

A Nucleoside That Contains a Rigid Nitroxide Spin Label: A Fluorophore in Disguise**

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Electron paramagnetic resonance (EPR) is a versatile technique for studying paramagnetic centers, such as paramagnetic metal ions that are bound to biological molecules.^[1] Although many biopolymers do not contain naturally occurring paramagnetic centers, EPR can nevertheless be employed to study their structure and dynamics after incorporation of stable paramagnetic spin labels, such as nitroxides.^[2] EPR has been particularly valuable when applied to the study of biopolymers that have proven difficult to study by other conventional techniques. Even in the presence of high-resolution structural information obtained from NMR spectroscopy or X-ray crystallography, EPR is a highly complementary technique and has, for example, yielded information about conformational changes that are coupled to biological function.^[3] To date, the main applications of EPR spectroscopy of biopolymers have been to ascertain intermediate to long distances,^[4–6] to determine solvent accessibility of individual residues,^[7] to measure dynamics over a range of timescales,^[4,8] and to use dynamics to provide structural insights into ligand binding.^[9] The field of EPR spectroscopy has seen a rapid growth over the last decade as a result of advances in instrumentation, theory, and spin-labeling techniques.^[10]

Investigation of the structure and dynamics of biopolymers by EPR spectroscopy requires incorporation of spin labels at specific sites. Proteins are conveniently labeled at cysteine amino acids that either preexist in the protein primary sequence or are incorporated through site-directed mutagenesis at selected sites.^[2] The labeling of nucleic acids has posed a greater challenge, but several methods have been developed for site-specific labeling at internal sites in nucleic acids, both at the sugar-phosphate backbone^[11] and base.^[6,12]

moieties. However, these spin labels are conjugated to the biopolymer of interest with a tether that has some degree of flexibility, which diminishes the accuracy of EPR-based measurements of distances and molecular motions. Thus, it is of interest to develop rigid spin labels that do not move independently of the biopolymer to which they are attached.^[13] Here we describe the synthesis and characterization of a nucleoside that contains a rigid nitroxide spin label and its incorporation into DNA. In addition to its valuable EPR spectroscopic properties, this label is particularly advantageous because it can be readily converted into a fluorescent probe, thereby enabling its use with two complementary spectroscopic techniques.^[14]

The design of the new spin label **Ç** (“C-spin”) was inspired by phenoxazine derivative **1**, an analogue of deoxycytidine, prepared by Matteucci and co-workers (Figure 1).^[15] Com-

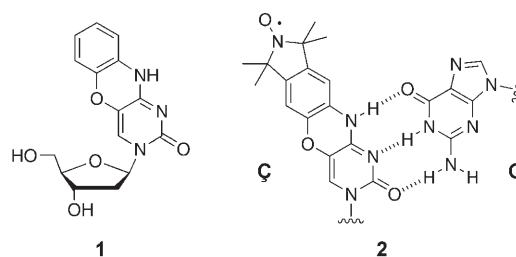


Figure 1. Design of the rigid nitroxide spin label (**Ç**), shown here base-paired with guanine (**G**) as **2**.

pound **1** has been used as a structural scaffold for a “G-clamp”, where a substituent on the terminal aromatic ring formed additional hydrogen bonds upon base-pairing with guanine.^[16] The G-clamp was shown by X-ray crystallography to be readily accommodated in a DNA duplex. In a similar manner, a nucleoside in which a nitroxide-bearing ring is fused to the terminal aromatic ring of **1** is expected to form a base pair with guanine (see **2**) and project the nitroxide into the major groove of a DNA duplex, where it will be fixed in space relative to the framework of a nucleic acid duplex.

The first stage in the synthesis of the rigid spin label was the preparation of isoindoline derivative **6** (Scheme 1). 5-Amino-1,1,3,3-tetramethylisoindoline (**3**)^[17] was converted into the corresponding phenol **4** by diazotization and hydrolysis.^[18] Nitration of **4** gave 5-hydroxy-6-nitro-1,1,3,3-tetramethylisoindoline (**5**), which was subsequently reduced to give **6** in 45% overall yield (four steps).

