Supplementary Information

A 2', 2'-disulfide-bridged dinucleotide conformationally locks RNA hairpins

Florian Gauthier, Frédéric Beltran, Annabelle Biscans, Françoise Debart, Christelle Dupouy* and Jean-Jacques Vasseur

Contents

General procedures ........................................................................................................... 3
Synthesis of 2'-O-AcSM ribonucleosides phosphoramidites 1a-c................................. 3
  Scheme S1: Synthesis of 2'-O-AcSM ribonucleoside phosphoramidites 1a-c ............ 3
  Synthesis conditions......................................................................................................3

1H, 13C, and 31P NMR spectra of nucleosides ............................................................ 9
  Figure S1: 400 MHz 1H-NMR and 100 MHz 13C-NMR spectra (CDCl3) of 3a .......... 9
  Figure S2: 400 MHz 1H-NMR and 100 MHz 13C-NMR spectra (CDCl3) of 3b ........ 10
  Figure S3: 400 MHz 1H-NMR and 100 MHz 13C-NMR spectra (CDCl3) of 3c ........ 11
  Figure S4: 400 MHz 1H-NMR and 100 MHz 13C-NMR spectra (DMSO-d6) of 4a .... 12
  Figure S5: 400 MHz 1H-NMR and 100 MHz 13C-NMR spectra (CDCl3) of 4b ........ 13
  Figure S6: 400 MHz 1H-NMR and 100 MHz 13C-NMR spectra (DMSO-d6) of 4c .... 14
  Figure S7: 400 MHz 1H-NMR and 100 MHz 13C-NMR spectra (CDCl3) of 5a ........ 15
  Figure S8: 400 MHz 1H-NMR and 100 MHz 13C-NMR spectra (CDCl3) of 5b ........ 16
  Figure S9: 400 MHz 1H-NMR and 100 MHz 13C-NMR spectra (CDCl3) of 5c ........ 17
  Figure S10: 121 MHz 31P-NMR spectrum (CD3CN) of 1a ...................................... 18
  Figure S11: 121 MHz 31P-NMR spectrum (CD3CN) of 1b ...................................... 19
  Figure S12: 121 MHz 31P-NMR spectrum (CD3CN) of 1c ...................................... 20

HPLC chromatograms & MALDI-TOF spectra of purified oligonucleotides .......... 21
  Figure S13: IEX-HPLC and MALDI-TOF MS analysis of purified RNA S1 .......... 21
  Figure S14: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H4-1 ......... 22
  Figure S15: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H4-2 ......... 23
  Figure S16: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H4-3 .......... 24
  Figure S17: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H5-1 .......... 25
  Figure S18: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H5-2 .......... 26
  Figure S19: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H5-3 .......... 27
Figure S20: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H5-4 ........................................ 28
Figure S21: IEX-HPLC and MALDI-TOF MS analysis of purified RNA DIS-1 ..................................... 29
Figure S22: IEX-HPLC and MALDI-TOF MS analysis of purified RNA DIS-2 ..................................... 30
Figure S23: IEX-HPLC and MALDI-TOF MS analysis of purified RNA DIS-2F ................................... 31

UV Melting curves .......................................................................................................................... 32
  Figure S24: Melting curves of D1, D2, D3 and D4 duplexes ............................................................ 32
  Figure S25: Melting curves of H4 hairpins ....................................................................................... 32
  Figure S26: Melting curves and first derivatives of H5 hairpins ....................................................... 33
  Figure S27: Melting curves and first derivatives of DIS ONs ............................................................ 34

Circular Dichroism spectra ............................................................................................................ 35
  Figure S28: Comparative CD spectra of H4 hairpins ..................................................................... 35
  Figure S29: Comparative CD spectra of H5 hairpins ..................................................................... 35
  Figure S30: Comparative CD spectra of DIS ONs ........................................................................... 36

Analyses of H4 & H5 hairpins by gel electrophoresis .................................................................... 37
  Figure S31: Gel electrophoresis analysis of H4 and H5 hairpins after incubation in buffer .......... 37
  Figure S32: Comparative gel electrophoresis of H4 and H5 with a 14-mer RNA duplex ............. 37

Enzymatic stability of RNA hairpins ............................................................................................. 38
  Figure S33: Gel electrophoresis of hairpins H4 and H5 incubated with SVPDE at 37°C ............... 38
  Figure S34: Enzymatic stability of modified H5 against SVPDE .................................................... 39

Fluorescence experiments ............................................................................................................. 40
  Figure S35: Fluorescence emission spectra of DIS-2F with gradient concentration of glutathione
  with an excess concentration of DIS. ............................................................................................ 40
  Figure S36: Glutathione concentration dependence of relative fluorescence intensity ((I-Io)/Io) of
  DIS-2F at the fluorescence emission maximum .......................................................................... 41
General procedures
CH$_3$CN, pyridine and DIEA were distilled over calcium hydride. All reactions were performed in anhydrous conditions under argon. NMR experiments were accomplished on Bruker DRX 400 and AM 300 spectrometers at 20°C. HRMS analyses were obtained with electrospray ionization (ESI) in positive or negative mode on a Q-TOF Micromass spectrometer.

