Hydrogen-bonding controlled rigidity of an isoindoline-derived nitroxide spin label for nucleic acids

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Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>General materials and methods</td>
<td>S2</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>S2</td>
</tr>
<tr>
<td>Synthesis and purification of oligonucleotides</td>
<td>S3</td>
</tr>
<tr>
<td>EPR measurements</td>
<td>S4</td>
</tr>
<tr>
<td>MALDI-Tof MS analysis of oligonucleotides</td>
<td>S4</td>
</tr>
<tr>
<td>CD measurements</td>
<td>S5</td>
</tr>
<tr>
<td>Tm measurements</td>
<td>S6</td>
</tr>
<tr>
<td>Comparison of the mobility of $^{1m}U$, $\zeta$ and $^{0x}U$ in DNA</td>
<td>S7</td>
</tr>
<tr>
<td>Base-pairing of $^{1m}U$ detected by EPR</td>
<td>S8</td>
</tr>
<tr>
<td>Synthesis of nucleoside $^{1m}U$ and the corresponding phosphoramidite 10A</td>
<td>S9</td>
</tr>
<tr>
<td>Compound 3</td>
<td>S9</td>
</tr>
<tr>
<td>Compound 4</td>
<td>S11</td>
</tr>
<tr>
<td>Compound 6</td>
<td>S13</td>
</tr>
<tr>
<td>Compound 9A</td>
<td>S15</td>
</tr>
<tr>
<td>Spin-labelled nucleoside $^{1m}U$</td>
<td>S17</td>
</tr>
<tr>
<td>Compound DMT- $^{1m}U$</td>
<td>S19</td>
</tr>
<tr>
<td>Phosphoramidite 10A</td>
<td>S21</td>
</tr>
<tr>
<td>Synthesis of $^{0x}U$ nucleoside and corresponding phosphoramidite</td>
<td>S23</td>
</tr>
<tr>
<td>Compound 8</td>
<td>S23</td>
</tr>
<tr>
<td>Compound 9B</td>
<td>S25</td>
</tr>
<tr>
<td>Spin-labelled nucleoside $^{0x}U$</td>
<td>S27</td>
</tr>
<tr>
<td>Compound DMT- $^{0x}U$</td>
<td>S29</td>
</tr>
<tr>
<td>Phosphoramidite 10B</td>
<td>S31</td>
</tr>
<tr>
<td>References</td>
<td>S33</td>
</tr>
</tbody>
</table>
General materials and methods

Thymidine was purchased from Rasayan Inc. USA. Other chemicals were purchased from Sigma Aldrich and used without further purification. Water was purified on MILLI-Q water purification system. Thin layer chromatography (TLC) was carried out using glass plates pre-coated with silica gel (0.25 mm, F-254) from Silicycle. Compounds were visualized by UV light and staining with p-anisaldehyde. Flash column chromatography was performed using ultra pure flash silica gel (Silicycle, 230-400 mesh size, 60 Å). Dichloromethane and pyridine were freshly distilled over calcium hydride prior to use. Anhydrous triethylamine, n-hexane and ethyl acetate were used directly as received. All moisture and air sensitive reactions were carried out in oven dried glassware under an inert argon atmosphere. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 MHz spectrometer. $^1$H NMR chemical shifts are reported in parts per million (ppm) relative to the residual proton signal of solvents CDCl$_3$ (7.26 ppm), $d_6$-DMSO (2.50 ppm), $d_4$-MeOH (3.31 and 4.84 ppm) for $^1$H NMR and CDCl$_3$ (77.0 ppm), $d_6$-DMSO (39.43 ppm), $d_4$-MeOH (49.05) for $^{13}$C NMR. $^{31}$P NMR chemical shifts are reported relative to 85% H$_3$PO$_4$ as an external standard. Commercial grade CDCl$_3$ was passed over basic alumina shortly before use with tritylated compounds. NMR spectra of compounds containing a nitroxide radical show significant broadening, sometimes to the extent that some nuclei are not seen in the spectra.$^{1,2}$ Therefore, integration of $^1$H NMR spectra of nitroxides is not reported. Mass spectrometric analyses of all organic compounds were performed on an HR-ESI-MS (Bruker, MicroTof-Q) in positive ion mode. UV-VIS spectra were recorded on a PerkinElmer Lambda 25 UV/VIS spectrometer. CD spectra were recorded on a JASCO J-810 spectropolarimeter at 25 °C with path length of 1 mm (Hellma), 10 scans, scanned from 500 nm to 200 nm with response of 1s, data pitch of 0.1 nm and band width of 1.0 nm. Molecular weight (MW) of oligonucleotides was determined by MALDI-TOF analysis (Bruker, Autoflex III).

