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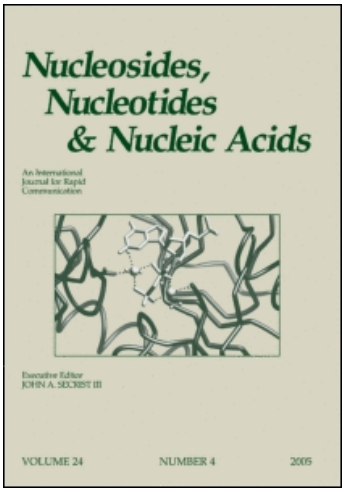

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THE SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING FLUORO-2'-DEOXYCYTIDINE FOR SECONDARY STRUCTURE DETERMINATION OF TANDEM TETRALOOP DNA ANALOGS

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ABSTRACT: A method to convert 5-FdU phosphoramidite to 4-triazol-5-FdU phosphoramidite was used to make deoxyoligonucleotides containing 5-FdC to follow a rare transition between a tandem hairpin and a single hairpin form, which results in moving a distant 5-FdC, 5-FdU bases into juxtaposition.

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Fluorinated pyrimidine analogs can provide a window into local DNA structure using ^{19}F NMR. 5-Fluorodeoxyuridine (5-FdU) has been used extensively for this purpose in looking at interactions between the fluorinated *lac* operator and the *lac* repressor[1], tRNA structure[2] and the structure of the T7 polymerase promoter[3]. 5-Fluorodeoxycytidine (5-FdC) has been used to study the mode of action of several methylases[4-8]. We have chemically synthesized DNA containing 5-FdC to probe DNA structure, as well as DNA-protein interactions.

In the past, most 5-FdC containing DNA was labeled through enzymatic means, which can be difficult and tedious. First 5-FdC would have to be chemically synthesized[9,10,11] and then converted to either di- or triphosphates for enzymatic synthesis[4] or protected, depending on the interest of the project[4,12]. Automated phosphoramidite chemistry would be more desirable for 5-FdC incorporation, so that 5-FdC could be placed at specific sites. However, incorporating 5-FdC synthetically into DNA has been

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very difficult due to the lability of the substituted base in protecting the amino group[4]. Because of this, many different approaches to the synthesis of 5-FdC phosphoramidite have been attempted with different protecting groups. For example, in MacMillan, et al. [12] the phosphoramidite TMP-FdU was synthesized, using a trimethylphenyl (TMP) group as the amide protecting group, while Schmidt et al.[13] uses a benzoyl group.

In this paper, the procedure from Xu, et al.[14] in which they use a post-synthetic method to synthesize DNA containing dC and 5-Me-dC from triazol-deoxyuridine and triazol-thymidine (respectively) phosphoramidite was applied to synthesize DNA containing 5-FdC with 4-triazol-5-FdU phosphoramidite as outlined in Figure 1.

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4-Triazolo-5-FdU phosphoramidite (**2**) was prepared from commercially available 5-FdU phosphoramidite (**1**) in high yield using the method for 4-triazolothymidine phosphoramidite preparation as described earlier [14]. The purity assessed by TLC was >95%. The coupling efficiency was estimated to be above 95% for the modified nucleotide incorporated into the oligonucleotide.

As a test, a pentadeoxynucleotide GCC^{F}AT (C^F = 5-FdC) was synthesized using standard 1 micromole scale phosphoramidite chemistry. The protected pentamer was cleaved from the controlled-pore-glass (CPG) support with concentrated aqueous ammonia at room temperature and heated to 55 °C for 6 hours (or room temperature for 24 hours) to substitute the 4-triazolo with an amino group and remove all the protected groups from the oligo (Figure 1). The crude oligonucleotide was purified by HPLC and the purity was confirmed by HPLC (see figure 2).

