

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

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Nucleosides spin-labelled with isoindoline-derived benzimidazole (^{Im}U) and benzoxazole (^{Ox}U) moieties were synthesized and incorporated into DNA oligonucleotides. Both labels display limited mobility in duplex DNA but ^{Im}U was less mobile, which was attributed to an intramolecular hydrogen bond between the N–H of the imidazole and O4 of the uracil nucleobase.

Long-range distance measurements using pulsed electron paramagnetic resonance (EPR) spectroscopy, such as pulsed electron–electron double resonance (PELDOR, also called DEER), are now routinely used in structural studies of biopolymers.¹ The distances are measured between two radicals that are usually incorporated using site-directed spin labelling (SDSL), in which aminoxyl radicals, usually called nitroxides, are linked to specific sites.² The majority of the currently available spin-labelling techniques rely on covalent attachment of the nitroxide using a tether that contains single bonds. Rotation about those single bonds usually results in displacement of the nitroxide relative to the anchoring site. This flexibility decreases the accuracy of distance measurements and renders EPR studies of dynamics more challenging. Therefore, considerable effort has been put into the preparation of spin labels with reduced mobility.³

Optimally, the spin label probe should be immobilized on the biopolymer. Such rigid labels have been prepared for nucleic acids, for example ζ^{3e} and ζm^{3i} (Fig. 1A) for DNA and RNA, respectively. In these cases, the nitroxide moiety has been fused to the nucleobase, which in turn is immobilized in a nucleic acid duplex through hydrogen bonding and base-stacking. These labels have the added advantage of enabling studies of orientation⁴ and dynamics.⁵ Syntheses of these rigid spin labels are non-trivial and, therefore, there is still a need for readily prepared labels with limited mobility.

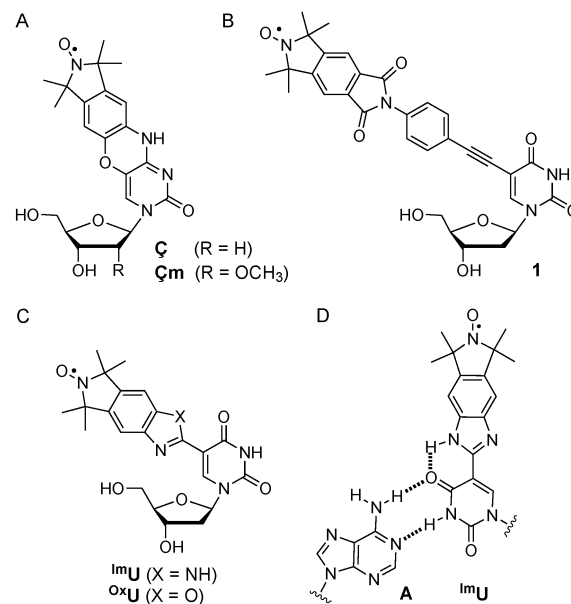


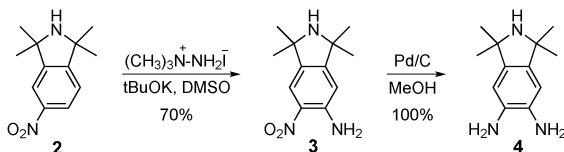
Fig. 1 Spin-labelled nucleosides. (A) Rigid spin labels ζ^{3e} and ζm^{3i} . (B) A nitroxide-derived nucleoside for conformationally unambiguous-spin labelling.⁸ (C) Isoindoline derived spin labels ^{Im}U and ^{Ox}U. (D) Base pairing of ^{Im}U with A. The intramolecular H-bond between the imidazole N–H and O4 of the uracil nucleobase restricts the rotation around the bond connecting the nitroxide to the base.

Godt and coworkers have coined the term “conformationally unambiguous spin labelling” for probes that have internal rotation about single bonds that do not change the spatial positioning of the nitroxide relative to the labelled molecule and demonstrated their usefulness for distance measurements in model compounds.^{3g} Nucleoside **1**^{3g} (Fig. 1B) is one example that illustrates the basis of the design: the N–O bond lies on the same axis as the rotatable bonds and, therefore, rotation around those bonds does not change the position of the nitroxide. However, this nucleoside is not suitable for nucleic acid labelling through chemical synthesis, because the phthalimide functional group is not stable under basic conditions,⁶ required for oligonucleotide deprotection.

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† In memory of Professor Har Gobind Khorana (1922–2011), acknowledging his legacy to the scientific community.

