Synthesis, Annealing Properties, ^{19}F NMR Characterization, and Detection Limits of a Trifluorothymidine-Labeled Antisense Oligodeoxyribonucleotide 21 mer†

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The synthesis and characterization are described of trifluorothymidine groups incorporated into an antisense 21 mer designed to target gene sequences that encode serine proteases in T-lymphocytes. ^1H NMR titration studies on 3',5'-*O*-TPDS-trifluorothymidine (3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)trifluorothymidine) with 3',5'-*O*-TPDS-2'-deoxyadenosine provided clear evidence of normal Watson–Crick base pairing via detection of the imino proton signals. The chemical shift of the imino proton is moved ca. 0.5 ppm upfield relative to the position with the natural nucleoside. ^1H NMR also confirmed normal annealing in the 21 mer with its complement in the imino proton detection with the most notable difference being that six AT signals move upfield into the region characteristic of Watson–Crick GC base pairs. 1D-NMR experiments confirm that a single species exists in solution and CD studies indicate that the duplex formed with its complement adopts B-form geometry. The 1D-NMR experiments show that two of three ordinary methyl groups in the hybrid duplex exist in a single conformation while the third methyl group is predominantly in a single conformation. Molecular modeling of the duplex formed between the trifluorothymidine antisense sequence and complementary mRNA indicates a stable A-type helix in which the CF_3 groups cause the thymidine base pairs to be displaced somewhat compared with the natural structure. The limits of ^{19}F NMR detection of the trifluorothymidine labeled 21 mer were determined to be ca. 10 μM at a 10:1 signal to noise ratio, i.e., satisfactory for projected in vivo NMR imaging studies.

Introduction

The conceptual basis for control of gene expression through the introduction of exogenous "antisense" DNA, as diagrammed in Figure 1, is very simple. The exogenous "antisense" DNA strand is taken up by the targeted cell and binds to complementary mRNA, creating an alternative fate for transcribed mRNA other than translation to a polypeptide product.[1] The field of antisense DNA has burgeoned in recent years owing to its potential for the control of gene expression although a number of challenging chemical problems remain before the full potential of this approach can be realized. Two fundamental problems have been the subject of intensive study, namely, the design of modified nucleic acids with improved properties of cellular uptake[2,3] and improved in vivo stability.[4,5] Much progress has been made in the design of analogues of nucleic acids which are readily taken up by eukaryotic cells and which are not susceptible to nuclease digestion, yet a number of studies indicate that there are nuances in the mechanism of action of antisense DNA not accounted for by the idealized situation depicted in Figure 1.

Homopolymers have been reported to curtail gene expression[6] and the correspondence between inhibition of translation and sequence specificity of antisense DNA is not always as expected.[7] Further, it has not been demonstrated that the antisense probe is selectively retained in the targeted cell line relative to control cell lines that do not express the mRNA whose translation is to be suppressed. In order to address these questions we have synthesized oligonucleotides in which the thymidine residues have been replaced by trifluorothymidine so that ^{19}F NMR may be used to follow the uptake and distribution of the oligomer probes and to gain information about the complexes that are formed intracellularly.

The fluorine nucleus has several properties that make it a useful candidate for incorporation into oligonucleotides and detection by NMR spectroscopy.[8] The van der Waals radius of fluorine is 1.35 Å compared with 1.10 Å for hydrogen so substitution of fluorine for hydrogen should not result in significant steric hindrance to the base pairing selectivity of nucleosides. The fluorine nucleus has a spin of $^1/_2$, is present at 100% natural abundance, and has a wide chemical shift dispersion that is sensitive to the chemical environment. There is no natural intracellular concentration of fluorine, so magnetic resonance studies on the uptake of fluorinated nucleosides and oligonucleotides do not suffer from a large background concentration. Fluorine has been incorporated into many sites of all four of the common nucleosides for pharmacological purposes[9] but the site of choice for the study of oligonucleotides taken up by cells is the α-methyl position of thymidine. By substituting the three hydrogens of the thymidine methyl group with fluorine, a 3-fold increase in concentration of the detected nucleus relative to the amount of administered oligonucleotide is achieved due to the degeneracy in chemical shift at the methyl position. This is important in order to permit detection of the relatively low concentrations desired and above which the

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(1) Stein, C.; Cohen, J. *Cancer Res.* **1988**, *448*, 2659–2668.
(2) Miller, P. S.; Ts'O, P. O. P. *Adv. Med. Chem.* **1988**, *23*, 295–312.
(3) Miller, P. S.; Yano, J.; Yano, E.; Carroll, C.; Jayaramau, K.; Ts'O, P. O. P. *Biochemistry* **1979**, *18*, 5234.
(4) Radhakrishnan, P. I.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 1253–1254.
(5) Morvan, F.; Rayner, B.; Imbach, J.-L.; Chang, D. K.; Lown, J. W. *Nucl. Acids Res.* **1986**, *14*, 5019–5035.
(6) Ho, P. T. C.; Sartorelli, A. C. *Proc. Am. Assoc. Cancer Res.* **1990**, *31*, 8.
(7) Letsinger, R. L.; Zhang, G.; Sun, D. K.; Ikeuchi, T.; Sarin, P. S. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6553–6556.
(8) Leibman, J. F., Greenberg, A., Dolbier, W. R., Jr., Eds. *Fluorine-Containing Molecules Structure, Reactivity, Synthesis, Applications*; VCH New York, 1988; Chapter 12.
(9) Hertel, L. W.; Kroin, J. S.; Misner, J. W.; Tustin, J. M. *J. Org. Chem.* **1988**, *53*, 2406–2408.