Synthesis of 2'-O-AcSM ribonucleosides phosphoramidites 1a-c

Synthesis conditions

2'-O-acetylthiomethyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-N4-acetyl cytidine 3a.
To a solution of 2'-O-methylthiomethyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl) cytidine 2a (3.00 g, 5.11 mmol, 1.0 equiv) in dry CH$_2$Cl$_2$ (34 mL) was added dropwise under argon a 1.0 M sulfuryl chloride solution in CH$_2$Cl$_2$ (8.00 mL, 7.67 mmol, 1.5 equiv). The mixture was stirred for 1.5h at room temperature. After completion of the reaction, the chloromethylether derivative was obtained as brown foam after evaporation of the solvent and was directly used in the next step. 18-crown-6 (1.01 g, 3.83 mmol, 0.75 equiv) was added to potassium thioacetate (1.00 g, 8.79 mmol, 1.72 equiv) solution in CH$_2$Cl$_2$ (13 mL). The solution was added dropwise to the chloromethyl ether derivative in dry CH$_2$Cl$_2$ (13 mL). After stirring at room temperature for 3h, the mixture was evaporated and diluted in ethyl acetate. The solution was filtered and
washed with water. The aqueous layer was then extracted with ethyl acetate. The organic layer was washed with brine and dried over Na₂SO₄. The solvent was concentrated under reduced pressure. The crude material was purified by silica gel column chromatography with cyclohexane/ethyl acetate (70/30). The desired compound 3a was obtained as white foam (1.89 g, 3.07 mmol, 60%). ¹H-NMR (400 MHz, CDCl₃) δ 10.06 (s, 1H, NH) ; 8.26 (d, J = 7.6 Hz, 1H, H₆) ; 7.41 (d, J = 7.2 Hz, 1H, H₃) ; 5.79 (s, 1H, H₁′) ; 5.46, 5.35 (2d, JAB = 11.2 Hz, 2H, OCH₂S) ; 4.25 (d, J = 13.6 Hz, 1H, H₅′) ; 4.16-4.07 (m, 3H, H₂, H₃, H₄) ; 3.96 (dd, J = 13.6 Hz, J = 2.0 Hz, 1H, H₅′′) ; 2.36 (s, 3H, SCOCH₃) ; 2.28 (s, 3H, CH₃ Ac) ; 1.09-0.98 (m, 28H, iPr). ¹³C-NMR (100 MHz, CDCl₃) δ 194.2 (SC=O) ; 171.0 (C=O) ; 162.9 (C=O), 144.3 (C₆) ; 96.3 (C₃) ; 89.5 (C₁′) ; 81.9 (C₄′) ; 80.7 (C₂′) ; 69.9 (OCH₂S) ; 67.3 (C₃′) ; 59.3 (C₅′) ; 30.9 (S(OCH₃)) ; 24.9 (CH₃) ; 17.4-16.8 (CH₃, iPr) ; 13.4-12.5 (CH, iPr). HRMS (ESI⁺) m/z calcd for C₂₆H₃₅N₃O₈S₂(M+H)+ 616.2544, Found 616.2550.

2’-O-acetylthiomethyl-3’,5’-O-(tetraisopropylsiloxane-1,3-diyl)-N6-phenoxyacetyl adenosine 3b. Using the same procedure as for the synthesis of 3a, starting from 2b (7.52 g, 10.70 mmol), compound 3b was obtained as a white foam (5.69 g, 7.78 mmol, 73%). Purification conditions: silica gel column chromatography with cyclohexane/ethyl acetate (60/40). ¹H-NMR (400 MHz, CDCl₃) δ 9.48 (s, 1H, NH) ; 8.75 (s, 1H, H₂) ; 8.26 (s, 1H, H₈) ; 7.36-7.03 (m, 5H, HAry) ; 6.02 (s, 1H, H₁′) ; 5.37 (s, 2H, OCH₂S) ; 4.87 (s, 2H, CH₂-PAC) ; 4.83 (dd, J = 9.6 Hz, J = 5.4 Hz, 1H, H₃′) ; 4.54 (d, J = 4.8 Hz, 1H, H₂′) ; 4.18 (dd, J = 13.2 Hz, J = 1.2 Hz, 1H, H₅′′) ; 4.11 (dt, J = 9.2 Hz, J = 2.0 Hz, 1H, H₄′) ; 4.01 (dd, J = 13.2 Hz, J = 2.4 Hz, 1H, H₃′′) ; 2.32 (s, 3H, CH₃) ; 1.10-1.06 (m, 28H, iPr). ¹³C-NMR (100 MHz, CDCl₃) δ 194.2 (SC=O) ; 166.6 (C=O) ; 157.0 (Cq PAC) ; 152.5 (C₂) ; 150.9 (C₄) ; 148.3 (C₆) ; 141.8 (C₈) ; 129.8, 122.4, 115.0 (Car) ; 122.9 (C₅) ; 88.6 (C₁′) ; 81.6 (C₄′) ; 80.5 (C₂′) ; 70.2 (OCH₂S) ; 69.3 (C₃′) ; 68.1 (CH₂-PAC) ; 59.8 (C₅′) ; 30.9 (CH₃) ; 17.4-16.9 (CH₃, iPr) ; 13.4-12.6 (CH, iPr). HRMS (ESI⁺) m/z calcd for C₃₃H₄₉N₅O₈S₂(M+H)+ 732.2919, Found 732.2924.

