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Conformationally restricted isoindoline-derived spin labels in duplex DNA: Distances and rotational flexibility by PELDOR

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Abstract: Three structurally related isoindoline-derived spin labels that have different mobilities were incorporated into duplex DNA to systematically study the effect of motion on orientation-dependent pulsed electron-electron double resonance (PELDOR) measurements. To that end, a new nitroxide spin label, ^{ExIm}**U**, was synthesised and incorporated into DNA oligonucleotides. ^{ExIm}**U** is the first example of a conformationally unambiguous spin label for nucleic acids, in which the nitroxide N-O bond lies on the same axis as the three single bonds used to attach the otherwise rigid isoindoline-based spin label to a uridine base. The CW-EPR measurements of ^{ExIm}**U** confirms a very high rotational mobility of the spin label in duplex DNA, when compared to the structurally related spin label ^{Im}**U**, which has restricted mobility due to an intramolecular hydrogen bond. The X-band CW-EPR spectra of ^{ExIm}**U** can be used to identify mismatches in duplex DNA. PELDOR distance measurements between pairs of the spin labels ^{Im}**U**, ^{Ox}**U** and ^{ExIm}**U** in duplex DNA showed a strong angular dependence for ^{Im}**U**, a medium dependence for ^{Ox}**U** and no orientation effect using ^{ExIm}**U**. Thus, precise distances can be extracted from ^{ExIm}**U** without having to take orientational effects into account.

Keywords: EPR spectroscopy, nucleic acids, DEER, site-directed spin labeling (SDSL), aminoxyl radical

Introduction

Electron paramagnetic resonance (EPR) spectroscopy has emerged as a powerful tool for investigating structure and dynamics of biopolymers, such as DNA and RNA under biological conditions.[1] Continuous wave (CW) EPR spectroscopy is useful to extract information about dynamics of specific sites through line-shape analyses of EPR spectra.[1b, 1e, 2] In CW-EPR, the dipolar coupling between spin centers results in line broadening and can be used to extract information about distances in the range of 5-20 Å.[3] Pulsed EPR methods, such as pulsed electron-electron double resonance (PELDOR), also called double electron-electron resonance (DEER), and double quantum coherence (DQC) have been used to measure long-range distances from 20-80 Å.[4] PELDOR is also useful for the determination of the relative orientation of spin labels, in particular at high field.[5]

Application of EPR spectroscopy to study nucleic acids requires incorporation of unpaired electron(s), since nucleic acids are inherently diamagnetic. The most commonly used spin labelling method is incorporation of aminoxyl (nitroxide) spin labels by covalent attachment to a nucleic acid.[6] Most of the spin labels that have been described are attached with a tether that has some degree of flexibility. Due to this flexibility, such labels can move independently of the biopolymer and are, therefore, not optimal probes for distance measurements. The recently developed rigid spin label **Ç** for DNA[7] and **Çm** for RNA[7b] (Figure 1c) are able to give accurate distances between two spin labels and provide information about their orientation in nucleic acids.[5b, 8] Native tyrosyl radicals have also been utilized for the same purpose in proteins.[5a]

Recently, we described isoindoline derived probes ^{Im}**U** and ^{Ox}**U**[9] (Figure 1a) and their incorporation into DNA. These probes are linked to the nucleobase by a single bond that lies on the axis of the nitroxide N-O bond and should be good probes for distance measurement in DNA duplexes, because rotation around the single bond does not cause displacement of the nitroxide relative to the DNA. The ^{Im}**U** spin label was shown by CW-EPR spectroscopy to be less mobile than the ^{Ox}**U** spin label due to intramolecular hydrogen-bonding between the imidazole N-H and O4 of **U** (Figure 1b).[9] Indeed, ^{Im}**U** displayed similar mobility to that of the rigid spin label **Ç** at low temperatures.[9] Inspired by these results, we decided to determine if ^{Im}**U** is useful for orientation-dependent distance measurements**,** which requires rigid labels.

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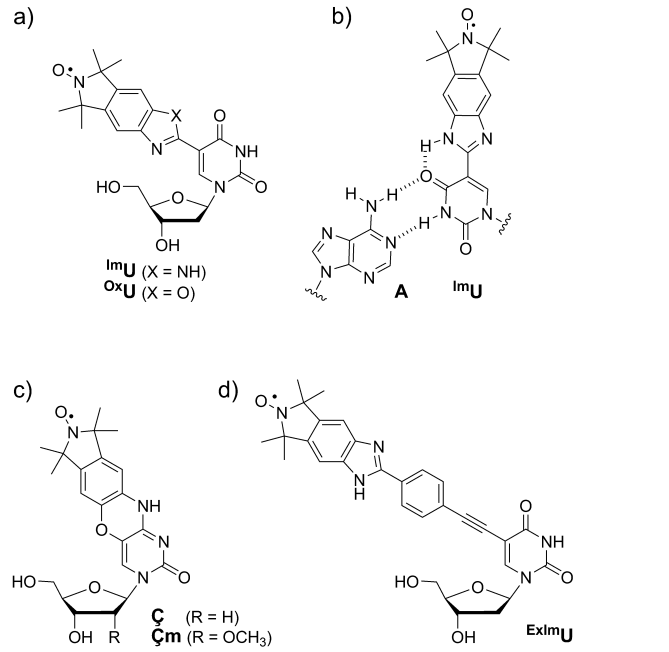


Figure 1. Spin-labelled nucleosides. a) Isoindoline-derived spin labels ^{Im}U and ^{Ox}U. b) Base pairing of ^{Im}U with **A**, showing the intramolecular hydrogen bond that restricts rotation around the bond connecting the nitroxide to the base. c) Rigid spin labels **Ç** and **Çm**. d) Extended benzimidazole spin label ^{ExIm}U.