0022-3263/91/1956-3602$02.50/0

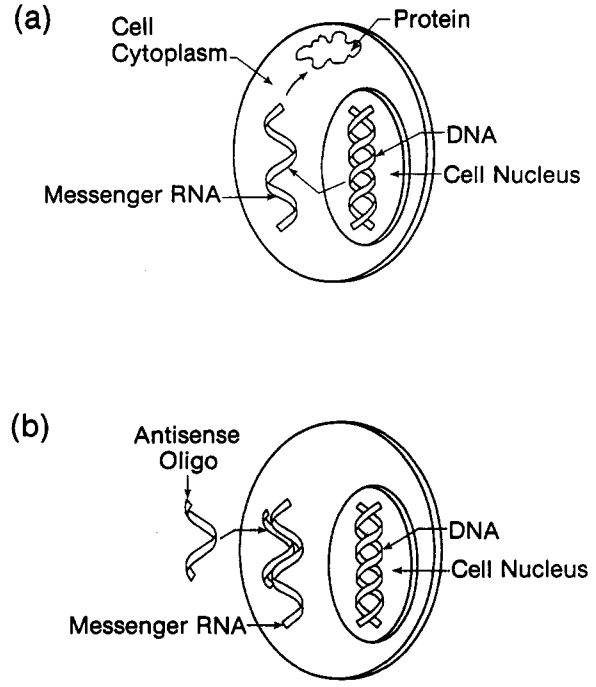


Figure 1. (a) Flow of information in the cell. Duplex DNA contained in the nucleus is transcribed to mRNA, which migrates to the cytoplasm and is translated to the ultimate effector molecule, a protein. (b) Translation of the mRNA to protein interupted by hybridization to an antisense DNA oligomer, supplied exogenously, followed by degradation by RNase H.

antisense DNA is toxic. The free rotation about the trifluoromethyl group assures that line widths remain narrow, even in complexes of high molecular weight. The isolation of the α-fluorines from other spin $^1/_2$ nuclei assures that fluorine signals are not split by couplings to ^{1}H that would require either decoupling of ^{1}H in the ^{19}F detected spectrum, together with concomitant heating in biological samples, or a loss in sensitivity for the multiplet signal.

We report the first synthesis of an oligonucleotide containing trifluorothymidine and describe the binding properties to its complement defined from Watson–Crick base pairing. The NMR chemical shift titration of the protected nucleoside 3′–5′ disiloxanediyl trifluorothymidine with 3′–5′ disiloxanediyl 2′-deoxyadenosine as determined by ^{1}H and ^{19}F NMR is also presented.

Experimental Section

5′-*O*-(4,4′-Dimethoxytrityl)-α,α,α-trifluorothymidine. To a stirred suspension of trifluorothymidine (5 mmol, 1.48 g) in 25 mL of anhydrous pyridine was added in five portions (1.2 mmol, 408 mg) 4,4′-dimethoxytrityl chloride at 1–2 h intervals. The reaction was monitored by TLC on silica, using $CHCl_3$ as eluent and visualized by UV or by spraying with 1 N HCl and gently heating. The reaction mixture was concentrated to 5 mL in vacuo and then diluted with 40 mL of chloroform and washed with 10% $NaHCO_3$ and water. The solution was concentrated to a thick oil under reduced pressure and applied to a 1.5 × 40 cm column of silica gel. The unreacted dimethoxytrityl chloride was eluted with chloroform and the product was eluted with a 90:10 chloroform/methanol solution. Fractions containing the product were combined and concentrated under reduced pressure and then redissolved in 2 mL of acetone and dried under high vacuum to give the title compound as a yellow foam: MS, CI m/z 597 (0.1, M^+), 303 (100, M − DmTr); ^{19}F NMR δ −68.207 (s, CF_3).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(methyl phosphoramidite)-α,α,α-trifluorothymidine. Diisopropylethylamine (6.3 mmol, 1.09 mL) was added to chloroform (6 mL) distilled from P_2O_5 and the solution was stirred at room temperature. 5′-*O*-(4,4′-Dimethoxytrityl)-α,α,α-trifluorothymidine (1.57 mmol, 0.9 g) and *N,N*-diisopropylmethylphosphonamidic chloride (1.9 mmol, 0.37 mL) were added successively and the reaction was stirred at room temperature. After 4 h, TLC on silica (90:10 $CHCl_3$/$(C_2H_5)_3N$) showed that the reaction was complete. The reaction mixture was diluted with ethyl acetate (40 mL) and then concentrated to 2 mL under reduced pressure and applied to a 1.5 × 40 cm column of silica gel. Elution was with 40:50:10 $CHCl_3/C_6H_{14}/(C_2H_5)_3N$. Fractions containing the product were collected and combined and the solvent was removed in vacuo. The product was dried under high vacuum to yield the title compound (830 mg, 74% yield) as a white foam: ^{1}H NMR δ 9.28 (br s, 1 H, H3), 8.07 (s, 1 H, H6), 7.35 (s, 2 H, phenyl of trityl), 7.34 (s, 3 H, phenyl of trityl), 7.26 (s, 2 H, phenyl of trityl), 7.24 (s, 2 H, phenyl of trityl), 6.81 (s, 2 H, phenyl of trityl), 6.79 (s, 2 H, phenyl of trityl), 6.18 (q, 1 H, H1′), 4.39 (m, 1 H, H5′), 4.10 (q, 1 H, H5″), 3.76 (s, 12 H, isopropyl methyls), 3.54 (d, 1 H, H3′), 3.34 (d, 1 H, H4′), 3.23 (s, 6 H, trityl methoxy), 2.40 (m, 2 H, H2′, H2″), 1.31 (d, J = 7.4, 3 H, methoxy on phosphorus).