2’-O-acetylthiomethyl-3’,5’-O-(tetraisopropylsiloxane-1,3-diyl)-N2-phenoxyacetyl guanosine 3c. To a solution of 2’-O-methylthiomethyl-3’,5’-O-(tetraisopropylsiloxane-1,3-diyl) guanosine 2c (6.67 g, 9.26 mmol, 1.0 equiv) in dry CH₂Cl₂ (100 mL) was added dropwise under argon a 1.0 M sulfuryl chloride solution in CH₂Cl₂ (11.10 mL, 11.10 mmol, 1.2 equiv) and 4-chloro-styrene (1.23 mL, 10.19 mmol, 1.1 eq). The mixture was stirred for 1h at room temperature. After completion of the reaction, the chloromethylether derivative was obtained as brown foam after evaporation of the solvent and was directly used in the next step. 18-crown-6 (1.84 g, 6.95 mmol, 0.75 equiv) was added to a CH₂Cl₂ (25 mL) potassium thioacetate solution.
(1.82 g, 15.93 mmol, 1.72 equiv). The solution was added dropwise to the chloromethyl ether derivative in dry CH₂Cl₂ (50 mL). After stirring at room temperature for 2 h, the mixture was evaporated and diluted in ethyl acetate. The solution was filtered and washed with water. The aqueous layer was then extracted with ethyl acetate. The organic layer was washed with brine and dried over Na₂SO₄. The solvent was concentrated under reduced pressure. The crude material was purified by silica gel column chromatography with CH₂Cl₂/Methanol (99/1). The desired compound 3c was obtained as white foam (4.52 g, 6.04 mmol, 65%). ¹H-NMR (400 MHz, CDCl₃) δ 11.84 (s, 1H, NH) ; 9.60 (s, 1H, NH) ; 8.01 (s, 1H, H₈) ; 7.36-6.97 (m, 5H, Ar) ; 5.88 (s, 1H, H'₁) ; 5.48, 5.39 (2d, AB, JₐB = 11.2 Hz, 1H+1H, OCH₂S) ; 4.72 (s, 2H, CH₂PAC) ; 4.44 (dd, J = 9.2 Hz, J = 4.4 Hz, 1H, H'₃) ; 4.33 (d, J = 4.4 Hz, 1H, H'₂) ; 4.19 (d, J = 13.2 Hz, 1H, H₅⁺) ; 4.11 (dt, J = 9.3 Hz, J = 2.0 Hz, 1H, H₄) ; 3.98 (dd, J = 13.2 Hz, J = 2.4 Hz, 1H, H₅⁻) ; 2.26 (s, 3H, CH₃) ; 1.09-0.99 (m, 28H, iPr). ¹³C-NMR (100 MHz, CDCl₃) δ 194.7 (SC=O) ; 169.8 (C=O) ; 156.5 (Cq PAC) ; 155.2 (C₆) ; 146.6 (C₄) ; 146.4 (C₃) ; 136.7 (C₂) ; 129.9, 122.9, 114.9 (Car) ; 122.2 (Cs) ; 87.8 (C₁') ; 81.7 (C₄') ; 80.9 (C₂') ; 70.2 (OCH₂S) ; 68.6 (C₃') ; 67.0 (CH₂ PAC) ; 59.6 (C₅) ; 30.8 (CH₃) ; 17.4-16.8 (CH₃Pr) ; 13.4-12.5 (CH₃iPr). HRMS (ESI⁺) m/z calcd for C₃₃H₄₉N₅O₉Si₂S (M+H)⁺ 748.2868, Found 748.2869.

2'-O-acetylthiomethyl-N4-acetyl cytidine 4a. To a solution of 3a (3.58 g, 5.82 mmol, 1.0 equiv) in anhydrous THF (83 mL) was added Et₃N-3HF solution (1.90 mL, 11.63 mmol, 2.0 equiv). After stirring for 2 h at room temperature, the reaction mixture was treated with triethylammoniumacetate buffer (2M, pH 7). The solvent was concentrated under reduced pressure. The crude material was coevaporated with water and ACN. The residue was purified by silica gel column chromatography with a step gradient of CH₂Cl₂ and methanol (0-5%). The desired compound 4a was obtained as white foam (1.61 g, 4.31 mmol, 74%). ¹H-NMR (400 MHz, DMSO-d₆) δ 10.88 (s, 1H, NH) ; 8.39 (d, J = 7.6 Hz, 1H, H₆) ; 7.19 (d, J = 7.6 Hz, 1H, H₅) ; 5.86 (d, J = 2.8 Hz, 1H, H₁') ; 5.25 (s, 2H, OCH₂S) ; 5.19 (m, 1H, OH) ; 4.09 (dd, J = 6.4 Hz, J = 5.2 Hz, 1H, H₃') ; 4.01 (dd, J = 5.2 Hz, J = 3.2 Hz, 1H, H₂') ; 3.88 (m, 1H, OH) ; 3.73 (dd, J = 12.4 Hz, J = 2.8 Hz, 1H, H₅') ; 3.57 (dd, J = 12.4 Hz, J = 3.2 Hz, 1H, H₄') ; 3.38 (d, J = 9.2 Hz, 1H, OH) ; 2.35 (s, 3H, SCOCH₃) ; 2.10 (s, 3H, CH₃ Ac). ¹³C-NMR (100 MHz, DMSO-d₆) δ 194.4 (SC=O) ; 171.0 (C=O) ; 162.4 (C=O) ; 154.6 (C₆); 145.2 (Cq); 95.4 (C₅); 87.9 (C₁') ; 84.4 (C₄'); 81.1 (C₂'); 69.1 (OCH₂S) ; 67.4 (C₃') ; 59.5 (C₅'); 30.8 (SCOCH₃) ; 24.3 (CH₃). HRMS (ESI⁺) m/z calcd for C₁₄H₁₉N₅O₅Si₂S (M+H)⁺ 748.2868, Found 748.2869.