Herein we report that the ^{Im}U spin label shows a strong orientation dependence by PELDOR, similar to the rigid spin labels **Ç**[5b] and **Çm**[8b]. Interestingly, ^{Ox}U, which does not have the possibility to restrict movement of the spin label around the single bond through hydrogen bonding like ^{Im}U, still shows orientational effects in the PELDOR time traces. A structurally-related spin label ^{ExIm}U (Figure 1d) was also synthesized and incorporated into DNA for comparison to ^{Im}U and ^{Ox}U. In ^{ExIm}U, rotation is possible around the two single bonds flanking the acetylene bond that lie on the same axis as the N-O bond. CW-EPR measurements of ^{ExIm}U confirmed the high rotational mobility of ^{ExIm}U in duplex DNA. As expected, ^{ExIm}U only showed a very minor orientation dependence in the PELDOR measurements. To our knowledge, ^{ExIm}U is the first example of a incorporation of a conformationally unambiguous spin label[10] into nucleic acids for distance measurements. Accurate distances can be measured by a single PELDOR experiment with the new spin label ^{ExIm}U, unlike rigid labels that requires summing up measurementes at several probe frequencies to disentangle distance and orientation effects.[5b, 11]

Results and Discussion

Syntheses of spin labelled phosphoramidites: The ^{Im}U and ^{Ox}U spin-labelled phosphoramidites were prepared by a previously reported procedure.[9] Synthesis of spin label ^{ExIm}U began with a Sonogashira coupling[12] of acetyl-protected 5-iodo 2′-deoxyuridine (**2**)[13] with 4-ethynylbenzaldehyde (**1**) to give compound **3** (Scheme 1). Treatment of compound **3** with 5,6 diamino 1,1,3,3-tetramethylisoindoline (**4**)[9] in the presence of NH_4Cl gave the extended benzimidazole derivative of 2′-deoxyuridine **(5)**.[14] Oxidation of **5** proved to somewhat challenging, similar to the previously reported spin labelled nucleosides ^{Ox}U and ^{Im}U,[9] but sodium azide facilitated *m*CPBA oxidation of **5** gave spin-labelled derivative **6** in good yields. Deprotection of the acetyl groups with methanolic NH_3 afforded the nucleoside ^{ExIm}U. The 5′-hydroxyl group was protected as a 4,4′-dimethoxytrityl ether and phosphitylation yielded the ^{ExIm}U phosphoramidite **7**, which was used for incorporation of ^{ExIm}U into DNA oligonucleotides.

Syntheses and characterisation of spin labelled oligonucleotides: Spin labelled oligonucleotides were prepared by solid phase synthesis and purified by denaturing polyacrylamide gel electrophoresis. Spin labels ^{Ox}U and ^{Im}U have previously been shown not to have any effect on B-conformation of duplex DNA and only a minor effect on DNA duplex stability.[9] ^{ExIm}U was incorporated into a 14-mer DNA (5'-d(GACCTCG^{ExIm}UATCG TG)), verified by MALDI-Tof analysis (Table S1). Circular dichroism (CD) spectra of both the unmodified and spin labeled 14-mer duplex possessed negative and positive molar ellipticities at ca. 250 and 280 nm, respectively, characteristic of a right-handed B-DNA (Figure S1). The ^{ExIm}U spin label slightly destabilized the DNA duplex by 6.5 °C (Table S2), similar to that reported by Korshun and co-workers for a structurally related compound.[15]

CW-EPR analysis: To study the motion of the spin-labelled nucleoside ^{ExIm}U within a nucleic acid, the CW-EPR spectra of the nucleoside ^{ExIm}U, as well as the ^{ExIm}U-labelled DNA single strand and duplex were compared (Figure 2, left column). Due to the rapid isotropic tumbling of the nucleoside in solution, its EPR spectrum shows narrow three lines. After incorporation into the 14-mer DNA, the tumbling of the nitroxide slows down and the resulting EPR spectrum is broader. However, there was not much difference between the spectral width of the single strand and the ^{ExIm}U-labelled duplex, indicating substantial mobility of the spin label independent of the nucleic acid. In contrast, the conformationally restricted ^{Im}U (Figure 2, right column) shows much lower mobility in duplex DNA, compared with single stranded DNA (Figure 2; see Figures S2 and S3 for simulated spectra and Figure S3 for measurements at other temperatures).