List of abbreviations

- mCPBA: *meta*Chloroperbenzoic acid
- DMTtCl: 4,4'-Dimethoxytrityl chloride
- EPR: Electron paramagnetic resonance
Synthesis and purification of oligonucleotides

All commercial phosphoramidites, columns and solutions were purchased from ChemGenes Corporation. Unmodified and spin-labelled oligonucleotides were synthesized by a trityl-off synthesis on a 1.0 μmol (1000 Å CPG columns) scale using phosphoramidites with standard base protection on an automated DNA synthesizer ASM800 (Biosset, Russia). The spin-labelled DNA was prepared by using previously reported protocols; the spin-labelled phosphoramidite was incorporated into the oligonucleotides by manual coupling by pausing the synthesizer program after completion of the prior cycle, removing the column from the synthesizer and running 200 μL of standard activator solution and 200 μL of a 0.05 M solution of spin-labelled phosphoramidite in 1,2 dichloroethane back and forth through the column for 10 min. Then the column was re-mounted on the synthesizer to complete the cycle. The oligos were deprotected from solid support using 33% aq. NH$_3$ at 55 °C for 8 h and purified by 20% denaturing polyacrylamide gel electrophoresis (DPAGE). The DNA bands were visualized by UV and excised from the gel, crushed and soaked in 10 mL buffer (250 mM NaCl, 10 mM Tris, 1 mM Na$_2$EDTA, pH 7.5) for 12 h. A polyethersulfone membrane (0.25 μm, disposable filter device from Whatman) was used for filtration of the DNA elution solutions, which were desalted using Sep-Pak cartridges (Waters Corporation) according to manufacturer’s instructions. Concentrations of oligonucleotides were calculated by Beer’s law based on measurements of UV absorbance at 260 nm using extinction coefficients (ε) which were determined by using UV WinLab oligonucleotide calculator (V2.85.04, PerkinElmer).

Figure S1 shows an analytical denaturing polyacrylamide gel of spin-labelled and unmodified (T instead of XU) oligonucleotides. Mobility of spin-labelled oligomers is slower, as seen from the lane 2 and 4 where spin-labelled and unmodified oligomers were co-spotted.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAC CTC G$^{\text{Im}}$UA TCG TG</td>
</tr>
<tr>
<td>2</td>
<td>GAC CTC GTA TCG TG + GAC CTC G$^{\text{Im}}$UA TCG TG</td>
</tr>
<tr>
<td>3</td>
<td>GAC CTC GTA TCG TG</td>
</tr>
<tr>
<td>4</td>
<td>GAC CTC GTA TCG TG + GAC CTC G$^{\text{Ox}}$UA TCG TG</td>
</tr>
<tr>
<td>5</td>
<td>GAC CTC G$^{\text{Ox}}$UA TCG TG</td>
</tr>
</tbody>
</table>

Fig. S1 Analytical gel of spin-labelled and unmodified oligonucleotides.
EPR measurements

2.0 nmol of spin-labelled, single-stranded DNA and 2.4 nmol of its complementary strand was dissolved in phosphate buffer (10 mM phosphate, 100 mM NaCl, 0.1 mM Na₂EDTA, pH 7.0) (10 μL, oligo final conc. 200 μM) and annealed using the following annealing protocol: 90 °C for 2 min, 60 °C for 5 min, 50 °C for 5 min, 40 °C for 5 min, 22 °C for 15 min. Samples (10 μL) were placed in a quartz capillary for EPR measurements. Continuous wave (CW) EPR spectra were recorded on a MiniScope MS200 spectrometer using 100 kHz modulation frequency, 1.0 G modulation amplitude and 2.0 mW microwave power. Each spectrum was scanned 100-120 times. The temperature was regulated by Magnettech temperature controller M01 with error ± 0.5 °C.