2'-O-acetylthiomethyl-N6-phenoxyacetyl adenosine 4b. Using the same procedure as for synthesis of 4a, starting from 3b (5.59 g, 7.65 mmol), compound 4b was obtained as white
foam (3.31 g, 6.77 mmol, 88%). Purification conditions: silica gel column chromatography with a step gradient of CH₂Cl₂ and methanol (0-5%). ¹H-NMR (400 MHz, CDCl₃) δ 9.63 (s, 1H, NH) ; 8.78 (s, 1H, H₂) ; 8.10 (s, 1H, H₅) ; 7.36-7.03 (m, 5H, HAr) ; 5.95 (d, J = 7.6 Hz, 1H, H₁) ; 4.98, 4.83 (2d, JAB = 11.2 Hz, 2H, OCH₂S) ; 4.88 (s, 2H, CH₂ PAC) ; 4.85 (dd, J = 4.8 Hz, J = 2.8 Hz, 1H, H₂) ; 4.64 (d, J = 4.8 Hz, 1H, H₃) ; 4.37 (s, 1H, H₄) ; 3.96 (dd, J = 12.8 Hz, J = 1.6 Hz, 1H, H₅); 3.77 (dd, J = 13.6 Hz, J = 1.2 Hz, 1H, H₅'); 2.22 (s, 3H, CH₃). ³¹C-NMR (100 MHz, CDCl₃) δ 194.4 (SC=O) ; 166.8 (C=O) ; 156.9 (Cq PAC) ; 152.1 (C₂) ; 150.6 (C₄) ; 149.1 (C₆) ; 143.6 (Cₘ) ; 129.8, 122.5, 114.9 (Car) ; 124.0 (C₅) ; 89.1 (C₁') ; 88.0 (C₄') ; 80.7 (C₂') ; 70.8 (C₃') ; 69.4 (OCH₂S) ; 68.1 (CH₂ PAC) ; 63.0 (C₅') ; 30.7 (CH₃). HRMS (ESI⁺) m/z calcd for C₃₂H₃₃N₅O₇S (M+H)⁺ 490.1396, Found 490.1396.

2'-O-acetylthiomyethyl-N2-phenoxyacetyl guanosine 4c. Using the same procedure as for synthesis of 4a, starting from 3c (4.52 g, 6.04 mmol), compound 4c was obtained as white foam (2.12 g, 4.20 mmol, 70 %). Purification conditions: silica gel column chromatography with a step gradient of CH₂Cl₂ and methanol (0-5%). ¹H-NMR (400 MHz, DMSO-d₆) δ 11.82 (s, 1H, NH) ; 9.25 (s, 1H, NH) ; 7.88 (s, 1H, H₅) ; 7.43-6.80 (m, 19H, Ar) ; 6.99 (d, J = 4.8 Hz, 1H, H₁') ; 5.21, 5.16 (2d, JAB = 11.2 Hz, 1H+1H, OCH₂S) ; 4.64-4.62 (m, 2H+1H, CH₂ PAC + H₂') ; 4.47 (q, J = 4.8 Hz, 1H, H₃') ; 4.22 (q, J = 4.0 Hz, 1H, H₄') ; 3.77 (s, 6H, OCH₃) ; 3.44 (dd, J = 10.4 Hz, J = 2.8 Hz, 1H, H₅') ; 3.37 (dd, J = 10.8 Hz, J = 4.0 Hz, 1H, H₅''); 2.27 (s, 3H, CH₃). ³¹C-NMR (100 MHz, DMSO-d₆) δ 194.4 (SC=O) ; 169.6 (C=O) ; 158.6, 144.4, 137.4, 135.5, 135.4 (Cq DMTr) ; 156.4 (Cq PAC) ; 155.3 (C₆) ; 147.8 (C₄) ; 146.3 (C₈) ; 137.8 (C₂) ; 130.1, 130.0, 129.9, 129.0, 128.2, 128.1, 127.9, 127.0, 125.3, 122.9, 114.8, 113.2 (Car) ; 122.1 (C₅) ; 86.7 (C₁') ; 85.8 (C₄') ; 84.2 (OCq, DMTr) ; 81.2 (C₂') ; 70.0 (OCH₂S) ; 69.8 (C₃') ; 66.9 (CH₂ PAC) ; 63.3 (C₅') ; 55.2 (OCH₃, DMTr) ; 30.9 (CH₃). HRMS (ESI⁺) m/z calcd for C₃₂H₃₃N₅O₇S (M+H)⁺ 808.2652, Found 808.2652.