3′,5′-*O*-TPDS-α,α,α-trifluorothymidine. The procedure of Robins et al.[10] for the preparation of 3′,5′-*O*-TPDS-uridine (TPDS = 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl) was followed without modification. To 0.296 g (1 mmol) of trifluorothymidine suspended in 10 mL of dry pyridine was added 320 μL of TPDS-Cl_2, and the mixture was stirred at room temperature for 3 h. Pyridine was evaporated and the residue was partitioned between EtOAc and H_2O. The organic phase was washed with 2 × 20 mL of cold 1 N HCl/H_2O, H_2O, saturated $NaHCO_3/H_2O$, and saturated NaCl/H_2O, dried (Na_2SO_4), and filtered, and the filtrate was concentrated. The residue was dissolved in 2 mL of 95:5 $CHCl_3/CH_3OH$ and chromatographed on a 1.5 × 40 cm column of silica gel, using the same solvent mixture as eluent to yield 380 mg (70% yield) of the product as a white solid: ^{1}H NMR δ 9.28 (br s, 1 H, N3), 8.21 (s, 1 H, H6), 5.98 (dd, J = 6.3, 0.5, 1 H, H1′), 4.45 (q, 1 H, H3′), 4.01 (qd, 2 H, H5′, H5″), 3.80 (m, 1 H, H4′), 2.55 (m, 1 H, H2″), 2.30 (m, 1 H, H2′), 1.60 (br s, 4 H, isopropyl methine), 1.10–1.00 (s, 24 H, isopropyl methyls); MS, CI m/z 556 (32.3).

3′,5′-*O*-TPDS-2′-deoxyadenosine. The procedure of Robins et al.[10] for the preparation of 3′,5′-*O*-TPDS-adenosine was followed without modification. The procedure was applied to 0.251 g (1 mmol) of 2′-deoxyadenosine to yield 410 mg (82% yield) of the title compound: ^{1}H NMR δ 8.30 (s, 1 H, H8), 8.06 (s, 1 H, H2), 6.30 (dd, 1 H, H1′, J = 3, 7), 5.60 (br s, 2 H, H6), 4.93 (q, 1 H, H3′, J = 8.8), 4.03 (br s, 2 H, H5′, H5″), 3.88 (m, 1 H, H4′), 2.66 (m, 2 H, H2′, H2″), 1.70 (br s, 4 H, isopropyl methine), 1.10 (s, 3 H, isopropyl methyl), 1.08 (s, 3 H, isopropyl methyl), 1.06 (s, 3 H, s, isopropyl methyl), 1.05 (s, 3 H, isopropyl methyl), 1.03 (s, 3 H, isopropyl methyl), 1.02 (s, 9 H, isopropyl methyls); MS, m/z 493.2537 (8.4, M^+ 493.2541), 450.1996 (75, M − iPr).

Synthesis of Oligonucleotides. To a large plastic support was added 0.34 g (15 μmol) of controlled pore glass derivatized with dG^{iBu}. The support was filled to 0.58 g with uncoated CPG. The column was inserted into an Applied Biosystems Model 380 solid state DNA synthesizer and a capping protocol consisting of a 10-min treatment with acetic anhydride/lutidine was carried out. All the nucleoside phosphoramidites, including the 5′-(dimethoxytrityl)-3′-(methyl phosphoramidite) derivative of trifluorothymidine, were prepared as 0.1 M solution in dry CH_3CN. The Applied Biosystems 10 μmol DNA synthesis cycle protocol was used without modification to synthesize the 21 mer 5′-dGAGGAT*CT*T*CAT*CT*T*CCCCGG (where T* is trifluorothymidine). This sequence, which was chosen as an antisense probe against the gene sequence that encodes a protease implicated in the cytotoxic action of T-cells,[11,12] was also synthesized with normal thymidine but with rG at the 3′-terminus rather than dG to allow chemical modification. The complement to this sequence, 5′-dCCGGGGAAGATGAAGATCCTC was also synthesized with normal thymidine. Average coupling yield, as determined by colorimetric analysis of the dimethoxytrityl group,

(10) Robins, M. J.; Wilson, J. S.; Hanskee, F. *J. Am. Chem. Soc.* **1983**, *105*, 4059–4065.
(11) Lobe, C. G.; Finlay, B. B.; Paranchych, W.; Paethau, V. H.; Bleackley, R. C. *Science* **1986**, *232*, 858–861.
(12) Lobe, C. G.; Shaw, J.; Fregeau, C.; Duggan, B.; Meier, M.; Brewer, A.; Upton, C.; McFadden, G.; Patient, R. K.; Paetkau, V.; Bleakley, R. C. *Nucl. Acids Res.* **1989**, *17*, 5765–5779.

