellow solid.
1H NMR (500 MHz, CDCl3) δ = 9.28 (s, 1H), 8.34 (d, J = 2.6 Hz, 1H), 8.04 (dd, J = 9.0, 2.6 Hz, 1H), 7.63 (s, 1H), 6.68 (d, J = 9.0 Hz, 1H), 5.58 (s, 1H), 4.53 (dd, J = 9.4, 5.2 Hz, 1H), 4.25 (d, J = 4.4 Hz, 1H), 4.20 (td, J = 10.3, 5.2 Hz, 1H), 3.91 – 3.83 (m, 1H), 3.73 (dd, J = 9.7, 4.4 Hz, 1H), 1.01 (s, 9H), 1.00 (s, 9H), 0.93 (s, 9H), 0.17 (s, 3H), 0.13 (s, 3H). 13C{1H} NMR (126 MHz, CDCl3) δ = 162.7, 154.9, 149.0, 143.1, 138.6, 133.3, 127.6, 115.2, 114.2, 108.4, 94.2, 76.0, 75.5, 75.0, 67.7, 27.4, 27.1, 26.0, 22.9, 20.5, 18.3. HRMS (ESI-TOF) m/z calcd for C29H47N4O8SSi2 [M + H]+: 667.2653, found: 667.2639.

3-{2-O-[tert-butyl(dimethyl)silyl]-β-D-ribofuranosyl}-7-nitro-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (4)

Compound 3 (9.24 g, 13.9 mmol) and PyBOP (14.9 g, 28.0 mmol) were charged in an oven-dried RBF. MeCN (267 mL) was added, and the RM was cooled to 0 °C. DBU (9.6 mL, 62.3 mmol) was added as a solution in MeCN (24 mL) with a syringe pump over 7 minutes. After a total of 1 hour the RM was allowed to return to RT. After a total of 4 hours, the reaction was quenched with aq. HCl (3.8 M, 100 mL) at 0 °C. The mixture was diluted with water (200 mL) and extracted with DCM (3 × 300 mL). The combined organic phases were dried on a phase separator, concentrated, loaded onto Celite, and washed on a short silica plug (eluent: EtOAc in heptane 1:1). The collected solution was concentrated, and the resulting orange solid (20.6 g) containing 3-{2-O-[tert-butyl(dimethyl)silyl]-3,5-O-(di-tert-butylsilylidene)-β-D-ribofuranosyl}-7-nitro-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (3a) was used in the next step without further purification.

Special safety measures for handling HF: BARRIER© chemical resistant gloves were used in combination with nitrile gloves, enhanced ventilation in fume hood. An open bottle of sat. aq. Ca(OAc)2 was kept in the fume hood in case a spill/exposure of HF-pyridine would occur.

The deprotection protocol was adapted from literature.[1] Crude 3a (20.6 g) was charged in an oven-dried RBF. CH2Cl2 (137 mL) was added and the RM was cooled to 0 °C. HF-pyridine (70% HF, 1.89 mL, 15.24 mmol) was added as a solution in pyridine (5 mL) over 1 minute. The reaction was quenched after a total of 6 hours with sat. aq. Ca(OAc)2 (50 mL) and stirred vigorously at RT for 20 minutes. The mixture was partitioned between CH2Cl2 (300 mL) and water (200 mL). The aqueous phase was extracted with CH2Cl2 (2 × 300 mL). The combined organic fractions were dried on a phase separator, and loaded onto Celite. The product was purified by flash column chromatography (330 g SNAP column, EtOAc in heptane: 20-80%) to afford 4.65 g (65% after two steps) of the title compound as a bright orange solid.

1H NMR (600 MHz, CDCl3) δ = 10.31 (s, 1H), 7.83 (dd, J = 8.9, 2.5 Hz, 1H), 7.81 (s, 1H), 7.79 (d, J = 2.5 Hz, 1H), 7.50 (d, J = 8.9 Hz, 1H), 5.58 (d, J = 3.1 Hz, 1H), 4.57 – 4.53 (m, 1H), 4.28 (q, J = 5.5 Hz, 1H), 4.11 (d, J = 5.8 Hz, 1H), 4.07 (dd, J = 12.1, 1.6 Hz, 1H), 3.89 (d, J = 11.3 Hz, 1H), 3.49 (s, 1H), 2.65 (d, J = 6.5 Hz, 1H), 0.93 (s, 9H), 0.21 (s, 3H), 0.16 (s, 3H). 13C{1H} NMR (151 MHz, CDCl3) δ = 160.5, 154.8, 144.2, 141.5, 137.5, 123.4, 121.5, 118.6, 118.3, 95.9, 93.6, 85.2, 75.1, 69.6, 61.2, 25.9, 18.2. HRMS (ESI-TOF) m/z calcd for C21H29N4O7SSi [M + H]+: 509.1526, found: 509.1525.