MALDI-Tof MS analysis of oligonucleotides

The incorporation of ImU spin label was confirmed by MALDI-Tof MS analysis. The instrument was calibrated with external standard prior to measuring the mass of spin-labelled oligonucleotide. The calculated monoisotopic mass of spin-labelled single strand GAC CTC GImUA TCG TG is 4467.84 and observed mass is 4468.07.

Fig. S2 MS spectrum of spin-labeled DNA 14-mer 5′-d(GAC CTC GImUA TCG TG).
CD measurements

To determine if the spin labels had any effect on the DNA duplex conformation, circular dichroism (CD) spectra of 14-mer unmodified and ImU and OxU spin-labelled duplex were recorded. The DNA samples (2.5 nmol of duplex) were dissolved in phosphate buffer (10 mM phosphate, 100 mM NaCl, 0.1 mM Na₂EDTA, pH 7.0), annealed and diluted to 200 µL with the same buffer. Both spectra possessed negative and positive molar ellipticities at ca. 250 and 280 nm respectively, which is characteristic of a right-handed B-DNA indicating that the spin-labelled nucleosides ImU (Figure 3A) and OxU (Figure 3B) do not alter the conformation of duplex DNA.

Fig. S3A CD spectra of an unmodified 14-mer DNA duplex (black) and spin-labelled ImU-containing DNA duplex (red).

Fig. S3B CD spectra of an unmodified 14-mer DNA duplex (black) and spin-labelled OxU-containing DNA duplex (blue).
Tm measurements

To determine if $^{1m}U$ and $^{Ox}U$ affect the stability of DNA duplexes, the thermal denaturation curves of unmodified and spin-labelled oligomers were determined. DNA samples (4.0 nmol of each strand) were dissolved in phosphate buffer (100 µL) (10 mM phosphate, 100 mM NaCl, 0.1 mM Na$_2$EDTA, pH 7.0), annealed and diluted to 1.0 mL with same phosphate buffer and degassed using argon. The samples were heated up from 20 °C to 90 °C (1.0 °C/min) and absorbance at 260 nm recorded. Table S1 shows melting temperatures ($T_m$s) of spin-labelled DNAs 2 and 3 and the unmodified 14 mer.

<table>
<thead>
<tr>
<th>Duplex</th>
<th>Sequence</th>
<th>$T_m$(°C)</th>
<th>$\Delta T_m$(°C)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-GAC CTC GTA TCG TG 3'-CTG GAG CAT AGC AC</td>
<td>59.5 ± 1.0</td>
<td></td>
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<tr>
<td>2</td>
<td>5'-GAC CTC $^{1m}UA$ TCG TG 3'-CTG GAG C AT AGC AC</td>
<td>55.5 ± 0.5</td>
<td>-4.0</td>
</tr>
<tr>
<td>3</td>
<td>5'-GAC CTC $^{Ox}UA$ TCG TG 3'-CTG GAG C AT AGC AC</td>
<td>53.5 ± 0.5</td>
<td>-6.0</td>
</tr>
</tbody>
</table>

$T_m$ is melting temperature of duplex and $\Delta T_m$ is difference between unmodified and modified oligos.
Comparision of the mobility of $^{15}$U, $\zeta$ and $^{18}$U in DNA

The mobility of the spin label $^{15}$U was compared with the rigid spin label $\zeta$ and spin label $^{18}$U in the same DNA sequence at various temperatures. The mobility of $^{15}$U-DNAs is identical with rigid spin label $\zeta$ at -10 °C (Figure S4). The mobility of $^{15}$U-modified DNA increased upon increasing the temperature and became nearly identical with that of $^{18}$U-modified DNA at 20 °C.

