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OLIGONUCLEOTIDES CONTAINING SPIN-LABELED 2'-DEOXYCYTIDINE AND 5-METHYL-2'-DEOXYCYTIDINE AS PROBES FOR STRUCTURAL MOTIFS OF DNA

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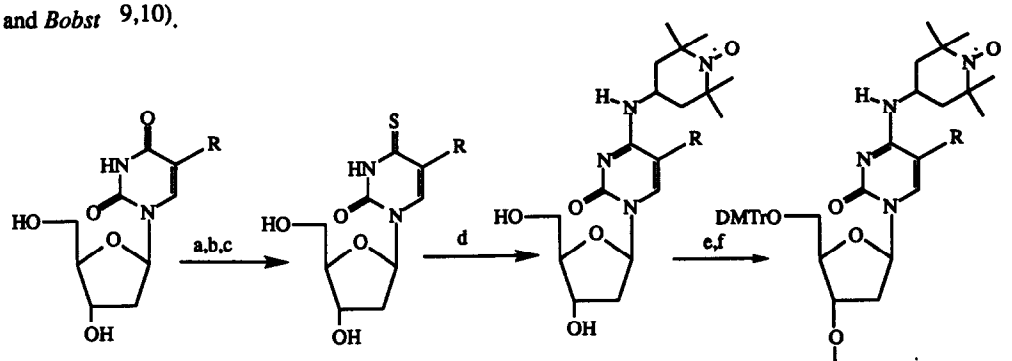
Abstract: The N^4-spin-labeled phosphoramidite of dC and 5-Me-dC can be applied to the specific incorporation of spin labels into synthetic DNA oligomers. In combination with EPR-spectroscopy, such labeled DNA can be used to detect hybridization processes.

The highly specific base-pairing properties of complementary nucleotide strands has found a wide-spread application in the DNA probe technology which can be applied to the detection of pathogens as well as genetic defects based on mutations[1-3]. The detection of the hybridization of a DNA probe to its target is preponderantly performed with radioactive labels. To widen the scope of application one needs to establish alternative reporter systems such as fluorescent molecules or enzymes (e.g. peroxidase and phosphatase) yielding colored or fluorescent products when reacting with appropriate substrates.

To distinguish hybridized from nonhybridized state, the current DNA probe technology is carried out mainly on solid support materials allowing for the removal of unhybridized probe by washing steps like in the well established Southern hybridization [4]. Homogeneous assay formats would eliminate these elaborate procedures. Most of the homogeneous assay formats for DNA detection are based on interactive label molecules, especially donor/acceptor combinations. The energy transfer takes place if two DNA probes, one of which is equipped with the energy donor and the other with the energy acceptor, are hybridizing at abuttal sites on the target [5-8].

It has been demonstrated that proper insertion of a paramagnetic probe in combination with electron paramagnetic resonance (EPR) spectroscopy into a single stranded DNA can be of high diagnostic value. The EPR spectrum of an unhybridized strand can be distinguished from that of the double stranded form in which the spin-labeled linear DNA fragment is engaged in hybridization to its complementary strand as shown by *Hopkins* and *Bobst* [9,10].



a: $(BzlO)_2CO$, pyridine; b: P_2S_5, pyridine; c: NH_3, MeOH;
d: 4-Amino-TEMPO, EtOH; e:DMTrCl, pyridine;
f: $NCCH_2CH_2OP(NPr^i_2)_2$, diisopropylammonium tetrazolide;

1a: R=H

1b: R=CH_3

2a: R=H

2b: R=CH_3

Scheme 1

In the same report it was shown that spin labels at mismatched or bulged positions within a duplex are clearly distinguishable from the ones engaged in perfect Watson-Crick base-pairing. As a consequence, spin-labelled DNA probes could possibly be applied in homogeneous assay formats. It would be possible to compensate for the relatively low sensitivity with amplification methods.

Furthermore, EPR spectroscopy generally provides information about local structure modifications within double-stranded DNA (loops, bulges, mismatches) and could possibly be applied to investigate sequence dependent dynamics of nucleic acids.