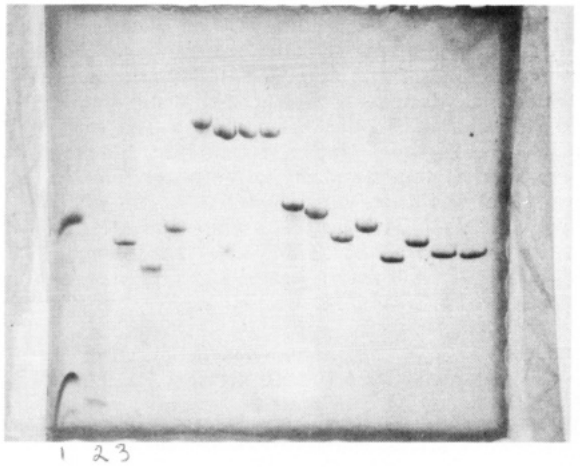


Figure 2. Photograph of 24% polyacrylamide gels run in 8 M urea showing the trifluorothymidine-containing oligomer (lane 2) and the control oligomer (lane 3).

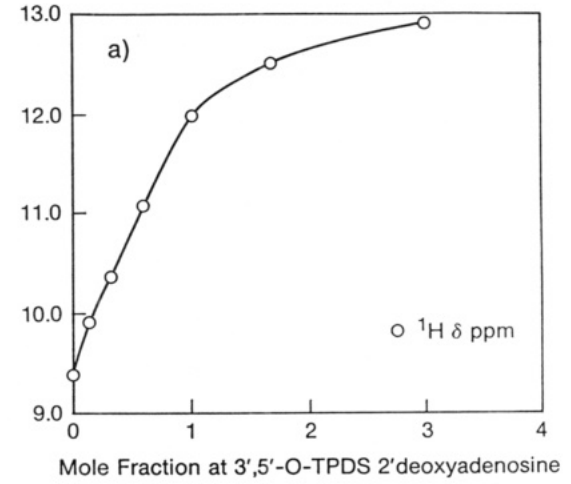
was >99% for A, C, and G and 94% for the trifluorothymidine additions to yield 51.9 mg of the 21 mer. The sequences were desalted on a 1 × 40 cm column of Sephadex G-25 with distilled H_2O at 1 mL/min and analyzed for purity using PAGE (Figure 2).

A 1 mL solution of the 21 mer containing trifluorothymidine having an absorbance of 2 at 260 nm was enzymatically degraded by treatment with 4 units of snake venom phosphodiesterase in 20 mM sodium acetate buffer, pH 6. Digestion was complete in 30 min at 37 °C as followed by a hyperchromic shift at 260 nm but was allowed to continue for 2 h. The pH of the solution was adjusted to 8.3 with 1 N NaOH, 4 units of alkaline phosphatase were added, and the reaction mixture was incubated at 37 °C for 14 h. The nucleoside mixture was analyzed by HPLC, using a 0.5 × 20 mm phenylglycine column as the stationary phase and an isocratic mobile phase consisting of 85% 20 mM sodium acetate, pH 6, 15% 60:40 CH_3CN/buffer. The nucleoside analysis gave the expected molar composition of 3:7:5:6 for A:C:G:T_f. Oligonucleotides containing trifluorothymidine are susceptible to enzymatic degradation with common nucleases and phosphatases and the trifluoromethyl group survives these procedures intact.

Preparation of Oligonucleotide NMR Samples. The sequence 5′-dCCGGGAAGATGAAGATCCTC, which is the complement to the antisense sequence, was dissolved in 300 μL of 40 mM sodium phosphate, 50 mM NaCl, pH 7.0. A 10-μL aliquot diluted to 1 mL had an A_{260} of 2.067. The antisense sequence, which contained trifluorothymidine, was dissolved and diluted in a similar manner and had an absorbance at 260 nm of 1.25 while the antisense sequence with normal thymidine had an A_{260} of 0.78. Equal amounts, as measured by absorbance at 260 nm, of the complement were combined separately with the fluorinated and standard antisense sequences. Total sample volume was 450 μL. The samples were transferred to Wilmad 507 NMR sample tubes for analysis.

Preparation of Samples for Base-Pairing Studies. A 0.1 M solution of 3′,5′-*O*-TPDS-trifluorothymidine was prepared in $CDCl_3$ as was a 0.1 M solution of 3′,5′-*O*-TPDS-2′-deoxyadenosine. Seven samples were prepared, each containing from 800 to 200 μL of the trifluorothymidine solution and from 0 to 600 μL of the 2′-deoxyadenosine solution incrementing in 100-μL amounts for each solution. The total volume and nucleoside concentration was constant in all seven samples.

