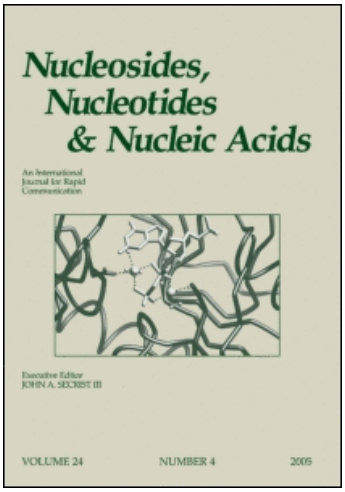

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CHEMICAL STABILITY OF 2′-DEOXY-5-METHYLISOCYTIDINE DURING OLIGODEOXYNUCLEOTIDE SYNTHESIS AND DEPROTECTION

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ABSTRACT

Previous studies have encountered difficulties with degradation of some isocytidine derivatives during solid-phase synthesis and deprotection of oligonucleotides. Here we investigate the degradation of a commonly used derivative, 2′-deoxy-5-methylisocytidine, during oligodeoxynucleotide synthesis and deprotection. A small, but detectable amount of hydrolytic deamination occurred at ca. 0.5% of 2′-deoxy-5-methylisocytidine residues using routine synthesis and deprotection conditions. Depyrimidination, or cleavage of the glycosylic bond, occurred to a far lesser extent during alkaline deprotection than previously suggested. In contrast to model studies of nucleoside monomers, significant depyrimidination was not observed, even at extended incubation times.

Key Words: Isocytosine; 5-Methyl-2′-deoxycytidine; Depyrimidination; Deamination

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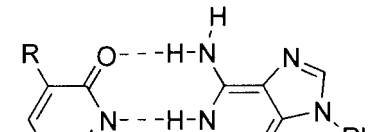
INTRODUCTION

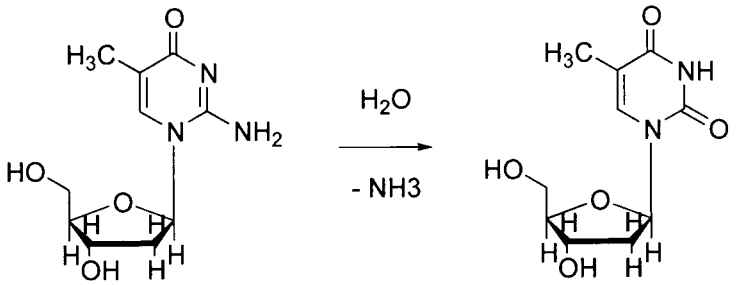
Nucleoside analogs bearing unnatural hydrogen bonding patterns have been proposed to expand the number of complementary nucleoside pairs available in nucleic acids.[1,2] Several nucleoside pairs with complementary hydrogen bonding patterns have been introduced into oligonucleotides.[3–5] The most studied of these analogs is the isocytidine-isoguanosine (isoC-isoG) pair (Fig. 1), which has extended the molecular recognition capacity of natural DNA as an additional nucleoside pair orthogonal to the two pairs found in nature. The pair has enabled polymerase-catalyzed replication of a six-letter genetic alphabet[3,6,7] and allowed ribosome-based translation with a novel codon-anticodon pair.[8] Hybridization of duplex DNA with isoC-isoG pairs retains discrimination analogous to natural DNA.[9–11] The potential for reduction of background signal with a six-letter alphabet has been exploited in a branched DNA assay employing oligonucleotides containing the isocytidine-isoguanosine pair.[12]

Despite the significant structural similarities that isoC and isoG share with natural nucleosides, use of this pair in automated solid-phase synthesis of oligonucleotides has required particular attention to protecting groups, and has occasionally involved slight alteration of reagents, synthesis cycles, and deprotection conditions.[9,10,13–15] Especially problematic has been the chemical instability of isoC derivatives noted during automated DNA synthesis and deprotection.[3,6,9,16] Because of the complexity of oligonucleotides, stability studies have utilized monomer nucleosides to model the behavior of isoC in oligonucleotide strands.[6,10,11,14,15] While investigations of monomer compounds are more easily executed and interpreted, they may inaccurately predict the properties of the corresponding compounds incorporated into oligonucleotides. In the present work the integrity



R = CH_3, H
R' = 2'-deoxyribose

Figure 1. An isoC-isoG pair in duplex DNA can form a three hydrogen bond recognition pattern distinct from C-G and T-A pairs. The 5-methyl (R = CH_3) derivative of isoC was used in this study.