3-{5-O-[bis(4-methoxyphenyl)(phenyl)methyl]-2-O-[tert-butyl(dimethyl)silyl]-β-D-ribofuranosyl}-7-nitro-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (4a)

Compound 4 (1.48 g, 2.91 mmol) was charged in an oven-dried RBF. Pyridine (9.7 mL) was added, and the RM was cooled to 0 °C. DMTr-Cl (1.13 g, 3.33 mmol) was added in one portion. After 30 minutes the RM was allowed to return to RT. The reaction was quenched after a total of 4 hours by adding MeOH (0.75 mL), and was stirred for 10 more minutes. The mixture was partitioned between CH2Cl2 (50 mL) and aq. NaHCO3 (8%, 50 mL). The aqueous phase was extracted with CH2Cl2 (3 × 50 mL). The
combined organic phases were dried on a phase separator, concentrated, and the residue loaded on Celite. The product was purified by flash column chromatography (50 g SNAP column; EtOAc in heptane: 10-60%) to provide 1.67 g (71%) of the title compound as a bright orange solid.

$^1$H NMR (600 MHz, CD$_3$CN) $\delta$ = 9.17 (s, 1H), 7.86 (dd, $J$ = 8.9, 2.5 Hz, 1H), 7.70 (s, 1H), 7.59 (d, $J$ = 2.5 Hz, 1H), 7.49 – 7.46 (m, 2H), 7.40 – 7.31 (m, 6H), 7.26 – 7.22 (m, 1H), 7.16 (d, $J$ = 8.9 Hz, 1H), 6.91 – 6.84 (m, 4H), 5.66 (d, $J$ = 2.6 Hz, 1H), 4.40 – 4.32 (m, 2H), 4.10 – 4.04 (m, 1H), 3.73 (s, 3H), 3.70 (s, 3H), 3.45 (dd, $J$ = 11.1, 3.2 Hz, 1H), 3.26 (dd, $J$ = 11.1, 1.8 Hz, 1H), 3.03 (d, $J$ = 7.1 Hz, 1H), 0.92 (s, 9H), 0.18 (s, 3H), 0.15 (s, 3H). $^{13}$C($^1$H) NMR (151 MHz, CD$_3$CN) $\delta$ = 160.8, 159.72, 159.66, 155.1, 145.4, 144.6, 143.2, 137.0, 136.8, 136.4, 131.0, 130.8, 129.05, 129.01, 128.0, 124.0, 122.3, 119.5, 117.9, 114.19, 114.18, 95.5, 91.5, 87.5, 83.7, 77.4, 70.6, 62.8, 55.82, 55.79, 26.1, 18.8, -4.4, -4.7. HRMS (ESI-TOF) m/z calcd for C$_{22}$H$_{47}$N$_4$O$_9$SSi [M + H]$^+$: 809.2676, found: 809.2675.

3-(5-O-[bis(4-methoxyphenyl)(phenyl)methyl]-2-O-[tert-butyldimethylsilyl]-3-O-((2-cyanoethoxy)[di(propan-2-yl)amino]phosphanyl)-ß-D-ribofuranosyl)-7-nitro-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (5)

Compound 4a (711 mg, 0.88 mmol) was charged in an oven-dried RBF. THF (10 mL) was added, followed by addition of DIPEA (0.61 mL, 3.51 mmol), and subsequent dropwise addition of CEP-Cl (0.39 mL, 1.75 mmol) over 1 minute. The RM was stirred at RT for 20 hours, upon which it was diluted with Et$_2$O (25 mL). The mixture was washed with cold NaHCO$_3$ (8%, 50 mL). The aqueous phase was extracted with cold Et$_2$O (25 mL). The combined organic phases were dried over Na$_2$SO$_4$, filtered and concentrated. The residue was re-dissolved in CH$_2$Cl$_2$ (3 mL) and applied on a samplet, which was beforehand treated with 2% TEA in heptane. The product was purified by flash column chromatography (50 g SNAP column, pre-treated with 2% TEA in heptane; EtOAc in heptane: 20-100%, both eluents contained 2% TEA) to provide 805 mg (91%) of the title compound as an orange solid.