NMR Spectroscopy. All NMR experiments were conducted at 400.13-MHz ^1H frequency. All experiments performed in H_2O utilized a Redfield 2–1–4 solvent suppressive pulse with the carrier frequency set 4022-Hz downfield of H_2O and with a spectral window of 8333 Hz. The total duration of the 90° pulse was 250 μs. ^{19}F observe experiments used a 45° tip angle (21 μs) and acquired either 512 or 2K data points over a 500-Hz spectral window, depending on whether high resolution or low resolution spectra were desired. T_1 values were measured by the inversion–recovery method and were 0.7 s for the trifluoromethyl resonances. An Ernst angle calculation gave an optimized acquisition time of 0.36 s for a 45° pulse, which corresponded to the acquisition of 512 data points. Spectra were not zero-filled nor weighted prior to Fourier transformation. Acquisition times were 4–12 h.



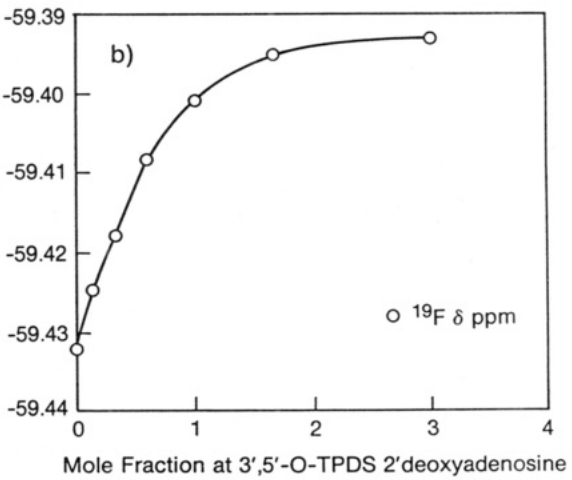


Figure 3. (a) Plot of the imino proton chemical shift in trifluorothymidine as a function of mole percent of deoxyadenosine at a constant nucleoside concentration (100 mM). (B) The corresponding ^{19}F chemical shifts for the trifluoromethyl resonance.

Molecular Modeling. Energy minimizations and molecular display were carried out by using the programs CHARMm and Quanta from Polygen.

Results

Nucleoside Base-Pairing Studies. The base-pairing properties of trifluorothymidine have not been explored and a number of points need to be established, namely, that specific hydrogen bonds are formed with adenosine in a manner similar to the natural nucleoside and, secondly, that the ^{19}F chemical shift of the methyl group is a sensitive monitor of the environment of the nucleoside. Such information is essential for further projected studies using trifluorothymidine as a probe for in vivo NMR studies of antisense oligonucleotides.

3′,5′-*O*-TPDS-trifluorothymidine was prepared as a 0.1 M solution in chloroform. NMR titrations were performed by preparing samples with from 0 to 3 molar equiv of 3′,5′-*O*-TPDS-2′-deoxyadenosine relative to the trifluorothymidine derivative at a constant (0.1 M) total nucleoside concentration to avoid concentration-dependent stacking phenomena. The ^1H NMR titration for the imino proton of thymidine is shown in Figure 3a and the ^{19}F titration

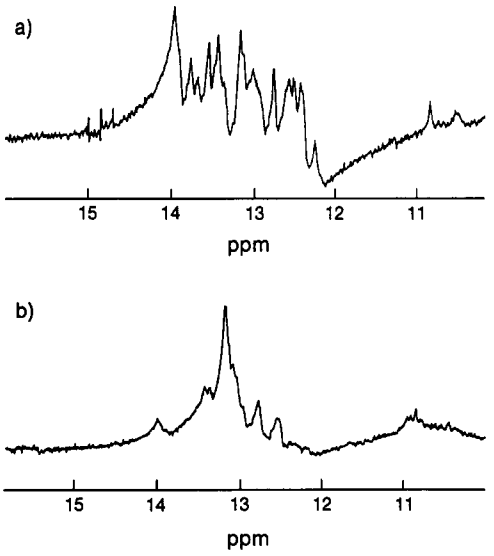


Figure 4. Plot of the imino proton region of the control oligonucleotide duplex (a) in 90:10 H_2O:D_2O. (b) Same region of the trifluorothymidine containing oligomer annealed to its DNA complement.

of the trifluoromethyl group is shown in Figure 3b. Both positions show a similar response to the increase of mole percent adenosine although the trifluoromethyl response is attenuated considerably owing to the 5 intervening bonds between it and the hydrogen-bonded imino proton. The line width of the fluoromethyl resonance is independent of base-pair formation due to the free rotation about the methyl group. The imino proton displays behavior similar to a normal uridine derivative base paired to adenosine in chloroform[13] with the exception that the limiting chemical shift is about 0.5 ppm upfield. The chemical shift of derivatized trifluorothymidine at a mole fraction of unity and at 0.1 M concentration is also about 0.5 ppm upfield relative to that of derivatized uridine, at 0.5 M concentration and a mole fraction of unity, indicating a similar downfield shift in response to base-pair formation but an intrinsically upfield shift in both the single component and the base-paired solutions.