2'-O-acetylthiomyethyl-5'-O-(4,4'-dimethoxytrityl)-N4-acetyl cytidine 5a A solution of 4a (1.61 g, 4.31 mmol, 1.0 equiv) in anhydrous CH₂Cl₂ (22 mL) was treated under argon with DIEA (1.21 mL, 6.89 mmol, 1.6 equiv) and dimethoxytrityl chloride (2.48 g, 7.32 mmol, 1.7 equiv) was added in small portions over 15 min. The mixture was stirred for 1.5h at room temperature. A saturated aqueous NaHCO₃ solution was added. The aqueous layer was then extracted with CH₂Cl₂. The organic layer was washed with water then brine and dried over Na₂SO₄. The solvent was concentrated under reduced pressure. The crude material was purified by silica gel column chromatography with a step gradient of CH₂Cl₂ and methanol (0-2%) containing 1% pyridine. The desired compound 5a was obtained as yellow foam (2.51 g, 3.72
mmol, 86%). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ 9.62 (s, 1H, NH); 8.46 (d, $J = 7.2$ Hz, 1H, H$_6$); 7.42-7.16 (m, 9H, Har DMTr); 7.13 (d, $J = 7.2$ Hz, 1H, H$_5$); 6.88-6.84 (m, 4H, Har DMTr); 5.96 (s, 1H, H$_7^\prime$); 5.50, 5.37 (2d$_{AB}$, $J_{AB} = 11.2$ Hz, 1H+1H, OCH$_2$S); 4.44 (m, 1H, H$_3^\prime$); 4.15 (d, $J = 5.2$ Hz, 1H, H$_2^\prime$); 4.04 (dt, $J = 9.2$ Hz, $J = 2.4$ Hz, 1H, H$_4^\prime$); 3.81 (d, $J = 1.6$ Hz, 6H, 2*OCH$_3$); 3.54 (qd, $J = 11.2$ Hz, $J = 2.0$ Hz, 2H, $H_5^\prime$+$H_5^{\prime\prime}$); 2.38 (s, 3H, SCOCH$_3$); 2.26 (s, 3H, CH$_3$ Ac). $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$ 194.9 (SC=O); 170.4 (C=O); 162.9 (C=O); 158.7, 155.0, 144.3, 135.5, 135.2 (Cq, Car); 144.6 (C$_6$); 130.1, 129.0, 128.2, 128.1, 128.0, 127.1, 125.3, 113.3 (CH, Car); 96.7 (C$_5$); 88.9 (C$_{11}$); 87.0 (OCq, DMTr); 83.1 (C$_4$); 81.3 (C$_2$); 69.7 (OCH$_2$S); 67.6 (C$_3^\prime$); 60.7 (C$_5^\prime$); 55.2 (OCH$_3$, DMTr); 31.0 (SCOCH$_3$); 24.9 (CH$_3$ Ac). HRMS (ESI$^+$) m/z calcd for C$_{33}$H$_{37}$N$_3$O$_9$S (M+H)$^+$ 676.2329, Found 676.2335.

2'-O-acetylthiomethyl-5'-O-(4,4'-dimethoxytrityl)-N6-phenoxyacetyl adenosine 5b. Using the same procedure as for synthesis of 5a, starting from 4b (3.10 g, 6.33 mmol), compound 5b was obtained as white foam (3.67 g, 4.63 mmol, 73%). Purification conditions: silica gel column chromatography with a step gradient of CH$_2$Cl$_2$ and methanol (0-2%) containing 1% pyridine. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ 9.48 (s, 1H, NH); 8.72 (s, 1H, H$_2$); 8.24 (s, 1H, H$_8$); 7.44-6.81 (m, 18H, HAr); 6.19 (d, $J = 4.8$ Hz, 1H, H$_1^\prime$); 5.18 (s, 2H, OCH$_2$S); 4.87 (s, 2H, CH$_2$ PAC); 4.85 (t, $J = 4.8$ Hz, 1H, H$_2^\prime$); 4.59 (q, $J = 5.2$ Hz, 1H, H$_3^\prime$); 4.25 (q, $J = 4.0$ Hz, 1H, H$_4^\prime$); 3.78, 3.77 (s+s, 3H+3H, 3*OCH$_3$); 3.52 (dd, $J = 10.4$ Hz, $J = 3.2$ Hz, 1H, H$_5^\prime$); 3.40 (dd, $J = 10.8$ Hz, $J = 4.0$ Hz, 1H, H$_5^{\prime\prime}$); 2.28 (s, 3H, CH$_3$). $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$ 194.4 (SC=O); 166.6 (C=O); 158.5, 144.5, 137.8, 135.6, 135.5 (Cq DMTr); 157.0 (Cq PAC); 152.5 (C$_2$); 151.5 (C$_4$); 148.3 (C$_6$); 142.2 (C$_8$); 130.0, 129.8, 129.0, 128.2, 128.1, 127.9, 126.9, 125.2, 122.4, 114.9, 113.2 (Car); 123.1 (C$_5$); 86.9 (C$_{11}$); 86.6 (OCq, DMTr); 84.2 (C$_4$); 80.7 (C$_2$); 70.0 (OCH$_2$S); 69.7 (C$_3^\prime$); 68.1 (CH$_2$ PAC); 62.9 (C$_5^\prime$); 55.2 (OCH$_3$, DMTr); 30.8 (CH$_3$). HRMS (ESI$^+$) m/z calcd for C$_{42}$H$_{41}$N$_3$O$_9$S (M+H)$^+$ 792.2703, Found 792.2711.