**Fig. S4** Comparison of EPR spectra of $^{15}$U (black), $\zeta$ (red) and $^{18}$U (blue) in duplex and single-stranded DNAs at various temperatures.
Base-pairing of $^{\text{Im}}$U detected by EPR

Comparing the EPR spectra of four duplexes, in which $^{\text{Im}}$U was paired with either A, T, G or C, yielded four distinguishable spectra (Figure S5A). The $^{\text{Im}}$U•A pair had the lowest mobility, while the $^{\text{Im}}$U•T pair had the highest mobility. Addition of Hg$^{2+}$ ions, known to form metallo base-pairs in T•T mismatches,$^4$ yielded an EPR spectrum that was nearly identical to the $^{\text{Im}}$U•A duplex (Figure S5B).

**Fig. S5** EPR spectra of $^{\text{Im}}$U-labelled DNA duplexs (14-mer) (A) with A (green), C (blue), G (black) and T (red). (B) $^{\text{Im}}$U paired with A (green), T (red) and after addition of Hg$^{2+}$ to the $^{\text{Im}}$U•T mismatch (magenta) at 25 °C.
1,1,3,3-Tetramethyl-6-nitroisoindolin-5-amine (3). To a solution of N,N,N-trimethylhydrazine iodide (1.83 g, 99.85 mmol) in DMSO (15 mL) was added t-BuOK (1.12 g, 99.85 mmol) and stirred reaction for 30 min. To this solution, compound 2 (1.0 g, 45.39 mmol) was added and the reaction stirred for 14 h at 22 °C. The reaction mixture was poured into 50 mL of ice-cold water, followed by extraction with CH₂Cl₂ (3 x 50 mL), the combined organic phases dried over anhydrous Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by flash silica gel column chromatography using a gradient elution (CH₂Cl₂:MeOH; 100:0 to 90:10) to give compound 3 as a yellow solid (0.750 g, 70% yield).

\[ ^{1}H\text{NMR} (d_{6}\text{-DMSO}): \delta 7.74 (s, 1H), 7.31 (s, 2H), 6.75 (s, 1H), 1.32 (d, J = 3.0 Hz, 12H). \]

\[ ^{13}C\text{NMR} (d_{6}\text{-DMSO}): \delta 158.45, 146.27, 138.14, 129.92, 117.60, 110.51, 61.63, 61.02, 31.84, 31.35. \]

HR-ESI-MS (M + H)⁺: calcd. for C₁₂H₁₈N₂O₂ 236.1394, found 236.1371.
$^1$H NMR spectrum of 3

$^{13}$C NMR spectrum of 3
1,1,3,3-Tetramethylisoindoline-5,6-diamine (4). To a solution of compound 3 (200.0 mg, 0.85 mmol) in MeOH (10 mL) was added 10% Pd/C (20.0 mg) and the mixture hydrogenated at 55 psi and 22 °C for 2 h. The reaction mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo to yield 1,1,3,3-tetramethylisoindoline-5,6-diamine (174.0 mg, 100%) which was used for the next reaction without further purification.

$^1$H NMR (CDCl$_3$): $\delta$ 6.46 (s, 2H), 1.40 (s, 12H).

$^{13}$C NMR (CDCl$_3$): $\delta$ 140.77, 134.21, 109.48, 109.42, 62.50, 32.05.

HR-ESI-MS (M + H)$^+$: calcd. for C$_{12}$H$_{18}$N$_3$O$_2$ 206.1652, found 206.1652.
$^1$H NMR spectrum of 4

$^{13}$C NMR spectrum of 4
Compound 6. 3',5'-Di-O-acetyl-5-formyl-2'-deoxyuridine (5) (346.0 mg, 1.02 mmol) and 1,1,3,3-tetramethylisoindoline-5,6-diamine (4) (174.0 mg, 0.85 mmol) were dissolved in MeOH (9 mL), K₃Fe(CN)₆ (402.0 mg, 1.22 mmol) added and the reaction stirred for 14 h at 22 °C. The reaction mixture was concentrated in vacuo and the crude product was purified by flash silica gel column chromatography using a gradient elution (CH₂Cl₂:MeOH; 98:02 to 90:10) to give compound 6 as a dark yellow solid (210.0 mg, 47% yield).