Scheme 1.

of the most commonly used isoC derivative, 2′-deoxy-5-methylisocytidine (d^{Me}isoC), was examined after authentic oligonucleotide synthesis and deprotection, specifically to determine the extent of degradation resulting from these processes.

Two modes of degradation, deamination and depyrimidination, have often been observed for isoC derivatives. Hydrolytic deamination was first reported in solid-phase syntheses with 2′-deoxyisocytidine[3] and 5-methylisocytidine.[16] However, deamination products have been appreciably suppressed in syntheses with 2′-deoxy-5-methylisocytidine.[7,9,14,15] Similar stabilization against deamination by the 5-methyl moiety of 2′-deoxy-5-methylcytidine is well-known.[17] Significant deamination of d^{Me}isoC monomers to thymidine (Sch. 1) has not been reported, even after extended incubation in alkaline deprotection conditions.[14,15] Depyrimidination, or cleavage of the glycosylic bond of isocytidine derivatives has been reported to occur during acidic detritylation and alkaline deprotection conditions in oligonucleotide synthesis. While isocytidine has been reported as relatively insensitive to treatment with either acid or base,[11] it has been generally observed that disoC and d^{Me}isoC monomers are susceptible to depyrimidination under acidic[9,11,14,15] and alkaline conditions.[11,15,18] Depyrimidination of d^{Me}isoC in oligonucleotides under acidic conditions has also been noted.[9] Significantly, depyrimidination of d^{Me}isoC in oligonucleotides under alkaline conditions has not been reported.

RESULTS AND DISCUSSION

Deamination

Deamination of 2′-deoxy-5-methylisocytidine within an oligonucleotide strand was measured in two distinct experiments. First, a novel design allowed analysis of the deamination of d^{Me}isoC in an intact oligonucleotide. An oligonucleotide sequence (**1**) was designed to form a hairpin (stem-loop) structure during capillary electrophoresis (CE) under modestly denaturing

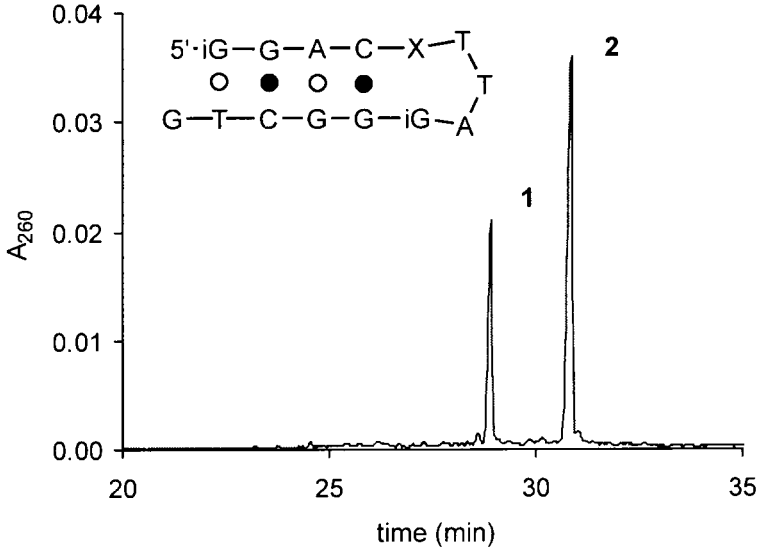


Figure 2. Oligonucleotides **1** and **2** (X = isoC and X = T, respectively) display a large difference in electrophoretic mobility. The difference is presumably due to stabilization of a hairpin structure through an isoC-isoG pair possible in **1**. Watson-Crick pairs (filled circles) and mispairs (circles) are indicated.

conditions. The oligonucleotide was empirically constructed such that the hairpin structure would be significantly disrupted by a change in a single pairing interaction; if the d^{Me}isoC in **1** is deaminated to thymidine, the resulting oligonucleotide (**2**) exhibits a substantial reduction in migration during CE (Fig. 2). The large shift in mobility allowed relative quantification of intact versus degraded d^{Me}isoC in the oligonucleotide.