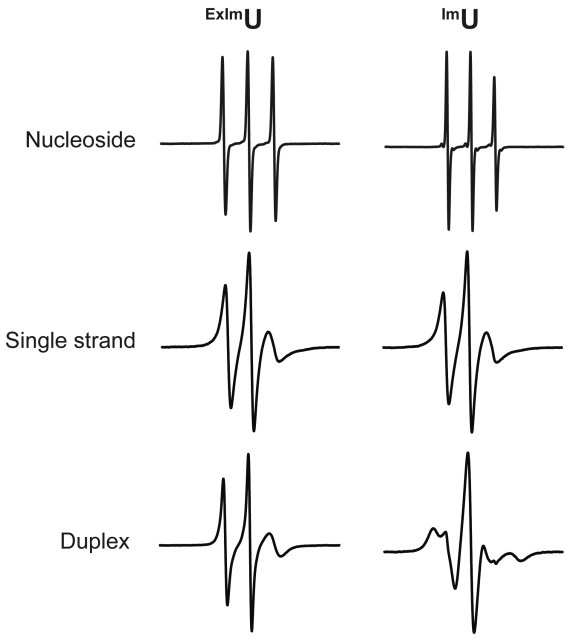
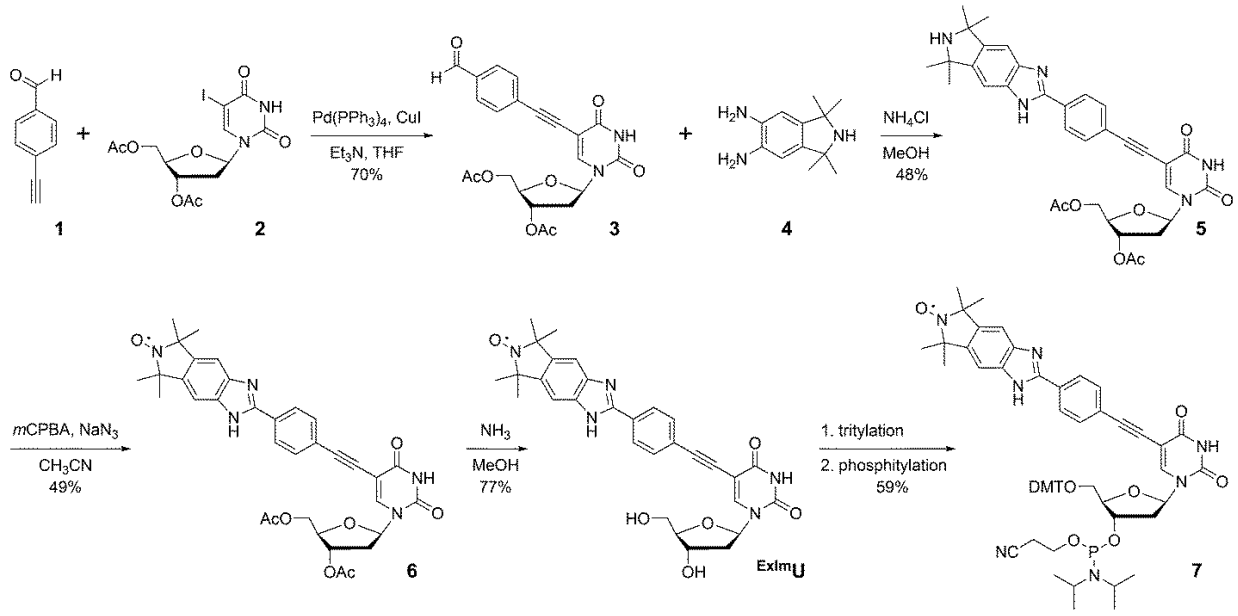


Figure 2. CW-EPR spectra of nucleosides ^{ExIm}U and ^{m}U (top) and after their incorporation into single stranded DNA (middle) and duplex DNA (bottom) at 20 °C (10 mM phosphate buffer, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0).

Spin labels have been used to study local structural perturbations in nucleic acids.[2c, 16] To investigate if the ^{ExIm}U spin label could be used to probe base-pairing in duplex DNA, four 14-mer duplexes containing either **A**, **T**, **G** or **C** paired with ^{ExIm}U were prepared. The EPR spectrum of ^{ExIm}U•**A** is the least mobile and markedly different from the others (Figure S4) ^{ExIm}U can, therefore, clearly distinguish between a "native"-like base pair and a mismatch.



Scheme 1. Synthesis of extended benzimidazole nucleoside ^{ExIm}U and its corresponding phosphoramidite.

Distance- and orientation-measurements by PELDOR: Two doubly-labeled duplex constructs were used for spin-labeling, containing either 7 base-pairs (DNA(1,9)) or 10 base-pairs (DNA(1,12)) between the labels. The distance between the ^{Im}U or the ^{Ox}U spin label pairs were similar for both DNA constructs, but their relative orientations were different (Figure 3, Table 1). The distances for the ^{ExIm}U duplexes were different than for ^{Im}U or ^{Ox}U. Molecular models of ^{Ox}U and ^{ExIm}U in both DNA constructs are shown in Figure S5.