2'-O-acetylthiomethyl-5'-O-(4,4'-dimethoxytrityl)-N2-phenoxyacetyl guanosine 5c. Using the same procedure as for synthesis of 5a, starting from 4c (0.85 g, 1.69 mmol), compound 5c was obtained as yellow foam (1.21 g, 1.50 mmol, 87%). Purification conditions: silica gel column chromatography with a step gradient of CH$_2$Cl$_2$ and methanol (0-1%) containing 1% pyridine. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ 11.82 (s, 1H, NH); 9.25 (s, 1H, NH); 7.88 (s, 1H, H$_8$); 7.43-6.80 (m, 19H, Ar); 6.99 (d, $J = 4.8$ Hz, 1H, H$_1^\prime$); 5.21, 5.16 (2d$_{AB}$, $J_{AB} = 11.2$ Hz, 1H+1H, OCH$_2$S); 4.64-4.62 (m, 2H+1H, CH$_2$ PAC + H$_2^\prime$); 4.47 (q, $J = 4.8$ Hz, 1H, H$_3^\prime$); 4.22 (q, $J = 4.0$ Hz, 1H, H$_4^\prime$); 3.77 (s, 6H, OCH$_3$); 3.44 (dd, $J = 10.4$ Hz, $J = 2.8$ Hz, 1H, H$_5^\prime$); 3.37 (dd, $J = 10.8$ Hz, $J = 4.0$ Hz, 1H, H$_5^{\prime\prime}$); 2.27 (s, 3H, CH$_3$). $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$ 194.4
2′-O-acetyltiethyl-3′-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-5′-O-(4,4′-dimethoxytrityl)-N6-acetyl cytidine 1a. To a solution of 5a (1.83 g, 2.71 mmol, 1.0 equiv) in anhydrous CH2Cl2 (20 mL) previously passed through an alumina column was added dropwise a mixture of N,N-diisopropylethylamine (0.94 mL, 5.42 mmol, 2.0 equiv) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (10.03 mL, 4.61 mmol, 1.7 equiv) in CH2Cl2 (4 mL). The mixture was stirred for 2h at room temperature under argon. After reaction completion, ethyl acetate previously washed with a saturated aqueous NaHCO3 solution was added and the reaction mixture was poured into saturated NaCl/NaHCO3 solution (1/1 v/v). The aqueous layer was extracted with ethyl acetate and organic layers were dried over Na2SO4. The solvent was concentrated under reduced pressure. The crude material was purified by silica gel column chromatography with an isocratic elution of Ethyl acetate/Cyclohexane (9/1) containing 1% pyridine. The desired phosphoramidite 1a was obtained as white foam (1.62 g, 1.85 mmol, 68%). 31P-NMR (121 MHz, CD3CN): δ 150.1, 148.7; HRMS (ESI+) m/z calcd for C42H41N5O10PS (M+H)+ 808.2652, Found 808.2652.

2′-O-acetyltiethyl-3′-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-5′-O-(4,4′-dimethoxytrityl)-N6-phenoxyacetyl adenosine 1b. Using the same procedure as for synthesis of 1a, starting from 5b (3.08 g, 3.88 mmol), compound 1b was obtained as white foam (3.20 g, 3.23 mmol, 83 %). Purification conditions: silica gel column chromatography with an isocratic elution of CH2Cl2/ethyl acetate (70/30) containing 1% pyridine. 31P-NMR (121 MHz, CD3CN): δ 149.9, 149.8; HRMS (ESI+) m/z calcd for C51H58N7O10PS (M+H)+ 992.3791, Found 992.3782.

2′-O-acetyltiethyl-3′-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-5′-O-(4,4′-dimethoxytrityl)-N2-phenoxyacetyl guanosine Using the same procedure as for synthesis of 1a, starting from 5c (0.86 g, 1.07 mmol), compound 1c was obtained as white foam (0.90 g, 0.89 mmol, 84 %). Purification conditions: silica gel column chromatography with an isocratic elution of CH2Cl2/ethyl acetate (50/50). 31P-NMR (121 MHz, CD3CN): δ 150.1, 149.8; HRMS (ESI+) m/z calcd for C51H59N7O10PS (M+H)+ 1008.3730, Found 1008.3731.
\(^1\)H, \(^{13}\)C, and \(^{31}\)P NMR spectra of nucleosides

Figure S1: 400 MHz \(^1\)H-NMR and 100 MHz \(^{13}\)C-NMR spectra (CDCl\(_3\)) of 3a
**Figure S2:** 400 MHz $^1$H-NMR and 100 MHz $^{13}$C-NMR spectra (CDCl$_3$) of 3b
Figure S3: 400 MHz $^1$H-NMR and 100 MHz $^{13}$C-NMR spectra (CDCl$_3$) of 3c
Figure S4: 400 MHz $^1$H-NMR and 100 MHz $^{13}$C-NMR spectra (DMSO-d$_6$) of 4a
Figure S5: 400 MHz $^1$H-NMR and 100 MHz $^{13}$C-NMR spectra (CDCl$_3$) of 4b
Figure S6: 400 MHz $^1$H-NMR and 100 MHz $^{13}$C-NMR spectra (DMSO-$d_6$) of 4c
Figure S7: 400 MHz $^1$H-NMR and 100 MHz $^{13}$C-NMR spectra (CDCl$_3$) of 5a
Figure S8: 400 MHz $^1$H-NMR and 100 MHz $^{13}$C-NMR spectra (CDCl$_3$) of 5b
Figure S9: 400 MHz $^1$H-NMR and 100 MHz $^{13}$C-NMR spectra (CDCl$_3$) of 5c
Figure S10: 121 MHz $^{31}$P-NMR spectrum (CD$_3$CN) of 1a
Figure S11: 121 MHz $^{31}$P-NMR spectrum (CD$_3$CN) of 1b
**Figure S12:** 121 MHz $^{31}$P-NMR spectrum (CD$_3$CN) of 1c
HPLC chromatograms & MALDI-TOF spectra of purified oligonucleotides

**Figure S13:** IEX-HPLC and MALDI-TOF MS analysis of purified RNA S1

IEX-HPLC analysis conditions: DNAPac® PA100, 4X250 mm, elution with a 20 min linear gradient of 20 to 100% of B in eluent A. Column temperature 75°C. Flow rate 1.5 mL min⁻¹. λ 260 nm.