$^{31}$P NMR: (202 MHz, CD$_3$CN) $\delta$ = 149.3, 149.0. LRMS (ES): m/z = 1009.3 ([M – H]$^-$, 100%).
Spectral Data

2'-O-\{\text{tert-butyldimethyl}silyl\}-3',5'-O-\{\text{di-tert-butyl}silylidene\}-5-iodouridine (1)

$^1$H NMR (500 MHz, CDCl$_3$)

$^{13}$C ($^1$H) NMR (126 MHz, CDCl$_3$)
2-amino-5-nitrobenzene-1-thiol (2)

$\text{H NMR (500 MHz, CDCl)}$

$\text{C}^{13}\text{H NMR (126 MHz, CDCl)}$

- S8 -
5-[(2-amino-5-nitrophenyl)sulfanyl]-2′-O-[tert-butyl(dimethyl)silyl]-3′,5′-O-(di-tert-butylsilylidene)uridine (3)

$^1$H NMR (500 MHz, CDCl₃)

$^{13}$C($^1$H) NMR (126 MHz, CDCl₃)

- S9 -
3-{2-O-[tert-butyl(dimethyl)silyl]-β-D-ribofuranosyl}-7-nitro-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (4)

$^1$H NMR (600 MHz, CDCl$_3$)

$^{13}$C$^{'(1)}$ NMR (151 MHz, CDCl$_3$)
3-{5-O-[bis(4-methoxyphenyl)(phenyl)methyl]-2-O-[tert-butyl(dimethyl)silyl]-β-D-ribofuranosyl}-7-nitro-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (4a)
3-(5-O-[bis(4-methoxyphenyl)(phenyl)methyl]-2-O-[tert-butyl(dimethyl)silyl]-3-O-[(2-cyanoethoxy)[di(propan-2-yl)amino]phosphanyl]-β-D-ribofuranosyl)-7-nitro-3H-pyrimido[5,4-b][1,4]benzothiazin-2(10H)-one (5)

$^{31}P$ NMR (202 MHz, CD$_3$CN)
Oligonucleotide synthesis and purification

The oligoribonucleotides used for the A-RNA benchmark study (Figure 2a in main text) were synthesised using an ÄKTA oligopilot plus 10 synthesiser (GE Healthcare), using a standard 5 μmole phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping and iodine oxidation. All β-cyanoethyl phosphoramidite monomers with 2′-O-tert-butyldimethylsilyl (TBDMS) protection (Sigma-Aldrich; tC0 was synthesised according to literature,[3] tCnitro was synthesised as shown in the synthesis section) were dissolved in anhydrous MeCN to a concentration of 0.1 M immediately prior to use. The coupling time for all phosphoramidite monomers was 20 minutes. Stepwise coupling efficiencies and overall yields were determined by automated trityl cation conductivity monitoring and in all cases were >96%. Oligoribonucleotides were cleaved from the support and base deprotected in a 1:3 EtOH/ammonia (aq. 26%) solution at 55 °C for 3 h and evaporated to dryness. Removal of TBDMS protecting groups was achieved by redissolving oligoribonucleotides in 625 μL dimethyl sulfoxide to which was added 625 μL 1 M triethylamine trihydrofluoride (Aldrich) and incubating at 65 °C for 2.5 hours prior to butanol precipitation. All oligoribonucleotides were purified by reverse-phase HPLC using a XBridge BEH column (C18, 19 × 150 mm, 5 μm, 130 Å pore), using an MeCN gradient with an aqueous phase of 100 mM triethylammonium acetate (pH 7.2). After HPLC purification, oligoribonucleotides were freeze-dried, then dissolved in phosphate buffer without the need for desalting.

The oligoribonucleotides sequences used for investigating the transition from A- to Z-form RNA were purchased from ATDBio (Southampton, UK).
### Table S1. Melting temperatures ($T_m$) of duplexes from the A-RNA benchmark study:

| Sample | RNA-duplex | $T_m$ (°C) | $\Delta T_m$ (°C) |
|--------|------------|------------|------------------|
| D0A0   | 5′-CGA CAC ACA CAA GGA GGA UUC C-3′ 3′-GCU GUG UGU GGU CCU GCU CCU AAG G-5′ | 78.9 ±0.2 | |
| D6A0   | 5′-CGA CAtCO ACA CAA GGA GGA UUC C-3′ 3′-GCU G UGU GUU GGU CCU GCU CCU AAG G-5′ | 80.3 ±0.1 +1.4 ±0.2 |
| D8A0   | 5′-CGA CAC AtCOA CAA GGA GGA UUC C-3′ 3′-GCU GUG UGU GUU GGU CCU GCU CCU AAG G-5′ | 79.8 ±0.3 +0.9 ±0.3 |
| D10A0  | 5′-CGA CAC ACA tCOAA GGA GGA UUC C-3′ 3′-GCU GUG UGU GUU GGU CCU GCU CCU AAG G-5′ | 81.1 ±0.2 +2.2 ±0.2 |
| D0A6   | 5′-CGA CAC ACA CAA GGA GGA G A UUC C-3′ 3′-GCU GUG UGU GGU GUU CCU GCU CCU AAG G-5′ | 80.3 ±0.3 +1.4 ±0.3 |
| D0A7   | 5′-CGA CAC ACA CAA GGA GGA G A UUC C-3′ 3′-GCU GUG UGU GGU GUU CCU GCU CCU AAG G-5′ | 82.8 ±0.2 +3.9 ±0.3 |
| D0A12  | 5′-CGA CAC ACA CAA G G A G A UUC C-3′ 3′-GCU GUG UGU GGU GUU GGU CCU GCU CCU AAG G-5′ | 80.6 ±0.2 +1.7 ±0.3 |
| D0A13  | 5′-CGA CAC ACA CAA G G A G A UUC C-3′ 3′-GCU GUG UGU GGU GUU GGU CCU GCU CCU AAG G-5′ | 83.1 ±0.1 +4.2 ±0.2 |
| D10A13 | 5′-CGA CAC ACA tCOAA G G A G G A UUC C-3′ 3′-GCU GUG UGU GGU GUU GGU CCU GCU CCU AAG G-5′ | 83.7 ±0.1 +4.8 ±0.2 |