The extent of 2′-deoxy-5-methylisocytidine conversion to thymidine was then corroborated by determination of the relative nucleoside composition of oligonucleotides. A 14mer oligonucleotide (**3**) containing only deoxycytidine and 2′-deoxy-5-methylisocytidine nucleosides was synthesized. Any T present in syntheses of **3** can only arise as a consequence of the deamination of d^{Me}isoC. Another 14mer (**4**), identical except for a single thymidine in place of one of the deoxycytidine nucleosides in **3**, was prepared to mix with **3** to form a standard curve. The synthetic oligonucleotides were enzymatically digested and absorbance corresponding to the component nucleosides was determined using reversed phase HPLC.

Oligonucleotides **1**–**4** were examined after three different deprotection incubation times in concentrated ammonium hydroxide (Table 1). Unlike previous work, a small amount of deamination of d^{Me}isoC was detected and quantified in the two experiments. Despite involving measurements at the limit of quantification of the experimental techniques,[19] the numbers were in reasonably good agreement at the two longer incubation times. Both

Table 1. Deamination of d^{Me}isoC After Solid Phase Synthesis and Deprotection[1] of Oligonucleotides at Various Incubation Times

Oligonucleotide	Method	24 h	48 h	72 h
1	Capillary Electrophoresis	1.5–1.9%[2]	0.58–0.72%	1.0–1.3%
3	Nucleoside Composition	0.45%	0.93%	1.2%

[1]In concentrated NH_4OH at 60°C.
[2]The incongruously high value at 24 h likely indicates incomplete deprotection.

experiments demonstrate that deamination of d^{Me}isoC during standard synthesis and deprotection conditions is minor. Even an extended incubation of 48 h caused deamination in less than 1% of d^{Me}isoC residues.

The apparent anomaly in the divergent measurement at 24 h incubation may be explained by a limitation of the electrophoresis experiment. Because a number of full-length side products, such as oligonucleotides with incomplete removal of a protecting group or degradation of a nucleoside other than d^{Me}isoC, may be unresolved from **2** in the electrophoresis, the amount of less mobile oligonucleotide represents an upper limit for the formation of any individual side product. The full-length side product diminished from 24 to 48 h, implying that much of the full-length side product at 24 h resulted from a small number of residual protecting groups, which decreased with longer incubation periods.

Depyrimidination

Oligonucleotides of less than full-length are the anticipated result of depyrimidination, as abasic sites resulting from depyrimidination are cleaved under alkaline deprotection conditions.[20] Unlike earlier experiments with d^{Me}isoC monomers,[11,15] CE analysis of oligonucleotides **1**–**4** demonstrated that d^{Me}isoC in all four oligonucleotides did not undergo significant depyrimidination at extended deprotection times. If the d^{Me}isoC in these oligonucleotides had degraded at a rate similar to the monomers, each d^{Me}isoC position would suffer ca. 20% depyrimidination over 72 h. Obviously, 20% depyrimidination at the six possible sites in **3** and **4** would result in very little full-length product and substantial amounts of shorter cleaved oligonucleotides. In fact, the fraction of A_{260} from shorter oligonucleotides may have increased only slightly during incubation from 24 to 72 h (Fig. 3).