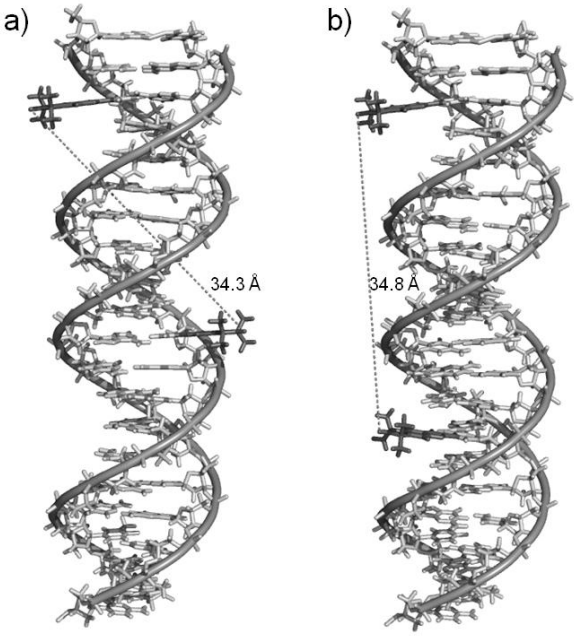


Figure 3. Molecular models of a) ^{Im}U-DNA(1-9) and b) ^{Im}U-DNA(1-12).

The dead-time-free four-pulse sequence was used for all PELDOR experiments.[17] The pump pulse was placed on the maximum of the nitroxide spectrum (Figure S6), thus exciting all orientations. The detection pulses were applied at a microwave

Table 1. Distance measurements in duplex DNA by PELDOR

Descriptor	Duplex	r [Å][a]
^{Im}U-DNA (1,9)	5′-d(GC^{Im}U AGT GCG C AC GCG CGA TC) 3′-d(CG A TCA CGC G ^{Im}UG CGC GCT AG)	34.3 / 34.3
^{Im}U-DNA (1,12)	5′-d(GC^{Im}U AGT CGC GCG C AC GCA TC) 3′-d(CG A TCA GCG CGC G ^{Im}UG CGT AG)	34.1 / 34.8
^{Ox}U-DNA (1,9)	5′-d(GC^{Ox}U AGT GCG C AC GCG CGA TC) 3′-d(CG A TCA CGC G ^{Ox}UG CGC GCT AG)	32.3 / 34.3
^{Ox}U-DNA (1,12)	5′-d(GC^{Ox}U AGT CGC GCG C AC GCA TC) 3′-d(CG A TCA GCG CGC G^{Ox}UG CGT AG)	34.1 / 34.8
^{ExIm}U-DNA (1,9)	5′-d(GC ^{ExIm}U AGT GCG C AC GCG CGA TC) 3′-d(CG A TCA CGC G ^{ExIm}UG CGC GCT AG)	40.1 / 38.4
^{ExIm}U-DNA (1,12)	5′-d(GC^{ExIm}U AGT CGC GCG C AC GCA TC) 3′-d(CG A TCA GCG CGC G^{ExIm}UG CGT AG)	35.8 / 35.3

[a] Measured / modelled distances between spin labels.

frequency with a frequency offset of 40 to 90 MHz from the pump pulse (Figure S6). Due to the narrow excitation bandwidth of the detection pulses (31 MHz) only a fraction of the nitroxide spectrum is excited. Thus, varying the frequency of the detection pulses causes a selection of different components of the hyperfine tensor A, originating from coupling with the ^{14}N of the nitroxide. If the orientation of the nitrogen hyperfine tensor is fixed with respect to the spin-spin distance vector r, as it is the case for rigid spin labels,[5b] selection of specific components of A also selects specific molecular orientations with respect to the external magnetic field. Since the dipolar interaction depends not only on the distance r, but also on the orientation of this vector with respect to the external magnetic field, different detection frequencies will result in different the rigidity of spin labels, since the dependence on the frequency offset is strongly reduced if the two spin labels have some conformational freedom with respect to each other and vanishes for very mobile labels.

The PELDOR time traces for the three spin labels in the two DNA constructs are shown in Figure 4. The PELDOR data clearly