A prerequisite for the structural investigations mentioned above is the possibility of site specific insertion of paramagnetic labels into DNA. This can be best executed by the preparation of a spin-labeled building unit which can be incorporated into solid-phase synthesis approaches of DNA fragments, preferably the phosphoramidite procedure. Since the linker which connects the spin label to the DNA has a great influence on the EPR spectra, the linkage should be sufficiently rigidly constrained to correlate its motion with that of the DNA it is attached to.

A preferred postion for the attachment of labels of different kind onto DNA building blocks is the C-5 position of uridine or 2'-deoxyuridine due to its relatively easy accessibility via the Pd-mediated olefination reaction [11,12] or the corresponding propargylation [13]. It has been demonstrated that a modification in this position leads only to a minimal perturbation in B-DNA structures [9,14]. Thus, a number of publications have appeared which have used the insertion of a spin label in the 5-position of dU and used this after chemical or enzymatic incorporation into DNA as indication of structural motifs within the DNA [15-19]. One report had the spin label pendant at one of the internal internucleotide linkages in the form of a phosphamide linkage [20]. All the labels applied were of the nitroxide type.

Our aim was to attach the spin label directly and without any spacer to the 4-position of cytidine which to our knowledge has not been reported. This position seems to be attractive since in B-DNA a non base-paired substituent on the N^4-position of dC protrudes directly into the central space of the major groove and thus the spin label should cause only a minor perturbation of the duplex DNA structure [14,21]. Furthermore the substitution at C-5 of dU has about the same effect on duplex destabilization as the substitution on N^4 of dC [14]. On the other hand positioning the label at the N^4-position via a direct linkage should sufficiently constrain the label and be very indicative for hybridization or structural changes in the DNA.

Commercially available 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino-TEMPO) was chosen as the spin label. The preparation of the spin-labeled phosphoramidites **2a** and **2b** is outlined in Scheme 1. 2'-Deoxyuridine or thymidine was transferred to the 3',5'-di-O-benzoyl derivative. Treatment with P_2S_5 lead to the corresponding 4-thioderivative [22] and further reaction with 4-aminotempo yields directly the spin-labeled nucleosides **1a** and **1b**. After regioselective protection of the 5'-position as 4,4'-dimethoxytrityl ether, the remaining hydroxy was phosphinylated with (2-cyanoethoxy)bis(diisopropylamino)phosphine in the presence of diisopropylammonium tetrazolide to yield the desired phosphoramidites **2a** and **2b** [23,24]. All steps in the reaction sequence proceeded in high yield exept the amination and the DMTr-protection (steps d and e) which has also been observed by others [19]. Primary alkylamines have been reported to react in good yield with 4-thio-dU and 4-thio-thymidine [25]. However, in the reaction with 4-amino-TEMPO the yield never exceeded 28 %. Prior transformation of the 4-thio nucleoside into the corresponding 4-thiocyanatoderivative [26] only slightly improved the reaction (34 %). Compounds **2a** and **2b** were both purified by short-column chromatography and obtained as a pure mixture of diastereoisomers. The structures of compounds **2a** and **2b** were confirmed by ^{1}H-NMR, EPR

and MS. The EPR-measurements of the phosphoramidites **2a** and **2b** also confirmed, that the synthetic manipulations had no influence on the spin-label attached to the N^4-position of dC or 5-Me-dC.

Next we synthesized oligonucleotide **3** by applying spin labeled phosphoramidite **2b** and standard solid phase approach on controlled pore glass as solid support [27]. Gel electrophoresis after deprotection revealed a very efficient incorporation of the spin-labeled phosphoramidite during the solid phase synthesis. EPR measurements with **3** indicated that synthesis, deprotection and purification had no impact on the label. Finally, we have synthesized the spin-labeled oligomers **4** and **5** by inserting the phosphoramidite **2b** and **2a** respectively and the unlabeled DNA fragments **6-8** (Table 1). Oligomers **6** and **7** are the corresponding unlabeled fragments with **7** having dU instead of dC, and **8** is the complementary sequence to **4**, **5** and **6**. After synthesis and deprotection, the oligomers were purified by gel electrophoresis followed by elution.

Melting experiments with these oligomers are demonstrated in Fig. 1. The duplex resulting from the complementary sequences **6** and **8** show the highest melting temperature. Insertion of a GU instead of a perfect matched GC causes only a slight destabilization as shown for the duplex composed of **7** and **8**. The introduction of the spin label into sequences **4** and **5** reduces stability during hybridization to sequence **8** resulting in a further decrease of the melting temperature. The additional methyl group in the spin labeled 5-Me dC as compared to dC seems to be negligible.