After purification by HPLC, the pentamer was digested with snake venom phosphodiesterase and bacterial alkaline phosphatase to generate individual nucleosides, and the products were resolved using HPLC. As can be seen in figure 3B, the enzymatic digestion of the pentamer resulted in five distinct peaks in the HPLC trace. Peaks 1, 4, 5, and 6 correspond to the non-modified nucleosides and peak 2 being the proposed 5-FdC (as seen in figure

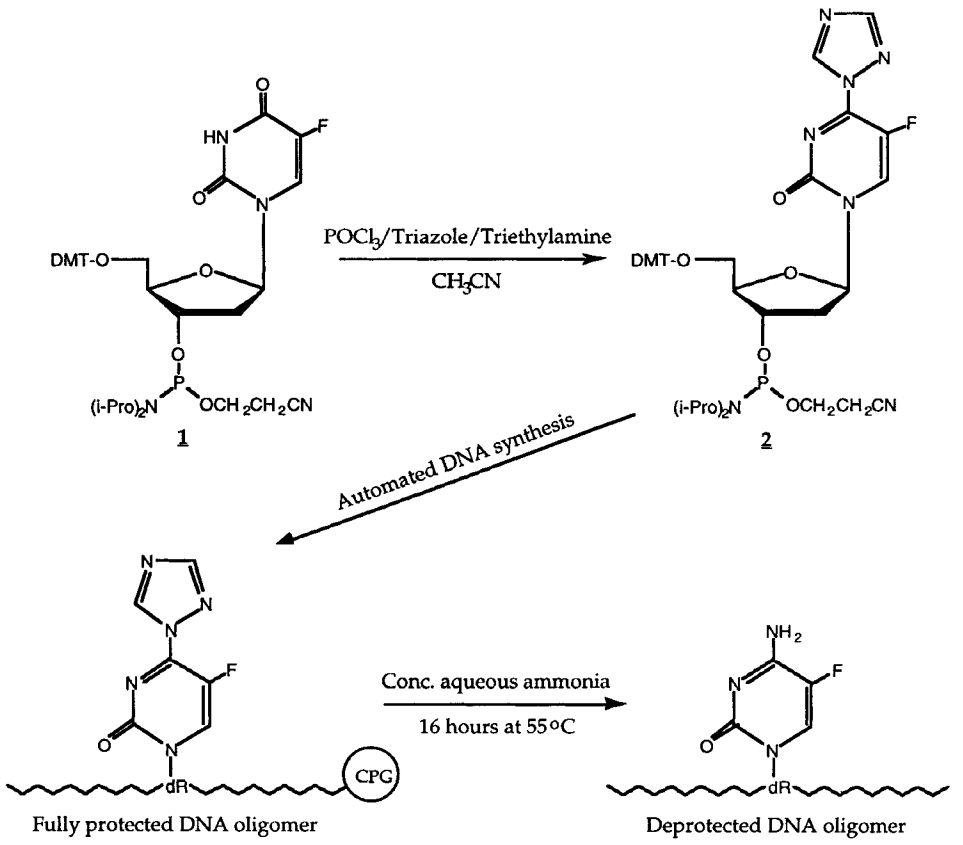

FIGURE 1 : The synthesis of 4-triazol-5-fluorodeoxyuridine phosphoramidite (2) and DNA containing 5-FdC. (1): 5-fluorodeoxyuridine phosphoramidite.

3A). The second peak was isolated and collected. It was confirmed to be 5-FdC by ^{1}H NMR and UV. A small peak was seen in the HPLC profile after 5-FdC eluted and it seems to correspond to where 5-FdU elutes.

Presently, a 32 base-pair *lac* promoter and adenovirus major late promoter sequence containing 5-FdC at specific sites was prepared by the method outlined here for ^{19}F NMR studies on the structure of DNA around a promoter site using by the method of Rastinejad, et al. [3]. Base composition analyses of selected 32-mers showed incorporation of 5-FdC (data not shown). This contradicts MacMillan, et al. [12], who stated that the triazolyl derviative

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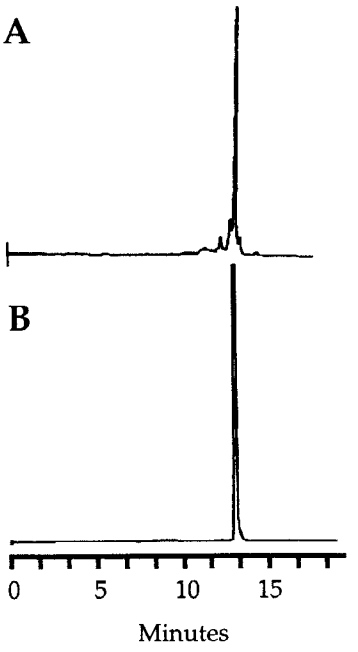