**Eluant A:** 25 mM Tris HCl, 20% ACN, pH 8

**Eluant B:** 25 mM Tris HCl, 200 mM NaClO₄, 20% ACN, pH 8
Figure S14: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H4-1

IEX-HPLC analysis conditions: DNAPac® PA100, 4X250 mm, elution with a 20 min linear gradient of 0 to 40% of B in eluent A. Column temperature 75°C. Flow rate 1.0 mL.min⁻¹. A 260 nm.

Eluant A : 25 mM Tris HCl, 5% ACN, pH 8
Eluant B : 25 mM Tris HCl, 400 mM NaClO₄, 5% ACN, pH 8
Figure S15: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H4-2

**IEX-HPLC analysis conditions:** DNAPac® PA100, 4X250 mm, elution with a 20 min linear gradient of 0 to 100% of B in eluent A. Column temperature 75°C. Flow rate 1.0 mL min⁻¹. \( \lambda \) 260 nm.

*Eluant A*: 25 mM Tris HCl, 20% ACN, pH 8

*Eluant B*: 25 mM Tris HCl, 200 mM NaClO₄, 20% ACN, pH 8
Figure S16: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H4-3

IEX-HPLC analysis conditions: DNAPac® PA100, 4X250 mm, elution with a 20 min linear gradient of 0 to 60% of B in eluent A. Column temperature 75°C. Flow rate 1.0 mL.min⁻¹. A 260 nm.

Eluant A : 25 mM Tris HCl, 5% ACN, pH 8

Eluant B : 25 mM Tris HCl, 400 mM NaClO₄, 5% ACN, pH 8
**Figure S17:** IEX-HPLC and MALDI-TOF MS analysis of purified RNA H5-1

**IEX-HPLC analysis conditions:** DNAPac® PA100, 4x250 mm, elution with a 20 min linear gradient of 0 to 45% of B in eluent A. Column temperature 75°C. Flow rate 1.0 mL.min⁻¹. λ 260 nm.

*Eluant A:* 25 mM Tris HCl, 5% ACN, pH 8

*Eluant B:* 25 mM Tris HCl, 400 mM NaClO₄, 5% ACN, pH 8
**Figure S18: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H5-2**

IEX-HPLC analysis conditions: DNAPac® PA100, 4x250 mm, elution with a 20 min linear gradient of 0 to 100% of B in eluent A. Column temperature 75°C. Flow rate 1.0 mL.min⁻¹. λ 260 nm.

*Eluant A*: 25 mM Tris HCl, 20% ACN, pH 8

*Eluant B*: 25 mM Tris HCl, 200 mM NaClO₄, 20% ACN, pH 8
Figure S19: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H5-3

IEX-HPLC analysis conditions: DNAPac® PA100, 4X250 mm, elution with a 20 min linear gradient of 0 to 60% of B in eluent A. Column temperature 75°C. Flow rate 1.0 mL.min⁻¹. A 260 nm.

Eluant A : 25 mM Tris HCl, 5% ACN, pH 8

Eluant B : 25 mM Tris HCl, 400 mM NaClO₄, 5% ACN, pH 8
**Figure S20**: IEX-HPLC and MALDI-TOF MS analysis of purified RNA H5-4

IEX-HPLC analysis conditions: DNAPac® PA100, 4X250 mm, elution with a 20 min linear gradient of 0 to 60% of B in eluent A. Column temperature 75°C. Flow rate 1.0 mL.min⁻¹. λ 260 nm.

**Eluant A**: 25 mM Tris HCl, 5% ACN, pH 8

**Eluant B**: 25 mM Tris HCl, 400 mM NaClO₄, 5% ACN, pH 8
**Figure S21:** IEX-HPLC and MALDI-TOF MS analysis of purified RNA DIS-1

**IEX-HPLC analysis conditions:** DNAPac® PA100, 4X250 mm, elution with a 20 min linear gradient of 0 to 60% of B in eluent A. Column temperature 75°C. Flow rate 1.0 mL.min⁻¹. A 260 nm.

**Eluant A :** 25 mM Tris HCl, 5% ACN, pH 8

**Eluant B :** 25 mM Tris HCl, 400 mM NaClO₄, 5% ACN, pH 8
Figure S22: IEX-HPLC and MALDI-TOF MS analysis of purified RNA DIS-2

IEX-HPLC analysis conditions: DNAPac® PA100, 4X250 mm, elution with a 20 min linear gradient of 0 to 60% of B in eluent A. Column temperature 75°C. Flow rate 1.0 mL.min⁻¹. A 260 nm.

Eluant A : 25 mM Tris HCl, 5% ACN, pH 8

Eluant B : 25 mM Tris HCl, 400 mM NaClO₄, 5% ACN, pH 8
**Figure S23**: IEX-HPLC and MALDI-TOF MS analysis of purified RNA DIS-2F

IEX-HPLC analysis conditions: DNAPac® PA100, 4X250 mm, elution with a 20 min linear gradient of 0 to 80% of B in eluent A. Column temperature 75°C. Flow rate 1.0 mL.min⁻¹. A 260 nm.

Eluant A : 25 mM Tris HCl, 5% ACN, pH 8

Eluant B : 25 mM Tris HCl, 400 mM NaClO₄, 5% ACN, pH 8
**UV Melting curves**

**Figure S24:** Melting curves of D1, D2, D3 and D4 duplexes  
Conditions: Buffer: 10 mM Na cacodylate, 100 mM NaCl, pH 7  
1.5µM in 1mL of buffer in a 1cm path length quartz cell,  
Heating-cooling-heating cycle in the 0-90°C temperature range with a 0.5°C.min\(^{-1}\) gradient  
Tm values from two independent experiments were accurate within ± 0.5°C.