¹H NMR (d₆-DMSO): δ 12.07 (s, 1H), 8.69 (s, 1H), 7.30 (s, 1H), 7.22 (s, 1H), 6.27 (t, J = 7.0 Hz, 1H), 5.27 (dt, J = 5.6, 2.7 Hz, 1H), 4.38 – 4.32 (m, 1H), 4.28 (d, J = 3.4 Hz, 2H), 2.49 – 2.42 (m, 1H), 2.28 (s, 3H), 2.09 (s, 3H), 1.41 (d, J = 4.0 Hz, 12H).

¹³C NMR (d₆-DMSO): δ 170.45, 169.97, 161.76, 149.38, 145.72, 143.47, 139.71, 104.58, 85.22, 81.80, 74.08, 63.59, 61.82, 37.08, 32.37, 20.72, 20.69.

HR-ESI-MS (M + H)⁺: calcd. for C₂₆H₃₂N₅O₇ 526.2223, found 526.2353.
$^1$H NMR spectrum of 6

$^{13}$C NMR spectrum of 6
**Compound 9A.** To a suspension of 6 (200.0 mg, 0.38 mmol) in CH$_3$CN (20 mL) was added NaN$_3$ (99.0 mg, 1.52 mmol) and the reaction stirred at 22 °C. After 30 min, mCPBA (131.0 mg, 0.76 mmol) was added. After 4 h the reaction mixture was concentrated *in vacuo* and the residue was purified by silica gel column chromatography using a gradient elution (CH$_2$Cl$_2$:MeOH; 100:0 to 95:5) to give compound 9A as a yellow solid (150.0 mg, 56.0% yield).

$^1$H NMR (d$_6$-DMSO): δ 12.50 (br s), 12.04 (br s), 8.59 (br s), 6.30 (br s), 5.28 (br s), 4.32 (br s), 2.33 (br s), 2.09 (br s), 1.23 (br s).

$^{13}$C NMR (d$_6$-DMSO): δ 170.14, 169.65, 161.56, 148.88, 140.92, 84.98, 81.61, 73.78, 63.39, 37.03, 20.68.

HR-ESI-MS (M + H)$^+$: calcd. for C$_{26}$H$_{30}$N$_5$O$_8$ 541.1267, found 541.2194.
$^1$H NMR spectrum of 9A

$^{13}$C NMR spectrum of 9A
**Spin-labelled nucleoside ImU.** A solution of 9A (150.0 mg, 0.28 mmol) in MeOH saturated with NH₃ (5.00 mL) was stirred at 22 °C for 16 h. The reaction mixture was concentrated *in vacuo* and the crude product purified by flash column chromatography, eluting with (CH₂Cl₂:MeOH; 98:2 to 92:8) to give ImU as a yellowish solid (98.0 mg, 77.0 %).

**¹H NMR** (d₆-DMSO): δ 12.46 (br s), 11.97 (br s), 9.55 (br s), 8.67 (br s), 8.00 (br s), 7.70 (br s), 7.26 (br s), 6.67 (br s), 6.48 (br s), 6.25 (br s), 6.12 (br s), 5.32 (br s), 5.04 (br s), 4.31 (br s), 3.90 (br s), 3.64 (br s), 3.33 (br s), 2.26 (br s), 1.76 (br s), 1.60 (br s), 1.33 (br s), 1.23 (br s), 0.83 (br s).