Imino Proton Studies on the 21 mer. The nucleoside base-pairing studies show that the chemical shifts of imino protons of trifluorothymidine are upfield relative to normal thymidine in duplex DNA. The imino protons of the oligonucleotide duplexes in 90:10 H_2O/D_2O were analyzed, using a Redfield 2-1-4 selective excitation pulse. The downfield region of the ^1H NMR spectrum for the duplex containing trifluorothymidine is shown in Figure 4b and that for the control duplex is shown in Figure 4a. The control duplex displays the spectrum expected for an oligonucleotide with 21 unique base pairs with 9 thymidine and 12 guanosine imino protons in the chemical shift ranges typical for each type of base pair. The oligonucleotide containing trifluorothymidine in place of thymidine has a different imino proton spectrum. The spectrum appears to contain many unresolved guanosine imino protoins and only a few thymidine imino protons. The upfield chemical shift of the trifluorothymidine imino protons base paired with adenosine moves the observed resonance into the region typical of Watson–Crick GC base pairs. A peak is still observed at 14 ppm at a frequency more typical of normal A-T base pairs which could be from the three thymidines in the strand containing normal thymidine. The broadness and severe overlap in the imino proton spectrum, which represents up to 21 protons, prohibits complete analysis and assignment. The guanosine imino protons, which are not directly affected by trifluoromethyl substitution at thymidine, are nonetheless affected by the perturbation it causes in the duplex. There is a pronounced broadening and apparent decreased intensity in the guanosine imino region with resonances centered about two or three frequencies rather than the random scatter of typical frequencies apparent in the control spectrum. While the imino proton spectrum is certainly affected by substitution with trifluorothymidine, it retains evidence that specific A-T, A-T_f, and G-C base pairs form. Trifluorothymidine-substituted oligonucleotides may differ in structural details from unsubstituted oligonucleotides, but they retain the capacity to hybridize complementary nucleic acid in a sequence specific manner, which 1D-NMR experiments confirm as a single species and CD studies indicate adopts B-form geometry. The 1D-NMR experiments show that two of three ordinary methyl groups in the hybrid duplex exist in a single conformation, while the third methyl group is predominantly in a single conformation.

Detection of Dilute Solutions of ^{19}F-Labeled Oligonucleotides. The practicality of using oligonucleotides incorporated with trifluorothymidine as probes of antisense activity rests on their ability to form stable duplexes with complementary nucleic acid sequences and for the probe to be readily detected by ^{19}F NMR spectroscopy at the relatively low concentrations occurring intracellularly. The upper limit of concentration for antisense probes intracellularly is about 10 μM. Above this concentration, no specificity in the control of gene expression could be achieved since the mRNA levels are lower than this. To verify that a 10 μM solution of oligonucleotide incorporated with trifluorothymidine is detectable, solutions of the single-stranded and duplex 21 mer trifluorothymidine containing antisense sequence were prepared. A 10:1 signal-to-noise ratio was considered satisfactory for detection and this could be acquired in about 4 h, using acquisition parameters of low digital resolution where all nine of the trifluoromethyl groups of the 21 mer were detected as a single peak. Under conditions of high digital resolution where chemical shift information is retained, spectra of 10:1 signal-to-noise were acquired in 12 h. The spectra are shown in Figure 5.

Melting and Circular Dichroism Studies on Duplex 21 mers. The incorporation of trifluorothymidine into oligonucleotides can impact stability of duplexes formed with complementary DNA or RNA in two ways. First, an intrinsic difference in the stability of the A-T_f base pair from a standard A-T base pair could result in a helix of reduced (or increased) stability. Second, the larger van der Waals radius of the trifluoromethyl group and its more negative electrostatic potential could disrupt helix geometry and destabilize the helix. The similar downfield shift of the imino proton in an A-T_f as an A-T base pair, relative to the monomeric shift, suggest that both types of base pairs are similar in stability. The detection of imino proton resonances in spectra of the hybrid duplex indicates that stable base pairs are formed and the detection of resonances at chemical shifts typical of thymidine in A-T base pairs and guanosine in G-C base pairs as well as trifluorothymidine in A-T_f base pairs indicates that sequence specific hybridization is still operative. There are clearly differences in the structural details of helices formed by normal and trifluorothymidine-substituted oligonucleotides, which will be the subject of a future NMR study. Thermal melting and circular dichroism studies

(13) Poulter, C. D.; Livingston, C. L. *Tetrahedron Lett.* **1979**, 755–758.