The DX and AY notation of the duplexes reflects the position of the donor, tCO (blue), and acceptor, tCnitro (tCn; orange) counting from the 5′-end. D0 and A0 refers to no donor and acceptor, respectively. Duplexes were formed by hybridization with the complementary strand as described in the “Materials and Methods” section. Melting temperatures were calculated as the maximum of the first derivative of the UV-melting curves and are reported with the standard error of the mean. Average effect of incorporating tCO: +1.5 °C; Average effect of incorporating tCnitro: +2.8 °C.

### Figure S1. CD spectra of duplexes from the A-RNA benchmark study:

Unmodified (D0A0) and modified duplexes containing either tCO (DXA0), tCnitro (D0AY) or both (DXAY). Measurements were performed at RT in phosphate buffer, pH 7.4, 123 mM Na+. 
Table S2. Amplitude-weighted fluorescence lifetimes from the A-RNA benchmark study.

| Duplex a  | Separation (no. bp.) | $\tau_1$ (ns) | $\alpha_1$ (%) | $\tau_2$ (ns) | $\alpha_2$ (%) | $\tau_3$ (ns) | $\alpha_3$ (%) | $<\tau>$ (ns) | $\chi^2$ |
|-----------|----------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------|
| D10A0     | -                    | 4.61          | 93.9          | 1.29          | 6.1           | 4.41          | 1.05          |
|           |                      | 5.66          | 17.3          | 4.21          | 82.7          | 4.46          | 1.02          |
| D10A6     | 9                    | 3.05          | 6.6           | 1.46          | 93.4          | 1.57          | 1.09          |
|           |                      | 2.30          | 20.9          | 1.38          | 79.1          | 1.57          | 1.06          |
| D10A7     | 8                    | 2.64          | 9.1           | 1.20          | 90.9          | 1.33          | 1.27          |
|           |                      | 2.24          | 15.7          | 1.17          | 84.3          | 1.33          | 1.00          |
| D10A12    | 3                    | 3.68          | 0.6           | 0.30          | 31.6          | 0.11          | 67.8          | 0.19          | 1.10    |
|           |                      | 3.35          | 0.6           | 0.28          | 35.6          | 0.10          | 63.8          | 0.19          | 1.24    |
| D10A13    | 2                    | 3.97          | 0.06          | 0.24          | 0.6           | 0.016         | 99.3          | 0.02          | 1.47    |
|           |                      | 3.65          | 0.05          | 0.22          | 0.8           | 0.018         | 99.1          | 0.02          | 1.92    |
| D8A0      | -                    | 8.67          | 1.7           | 4.43          | 98.3          | 4.50          | 1.08          |
|           |                      | 4.67          | 90.4          | 2.51          | 9.6           | 4.47          | 0.93          |
| D8A6      | 11                   | 4.29          | 20.6          | 2.99          | 79.4          | 3.25          | 1.01          |
|           |                      | 3.49          | 79.6          | 2.12          | 20.4          | 3.21          | 0.91          |
| D8A7      | 10                   | 3.29          | 15.7          | 1.79          | 84.3          | 2.03          | 1.10          |
|           |                      | 3.41          | 13.6          | 1.82          | 86.4          | 2.04          | 0.91          |
| D8A12     | 5                    | 3.96          | 1.7           | 0.55          | 19.2          | 0.15          | 79.1          | 0.29          | 1.23    |
|           |                      | 3.98          | 1.8           | 0.57          | 18.4          | 0.16          | 79.8          | 0.30          | 1.11    |
| D8A13     | 4                    | 4.18          | 0.8           | 0.40          | 19.8          | 0.10          | 79.4          | 0.19          | 1.40    |
|           |                      | 4.15          | 0.8           | 0.41          | 21.4          | 0.11          | 77.8          | 0.21          | 1.38    |
| D6A0      | -                    | 4.77          | 87.8          | 2.74          | 12.2          | 4.52          | 0.91          |
|           |                      | 6.29          | 7.5           | 4.32          | 92.5          | 4.47          | 0.97          |
| D6A6      | 13                   | 4.57          | 89.8          | 2.28          | 10.2          | 4.34          | 1.00          |
|           |                      | 4.51          | 92.3          | 2.43          | 7.7           | 4.35          | 1.07          |
| D6A7      | 12                   | 4.47          | 83.0          | 2.62          | 17.0          | 4.16          | 0.99          |
|           |                      | 4.35          | 91.8          | 1.99          | 8.2           | 4.16          | 1.06          |
| D6A12     | 7                    | 2.61          | 67.7          | 1.27          | 32.3          | 2.18          | 1.02          |
|           |                      | 2.62          | 67.8          | 1.32          | 32.2          | 2.20          | 0.95          |
| D6A13     | 6                    | 1.79          | 36.5          | 0.94          | 63.5          | 1.25          | 1.35          |
|           |                      | 0.94          | 65.8          | 1.81          | 34.2          | 1.24          | 1.12          |