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FIGURE 2 : A. The HPLC trace of the crude 5-mer, GCC^{F}AT (C^F = 5-FdC) after complete deprotection with concentrated ammonia solution. **B.** The HPLC trace of the purified 5-mer, GCC^{F}AT (C^F = 5-FdC). The fully deprotected trityl-off 5-mers were purified by HPLC using a Beckman Ultrasphere C18 column (10 x 150 mm, 2.5 mL/min) with a gradient as follows: 0% to 30% buffer B in 20 minutes, 30% to 50% buffer B in 5 minutes, 50% buffer B for 5 minutes and back to 100% buffer A in 5 minutes. The buffers were as follows: buffer A: 2.5% CH_3CN/ 0.1 M ammonium bicarbonate; buffer B: 50% CH_3CN/ 0.1 M ammonium bicarbonate..

of 5-FdU was unstable toward acid and was therefore deemed unsuitable for automated DNA synthesis. Based on our results, the triazolyl group on the 5-FdC substituted DNA is no more sensitive to trichloroacetic acid during automated DNA synthesis than other base protected groups.

To illustrate the utility of this FdC (C^F) synthesis, we have incorporated both FdU (U^F) and FdC into DNA analogs of a RNA sequence which forms a hairpin by solution NMR[15,16] and a double helical conformation by X-ray diffraction[17]. To establish the double helical form with the unusual C-U base-pairs is present in solution as has been seen with the RNA sequence[18], we

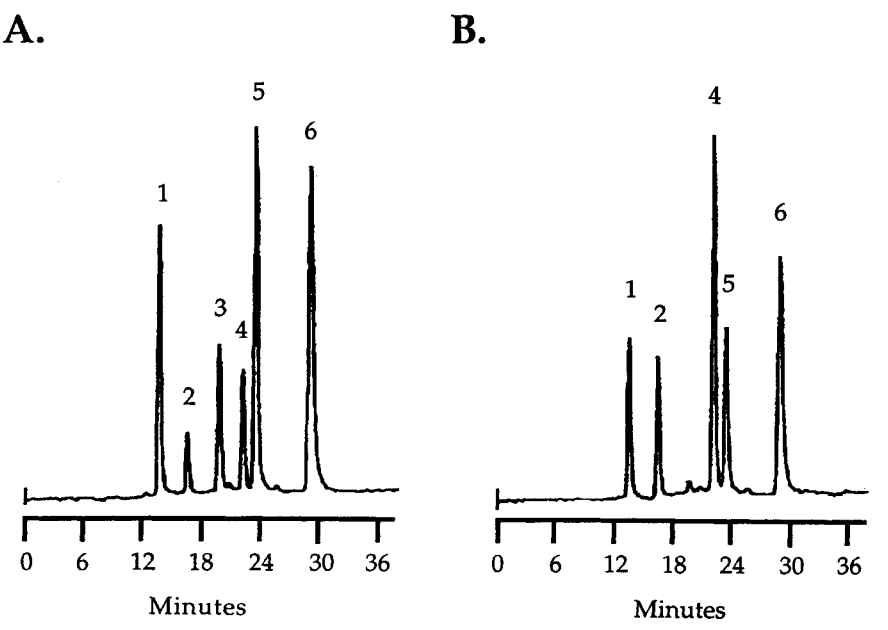

FIGURE 3: **A**. HPLC trace of deoxyribonucleosides and analogs of deoxyribonucleosides. dA, dC, dG, dT and 5-FdU nucleosides were purchased. 5-FdC nucleoside came from digestion of the 5-mer, GCC^{F}AT (C^F = 5-FdC). **B**. HPLC trace of the digested 5-mer oligonucleotide, GCC^{F}AT (C^F = 5-FdC). All peak labels in this figure are: 1=dC, 2=5-FdC, 3=5-FdU, 4=dG, 5=dT and 6=dA.

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have situated the FdC and the FdU to see J coupling between the two fluorines so that the distance between them is approximately 9 Angstroms when the two tandem tetraloops (see III on figure 4) form one hairpin with the C^F-U^F mismatch. Fluorine is known to have long range through-space coupling, up to 25 Angstroms, between the two fluorine atoms as been seen in fluorine containing organic compounds[19,20,21].