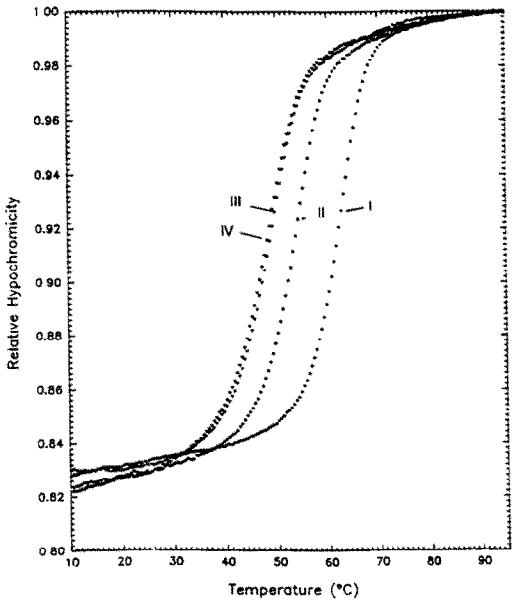


Table 1

3 d(C^{Me}TTTTTTTTT)$_3$'

4 d(CACTGGCC*^{Me}GTCGTTTT)$_3$'

5 d(CACTGGCC*GTCGTTTT)$_3$'

6 d(CACTGGCCGTCGTTTT)$_3$'

7 d(CACTGGCdUGTCGTTTT)$_3$'

8 d(GTGACCGGCAGCAAAA)$_5$'

Fig. 1: Melting profiles of the different hybridizations.
I: **6**+**8**: 62.6 °C; II: **7**+**8**: 54.2 °C; III: **5**+**8**: 49.7 °C;
IV: **4**+**8**: 48.8 °C; 10 mM phosphate pH 7.0; 100 mM NaCl;
0.1 mM EDTA; 2μM oligomer.

The label does not severely alter the helix to single strand transition.The results of the EPR-measurements obtained with the single stranded oligomer **4** and the duplex composed of **4** and **8** are shown in Fig. 2[18]. The EPR spectra of **4** at different temperatures are characteristic for a label undergoing a relatively fast molecular movement. As expected for a single stranded DNA, the line intensity gradually increases with temperature from 20 °C to 85 °C. In contrast to this we observe with the duplex at temperatures below 65 °C relatively broad lines in the EPR spectra indicating a strong restriction of the label mobility as compared to the one observed in **4**.

However, at temperatures above 65 °C the EPR line-widths and intensities resemble that of the single stranded oligomer indicating the separation of the two strands.

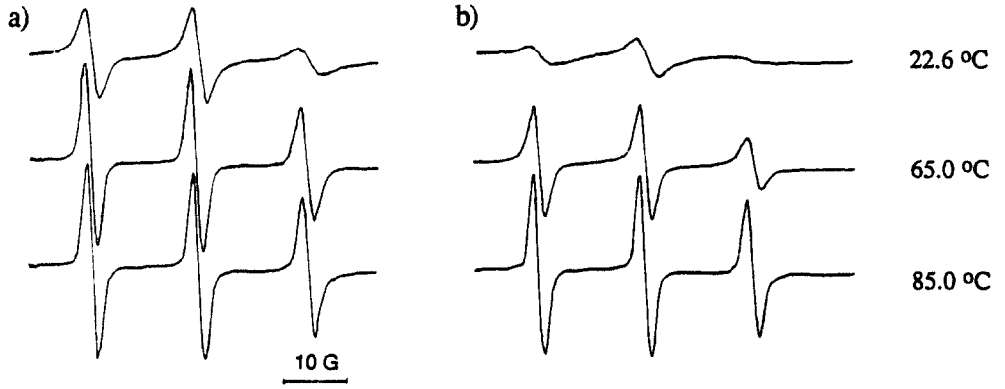


Fig.2: EPR spectra of single-stranded oligomer **4** (a) and of the hybrid composed of **4** and **8** (b). 1 M NaCl; 10 mM phosphate pH 7.0; conc. **4**: 100 μM; conc. **8**: 200 μM.

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