**Figure S25:** Melting curves of H4 hairpins  
Conditions: Buffer: 10 mM Na cacodylate, 100 mM NaCl, pH 7  
1.5µM in 1mL of buffer in a 1cm path length quartz cell,  
Heating-cooling-heating cycle in the 0-90°C temperature range with a 0.5°C.min\(^{-1}\) gradient  
Tm values from two independent experiments were accurate within ± 0.5°C.
Figure S26: Melting curves and first derivative of H5 hairpins

Conditions: Buffer: 10 mM Na cacodylate, 100 mM NaCl, pH 7
1.5µM in 1mL of buffer in a 1cm path length quartz cell,
Heating-cooling-heating cycle in the 0-90°C temperature range with a 0.5°C.min⁻¹ gradient
Tm values from two independent experiments were accurate within ± 0.5°C.
Figure S27: Melting curves (1) and first derivatives (2a, 2b, 2c) of DIS ONs

Conditions: Buffer: 20 mM sodium cacodylate, 25 mM KCl, 5 mM MgCl₂, pH 7

1.5µM in 1mL of buffer in a 1cm path length quartz cell.

Heating-cooling-heating cycle in the 0-90°C temperature range with a 0.5°C.min⁻¹ gradient.

Tm values from two independent experiments were accurate within ± 0.5°C.
Circular Dichroism spectra

Figure S28: Comparative CD spectra of H4, H4-1, H4-2 and H4-3

Solutions used for Tm experiments conserved at -80°C before CD analyses.
Recorded on a Jasco J-815 spectropolarimeter. Measurements performed at 1°C, wavelength range 340-200 nm, scanning speed of 100 nm.min\(^{-1}\).
Raw data were acquired over 2 scans.

Figure S29: Comparative CD spectra of H5, H5-1, H5-2, H5-3 and H5-4 hairpins.

Solutions used for Tm experiments conserved at -80°C before CD analyses.
Recorded on a Jasco J-815 spectropolarimeter. Measurements performed at 1°C, wavelength range 340-200 nm, scanning speed of 100 nm.min\(^{-1}\).
Raw data were acquired over 2 scans.
**Figure S30:** Comparative CD spectra of **DIS, DIS-1** and **DIS-2** ONs.
Solutions used for Tm experiments conserved at -80°C before CD analyses.
Recorded on a Jasco J-815 spectropolarimeter. Measurements performed at 1°C, wavelength range 340-200 nm, scanning speed of 100 nm.min⁻¹.
Raw data were acquired over 2 scans.
Analyses of H4 & H5 hairpins by gel electrophoresis

Figure S31: Gel electrophoresis (15% polyacrylamide) of H4 and H5 5µM in Na-cacodylate 10 mM, NaCl 100 mM, pH 7.
Migration in TBE buffer at 4°C. Staining with GelRed 3X (VWR) in a 0.1M NaCl solution for 1h, revelation with a UV transilluminator.
(1: H4, 2: H4-1, 3: H4-2, 4: H4-3, 5: H5, 6: H5-1, 7: H5-2, 8: H5-3, 9: H5-4)

Figure S32: Comparative gel electrophoresis (15% polyacrylamide) of H4 and H5 5µM with a 14-mer RNA duplex in Na-cacodylate 10 mM, NaCl 100 mM, pH 7.
Migration in TBE buffer at 4°C. Staining with GelRed 3X (VWR) in a 0.1M NaCl solution for 1h, revelation with a UV transilluminator.
Enzymatic stability of RNA hairpins

**Figure S33:** Gel electrophoresis (15% polyacrylamide) of RNAs (48 µM) incubated with SVPDE (2 mU) in a 450 mM ammonium citrate buffer at 37°C. Migration in TBE buffer 1X at 4°C. Staining with GelRed 3X (VWR) in a 0.1 M NaCl solution for 1h, revelation with a UV transilluminator. 1: 1 min, 2: 5 min, 3: 15 min, 4: 30 min, 5: 1h, 6: 3h, 7: 6h, 8: 24h, 9: reference without enzyme 24h.
Figure S34: Enzymatic stability of H5 against SVPDE evaluated by gel electrophoresis. Band analysis was performed using ImageJ Software (Broken Symmetry Software V:1.4.3.67). Percentages of intact RNA were calculated on Microsoft Office Excel by comparison with the reference incubated without enzyme (lane 9 of the gels).
Fluorescence experiments

**Figure S35:** Fluorescence emission spectra of DIS-2F, 2 µM in 100 nM to 1 mM glutathione, 20 mM sodium cacodylate, 25 mM KCl, 5 mM MgCl$_2$, pH 7 with a 20 µM concentration of DIS (ratio 10:1). Fluorescence emission spectra at 25°C from 400 to 600 nm, excitation wavelength of 494 nm in a 100 µL quartz cell with a 1cm light path. Raw data were acquired in triplicates over 2 scans.
**Figure S36**: Glutathione concentration dependence of relative fluorescence intensity \(((I-\text{lo})/\text{lo})\) of DIS-2F, 2 µM at the fluorescence emission maximum \((\lambda=515\text{nm})\) (A: complete curve with GSH concentration ranging from 0.1 µM to 1000 µM, Log-10 scale was used for the concentration axis, B: linear domain from 1 µM to 100 µM)

Error bars are the standard deviation of the three repetitive independent experiments.

Buffer: 20 mM sodium cacodylate, 25 mM KCl, 5 mM MgCl₂, pH 7 with a 20 µM concentration of DIS (ratio 10:1).