As a control for J coupling, two tandem hairpins were synthesized where one has only one fluorine label (indicated with a I in figure 4), just the FdU, and the other has two fluorine labels (indicated with a II in figure 4) where the FdU and the FdC will be over 25 Angstroms from each other in either the tandem hairpin form or the single hairpin form. In these controls,

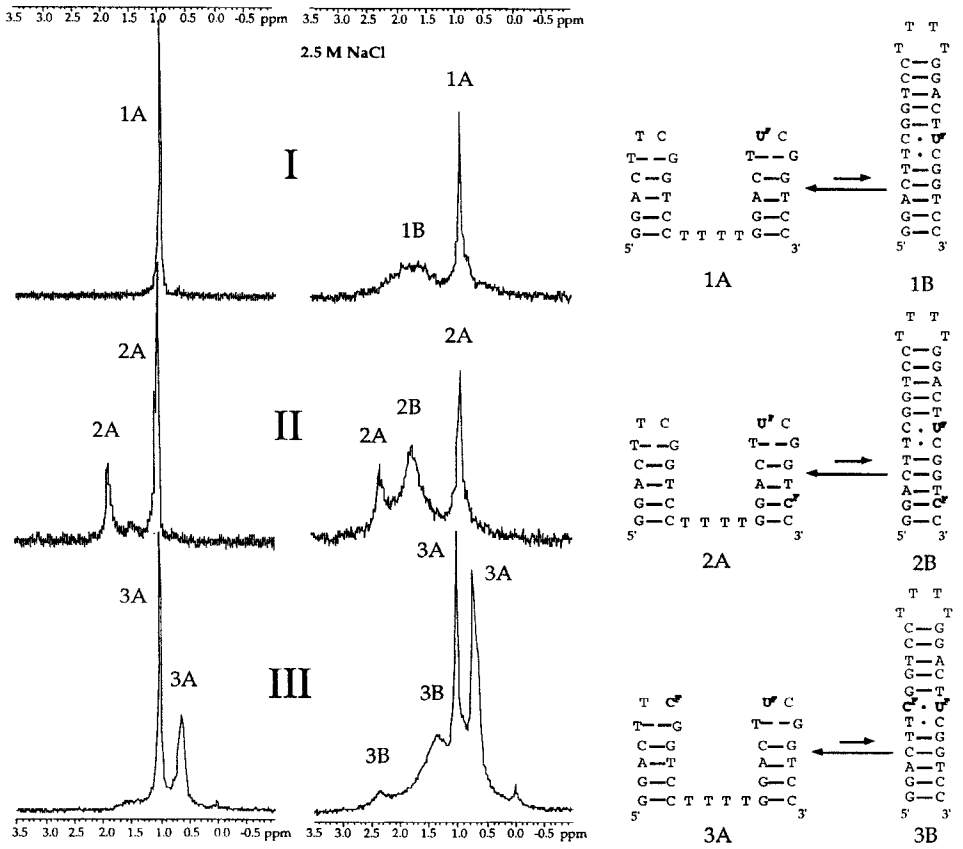

FIGURE 4: ^{19}F 1 D NMR spectra of three ^{19}F labeled tethered DNA tetraloops. Spectra on the left are done in 100 mM sodium citrate and 50 mM Tris, pH 6.7 and the spectra on the right are done in buffer plus 2.5 M NaCl. FdU is indicated by U^F and FdC is indicated by C^F.

no J coupling was seen, but the single hairpin form was observed (peaks 1B and 2B) in both controls by the presence of a large broad peak which is indicative of fluorine entering a different enviroment. For RNA, high salt concentration in the buffer shifts the equilibrium from the two tethered hairpins to one large hairpin[18]. Figure 4 shows that this is also the case for DNA of the same sequence. The spectra with and without 2.5 M NaCl are shown.

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The difference due to the presence of the single hairpins is also reflected in the J coupling between the two fluorines shown in peaks 3B of III in figure 4. To demonstrate the coupling of the two fluorines, we have irradiated the peaks and present the difference spectra in figure 5. Irradiation of peaks 3 and 4, shows the presence of an uncoupled species, which corresponds to the presence of the tandem hairpin form. Irradiation of peak 2 shows the presence of hidden peaks, which would correspond to two doublets indicating the single hairpin form.