aDuplexes were formed as described in the “Materials and Methods” section and measured at RT in phosphate buffer, pH 7.4, 123 mM Na+. Each duplex was prepared and measured twice, the individual fits are shown in the table.
Figure S2. Fluorescence spectra of duplexes from the A-RNA benchmark study: Unmodified (D0A0) and modified duplexes containing either tC (“DXA0), tC nitro, (D0AY) or both (DXAY). Measurements were performed at RT in phosphate buffer, pH 7.4, 123 mM Na+.

Table S3. Steady-state and lifetime FRET-efficiency values from the A-RNA benchmark study.

| Duplex  | Separation (no. bp.) | Steady-state | TCSPC | Average |
|---------|---------------------|-------------|-------|---------|
| D10A13  | 2                   | 0.99        | 1.00  | 0.99    |
| D10A12  | 3                   | 0.96        | 0.96  | 0.96    |
| D8A13   | 4                   | 0.95        | 0.96  | 0.95    |
| D8A12   | 5                   | 0.93        | 0.93  | 0.93    |
| D6A13   | 6                   | 0.73        | 0.72  | 0.73    |
| D6A12   | 7                   | 0.51        | 0.51  | 0.51    |
| D10A7   | 8                   | 0.71        | 0.70  | 0.71    |
| D10A6   | 9                   | 0.66        | 0.65  | 0.65    |
| D8A7    | 10                  | 0.54        | 0.55  | 0.54    |
| D8A6    | 11                  | 0.28        | 0.28  | 0.28    |
| D6A7    | 12                  | 0.08        | 0.08  | 0.08    |
| D6A6    | 13                  | 0.05        | 0.03  | 0.04    |

aDuplexes were formed as described in the “Materials and Methods” section and measured at RT in phosphate buffer, pH 7.4, 123 mM Na+.
Table S4. Quantum yield of tC\(^0\) in the donor positions used for the A-RNA benchmark study.

| Duplex\(^a\) | RNA-duplex | \(\Phi_f\) (%)\(^b\) |
|-------------|-------------|---------------------|
| D6A0        | 5′-CGA CATC\(^0\) ACA CAA GGA CGA GGA UUC C-3′ 3′-GCU GU G UGU GUU CCU GCU CCU AAG G-5′ | 19.2 |
| D8A0        | 5′-CGA CAC ATC\(^0\)A CAA GGA CGA GGA UUC C-3′ 3′-GCU GUG U GUU CCU GCU CCU AAG G-5′ | 18.9 |
| D10A0       | 5′-CGA CAC ACA tC\(^0\)AA GGA CGA GGA UUC C-3′ 3′-GCU GUG UGU G UU CCU GCU CCU AAG G-5′ | 19.1 |
| Average     |             | 19.1               |

\(^a\)Duplexes were formed as described in the “Materials and Methods” section and measured at RT in phosphate buffer, pH 7.4, 123 mM Na\(^+\). \(^b\)Quantum yields were determined with quinine sulfate as reference (\(\Phi_f = 54.6\%\) in 0.5 M H\(_2\)SO\(_4\)) using an excitation wavelength of 356 nm.

