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Synthesis and Characterization of π-Stacked Phenothiazine-Labeled Oligodeoxynucleotides

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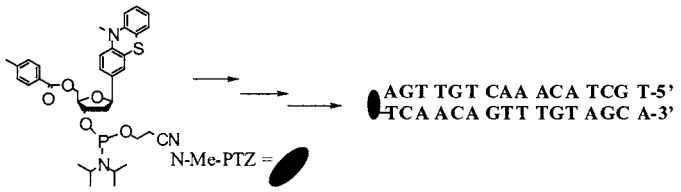
ABSTRACT



A facile procedure for the incorporation of *N*-methyl phenothiazine as the terminal nucleoside in oligodeoxynucleotides is reported. The phenothiazine nucleoside analogue is synthesized and then incorporated into DNA using an automated DNA solid-phase synthesizer. Phenothiazine-labeled oligodeoxynucleotides form stable B-form duplexes with higher melting temperatures compared to unlabeled DNA duplexes.

Incorporation of nonnatural nucleosides into oligonucleotides provides research opportunities for (1) sequencing genomes and identifying genetic abnormalities,[1−11] (2) regulating gene and protein expression through antisense oligonucleotides,[12−17] (3) probing DNA−DNA, DNA−RNA, DNA−protein interactions,[18−23] (4) characterizing charge-transfer reactions in DNA,[24−30] and (5) assessing the role of hydrogen bonding and π-stacking in DNA stability.[18,31−36] As expected, the

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chemical and physical properties of the nonnatural nucleoside dictate the specific use. We are interested in nonnatural nucleosides that are redox active and of potential use as probes for electrochemical-based hybridization assays and for DNA-mediated charge-transfer studies. To date, we have prepared oligodeoxynucleotides labeled at the C5-position of uridine,[37−40] the C8-position of 2′-deoxyadenosine,[41] 5′-terminal phosphate,[42,43] and the 5′-position of thymidine[44,45] with spectroscopic and redox-active chromophores. Nonnatural nucleosides, where the base is completely substituted by a redox probe, are less common. Phenothiazine (PTZ) is a low-potential reductant ($PTZ^{+\bullet}$/PTZ; 0.59 V vs SCE)[46] that possesses a spectroscopically well-characterized one-electron oxidized product, $PTZ^{+\bullet}$.[47,48] This redox probe has also been previously used to study photoinduced charge-transfer reactions.[46,48,49] Herein we describe the synthesis and characterization of a novel phenothiazine-nucleoside analogue and the incorporation of this nonnatural PTZ-nucleoside in oligodeoxynucleotides.

The phenothiazine 2′-deoxynucleoside analogue, 1-(β)-(10-methyl-phenothiazin-3-yl)-5-(*O*-*p*-toluoyl)-2-deoxy-D-ribose, investigated possesses a carbon−carbon linkage (see Figure 1). 3,5-Di-toluoyl-1-α-chloro-2-deoxy-D-ribose, **3**, was

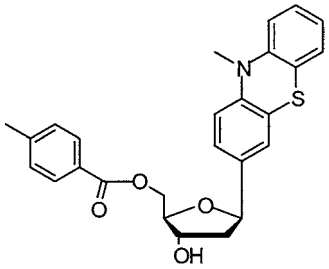
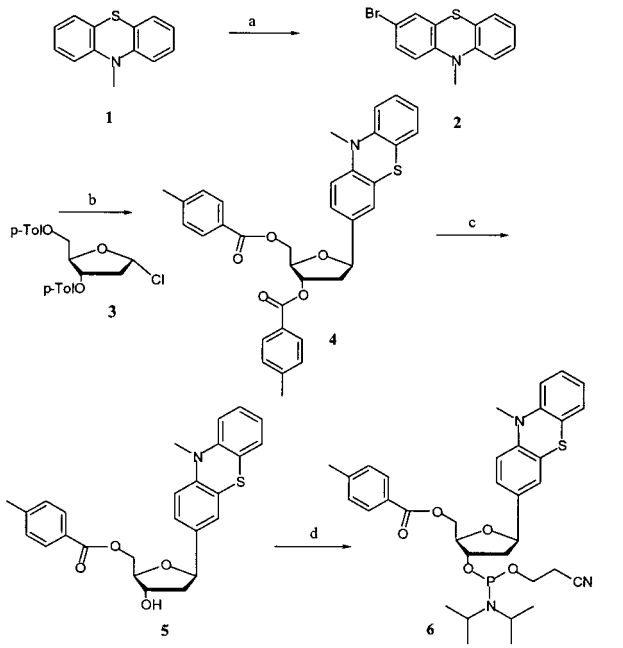