**¹³C NMR** (d₆-DMSO): δ 170.89, 161.54, 148.84, 141.45, 87.37, 84.82, 70.01, 60.96, 22.21.

**HR-ESI-MS (M + H)+:** calcd. for C₂₂H₂₇N₅O₆ 457.1959, found 457.1959.
$^1$H NMR spectrum of $^{1m}$U

$^{13}$C NMR spectrum of $^{1m}$U
**Compound DMT-ImU.** ImU (90.0 mg, 0.20 mmol), DMTCl (120.0 mg, 0.36 mmol) and DMAP (2.4 mg, 0.02 mmol) were weighed into a round bottom flask and kept under vacuum for 16 h. Pyridine (4.0 mL) was added and the solution was stirred at 22 °C for 2 h. MeOH (100 µL) was added and the solvent was removed in vacuo to give a crude orange solid. The solid was purified by column chromatography using a gradient of (CH₂Cl₂:MeOH; 100:0 to 97.5:2+0.5% Et₃N), using a column that was prepared in 99.5% CH₂Cl₂ + 0.5% Et₃N. Compound DMT-ImU was obtained as a yellow solid (100.0 mg, 67.0%).

**¹H NMR** (d₆-DMSO): δ 12.47 (br s), 8.61 (br s), 7.29 (br s), 6.76 (br s), 6.20 (br s), 5.36 (br s), 4.19 (br s), 4.02 (br s), 3.61 (br s), 3.31 (br s), 2.62 (br s), 2.36 (br s), 1.76 (br s), 1.23 (br s), 1.01 (br s).

**¹³C NMR** (d₆-DMSO): δ 161.77, 157.59, 157.56, 148.98, 144.38, 135.21, 135.12, 129.43, 129.35, 127.50, 127.45, 126.37, 112.81, 85.85, 85.78, 85.52, 70.10, 63.14, 55.27, 55.23, 55.19, 45.42, 10.51.

**HR-ESI-MS** (M + H)⁺: calcd. for C₄₃H₄₅N₅O₈ 759.3263, found 759.3264.
$^1$H NMR spectrum of DMT-$^{1m}$U

$^{13}$C NMR spectrum of DMT-$^{1m}$U
**ImU phosphoramidite (10A).** DMT-ImU (50.0 mg, 0.07 mmol) and diisopropyl ammonium tetrazolide (15.0 mg, 0.09 mmol) were dissolved in pyridine (2 mL) and the pyridine removed *in vacuo*. The residue was kept under vacuum for 19 h, followed by dissolution in CH₂Cl₂ (3.5 mL), CH₃CN (2.0 mL) and addition of 2-cyanoethyl N,N,N',N'-tetraisopropyl phosphoramidite (26.0 mg, 0.09 mmol). The reaction mixture was stirred at 22 °C for 2 h, diluted with CH₂Cl₂ (10 mL) and washed successively with saturated aq. NaHCO₃ (3x10 mL) and saturated aq. NaCl (2 x 10 mL). The organic solution was dried over anhydrous Na₂SO₄, filtered and concentrated *in vacuo* to give a crude solid. The solid was purified by precipitation by first dissolving it in CH₂Cl₂ (1 mL) followed by addition of n-hexane (80 mL). The liquid was decanted and the operation repeated twice to furnish phosphoramidite 10A as a yellow solid (48.0 mg, 76%).

¹H NMR (CDCl₃): δ 11.28 (br s), 8.96 (br s), 8.12 (br s), 7.50 (br s), 7.42 (br s), 7.26 (br s), 6.80 (br s), 6.35 (br s), 5.33 (br s), 4.71 (br s), 4.58 (br s), 4.30 (br s), 3.72 (br s), 3.48 (br s), 2.67 (br s), 2.49 (br s), 2.21 (br s), 1.30 (br s), 1.22 (br s), 0.92 (br s).

³¹P NMR (CDCl₃): δ 149.41, 149.02.