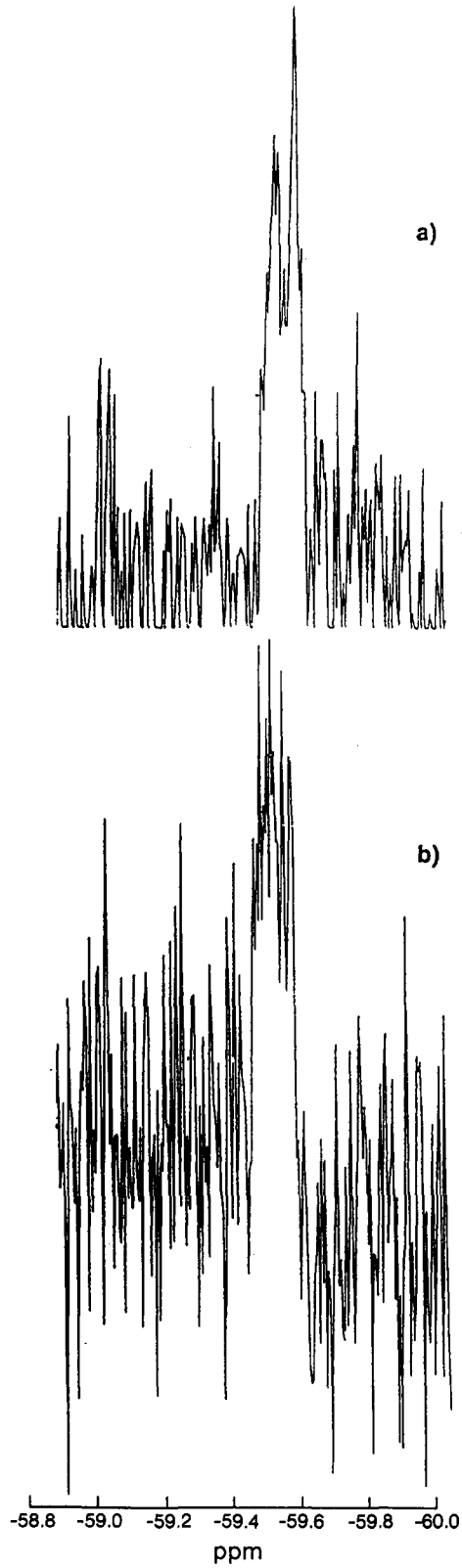


Figure 5. Plot of ^{19}F NMR spectra of the trifluorothymidine-containing oligonucleotide duplex at 10 μM concentration: (a) low digital resolution conditions; (b) high digital resolution. Signal-to-noise ratio is better than 10:1 in both spectra. Spectrum a was acquired in 4 h and spectrum b was acquired in 12 h.

were conducted on the antisense 21 mers (with trifluorothymidine and thymidine) annealed to their complements to determine the effect of trifluoromethyl substitution on helix formation and stability in nucleic acids. The samples were prepared in 10 mM PO_4^{2-}, 40 mM NaCl, pH 7. The thermal melting profile appears in Figure 6 and the circular

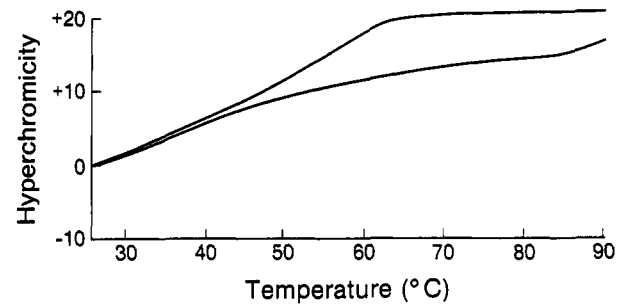


Figure 6. Plot of UV absorbance versus temperature for the trifluorothymidine-containing oligomer annealed with its DNA complement and for the control oligomer annealed to the complement. Increase in hyperchromicity is very similar from room temperature up to 50 °C.

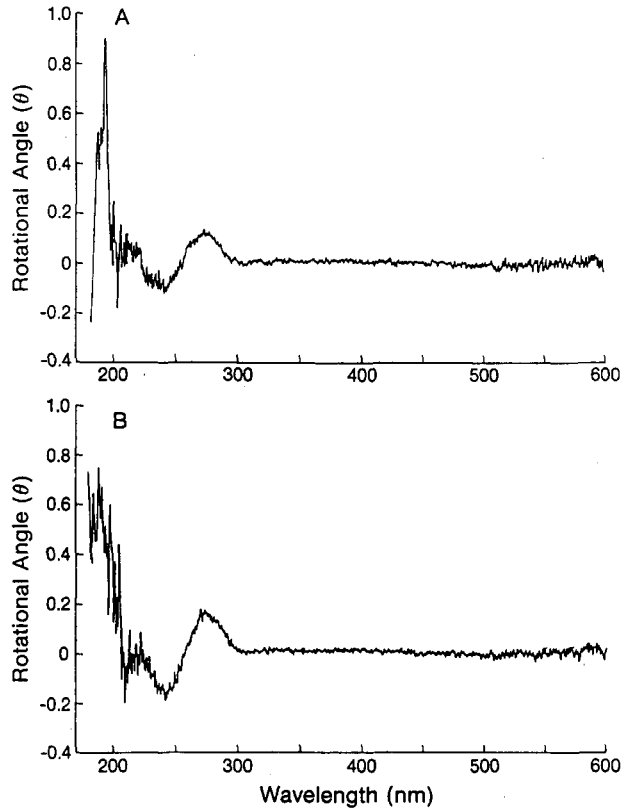


Figure 7. Circular dichroism spectra of the trifluorothymidine-containing oligomer annealed to its complement (a) and the control oligomer annealed to complement (b). The null, maxima, and minima of the two spectra are identical, indicating similar secondary structure. Spectra are typical of B-DNA. Instrumental correction factor is 3.03×10^{-5} M deg.

dichroism spectrum appears in Figure 7. The figures indicate that trifluorothymidine does not significantly effect the stability or the structure of duplex DNA. The trifluorothymidine and normal antisense 21 mer each have virtually identical thermal melting profiles up to 50 °C. Above 50 °C there are differences in the hyperchromicity of the two duplexes. Since most studies are conducted at or below physiological temperature, the differences in helix stability in trifluorothymidine-substituted versus normal duplexes are negligible. Both duplexes clearly adopt B-DNA geometry as evidenced by the negative 220–250-nm and positive 250–300-nm absorbances of equal intensity. Both spectra display the same nulls, maxima, and minima. These studies indicate that trifluoromethyl substitution is not detrimental to the helix-forming properties or the duplex stability of oligonucleotides containing the substituted residues.