EXPERIMENTAL

Materials and Methods: Deoxyadenosine, deoxycytidine, deoxyguanosine and thymidine were purchased from Sigma. 5-Fluorodeoxyuridine was purchased from Aldrich Chemical Co. 5'-O-(4,4'-dimethoxytrityl)-5-fluoro-2'-deoxyuridine-3'-O-N,N'-diisopropyl-β-cyanoethylphosphoramidite (5-FdU phosphoramidite) was purchased from BioGenex, Inc. 1,2,4-triazole and phosphorous oxychloride were obtained from Fluka. Snake venom phosphodiesterase and bacterial alkaline phosphatase were purchased from United States Biochemicals. Standard phosphoramidites for DNA synthesis were purchased from Millipore. Silica gel plates containing a 254 nm indicator (Whatman) were used. ^{1}H spectroscopy was performed on a Bruker DMX 500 MHz NMR Spectrometer.

5'-O-(4,4'-Dimethoxytrityl)-4-(1,2,4-triazol-1-yl)-5-fluoro-2-pyrimidon-1-yl-β-D-2'-deoxyribofuranoside-3'-O-N,N'-diisopropyl-β-cyanoethylphosphoramidite (2)

Compound **2** was synthesized using a modification of the procedure described by Xu, et al. (14) (Figure 1A). The phosphoryl tristriazolide reagent was made first. 1,2,4-Triazole (1.38 g) , in 20 ml anhydrous acetonitrile, was mixed with phosphorous oxychloride (0.4 ml) in an ice bath. Triethylamine (TEA) (3 mL) was added and stirred for 30 minutes. 0.25g (0.33 mmoles) of 5-FdU phosphoramidite (**1**) was dissolved in 5 mL anhydrous acetonitrile and

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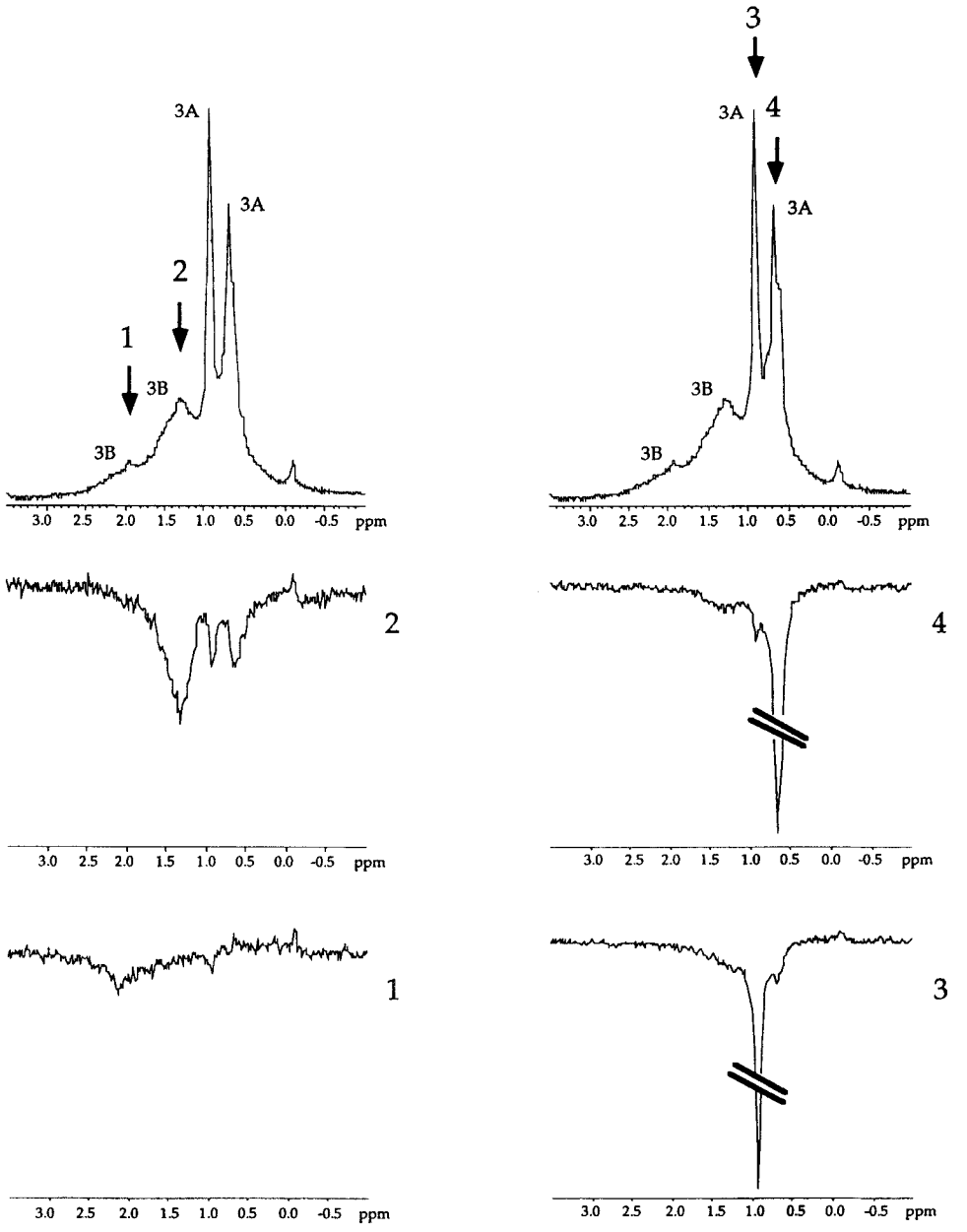