HR-ESI-MS (M + H)⁺: calcd. for C₅₂H₆₂N₇O₉P 959.4341, found 959.4368.
$^1$H NMR spectrum of 10A

$^{31}$P NMR spectrum of 10A
**Compound 8.** To a solution of compound 7 (349.0 mg, 1.69 mmol) in MeOH (18 mL) was added 3',5'-di-O-acetyl-5-formyl-2'-deoxyuridine (5) (633.0 mg, 1.86 mmol) and the reaction heated at 40 °C for 2 h, followed by cooling to 0 °C and adding the iodobenzene diacetate (599.0 mg, 1.86 mmol) all at once. Reaction was allow warm slowly to 22 °C and then stirred for 16 h. The solvent was removed *in vacuo* and the crude mixture purified by silica gel column chromatography using a gradient elution (CH$_2$Cl$_2$:MeOH; 100:0 to 80:20) to give compound 8 as a grey coloured solid (350.0 mg, 40% yield).

$^1$H NMR (d$_6$-DMSO): $\delta$ 8.54 (s, 1H), 7.51 (s, 1H), 7.45 (s, 1H), 6.21 (t, $J$ = 6.9 Hz, 1H), 5.30 – 5.21 (m, 1H), 4.39 – 4.22 (m, 3H), 2.49 – 2.40 (m, 2H), 2.13 (s, 3H), 2.08 (s, 3H), 1.41 (s, 12H).

$^{13}$C NMR (d$_6$-DMSO): $\delta$ 170.19, 169.95, 159.04, 158.35, 149.53, 149.29, 143.05, 140.40, 111.47, 103.38, 102.52, 85.72, 81.94, 73.96, 63.51, 61.99, 61.90, 37.02, 32.26, 32.12, 20.69, 20.55.

HR-ESI-MS (M + H)$^+$: calcd. for C$_{26}$H$_{31}$N$_4$O$_8$ 527.2136, found 527.2146.
$^{1}H$ NMR spectrum of 8

$^{13}C$ NMR spectrum of 8
**Compound 9B.** To a suspension of 8 (175.0 mg, 0.33 mmol) in CH$_3$CN (18 mL) was added NaN$_3$ (87.0 mg, 1.33 mmol) and the reaction stirred at 22 °C. After 30 min mCPBA (115.0 mg, 0.66 mmol) was added. After 14 h the reaction mixture was concentrated *in vacuo*. The residue was purified by silica gel column chromatography using a gradient elution (CH$_2$Cl$_2$:MeOH; 100:0 to 96:4) to give compound 9B as a yellow solid (90.0 mg, 50% yield).

$^1$H NMR (CDCl$_3$): δ 8.81 (br s), 8.25 (br s), 8.16 (br s), 7.69 (br s), 7.55 (br s), 7.26 (br s), 6.57 (br s), 5.49 (br s), 5.41 (br s), 4.60 (br s), 2.83 (br s), 2.48 (br s), 2.35 (br s), 2.29 (br s).

$^{13}$C NMR (CDCl$_3$): δ 165.88, 155.97, 154.20, 144.51, 138.29, 130.10, 129.07, 125.92, 125.57, 124.06, 98.93, 82.02, 78.84, 76.68, 70.14, 59.95, 34.57, 16.98, 16.85.

HR-ESI-MS (M + Na)$^+$: calcd. for C$_{26}$H$_{29}$N$_4$O$_9$Na 564.1827, found 564.1779.
$^1$H NMR spectrum of 9B

$^{13}$C NMR spectrum of 9B
**Spin-labelled nucleoside Ox**. A solution of 9B (174.0 mg, 0.32 mmol) in MeOH saturated with NH$_3$ (4.00 mL) was stirred at 22 °C for 14 h. The reaction mixture was concentrated *in vacuo* and the residue purified by flash column chromatography eluting with (CH$_2$Cl$_2$:MeOH; 98:2 to 92:8) to give Ox as yellow solid (118.0 mg, 80.0%).

$^1$H NMR (d$_6$-DMSO): δ 8.89 (br s), 8.50 (br s), 7.88 (br s), 7.52 (br s), 7.30 (br s), 6.67 (br s), 6.20 (br s), 5.71 (br s), 5.35 (br s), 5.18 (br s), 4.31 (br s), 3.90 (br s), 3.65 (br s), 2.67 (br s), 2.26 (br s).