Figure 1. Chemical structure of 1-(β)-(10-methyl-phenothiazin-3-yl)-5-(*O*-*p*-toluoyl)-2-deoxy-D-ribose.

first prepared by treating 2-deoxy-D-ribose with anhydrous HCl gas in methanol for 2 h and then protecting the 3,5-hydroxyl groups with *p*-toluoyl chloride followed by reaction

Scheme 1. Synthesis of a Phenothiazine Nucleoside Analogue Possessing a C−C Linkage[a]



[a] Reagents: (a) bromine, acetic acid/sodium acetate, 27 °C, 2 h, 66% yield; (b) Mg, THF, 25 °C, 1.6 h, 13% yield; (c) NaOMe, MeOH, 25 °C, 1.5 h, 24% yield or (i) NaOMe, MeOH, 25 °C, 8 h and (ii) *p*-toluoyl chloride, pyridine, 25 °C, 16 h, 27% total yield; (d) DIPEA, ACN, 2-cyanoethyl-*N*,*N*′-diisopropyl-chlorophosphoramidite, 25 °C, 1 h, >95% yield (TLC).

with HCl.[50−52] 1-(β)-(10-methyl-phenothiazin-3-yl)-5-(*O*-*p*-toluoyl)-2-deoxy-D-ribose, **5**, was prepared as shown in Scheme 1. Bromination of 10-methyl-phenothiazine, **1**, in acetic acid/sodium acetate buffer gave 3-bromo-10-methyl-phenothiazine, **2**, in 66% yield.[53] Next, the Grignard of phenothiazine was coupled with 1-chloro-3,5-di-(*O*-*p*-toluoyl)-2-deoxy-D-ribose, **3**. Among the various reaction solvents (ethyl ether, toluene, and THF), temperatures (25, 40, and 70 °C), and time scales (1, 2, 6, and 12 h) used for the Grignard reaction, only THF at refluxing temperature, 70 °C, yielded the phenothiazine-derivatized Grignard reagent within 2 h. This reaction condition reflects the relatively lower reactivity of the aryl bromide toward magnesium compared to that of an alkyl bromide. The 1-(α,β)-(10-methyl-phenothiazin-3-yl)-3,5-di(*O*-*p*-toluoyl)-

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2-deoxy-D-ribose was obtained in 57% yield. The α and β anomers were separated by column chromatography with the α anomer being the principal product (75%). The stereochemistry at the anomeric position of **4** was identified by ^{1}H NMR and NOE experiments (see SI for spectra). Additional β anomer could be obtained by acid-catalyzed epimerization of the α anomer following the procedure published by Kool (60−65% yield).[54]

Two approaches were explored to obtain the desired PTZ-nucleoside, **5**. First, both the 5′- and 3′-hydroxyl groups were deprotected. Attempts to protect the 5′-hydroxyl with DMT−Cl afforded the starting material. Thus, the 5′-hydroxyl was selectively protected using 1 equiv of *p*-toluoyl chloride. The product 1-(β)-(10-methyl-phenothiazin-3-yl)-5-(*O*-*p*-toluoyl)-2-deoxy-D-ribose was obtained in 27% yield. Alternatively, **5** can be obtained by removing only one of the *p*-toluoyl groups of **4**. This was accomplished using 0.8 equiv of NaOMe for 1.5 h. TLC detected three new compounds in the reaction mixture. On the basis of ^{1}H NOE experiments, these compounds were identified as the 5′-toluoyl-protected β anomer, 3′-toluoyl-protected β anomer, and unprotected β anomer. The desired product, **5**, was isolated in 24% yield.

As mentioned earlier, we used the ^{1}H NOE technique to assign the stereochemistry at the anomeric, C-1′, position. The NOE is particularly useful in this regard, since one-dimensional spectra may be difficult to analyze given the multiple conformations that the ribose may adopt.[54,55] For example, with the β anomer, irradiation of the H-1′ proton resonances at 5.16 ppm affords NOE on H-2′ and H-4′, while for the α anomer, irradiation of the H-1′ proton resonances affords NOE on H-2′ and H-3′ but not H-4′. Consequently, the α and β anomers of the PTZ-2-deoxynucleoside could be distinguished (see SI for spectra).