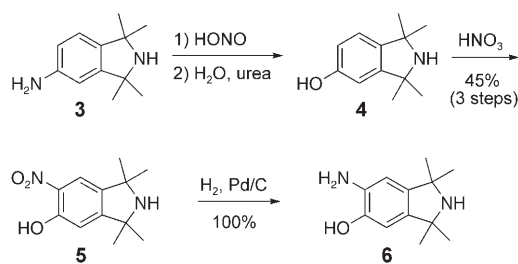
To incorporate the isoindoline moiety into a nucleoside, 3',5'-diacetyl-5-bromo-2'-deoxyuridine was activated with PPh₃ and CCl₄,^[15] followed by reaction with **6** in the presence

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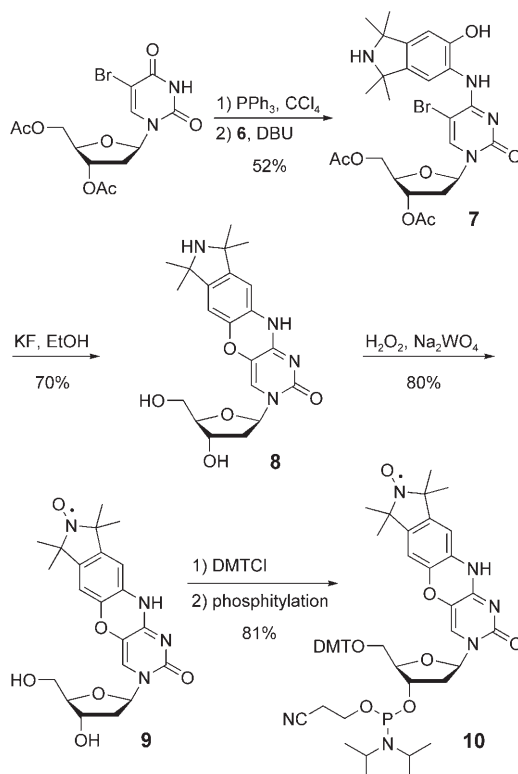
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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.



Scheme 1. Synthesis of isoidolone **6**.

of DBU (Scheme 2). Alternatively, 2',3',5'-triacetyl-5-bromouridine can be used, which would facilitate preparation of a rigid spin label for RNA studies. This one-pot procedure yielded conjugate **7**, which was heated in the presence of KF



Scheme 2. Synthesis of spin-labeled nucleoside **9** and its corresponding phosphoramidite **10** for DNA synthesis. DBU: 1,8-diazabicyclo-[5.4.0]undec-7-ene; DMT: dimethoxytrityl.

to afford the ring-closed phenoxazine derivative **8**. Subsequent oxidation with hydrogen peroxide in the presence of sodium tungstate gave the spin-labeled nucleoside **9**. Protection of the 5'-hydroxy group as a dimethoxytrityl ether and phosphitylation yielded phosphoramidite **10**, which was used for the incorporation of the spin-labeled nucleoside into DNA.

The spin-labeled DNA 14-mer 5'-d(GACCTCG- $\dot{\text{C}}$ ATCGTG) was prepared by automated oligonucleotide synthesis and purified by gel electrophoresis (Supporting Information). Mass spectrometric analysis of the oligomer

verified the incorporation of the modified nucleoside, and EPR spectroscopy confirmed the presence of the nitroxide. EPR spectroscopic analysis of the spin-labeled nucleoside **9** and the spin-labeled oligomer is shown in Figure 2. The EPR

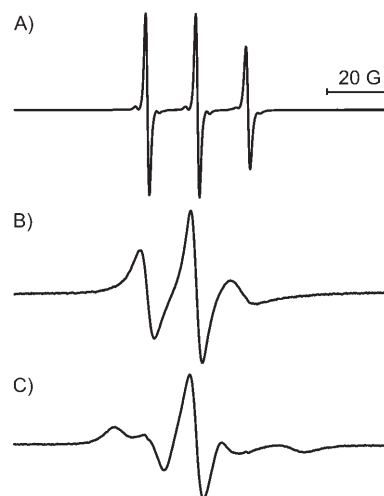
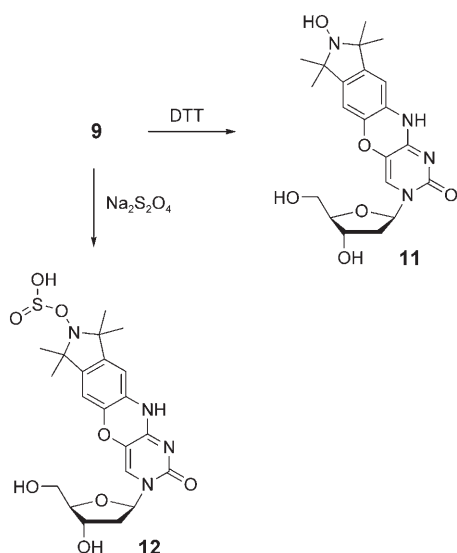