CONCLUSION

The negligible deamination of d^{Me}isoC measured here during oligonucleotide deprotection (ca. 0.5% at 24 h) resembles previous results with

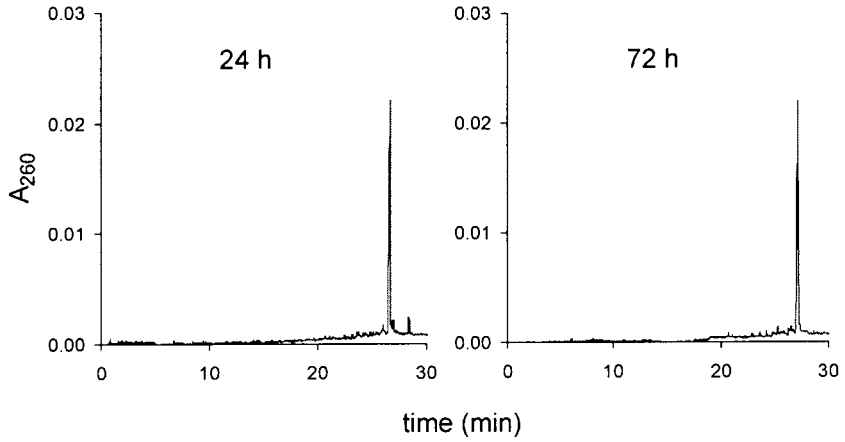


Figure 3. CE analysis of oligonucleotide **3** after incubation with standard deprotection conditions (conc. NH_4OH, 60°C) for 24 and 72 h. Significant depyrimidination would cause excessive cleavage of **3**, which was not observed during incubation to 72 h.

d^{Me}isoC monomers, in which little or no deamination was reported.[14,15] This small amount of deamination of d^{Me}isoC is inconsequential for many oligonucleotides containing d^{Me}isoC, such as those used as probes[12] or in primer extension experiments.[3,6,7] However, if d^{Me}isoC is to be used as a component of a six-nucleobase *amplification* system, even such a small amount of deamination may be important. If a polymerase displays bias against replicating d^{Me}isoC (e.g., pausing at d^{Me}isoC template positions), deamination of d^{Me}isoC to thymidine in a template could give rise to sequences that are preferentially amplified. The resulting amplification products that are depleted in d^{Me}isoC may then appear to be the result of polymerase infidelity,[3,6] instead of contamination of the initial oligonucleotide template. Our studies suggest that in syntheses where maximum suppression of deamination is desired, more rapidly cleaved protecting groups on d^{Me}isoC may be advantageous.[15]

In contrast, previous depyrimidination measurements on d^{Me}isoC monomers had little predictive value for oligonucleotides. No significant depyrimidination of d^{Me}isoC was observed during 24 to 72 h incubations. The reduced depyrimidination of d^{Me}isoC in oligonucleotides vis-à-vis d^{Me}isoC nucleosides parallels depurination of 2′-deoxyadenosine under acidic conditions. Considerable stabilization against depurination was provided by 3′ and 5′ phosphotriester substitution of 2′-deoxyadenosine, presumably through an electron withdrawing effect.[21] The absence of depyrimidination means the choice of protecting groups for d^{Me}isoC need not be routinely restricted to avoid depyrimidination during extended alkaline deprotection times.[15]

EXPERIMENTAL SECTION

Oligodeoxynucleotides **1**–**4** containing d^{Me}isoC and disoG were synthesized using the 3′-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidites of 5′-dimethoxytrityl-N2-di-n-butylaminomethylidene-5-methyl-2′-deoxyisocytidine and 5′-dimethoxytrityl-O2-diphenylcarbamoyl-N6-diisobutylaminomethylidene-2′-deoxyisoguanosine. The oligonucleotides were individually synthesized[9,10,14] on an ABI 394 synthesizer and split into three portions. The portions were deprotected in ammonium hydroxide solution at 60°C and were individually removed from heating after 24, 48, or 72 h. The solvent was removed under vacuum and the residue was redissolved and ethanol precipitated. Oligonucleotide identity was verified by MALDI-TOF on a PerSeptive Biosystems Voyager-DE mass spectrometer using a 3-hydroxypicolinic acid matrix. Nucleoside composition was determined by digestion with snake venom phosphodiesterase I (Amersham Pharmacia Biotech) and alkaline phosphatase (Roche Molecular Biochemical). The resulting nucleosides were separated by reversed-phase HPLC (Hewlett Packard Series 1100 with diode array detector and Rainin Microsorb MV C-18, 3 μm, 4 × 100 mm column) using a gradient of ammonium acetate, pH 7.0 and acetonitrile. CE was performed at 40°C using a polyacrylamide-filled capillary containing 7 M urea on a Beckman P/ACE System 2050.