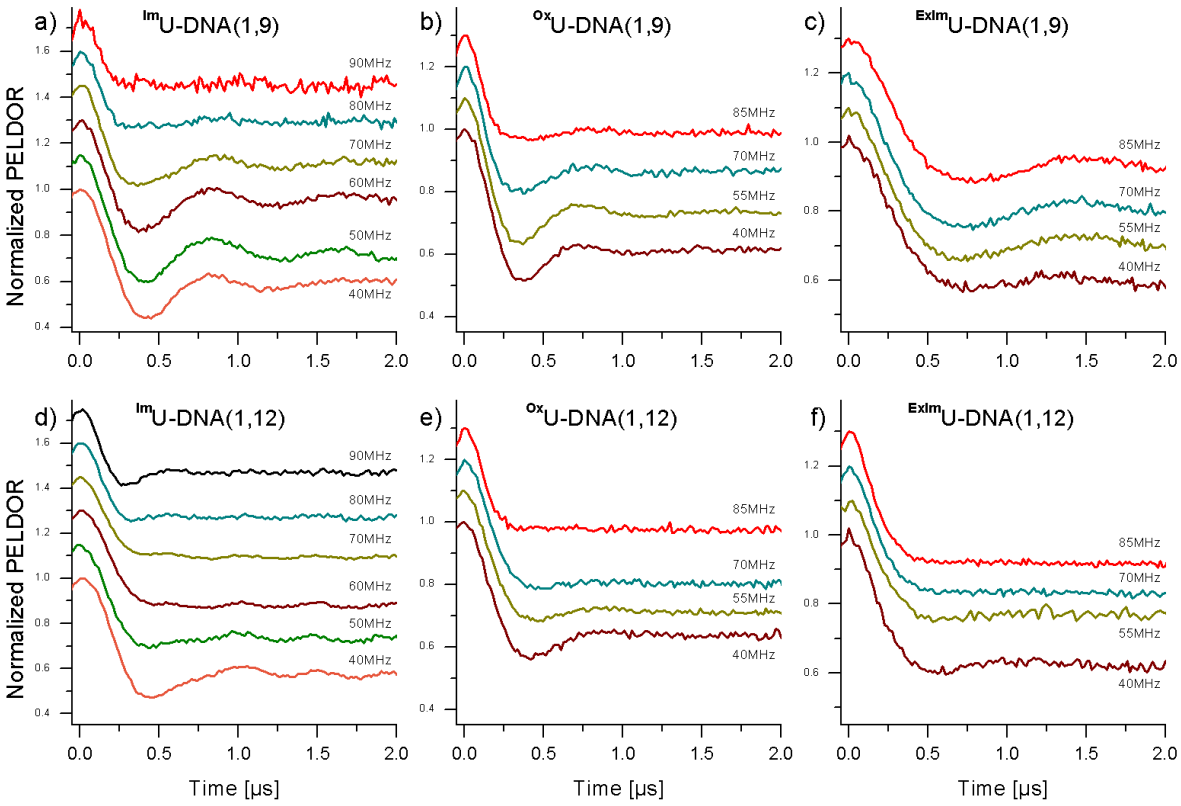


Figure 4. PELDOR time traces at different frequency offsets (see Figure S5) for a) ^{Im}U-DNA(1,9), b) ^{Ox}U-DNA(1,9), c) ^{ExIm}U-DNA(1,9), d) ^{Im}U-DNA(1,12), e) ^{Ox}U-DNA(1,12) and f) ^{ExIm}U-DNA(1,12). Offset between fixed pump and different detection frequency is indicated at the PELDOR time traces.

shows orientation dependence for both ^{Im}U and ^{Ox}U, as judged by the degree of variation between the time traces recorded at different offsets.The ^{Im}U label has the strongest orientation dependence of the three, followed by ^{Ox}U and ^{ExIm}U. This is in agreement with CW-EPR data that showed that ^{Im}U is less mobile than ^{Ox}U, presumably because an intramolecular hydrogen bond between the imidazole hydrogen and O4 of the nucleobase (Figure 1b) limits the rotation around the single bond connecting the nitroxide to the base.[9] As expected, negligible orientation dependence was observed for ^{ExIm}U, which has free rotation around the two bonds flanking the acetylene linker.

For evaluation of the distance between two rigid spin labels that display orientation dependence, like Ç,[5b] the time traces from different frequency offsets need to be summed up and the intermolecular decay function has to be removed before performing a Tikhonov regularization. This was demonstrated for ^{Im}U, where the 70 MHz offset (normally used for single-measurement distance determination) gave a distance distribution indicating two distances, whereas the summed traces gave a relatively sharp single distance distribution below 4 nM (Figure 5a). In contrast, the spin label ^{ExIm}U, which showed a very small orientation dependency, allows extraction of distances directly from a single measurement at a fixed frequency offset (Figure 5b).

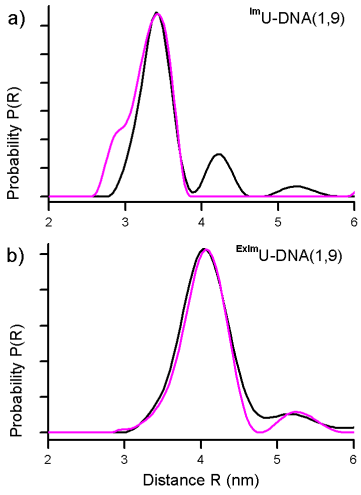


Figure 5. Evaluation of interspin distances in doubly spin-labelled DNA duplexes from PELDOR data for a) ^{Im}U-DNA(1,9) and b) ^{ExIm}U-DNA(1,9). Distance probabilities derived from the experimental data by Tikhonov regularisation from one PELDOR time trace (70 MHz offset, magenta line) and all PELDOR time traces (sum of all offset, black line)

Conclusion

The new spin label ^{ExIm}U was prepared and incorporated into DNA oligonucleotides for distance measurements by pulsed EPR spectroscopy. CW-EPR spectra of ^{ExIm}U in duplex DNA, confirm its high mobility, compared to the previously reported structurally related spin label ^{Im}U, which has restricted rotational mobility due to an intramolecular hydrogen bond. The ^{ExIm}U spin label can distinguish between pairing with its Watson-Crick partner **A** and any of the other mismatches by CW-EPR spectroscopy. PELDOR distance measurements using the spin labels ^{Im}U, ^{Ox}U and ^{ExIm}U show that ^{ExIm}U exhibits only negligible orientation dependence, according to its design, unlike ^{Im}U and ^{Ox}U. Thus, PELDOR measurements using ^{ExIm}U in nucleic acids allow distance determination from a single measurement with a fixed detection frequency.