FIGURE 5: The ^{19}F NMR difference spectra of the double labeled J coupled tethered DNA tetraloop irradiated at the indicated for 100 ms. The control 1 D spectrum is shown on the top and the peaks irradiated are indicated by arrows and numbers.

was added dropwise into the phosphoryl tristriazolide reagent. This was stirred for 1 hour. The reaction was monitored by thin layer chromatography (TLC) (98% CH_2Cl_2, 1% MeOH, 1% TEA) ; the product appears as a dark blue spot under long-wave UV light and migrates slightly faster (R_f = 0.32 and 0.34) than the starting material, 1 (R_f = 0.19 and 0.23).

The reaction was quenched with 30 mL of a saturated $NaHCO_3$ solution and then extracted with 50 mL of dichloromethane. The organic layer was washed with a saturated $NaHCO_3$ solution and a saturated NaCl solution, dried with anhydrous Na_2SO_4 and rotovapped until dry. The residue was co-evaporated with toluene and precipitated in *n*-pentane cooled in a salt-ice bath. The precipitate was dissolved in anhydrous benzene and lyophilized to yield 0.25 grams (94% yield) of a light yellow crystalline powder (average yield 90-95%).

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Oligonucleotide Synthesis and Purification

The DNA was synthesized on an Expedite 8909 automatic DNA synthesis system at 1.0 micromole scale using standard base protected 2-cyanomethyl phosphoramidites. The modified phosphoramidite (2) was incorporated into the DNA automatically with a standard coupling time. After synthesis, the trityl-on protected DNA containing a 5-fluorodeoxyuridine triazole was cleaved from the column with 2 ml concentrated aqueous ammonia (28%) and deprotected at 55°C for 6 hours for the 5-mers and 16 hours for the other oligonucleotides. The fully deprotected trityl-on oligos were purified by HPLC using a PRP-1 Hamilton column (4.1x150 mm, 2 mL/min) with a gradient as follows: 5 min 88% 0.1 M triethylamine acetate, pH 7.6 and 12% acetonitrile which washed off the trityl-off DNA, 3 min 100% 0.1 M triethylamine acetate, 5 min 2% trifluoroacetic acid which removed the trityl group, 15 min 100% 0.1 M triethylamine acetate to 80% 0.1 M triethylamine acetate and 20% acetonitrile. The full length oligonucleotide was collected, dried down, and precipitated with ethanol. For a 1 micromole scale synthesis of a 5-mer oligonucleotide, our yield was 0.48 micromoles after HPLC purification. The purity of the 5-mer was confirmed by HPLC (figure 2B).

Composition analysis

The synthetic DNA containing 5-fluorodeoxycytidine (5-FdC) was confirmed by DNA digestion analysis. Oligonucleotides (~2 OD) were digested with snake venom phosphodiesterase (0.1 unit) and bacterial alkaline phosphatase (0.2 unit) in 55 μl digestion buffer (30 mM Tris, pH 7.6, 15 mM $MgCl_2$) at 37°C overnight. The enzymes were precipitated with ethanol and the digestion products were resolved by HPLC using a Beckman Ultrasphere C18 column (10x250 mm, 2.5 mL/min) with a gradient of 100% 0.1 M triethylamine acetate, pH 7.6 to 90% 0.1 M triethylamine acetate and 10% acetonitrile over 30 minutes. The retention times were as follows (figure 3): dC, 13.5 min; 5-FdC, 16.5 min; 5-FdU, 19.7 min; dG, 21.2 min; dT, 23.4 min; dA, 28.8 min. Authentic compounds of dA, dC, dG, dT and 5-FdU were purchased to be used as references. For figure 3A, they were dissolved in distilled water, then resolved on the C18 column.