Oligonucleotides 1 and 2

A sample of oligonucleotide **1** was spiked with **2** and used to determine that **2** trailed **1** by a substantial 1.9 min in CE analysis. The syntheses of oligonucleotide **1** incubated at each of the three deprotection times were analyzed by CE and the integrated absorbance at 260 nm of peaks corresponding to **1** and **2** in the individual samples were quantified. The relative amount of **2** found in each crude synthetic sample of **1** was calculated as a range to account for any hypochromic effect in ''duplex-like'' oligonucleotide **1**. First, the amount of deamination was calculated assuming identical extinction coefficients for **1** and **2**, and then with an extinction coefficient for **1** reduced by 20% relative to **2**.[22]

Oligonucleotides 3 and 4

Samples of oligonucleotides **3** and **4** (0.2 A_{260} units) were completely digested to component nucleosides with snake venom phosphodiesterase I and alkaline phosphatase. The digested samples were analyzed using reversed phase HPLC, and the areas under the peaks corresponding to T, dC and d^{Me}isoC were quantified. No trace of deamination of dC to give

2′-deoxyuridine was observed under these conditions. Dividing the integrated absorbance at 260 nm associated with the T nucleoside peak (A_T) by the corresponding absorbance of the 2′-deoxycytidine nucleoside (A_C), the ratio A_T/A_C was obtained for each oligonucleotide sample. A range of A_T/A_C ratios was generated with the 24 h incubation samples by diluting a solution of **4** with a solution of **3**. The data were corrected for conversion of a small amount of d^{Me}isoC to T (any T found in digests of **3**) and the different numbers of dC residues in **3** and **4**. An equation describing a regression line best fitting the data ($R^2 = 0.9998$) was obtained for the measured absorbance ratio A_T/A_C vs. the molar ratio n_T/n_C.[23] The ratio n_T/n_C for **3** was then calculated from the observed A_T/A_C ratio, and the proportion of d^{Me}isoC residues suffering deamination was determined from the ideal ratio n_C/n_{isoC} for **3**.

SEQUENCES

1: d-5′-iGGA CiCA TTiG GGC TG
2: d-5′-iGGA CTA TTiG GGC TG
3: d-5′-iCiCC CCiC iCCiC CCiC C
4: d-5′-iCiCC TCiC iCCiC CCiC C
iC = 5-methylisocytidine
iG = isoguanosine

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19. A series of solutions with a small amount of T spiked into samples with a 7:6 molar ratio (as in **3**) of dC to d^{Me}isoC (total 0.2 A_{260} units) were injected on the HPLC system. With ε_{isoC} as 6300 $M^{-1}{\cdot}cm^{-1}$[7], the limit of identification of the T peak UV spectrum was estimated at ca. 0.5% for n_T/n_{isoC}.

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23. The slope of this line is the ratio of the extinction coefficients of dC and T ($\varepsilon_C/\varepsilon_T$). If ε_C and ε_T at pH 7 are taken as 7600 and 8800 $M^{-1}\cdot cm^{-1}$, respectively[15], $\varepsilon_C/\varepsilon_T$ is 0.86, reasonably close to the measured slope of 0.9069. Alternatively, using a plot of n_T/n_{isoC} against A_T/A_{isoC} after solving through iteration yields deamination virtually identical to the results in Table 1. The slope of this line ($\varepsilon_{isoC}/\varepsilon_T$) can be used to estimate ε_{isoC} at 260 nm for d^{Me}isoC. If ε_T at pH 7 is taken as 8800 $M^{-1}\cdot cm^{-1}$, ε_{isoC} is 6500 $M^{-1}\cdot cm^{-1}$, in agreement with previously used values of 6300 $M^{-1}\cdot cm^{-1}$ (7) and 6100 $M^{-1}\cdot cm^{-1}$[15].

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