Experimental Section

General: All chemicals, except 2'-deoxyuridine and thymidine, were purchased from Sigma Aldrich, Acros or Fluka and used without further purification. 2'-deoxyuridine and thymidine were purchased from Rasayan Inc. USA. 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). Water was purified on EASYpure RoDi Water Purification Systems. CH_2Cl_2 and pyridine were freshly distilled over calcium hydride prior to use. Anhydrous Et_3N, n-hexane and EtOAC were used directly as received. All moisture-and air-sensitive reactions were carried out in oven dried glassware under an inert atmosphere of argon. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 MHz spectrometer. 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), for ^{1}H NMR and $CDCl_3$ (77.0 ppm), [D_6]-DMSO (39.43 ppm), for ^{13}C NMR. ^{31}P NMR chemical shifts are reported relative to 85% H_3PO_4 as an external standard. Commercial grade $CDCl_3$ was passed over basic alumina shortly before use with tritylated compounds. Mass spectrometric analyses of all organic compounds were performed on HRMS-ESI (Bruker, MicroTof-Q) in positive ion mode.

DNA oligonucleotide synthesis, purification and characterization: The DNA oligonucleotides were synthesized by phosphoramidite chemistry on an automated ASM800 DNA/RNA synthesizer (Biosset, Novosibirsk, Russia) by using trityl-off protocol and phosphoramidites with standard protecting groups on 1.0 µmol scale, 1000 Å CPG columns. All commercial phosphoramidites, CPG columns and solutions were purchased from ChemGenes Corporation (Wilmington, MA). The spin-labelled DNA was prepared by using previously reported protocols;[18] the spin-labelled phosphoramidite was manually incorporated into the oligonucleotides 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 10-12 min. After manual coupling, the column was re-mounted on the synthesizer and the synthesis cycle completed. Treatment with 33% aq. NH_3 at 55 °C for 8 h deprotected the oligos, that were subsequently purified by 20% denaturing polyacrylamide gel electrophoresis (DPAGE). The DNA oligonucleotides bands were visualized under UV light and excised from the gel, crushed and eluted from gel with 2 x 10 mL Tris buffer (Tris (10 mM, pH 7.5), NaCl (250 mM), Na_2EDTA (1 mM)). The DNA elution solutions were filtered through 0.45 mM cellulose acetate membrane (Whatman) and desalted using a Sep-Pak cartridge (Waters Corporation). The dried oligonucleotides were dissolved in 400 µL of sterile water and their final concentrations were calculated according to Beer's law based on UV absorbance of oligonucleotides at 260 nm. Extinction coefficients were determined by using the UV WinLab oligonucleotide calculator (V2.85.04; PerkinElmer). Molecular weights (MW) of oligonucleotides were determined by MALDI-TOF analysis (Bruker, Autoflex III) after calibration with an external standard. UV-VIS spectra were recorded on a PerkinElmer Lambda 25 UV/VIS spectrometer. CD spectra were recorded on a JASCO J-810 spectropolarimeter at 20 °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.

CW-EPR measurements and sample preparation: 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 a Magnettech temperature controller M01 with an error ± 0.5 °C. The sample was prepared by dissolving 2.0 nmol of spin-labelled, single-stranded DNA and 2.4 nmol of its complementary strand in phosphate buffer (10 mM phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0) (10 µL, oligo final conc. 200 µM). The resulting mixture was 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 prior to EPR measurements.

PELDOR sample preparation: The DNA samples for PELDOR measurement were prepared by annealing 10 nmoles of each strand with 10 nmoles of its complementary strand in 100 µL of 10 mM phosphate buffer, pH 7.0, 100 mM NaCl and 0.1 mM EDTA, followed by evaporation of the water. The annealed dried samples were dissolved in 100 µL of 20% ethylene glycol/H_2O before the PELDOR measurements.

PELDOR data collection: The dead-time free four-pulse PELDOR sequence was used for all experiments.[17] A Bruker Elexsys E580 X-band spectrometer equipped with Flexline MS-3 probe in an Oxford CF935 cryostat and a PELDOR frequency unit was used. Microwave pulses were amplified by a 1 kW TWT amplifier (ASE 117x). Typical pulse lengths were 32 ns ($\pi/2$ and π) for the probe pulses and 12 ns (π) for the pump pulse. The delay between the first and second probe pulses was varied between 132 and 196 ns in 8 ns steps to reduce contributions from proton modulations. The pulse separation between the second and third probe pulses was between 2.5 and 3.0 µs, depending on the sample preparation. The frequency of the pump pulse was fixed to the central peak of the nitroxide powder spectrum to obtain maximum pumping efficiency. The probe frequency was chosen 40-90 MHz above this frequency (Figure S5). This range corresponds to the smallest frequency offset that avoids strong pump/probe frequency overlap, and therefore large proton modulation artifacts. The 90 MHz offset is the frequency offset that excites the edge of the nitroxide spectrum. All experiments were carried out at 50K.