Figure 2. EPR spectra, recorded in aqueous solution (10 mM phosphate buffer, pH 7.0, 100 mM NaCl, 0.1 mM EDTA) at 20°C: A) spin-labeled nucleoside **9**; B) spin-labeled oligomer 5'-d(GACCTCG- $\dot{\text{C}}$ ATCGTG); and C) spin-labeled duplex 5'-d(GACCTCG $\dot{\text{C}}$ ATCGTG)·5'-d(CACGATGCGAGGTC).

spectral width ($2A_{zz}$), which spans the crest of the lowfield peak to the trough of the highfield peak, is sensitive to the motion of the nitroxide; generally speaking, the broader the spectrum, the slower the motion.^[19] The EPR spectrum of **9** (Figure 2A) shows three narrow lines, consistent with rapid isotropic tumbling of the nucleoside in solution, whereas the spectrum of the spin-labeled 14-mer (Figure 2B) reflects slower, more anisotropic tumbling of the spin label within the oligomer. There is a further dramatic change in the EPR spectrum observed upon annealing the spin-labeled DNA to its complementary strand (Figure 2C). In fact, the spectrum is similar to what one would expect for a rigid cylinder with the dimensions of a 14-mer duplex (see Supporting Information). This EPR data indicates the successful design of the spin label: The nitroxide is lodged within the duplex, presumably by forming a stable base pair with guanine and stacking on the neighboring bases. Thermal denaturation studies provided further support for this interpretation; the melting temperature (T_M) of the spin-labeled 14-mer duplex is approximately 1°C lower than that of the unlabeled oligomer, whereas the T_M is 10–15°C lower when $\dot{\text{C}}$ is paired with A, C, or T (data not shown).

Nucleoside **8** was observed to be highly fluorescent, whereas the corresponding nitroxide **9** did not exhibit fluorescence. The lack of fluorescence in the spin label was not unexpected, because nitroxides are known to effectively quench fluorescence.^[20] Since nitroxides are readily reduced, these observations suggested that reduction of the nitroxide spin label with a mild reducing agent would reactivate the fluorescence properties of the probe. In fact, reduction of **9** with either DTT or sodium dithionite yielded fluorescent



Scheme 3. Reduction of the nitroxide functional group with mild reducing agents to afford the fluorescent nucleosides **11** and **12**. DTT: dithiothreitol.

nucleosides **11** and **12**, respectively (Scheme 3). Hydroxylamine **11** was readily oxidized back to the nitroxide upon exposure to oxygen, whereas the sulfurous ester **12** was not affected by incubation with oxygen. Thus, reduction of the spin label within the 14-mer DNA using sodium dithionite gave a fluorescent oligomer (Supporting Information) and provided further evidence for incorporation of the spin label into the DNA. Nucleosides **8**, **11**, and **12** display properties that make them attractive probes for biophysical studies of nucleic acids by fluorescence spectroscopy (Table S1 in the Supporting Information). Their UV absorption maxima lie around 360 nm, away from where the normal nucleotides absorb, and they exhibit a large Stokes shift with fluorescence emission maxima around 450 nm. Even after incorporation into DNA, they exhibit moderate fluorescence; the quantum yields of DTT-reduced single-stranded and duplex DNA were determined to be 0.09 and 0.03, respectively.

In summary, we have prepared a rigid, spin-labeled nucleoside using a convergent synthetic strategy that can also be applied for the synthesis of the corresponding ribonucleoside. EPR spectroscopic analysis of a DNA that contains the rigid spin label verified its limited mobility within a DNA duplex. The rigid nitroxide spin label has several advantages over previously reported spin labels for nucleic acids. First, more accurate distance measurements between two rigid spin labels will be possible than between nitroxides that are linked with a flexible tether. Second, fixing two labels in space, relative to each other, will enable the determination of relative orientations of the two labels and thereby provide more detailed structural information.^[21] Third, the nucleoside becomes fluorescent upon reduction of the nitroxide with a mild reducing agent; this is, to our knowledge, the first example of a spectroscopic probe that can be used for structural studies by both EPR and fluorescence spectroscopies. Furthermore, the dual spectroscopic activity of the spin label enables the preparation of nucleic acids that

contain a redox-active sensor. More detailed characterization of the new bifunctional spectroscopic probe and its application for the studies of the structure and dynamics of nucleic acids will be reported in due course.

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