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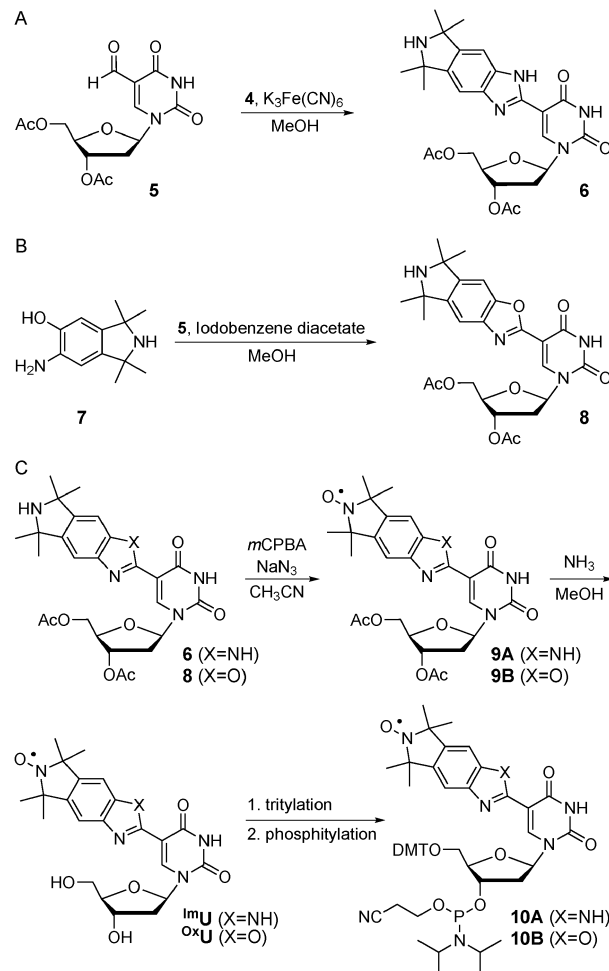
Scheme 1 Synthesis of diamino isoindoline **4**.

In this communication we describe two nucleosides that contain nitroxide spin labels with limited mobility, an isoindoline-derived benzimidazole (**ImU**) and a benzoxazole (**OxU**) (Fig. 1C). In addition to the ease of synthesis, in particular of **ImU**, they enable base-pairing with A, whereas **C** pairs with G. The nitroxides are linked to the nucleobase by a single bond that nearly lies on the axis of the N–O bond and should, therefore, be good probes for distance measurements. We show by continuous wave (CW) EPR that these labels have limited mobility in DNA duplexes. Furthermore, we demonstrate that **ImU** is less mobile than **OxU**, presumably due to an intramolecular hydrogen bond between the N–H of the imidazole moiety and the O4 of the nucleobase (Fig. 1D), which restricts rotation around the single bond linking the label to the base.

The synthesis of **ImU** started with the preparation of 4,5-diamino-1,1,3,3-tetramethylisoindoline⁴ (**4**, Scheme 1), which had previously been prepared in four steps from **2**.⁶ We discovered that **4** could be synthesized in only two steps by direct amination of 5-nitro-1,1,3,3-tetramethylisoindoline (**2**)⁷ with 1,1,1 trimethylhydrazinium iodide, followed by hydrogenation.

Reaction of diamine **4** with 3',5'-di-*O*-acetyl-5-formyl-2'-deoxyuridine (**5**)⁸ in the presence of $K_3Fe(CN)_6$ ⁹ yielded the benzimidazole derivative **6** (Scheme 2A). Oxidation of **6** proved to be somewhat challenging; different oxidizing agents under a variety of conditions led to decomposition of the starting material. We argued that oxidation of the uracil 5,6-double bond could be the cause of the side reaction(s) and might be avoided by inclusion of a nucleophile that could add reversibly to the 6-position.¹⁰ Indeed, sodium azide facilitated *m*CPBA oxidation of **6** to give **9A** in a moderate yield. Deprotection, tritylation and phosphitylation subsequently gave the **ImU** spin-labelled phosphoramidite **10A**. The phosphoramidite of **OxU** was prepared in a similar manner (Scheme 2B and C), except that the isoindoline-derived amino phenol **7**^{3e} was oxidatively coupled to **5** in the presence of iodobenzene diacetate to yield **8** (Scheme 2B).¹¹

The spin labelled nucleosides **ImU** and **OxU** were incorporated into the 14-mer DNA oligonucleotide 5'-d(GAC CTC G^XUA TCG TG) by solid-phase synthesis and purified by denaturing polyacrylamide gel electrophoresis (DPAGE). DPAGE analysis showed that these oligomers migrated slower than the unmodified 14-mer (T instead of ^XU) (Fig. S1, ESI†), consistent with incorporation of the spin labels, which was confirmed by mass spectrometry (Fig. S2, ESI†). Circular dichroism (CD) spectra of the **ImU** and **OxU** containing 14-mer duplexes were consistent with right-handed B-DNA (Fig. S3, ESI†). **ImU** and **OxU** slightly destabilized the DNA duplexes, as judged by a decrease in the melting temperature (T_M) of 4 and 6 °C, respectively.



Scheme 2 (A) Synthesis of benzimidazole derivative **6**. (B) Synthesis of benzoxazole derivative **8**. (C) Synthesis of nucleosides **ImU** and **OxU** and their corresponding phosphoramidites. Yields: **6** (47%), **8** (40%), **9A** (56%), **9B** (50%), **ImU** (77%), **OxU** (80%), **10A** (51%), **10B** (44%).