Compound 3: To a solution of 3′,5′-di-*O*-acetyl-5-iodo-2′-deoxyuridine (100 mg, 0.23 mmol), and 4-ethynylbenzaldehyde (60 mg 0.46 mmol) in THF (2 mL) was added CuI (5 mg, 0.026 mmol) and Et_3N (0.5 mL). The suspension was degassed by bubbling argon gas through the solution for 5 min, after which $Pd(PPh_3)_4$ (27 mg, 0.023 mmol) was added. After stirring the reaction for 3 h at 22 °C, the solvent was removed *in vacuo* and the crude product purified by flash silica gel column chromatography using gradient elution (CH_2Cl_2:MeOH; 100:00 to 98:02) to give compound **3** as a yellow solid (70 mg, 70% yield). R_f=0.45 (5% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz,$CDCl_3$): δ=10.02 (s, 1H), 8.48 (s, 1H), 7.94 (s, 1H), 7.85 (d, J = 8.2 Hz, 2H), 7.72 – 7.60 (m, 2H), 6.32 (dd, J = 7.8, 5.9 Hz, 1H), 5.30 – 5.21 (m, 1H), 4.61 – 4.11 (m, 3H), 2.68 – 2.52 (m, 1H), 2.36 – 2.19 (m, 1H), 2.17 (s, 3H), 2.13 (s, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ=191.28, 170.32, 170.01, 160.55, 148.93, 141.97, 135.72, 132.13, 132.05, 131.87, 129.54, 128.51, 128.48, 128.39, 100.25, 92.96, 85.75, 83.95, 82.73, 73.86, 63.71, 38.37, 20.84, 20.79; HRMS (ESI): *m/z* calcd. for $C_{22}H_{20}N_2O_8Na$: 463.1112 [M + Na]$^+$; found 463.1121.

Compound 5: Compound **3** (749 mg, 1.7 mmol) and 1,1,3,3-tetramethylisoindoline-5,6-diamine (**4**) (349 mg, 1.7 mmol) were dissolved MeOH (15 mL). NH_4Cl (364 mg, 6.8 mmol) was added and the resulting solution stirred at 60 °C for 2 h and then at 22 °C for 14 h. After removing the solvent *in vacuo*, the crude product was purified by flash silica gel column chromatography using a gradient elution (CH_2Cl_2:MeOH; 98:02 to 85:15) to give compound **5** as a dark yellow solid (577 mg, 48% yield). R_f=0.25 (25% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, [D_6] DMSO): δ=13.41 (s, 1H), 8.25 (d, J = 8.3 Hz, 2H), 8.14 (s, 1H), 7.77 – 7.57 (m, 3H), 7.47 (s, 1H), 6.18 (t, J = 7.0 Hz, 1H), 5.22 (dd, J = 6.3, 3.0 Hz, 1H), 4.34 – 4.17 (m, 3H), 2.56 (dt, J = 14.5, 7.3 Hz, 1H), 2.44 – 2.31 (m, 1H), 2.11 (s, 3H), 2.07 (s, 3H), 1.77 (s, 12H); ^{13}C NMR (100.6 MHz, [D_6] DMSO): δ=169.99, 169.94, 161.11, 151.80, 149.25, 143.69, 131.51, 129.70, 126.79, 123.54, 111.73, 104.21, 98.50, 91.73, 84.99, 84.07, 81.49, 73.63, 66.56, 66.45, 63.45, 40.05, 39.84, 39.64, 39.43, 39.22, 39.01, 38.80, 36.18, 29.00, 20.68, 20.52; HRMS (ESI): *m/z* calcd. for $C_{34}H_{36}N_5O_7$: 626.2596 [M + H]$^+$; found 626.2596.

Compound 6: To a suspension of **5** (50 mg, 0.08 mmol) in CH_3CN and MeOH (5 + 1 mL) was added NaN_3 (21 mg, 0.32 mmol) and the suspension stirred at 22 °C. After 30 min, *m*CPBA (28 mg, 0.16 mmol) was added. After 3 h, the reaction mixture was concentrated *in vacuo* and the residue purified by silica gel column chromatography using a gradient elution (CH_2Cl_2:MeOH; 100:0 to 95:5) to give compound **6** as a yellow solid (25 mg, 49% yield). R_f=0.70 (10% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, [D_6] DMSO): δ=13.43 (br s), 11.84 (br s), 8.13 (br s), 7.90 (br s), 7.70 (br s), 7.54 (br s), 6.19 (br s), 5.23 (br s), 4.31 (br s), 2.07 (br s); ^{13}C NMR (100.6 MHz, [D_6] DMSO): δ=169.75, 169.71, 165.72, 160.95, 149.02, 143.62, 133.05, 132.49, 130.73, 130.44, 128.55, 127.66, 127.31, 98.14, 90.91, 84.79, 81.33, 73.45, 63.34, 59.86, 36.09, 20.66, 20.48, 13.84; HRMS (ESI): *m/z* calcd. for $C_{34}H_{35}N_5O_8$: 641.2480 [M + H]$^+$; found 641.2465.

^{ExIm}U: A solution of **6** (150 mg, 0.23 mmol) in methanolic NH_3 (3 mL) was stirred at 22 °C for 14 h, after which the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography (CH_2Cl_2:MeOH; 98:2 to 90:10) to give **^{ExIm}U** as yellowish solid (100 mg, 77% yield). R_f=0.30 (10% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, [D_6] DMSO): δ=13.40 (br s), 11.79 (br s), 8.44 (br s), 8.12 (br s), 7.71 (br s), 6.15 (br s), 5.29 (br s), 5.21 (br s), 4.29 (br s), 3.84 (br s), 3.67 (br s), 2.19 (br s); ^{13}C NMR (100.6 MHz, [D_6] DMSO): δ=161.13, 149.07, 144.05, 130.76, 127.24, 124.14, 97.48, 90.64, 87.30, 84.62, 84.50, 69.57, 60.55; HRMS (ESI): *m/z* calcd. for $C_{30}H_{31}N_5O_6$: 557.2269 [M + H]$^+$; found 557.2285.