Figure S3. CD spectra of duplexes from the A- to Z-RNA study. a) Unmodified duplex D0A0 measured in phosphate buffer, pH 7.4, with different concentrations of NaClO\(_4\) (0-9 M) at 45 °C. b) Duplex D0A0 measured in phosphate buffer, pH 7.4, with 8 M NaClO\(_4\) first at 45 °C and then at 22 °C over time (0 minutes to 18 hours). The Z-form peak at 280 nm remains unchanged over this time interval indicating that the Z-form is stable for at least 18 h at 22 °C. c) Unmodified duplex D0A0 and all modified duplexes used for the A- to Z-RNA study, measured in phosphate buffer, pH 7.4, with 8 M NaClO\(_4\) at 22 °C.
**Figure S4. Fluorescence spectra of duplexes from the A- to Z-RNA study**: Duplexes containing either tC\(^0\) alone (DXA0, black line) or both tC\(^0\) and tC\(^\text{nitro}\) (DXAY). a) A-form duplexes. b) Z-form duplexes induced by 8 M NaClO\(_4\). The duplexes were prepared as described in the “Materials and Methods” section and measured at RT. A-form duplexes were measured in phosphate buffer, pH 7.4, 123 mM Na\(^+\). Z-form duplexes were measured in phosphate buffer, pH 7.4, with 8 M NaClO\(_4\). The Z-form fluorescence emission spectra were corrected for variations in the concentration arising from the mixing with solid NaClO\(_4\). To illustrate the variation of fluorescence intensity during the transition, the vertical scales are identical for both salt conditions.

**Figure S5. Measured FRET-efficiency between tC\(^0\) and tC\(^\text{nitro}\) for A-form duplexes in the A- to Z-RNA study** (same strand - red squares and opposite strand - black triangles), together with theoretical curves for duplexes containing donor and acceptor in the same strand (red) and in opposite strand (black). Measurements were performed at RT in phosphate buffer, pH 7.4, 123 mM Na\(^+\).
Table S5. Amplitude-weighted fluorescence lifetimes for A-form duplexes in the A- to Z-RNA study.

| Duplex<sup>a</sup> | Separation (no. bp.) | $\tau_1$ (ns) | $\alpha_1$ (%) | $\tau_2$ (ns) | $\alpha_2$ (%) | $\tau_3$ (ns) | $\alpha_3$ (%) | $<\tau>$ (ns) | $\chi^2$ |
|-------------------|---------------------|---------------|----------------|---------------|----------------|---------------|----------------|--------------|----------|
| D2A0              | -                   | 4.81          | 89.6           | 2.30          | 10.4           |               |                | 4.55         | 1.05     |
|                   |                     | 4.82          | 92.6           | 0.92          | 7.4            |               |                | 4.53         | 1.10     |
| D2A6              | 3                   | 4.48          | 3.0            | 0.36          | 20.7           | 0.081         | 76.3           | 0.27         | 1.43     |
|                   |                     | 4.48          | 2.9            | 0.42          | 15.8           | 0.10          | 81.3           | 0.28         | 1.10     |
| D2A8              | 5                   | 4.34          | 4.2            | 0.95          | 24.4           | 0.38          | 71.4           | 0.68         | 1.05     |
|                   |                     | 4.32          | 3.6            | 0.95          | 24.8           | 0.37          | 71.6           | 0.66         | 0.91     |
| D2A9              | 6                   | 4.09          | 6.9            | 1.21          | 64.3           | 0.37          | 28.8           | 1.17         | 1.04     |
|                   |                     | 4.04          | 6.0            | 1.25          | 62.5           | 0.45          | 31.5           | 1.17         | 1.02     |
| D2A10             | 7                   | 3.94          | 81.9           | 1.86          | 18.1           |               |                | 3.56         | 1.07     |
|                   |                     | 3.89          | 86.3           | 1.38          | 13.8           |               |                | 3.55         | 1.06     |
| D2A12             | 9                   | 3.89          | 26.8           | 2.11          | 73.2           |               |                | 2.59         | 1.21     |
|                   |                     | 3.82          | 28.4           | 2.09          | 71.6           |               |                | 2.58         | 1.16     |
| D2A13             | 10                  | 4.32          | 18.6           | 1.86          | 81.4           |               |                | 2.32         | 1.10     |
|                   |                     | 4.34          | 16.2           | 1.90          | 83.8           |               |                | 2.30         | 1.01     |
| D4A0              | -                   | 4.58          | 93.0           | 1.12          | 7.0            |               |                | 4.34         | 1.08     |
|                   |                     | 4.64          | 93.2           | 1.20          | 6.8            |               |                | 4.41         | 1.13     |
| D4A9              | 4                   | 4.07          | 3.6            | 0.51          | 27.2           | 0.13          | 69.2           | 0.38         | 1.04     |
|                   |                     | 4.05          | 3.0            | 0.50          | 28.1           | 0.12          | 68.9           | 0.35         | 1.17     |
| D4A13             | 8                   | 3.90          | 14.8           | 1.37          | 85.2           |               |                | 1.74         | 1.42     |
|                   |                     | 3.67          | 16.2           | 1.35          | 83.8           |               |                | 1.72         | 1.28     |