The site-specific incorporation of this phenothiazine nucleoside analogue into oligodeoxynucleotides was accomplished using the phosphoramidite method. The free 3′-hydroxyl group of **5** was reacted with 2-cyanoethyl-*N*,*N*′-diisopropylchlorophosphoramidite in dry CH_3CN to afford the phenothiazine-labeled phosphoramidite **6** (see Scheme 1). Standard solid-phase oligodeoxynucleotide syntheses were then performed,[56,57] except that the PTZ-2′-deoxynucleoside phosphoramidite, **6**, was introduced in the last coupling step. Collection and analysis of the DMT fractions during automated synthesis showed efficient phosphoramidite couplings throughout the procedure for the standard pyrimidine and purine nucleosides (>98%). Once synthesized, the PTZ-labeled oligodeoxynucleotides were cleaved from the column, and the nitrogenous bases and phosphate groups were deprotected in 30% ammonium hydroxide at 55 °C for 8 h. Finally, these synthetic oligodeoxynucleotides (see Table 1) were purified by RP-HPLC (overall yield = 28%) and analyzed by MALDI mass spectrometry.

The thermal denaturation profiles for three different PTZ-labeled DNA duplexes (**7·10**, **8·9**, and **7·8**) and one unlabeled duplex (**9·10**) were determined using an optical method. As shown in Figure 2, duplex **7·8**, containing phenothiazine at both duplex ends, possesses the highest melting temperature

Table 1. List of Oligodeoxynucleotides Synthesized and Melting Temperatures

7	5′-PTZ12-TCA ACA GTT TGT AGC A-3′
8	5′-PTZ12-TGC TAC AAA CTG TTG A-3′
9	5′-TCA ACA GTT TGT AGC A-3′
10	5′-TGC TAC AAA CTG TTG A-3′

duplex	$T_m \pm 1$ °C
7·8	55.0
7·10	52.2
8·9	52.1
9·10	48.3

(55 °C). Duplex **9·10**, with no modification on either end, has the lowest melting temperature (48 °C), while duplexes **7·10** and **8·9**, with phenothiazine attached to one strand, have the same intermediate melting temperature (52 °C). Thus, labeling a DNA duplex with this phenothiazine nucleoside analogue, **5**, stabilizes the structure. Labeling a DNA duplex with 5′-amino-phenothiazine[45] or 2-phenothiazin-10-yl-ethanol[43] affords a smaller change in melting temperature compared to the unlabeled duplex. This PTZ nucleobase interacts more strongly with the duplex than the other two PTZ derivatives, and this likely reflects the different attachment mode and relative orientation to the duplex. The 7 °C increase in T_m observed for the duplex possessing two β-PTZ-nucleosides is similar to that observed when two β-benzene-nucleosides are incorporated in an ODN.[33] These data are consistent with a free energy change (ΔΔG°) of 1.0−1.5 kcal/mol.[33]

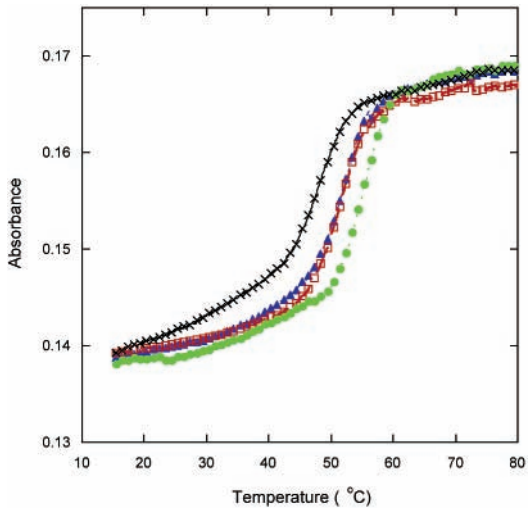

Figure 2. Melting curve of DNA duplexes **9·10** (black), **8·9** (red), **7·10** (blue), and **7·8** (green).

The formation of structurally well-defined DNA duplexes with phenothiazine-labeled oligodeoxynucleotides is also supported by circular dichroism (CD) spectroscopy.[58] The PTZ-labeled (**7·8** and **8·9**) and unlabeled (**9·10**) duplexes possess similar CD spectra (see Figure 3). The characteristic

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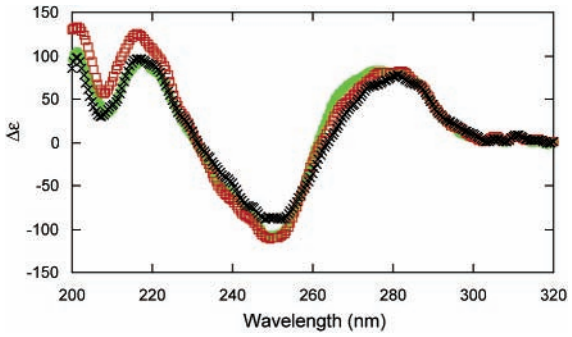

Figure 3. CD spectra of DNA duplexes **9·10** (black), **8·9** (red), and **7·8** (green).

positive and negative absorption at 280 and 240 nm, respectively, indicate B-form DNA for the secondary structure of the DNA duplexes.