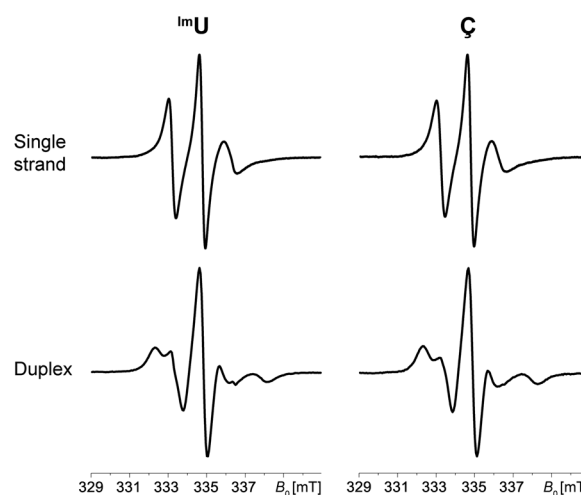


Fig. 2 CW EPR spectra of single stranded (upper) and duplex DNA (lower) containing **ImU** (left) and the rigid spin label **C** (right) at 20 °C (10 mM phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA , pH 7.0).

The EPR spectra of **ImU**-labelled single stranded and duplex DNA are shown in Fig. 2. Broadening of the spectrum upon

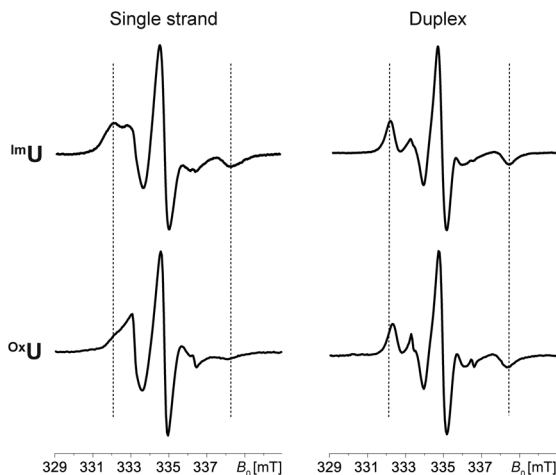


Fig. 3 CW EPR spectra of ^{1m}U - and ^{Ox}U -containing single-stranded (left) and duplex DNA (right) at 0 °C (10 mM phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA , pH 7.0).

duplex formation is striking and indicates that there is limited motion of the label independent of the nucleic acid itself. In fact, at 20 °C the mobility of the ^{1m}U is similar to that of the rigid spin label \dot{C} in the same DNA sequence (Fig. 2) and at –10 °C the spectra of the two become superimposable (Fig. S4, ESI†). We postulate that the low mobility of ^{1m}U is in part due to an intramolecular hydrogen bond between the imidazolic hydrogen and the O4 carbonyl of uracil (Fig. 1D).

To investigate if hydrogen bonding played a role in the limited mobility of ^{1m}U , we compared the spectra of ^{1m}U - and ^{Ox}U -labelled single strands and duplexes (Fig. 3). The only difference between these two labels is that the imidazole N–H of ^{1m}U has been replaced by O in ^{Ox}U . Therefore, ^{Ox}U should be more mobile if hydrogen bonding between the imidazole N–H and the O4 of the uracil reduces the motion about the single bond that connects the nitroxide to the nucleobase. Comparison of the EPR spectra of ^{1m}U - and ^{Ox}U -labelled DNAs shows that there is not much difference between the two labels at 25 °C, while the spectra of the ^{1m}U -labelled DNA are noticeably broader than the spectra of the ^{Ox}U oligomers at 0 °C (Fig. 3). These data clearly indicate that hydrogen bonding between the imidazole N–H and O4 of the uracil contributes towards reducing the motion of the spin label at lower temperatures.

Spin labels with limited mobility can be used to study local structural perturbations in nucleic acids.¹² To determine if EPR could be used to detect base-pairing of ^{1m}U , four DNA 14-mer duplexes were prepared, in which ^{1m}U was paired with either A, T, G or C. Overlay of the four EPR spectra revealed that they could indeed all be distinguished from each other (Fig. S5A, ESI†), although ^{1m}U -G and ^{1m}U -C were similar. Since T–T mismatches are able to form metallo base-pairs with mercuric ions,¹³ it was not surprising that the EPR spectrum of the ^{1m}U -T-pair became nearly identical to that of ^{1m}U -A upon addition of Hg^{2+} -ions (Fig. S5B, ESI†). Thus, ^{1m}U can clearly

detect its base-pairing by EPR spectroscopy, due to the limited motion of the spin label relative to the base.

In summary, we have synthesized novel nitroxide-labelled benzimidazole (^{1m}U) and benzoxazole (^{Ox}U) derivatives of 2'-deoxyuridine as spin probes for nucleic acids. Both ^{1m}U and ^{Ox}U had limited mobility in duplex DNA, in particular ^{1m}U , indicating that rotation around the single bond linking the spin label to the uracil is restricted. This is, to our knowledge, the first example of using intramolecular hydrogen-bonding to restrict spin label mobility. ^{1m}U should not only be a good label for accurate distance measurements in oligonucleotides, but also yield information about the relative orientation of the labels.⁴ Distance measurements by pulsed EPR using these spin probes are in progress and the results will be reported in due course.

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