<sup>a</sup>Duplexes were formed as described in the “Materials and Methods” section and measured at RT in phosphate buffer, pH 7.4, 123 mM Na<sup>+</sup>. Each duplex was prepared and measured twice. The individual fits are shown in the table.
Table S6. Amplitude-weighted fluorescence lifetimes for the Z-form in the A- to Z-RNA study

| Duplex<sup>a</sup> | Separation (no. bp.) | $\tau_1$ (ns) | $\alpha_1$ (%) | $\tau_2$ (ns) | $\alpha_2$ (%) | $\tau_3$ (ns) | $\alpha_3$ (%) | $<\tau>$ (ns) | $\chi^2$ |
|---------------------|---------------------|--------------|---------------|--------------|---------------|--------------|---------------|--------------|--------|
| D2A0                | -                   | 4.95         | 60.8          | 2.71         | 39.2          |              |               | 4.07         | 0.97   |
|                     |                     | 4.71         | 65.3          | 2.03         | 34.7          |              |               | 3.78         | 1.18   |
| D2A6                | 3                   | 4.40         | 3.9           | 0.84         | 43.8          | 0.27         | 52.3          | 6.68         | 1.16   |
|                     |                     | 4.34         | 3.4           | 0.89         | 39.3          | 0.30         | 57.3          | 0.67         | 0.85   |
| D2A8                | 5                   | 4.19         | 6.7           | 1.69         | 76.2          | 0.34         | 17.1          | 1.63         | 1.06   |
|                     |                     | 3.92         | 6.9           | 1.66         | 75.4          | 0.24         | 17.7          | 1.57         | 0.95   |
| D2A9                | 6                   | 4.16         | 58.6          | 2.22         | 41.4          |              |               | 3.36         | 1.13   |
|                     |                     | 4.08         | 60.5          | 2.18         | 39.5          |              |               | 3.33         | 1.22   |
| D2A10               | 7                   | 4.26         | 71.7          | 1.90         | 28.3          |              |               | 3.59         | 1.26   |
|                     |                     | 4.22         | 70.3          | 1.95         | 29.7          |              |               | 3.54         | 1.14   |
| D2A12               | 9                   | 4.11         | 49.5          | 2.35         | 50.5          |              |               | 3.22         | 1.26   |
|                     |                     | 3.83         | 63.5          | 2.06         | 36.5          |              |               | 3.18         | 1.05   |
| D2A13               | 10                  | 4.68         | 53.4          | 2.39         | 46.6          |              |               | 3.61         | 1.17   |
|                     |                     | 4.62         | 52.3          | 2.45         | 47.7          |              |               | 3.58         | 1.17   |
| D4A0                | -                   | 4.61         | 84.8          | 2.18         | 15.2          |              |               | 4.24         | 1.09   |
|                     |                     | 4.52         | 92.8          | 2.17         | 7.2           |              |               | 4.35         | 1.25   |
| D4A9                | 4                   | 4.40         | 18.1          | 1.27         | 24.7          | 0.43         | 57.2          | 1.36         | 1.21   |
|                     |                     | 4.33         | 16.3          | 1.18         | 28.8          | 0.40         | 54.9          | 1.27         | 0.96   |
| D4A13               | 8                   | 4.22         | 81.9          | 1.92         | 18.1          |              |               | 3.81         | 1.09   |
|                     |                     | 4.14         | 82.2          | 1.80         | 17.8          |              |               | 3.72         | 1.08   |

<sup>a</sup>Duplexes were formed as described in the “Materials and Methods” section and measured at RT in phosphate buffer with 8 M NaClO₄, pH 7.4, 123 mM Na⁺. Each duplex was prepared and measured twice. The individual fits are shown in the table.
Table S7. Steady-state and lifetime FRET-efficiency values from the A- to Z-RNA study