The UV−vis absorption spectrum of 1-(10-methyl-phenothiazin-3-yl)-5-(*O*-*p*-toluoyl)-2-deoxy-D-ribose, **5**, contains π-to-π^* and weak π-to-n absorptions at 250 and 315 nm (ϵ = 5000 M^{-1} cm^{-1}), respectively, in methanol. These absorption bands are relatively insensitive to solvent polarity with a λ_{max} change of only a few nanometers between solvents such as methanol and benzene. The absorption bands of PTZ in the labeled duplex are masked by the intense DNA absorption. Excitation of **5** at 315 nm produces a broad emission centered at approximately 451 nm in 4:1 CH_3CN/H_2O and agrees with the reported literature value.[59] Excitation of DNA duplexes **7·8** or **8·9** at 315 nm affords a broad emission centered at 397 nm. For duplex **8·9**, the emission λ_{max} shifted from 397 nm at 25 °C to 437 nm at 85 °C (Figure 4). The temperature dependence of the emission is similar to the absorption melting temperature curves, demonstrating that the PTZ is π-stacked with the duplex. The PTZ emission shifts to the blue upon hybridization consistent with a more nonpolar environment. Similar affects are observed in proteins where the local environment around a chromophore changes.[60] The quantum yields for **5**, **8·9**, and **7·8** were 0.0025, 0.0027, and 0.0019, respectively.

Next, we measured the emission anisotropy for duplex **7·8**. PTZ fluorescence of **7·8** (λ_{ex} = 315 nm) exhibited a steady-state anisotropy of 0.05. Emission polarization of PTZ is in accord with its short fluorescence lifetime (~2 ns)[61] and restricted rotation due to being linked to DNA as opposed to freely rotating in solution. No anisotropy was observed with free PTZ. For comparison, the steady-state fluorescence anisotropy of a fluorescein dye labeled at the 5′-terminus (three-carbon amide linker) of a 33-mer duplex is 0.09.[62]

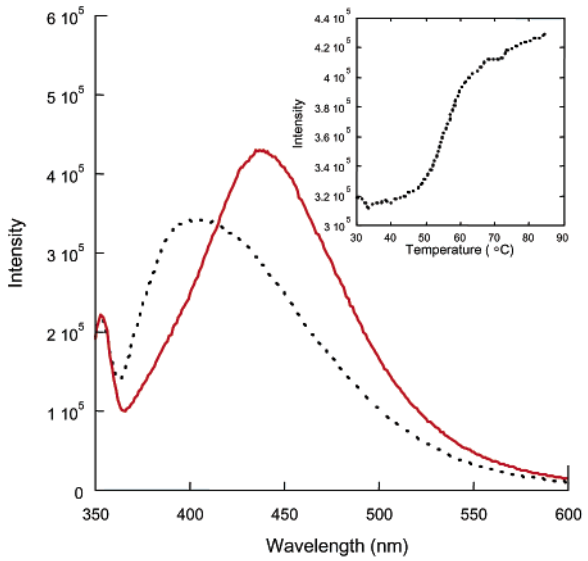

Figure 4. Emission spectra for **8·9** at 25 °C (black) and 85 °C (red) in 5 mM phosphate buffer 50 mM NaCl, pH 7. (Excitation at 315 nm) Inset: temperature dependence for the emission at 437 nm.

The anisotropy data are consistent with restricted rotation of the PTZ at the duplex end.

The synthesis of 1-(10-methyl-phenothiazin-3-yl)-5-(*O*-*p*-toluoyl)-2-deoxy-D-ribose and the successful incorporation of this C-nucleoside in ODNs are described. The PTZ-labeled oligodeoxynucleotides form stable B-form DNA duplexes with complementary sequences, and these duplexes have elevated melting temperatures compared to unlabeled duplexes. The photophysical properties of the phenothiazine-nucleoside are sensitive to duplex formation, and the PTZ is π-stacked with the DNA duplex. This work as well as work by others in this area demonstrates that a wide variety of structurally and electronically different nonnatural nucleosides can be incorporated into DNA at various locations. Such derivatives are of potential use for mechanistic biochemical and biophysical studies as well as for therapeutic applications.

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Supporting Information Available: Detailed synthetic procedures and ^{1}H NMR and NOE spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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