5′-Dimethoxytritylated spin-labeled nucleoside (DMT-^{ExIm}U): Spin-labeled nucleoside **^{ExIm}U** (50 mg, 0.09 mmol), DMTrCl (61 mg, 0.18 mmol) and DMAP (1.0 mg, 0.008 mmol) were weighed into a round-bottomed flask and kept *in vacuo* for 16 h. Pyridine (2 mL) was added and the solution was stirred at 22 °C for 2 h. MeOH (100 µL) was added and the solution stirred for 10 min, after which the solvent was removed *in vacuo* to give a crude orange solid. The solid was purified by column chromatography using a gradient elution (CH_2Cl_2:MeOH; 100:0 to 93.5:6+0.5% Et_3N), using a column that was prepared in 99.5% CH_2Cl_2 + 0.5% Et_3N. **DMT-^{ExIm}U** was obtained as a yellow solid (58 mg, 75% yield). R_f=0.65 (15% MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, [D_6] DMSO): δ=8.14 (br s), 7.70 (br s), 7.45 (br s), 7.31 (br s), 6.87 (br s), 6.17 (br s), 6.16 (br s), 5.74 (br s), 5.42 (br s), 4.33 (br s), 3.98 (br s), 3.66 (br s), 3.05 (br s), 2.10 (br s), 1.90 (br s), 1.19 (br s); ^{13}C NMR (100.6 MHz, [D_6] DMSO): δ=161.33, 157.96, 149.19, 144.57, 143.21, 135.43, 135.30, 129.56, 127.84, 127.54, 113.16, 85.99, 85.84, 85.16, 70.31, 63.45, 54.92, 45.32, 40.05, 8.42; HRMS (ESI): *m/z* calcd. for $C_{51}H_{49}N_5O_8$: 859.3576 [M + H]$^+$; found 859.3606.

^{ExIm}U phosphoramidite (7): DMT-^{ExIm}U (25 mg, 0.03 mmol) and diisopropyl ammonium tetrazolide (8 mg, 0.05 mmol) were dissolved in pyridine (1 mL) and the pyridine removed *in vacuo*. The residue was kept *in vacuo* for 19 h, followed by dissolution in CH_2Cl_2 (2 mL) and CH_3CN (1 mL), followed by addition of 2-cyanoethyl N,N,N',N'-tetraisopropyl phosphoramidite (27 mg, 0.09 mmol). The reaction mixture was stirred at 22 °C for 3 h, diluted with CH_2Cl_2 (10 mL) and washed successively with saturated aq. $NaHCO_3$ (3 x 10 mL) and saturated aq. NaCl (2 x 10 mL). The organic solution was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude solid was purified by precipitation by dissolution in CH_2Cl_2 (0.5 mL), followed by addition of n-hexane (50 mL). The liquid was decanted and the operation repeated thrice to furnish phosphoramidite **7** as a yellow solid (24 mg, 78% yield). R_f=0.50 (100% EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.42 (br s), 7.48 (br s), 7.39 (br s), 6.81 (br s), 6.41 (br s), 4.71 (br s), 4.28 (br s), 4.23 (br s), 3.68 (br s), 3.34 (br s), 2.64 (br s), 2.45 (br s), 1.29 (br s), 1.19 (br s), 1.09 (br s), 0.91 (br s); ^{13}P NMR (162 MHz, $CDCl_3$): δ=149.16, 148.81; HRMS (ESI): *m/z* calcd. for $C_{60}H_{66}N_7O_9P$: 1059.4654 [M + H]$^+$; found 1059.4601.

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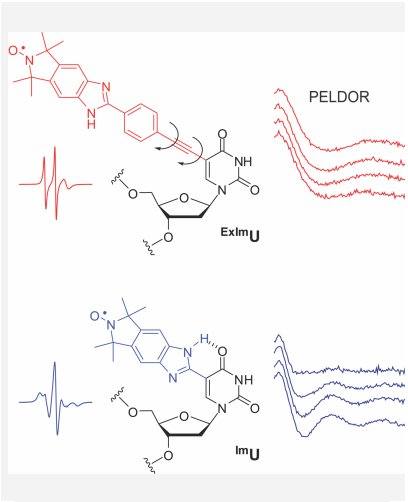
Entry for the Table of Contents (Please choose one layout only)

Layout 1:

Spin labels on an axis and EPR

Dnyaneshwar B. Gophane, Burkhard Endeward, Thomas F. Prisner and Snorri Th. Sigurdsson … Page – Page

Conformationally restricted isoindoline-derived spin labels in duplex DNA: Distances and rotational flexibility by PELDOR



The conformationally unambiguous spin label ^{ExIm}U was prepared and shown by CW-EPR spectroscopy to have a much higher mobility in duplex DNA than the structurally related spin labels ^{Ox}U and ^{Im}U. ^{ExIm}U showed negligible angular dependency in nanometer distance measurements by pulsed EPR (PELDOR/DEER). Thus, ^{ExIm}U can be used for accurate distance determinations at a single detection frequency, while ^{Im}U can be used for orientation-dependent measurements.