$^{13}$C NMR (d$_6$-DMSO): δ 158.95, 149.08, 144.43, 132.94, 132.17, 130.38, 128.54, 127.65, 100.50, 87.57, 85.30, 69.69, 60.60.

HR-ESI-MS (M + Na)$^+$: calcd. for C$_{22}$H$_{23}$N$_4$O$_7$Na 480.1615, found 480.1603.
$^1$H NMR spectrum of $^{\text{Ox}}$U

$^{13}$C NMR spectrum of $^{\text{Ox}}$U
Compound DMT-OxU. OxU (45.0 mg, 0.10 mmol), DMTrCl (67.0 mg, 0.20 mmol) and DMAP (1.2 mg, 0.01 mmol) were weighed into a round bottom flask and kept in vacuo for 12 h. Pyridine (1.5 mL) was added and the solution was stirred at 22 °C for 3 h. MeOH (100 μL) was added and the solvent was removed in vacuo to obtain residue. The residue was purified by silica gel column chromatography using a gradient of (CH₂Cl₂:MeOH; 100:0 to 97.5:2 + 0.5% Et₃N). The column was prepared in 99.5% CH₂Cl₂ + 0.5% Et₃N. Compound DMT-OxU was obtained as a yellow solid (45.0 mg, 60.0%).

¹H NMR (CDCl₃): δ 9.03 (br s), 7.26 (br s), 6.56 (br s), 4.58 (br s), 3.55 (br s), 2.95 (br s), 1.25 (br s), 1.16 (br s).

¹³C NMR (CDCl₃): δ 162.99, 156.61, 156.04, 156.01, 146.79, 142.08, 140.98, 133.46, 133.03, 127.72, 127.61, 127.53, 127.32, 125.99, 125.90, 125.62, 124.76, 110.83, 110.79, 100.24, 84.22, 60.46, 53.56, 43.62, 27.27, 6.68.

HR-ESI-MS (M + Na⁺): calcd. for C₄₃H₄₃N₄O₉Na 782.2922, found 782.2912.
$^1$H NMR spectrum of DMT-O$_x$U

$^{13}$C NMR spectrum of DMT-O$_x$U
**Ox Phosphoramidite (10B).** A mixture of diisopropyl ammonium tetrazolide (9.4 mg, 0.05 mmol) and DMT-**Ox**U (28.0 mg, 0.04 mmol) was dissolved in pyridine (2 mL), evaporated in vacuo and residue was kept under vacuo for 17 h. CH₂Cl₂ (2.0 mL) and 2-cyanoethyl N,N,N',N'-tetraisopropyl phosphoramidite (33.30 mg, 0.11 mmol) was added. The reaction mixture was stirred at 22 °C for 16 h, diluted with CH₂Cl₂ (10 mL) and washed successively with saturated aq. NaHCO₃ (3 x 10 mL) and saturated aq. NaCl (2 x 10 mL). The organic solution was dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo to give a solid that was purified by precipitation by first dissolving it in CH₂Cl₂ (0.5 mL), followed by addition of petroleum ether (50 mL). The liquid was decanted and the operation repeated three times to furnish phosphoramidite 10B as a yellowish solid (26 mg, 74%).

**¹H NMR** (CDCl₃): δ 9.05 (br s), 7.45 (br s), 7.26 (br s), 7.12 (br s), 6.61 (br s), 6.34 (br s), 4.59 (br s), 4.12 (br s), 4.07 (br s), 3.75 (br s), 3.55 (br s), 3.43 (br s), 3.26 (br s), 3.06 (br s), 2.52 (br s), 2.28 (br s), 1.29 (br s), 1.18 (br s), 1.04 (br s), 0.89 (br s), 0.79 (br s).

**¹³P NMR** (CDCl₃): δ 149.43, 148.97, 14.40, 2.78.

**HR-ESI-MS (M + Na)⁺**: calcd. for C₅₂H₆₀N₆O₁₀PNa 982.4001, found 982.4007.
$^1$H NMR spectrum of 10B

$^{31}$P NMR spectrum of 10B
References