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After more DNA was digested, the 5-FdC peak was collected and analyzed by UV and ^{1}H-NMR. The nucleoside had the following spectroscopic properties. ^{1}H-NMR (DMSO-d_6): δ (ppm) 8.08 (1H, d, H6), 7.73 (1H, s, NH_2), 7.49 (1H, s, NH_2), 6.09 (1H, t, H1'), 5.22-5.11 (2H, m, OH), 4.20 (H, m, H3'), 3.76 (H, m, H4'), 3.58 (2H, m, H5', H5") 2.10–1.98 (2H, m, H2', H2"); UV absorption: λ_{max}= 280 nm, λmin = 257 nm.

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REFERENCES

1. Rastinejad, F.; Artz, P.; Lu, P. *J. Mol. Biol.* **1993**, 233, 389-399.

2. Chu, W. C.; Horowitz, J. *Biochemistry* **1991**, 30, 1655-1663.

3. Rastinejad, F.; Lu, P. *J. Mol. Biol.* **1993**, 232, 105-122.

4. Osterman, D. G.; DePillis, G. D.; Wu, J. C.; Matsuda, A.; Santi, D. V. *Biochemistry* **1988**, 27, 5204-5210.

5. Oretskaya, T. S.; Gromova, E. S.; Shabarova, Z. A.; Bhagwat, A. S. *Nucleic Acids Res.* **1993**, 21, 295-301.

6. Chen, L.; MacMillan, A. M.; Chang, W.; Ezaz-Nikpay, K.; Lane, W. S.; Verdine, G. L. *Biochemistry* **1991**, 30, 11018-11025.

7. Friedman, S.; Ansari, N. *Nucleic Acids Res.* **1992**, 20, 3241-3248.

8. Som, S.; Friedman, S. *J. Biol. Chem.* **1994**, 269, 25986-25991.

9. Krug, A.; Schmidt, S.; Lekschas, J.; Lemke, K.; Cech, D. *J. prakt. Chem.* **1989**, 331, 835-842.

10. Cech, D.; Meinert, H.; Etzolt, G.; Langen, P. *J. prakt. Chem.* **1973**, 315, 149-153.

11. Duschinsky, R.; Gabriel, J.; Hoffer, M.; Berger, J.; Titsworth, E.; Grunberg, E.; Burchenal, J. H.; Fox, J. J. *J. Med. Chem.* **1966**, 9, 566-572.

12. MacMillan, A. M.; Chen, L.; Verdine, G. L. *J. Org. Chem.* **1992**, 57, 2989-2991.

13. Schmidt, S.; Pein, C.-D.; Fritz, H.-J.; Cech, D. *Nucleic Acids Res.* **1992**, 20, 2421-2426.

14. Xu, Y.-Z.; Zheng, Q.; Swann, P. F. *J. Org. Chem.* **1992**, 57, 3839-3845.

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15. Cheong, C.; Varani, G.; Tinoco, I. Jr. *Nature* **1990**, 346, 680-682.

16. Varani, G.; Cheong, C.; Tinoco, I. Jr. *Biochemistry* **1991**, 30, 3280-3289.

17. Holbrook, S. R.; Cheong, C.; Tinoco, I. Jr.; Kim, S.-H. *Nature* **1991**, 353, 579-581.

18. Kanyo, J. E.; Duhamel, J.; Lu, P. *Nucleic Acids Res.* **1996**, in press.

19. Jonas, J.; Borowski, L.; Gutowsky, H. S. *J. Chem. Phys.* **1967**, 47, 2441-2448.

20. Hilton, J.; Sutcliffe, L. H. *Progress in Nuclear Magnetic Resonance Spectroscopy*, **1975**, Pergamon Press, London, 10, part 1, 27-39.

21. Hilton, J.; Sutcliffe, L. H. *J. Chem. Soc. Faraday II* **1976**, 71, 1395-1396.

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