| Duplex | tC\textsuperscript{O},tC\textsubscript{nitro} separation (no. bp.) | RNA-duplex\textsuperscript{b} | FRET-efficiency | Lifetime | Steady-state\textsuperscript{c} |
|--------|---------------------------------------------------------------|---------------------------------|-----------------|----------|-------------------------------|
|        |                                                               |                                  | A-form  | Z-form  | A-form  | Z-form  |
| D2A6   | 3                                                             | 5\textsuperscript{	extprime}-GtC\textsuperscript{O}GCCGCGCGGC-3\textprime | 0.94    | 0.83    | 0.94    | 0.84    |
| D4A9   | 4                                                             | 5\textsuperscript{	extprime}-GCGC   G CGC-3\textprime | 0.92    | 0.69    | 0.92    | 0.67    |
| D2A8   | 5                                                             | 5\textsuperscript{	extprime}-GtC\textsuperscript{O}GCCGCGCGGC-3\textprime | 0.85    | 0.59    | 0.88    | 0.62    |
| D2A9   | 6                                                             | 5\textsuperscript{	extprime}-GtC\textsuperscript{O}GCCGCGCGGC-3\textprime | 0.74    | 0.15    | 0.77    | 0.23    |
| D2A10  | 7                                                             | 5\textsuperscript{	extprime}-GtC\textsuperscript{O}GCCGCGCGCG-3\textprime | 0.22    | 0.09    | 0.34    | 0.29\textsuperscript{d} |
| D4A13  | 8                                                             | 5\textsuperscript{	extprime}-GtC\textsuperscript{O}GCCGCGCGCG-3\textprime | 0.60    | 0.12    | 0.63    | 0.04    |
| D2A12  | 9                                                             | 5\textsuperscript{	extprime}-GtC\textsuperscript{O}GCCGCGCGCG-3\textprime | 0.43    | 0.18    | 0.57    | 0.46\textsuperscript{d} |
| D2A13  | 10                                                            | 5\textsuperscript{	extprime}-GtC\textsuperscript{O}GCCGCGCGCGCG-3\textprime | 0.49    | 0.08    | 0.53    | 0.10    |

\textsuperscript{a}The DX and AY notation of the duplexes reflects the position of the donor, tC\textsuperscript{O} (blue), and acceptor, tC\textsubscript{nitro} (tC\textsubscript{n}; orange) counting from the 5\textsuperscript{	extprime}-end of the GC-repeat of the donor-containing sequence. \textsuperscript{b}Placement of the donor and acceptor in the GC-repeat of the sequences used for the A- to Z-RNA study. For the full structure, see main text Figure 2b. The duplexes were prepared as described in the “Materials and Methods" section and measured at RT. A-form duplexes were measured in phosphate buffer, pH 7.4, 123 mM Na\textsuperscript{+}. Z-form duplexes were measured in phosphate buffer, pH 7.4, with 8 M NaClO\textsubscript{4}. \textsuperscript{c}Z-form steady-state FRET efficiencies were corrected for variations in the concentration arising from the mixing with solid NaClO\textsubscript{4}. \textsuperscript{d}The amount of dark species for separations seven and nine is estimated to be roughly 20% and 30% respectively, based on the difference in FRET efficiency from lifetime and steady-state measurements.

Table S8. Quantum yield of tC\textsuperscript{O} in the donor positions used in the A- to Z-RNA study

| Duplex | RNA-duplex\textsuperscript{b} | A-form \(\Phi_f\) (%)\textsuperscript{c} | Z-form \(\Phi_f\) (%)\textsuperscript{c} |
|--------|--------------------------------|-----------------------------------------------|-----------------------------------------------|
| D2A0   | 5\textsuperscript{	extprime}-GtC\textsuperscript{O}GCCGCGCGCGCGCG-3\textprime | 21.6                                          | 21.2                                          |
| D4A0   | 5\textsuperscript{	extprime}-GCGtC\textsuperscript{O}GCCGCGCGCGCGCG-3\textprime | 20.2                                          | 21.4                                          |
| Average|                                                               | 20.9                                          | 21.3                                          |

\textsuperscript{a}Duplexes were formed as described in the experimental section and measured at RT. \textsuperscript{b}Placement of the donor in the GC-repeat of the sequences used for the A- to Z-RNA study. For the full structure, see main text Figure 2b. A-form duplexes were measured at RT in phosphate buffer, pH 7.4, 123 mM Na\textsuperscript{+}. Z-form duplexes were measured at RT in phosphate buffer, pH 7.4, with 8 M NaClO\textsubscript{4}. \textsuperscript{c}Quantum yields were determined with quinine sulfate as a reference (\(\Phi_f = 54.6\%) in 0.5 M H\textsubscript{2}SO\textsubscript{4} using an excitation wavelength of 356 nm.
References

[1] V. Serebryany, L. Beigelman, *Tetrahedron Lett.* **2002**, *43*, 1983-1985.

[2] O. Demeter, E. A. Fodor, M. Kállay, G. Mező, K. Németh, P. T. Szabó, P. Kele, *Chem. Eur. J.* **2016**, *22*, 6382-6388.

[3] A. F. Füchtbauer, S. Preus, K. Börjesson, S. A. McPhee, D. M. J. Lilley, L. M. Wilhelmsson, *Sci. Rep.* **2017**, *7*, 2393.