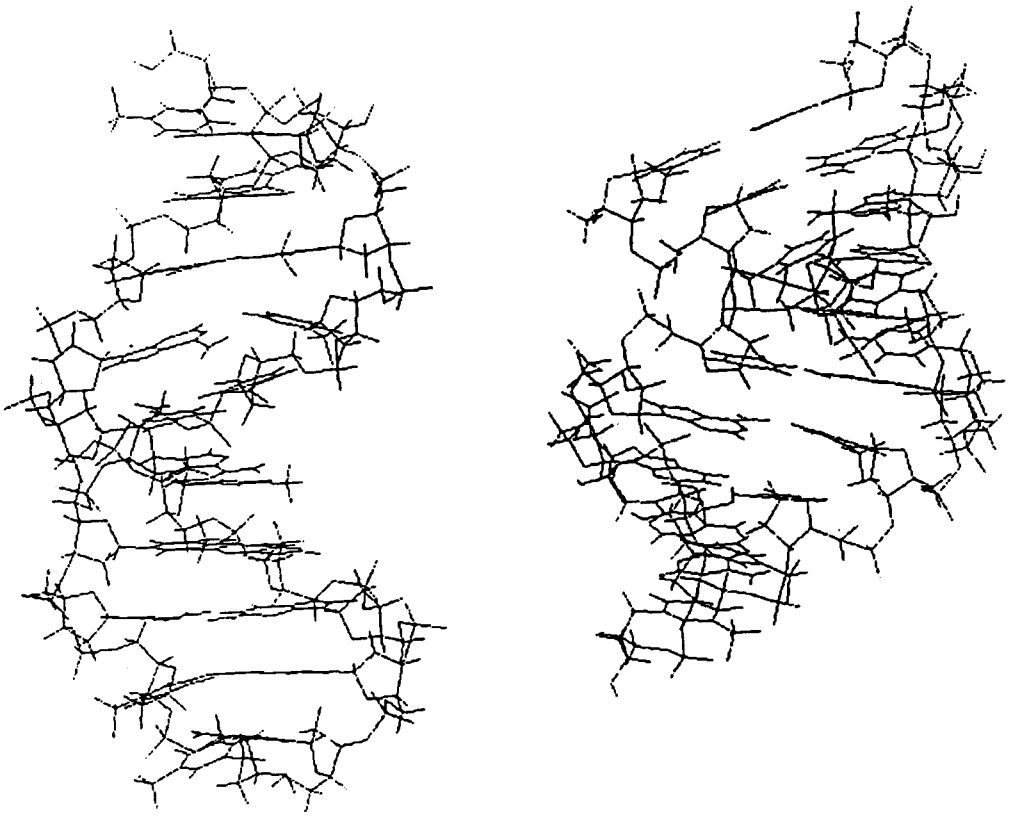


Figure 8. Energy-minimized duplexes of the central 10 mer 5'-dTfCTfTfCATfCTfTfTf (Tf = trifluorothymidine) of the 21 mer antisense oligonucleotide annealed with complementary DNA (a) and complementary RNA (b). The DNA duplex was minimized from B-form geometry and the DNA–RNA hybrid was minimized from A-form geometry. The trifluoromethyl group protrudes into the major groove but does not disturb helical geometry.

Molecular Modeling of Trifluorothymidine Oligonucleotides. A 10 base-pair fragment from the interior of the antisense DNA 21 mer was chosen for molecular modeling studies. The 10 mer chosen (5'-dTCTTCATCTT) contained all six of the thymidine positions of the antisense 21 mer, assuring that the effects of trifluoromethyl substitution were incorporated into the model adequately. The truncated sequence allowed for faster computations and made visual interpretation of the results more facile. The 10 mers with and without trifluorothymidine were base paired with complementary DNA using B-form geometry and RNA using A-form geometry.

Carbon–fluorine bond lengths of 1.37 Å and partial charges of −0.28 eu were obtained for the trifluorothymidine residue, using the PCMODEL molecular mechanics program, and were restrained to these values in the final structure of the oligonucleotide. The trifluorothymidine residue topology files replaced the thymidine files in duplexes with complementary DNA and RNA, respectively. 10 mers with normal thymidine served as controls. The structures were energy minimized and examined for distortion from normal helix geometry. The trifluoromethyl group extends out into the major groove of the DNA duplex and the DNA–RNA hybrid duplex but does not interfere with base pairing or base stacking, nor does it significantly affect helical dimensions. The energy-minimized duplex DNA and DNA–RNA hybrid containing trifluorothymidine are shown in Figure 8.

Discussion

The utility of antisense DNA in control of gene expression has been well documented but additional analytical methods are necessary in order to gain a complete understanding of the mechanism by which such probes operate and to design new analogues of nucleic acids which will be more effective antisense agents. A number of methodologies can be envisioned, including covalently attaching a fluorescent probe,[14] but NMR in general, and ^{19}F NMR in particular, offers the potential advantage of providing chemical and structural information. The relaxation times of ^{19}F resonances are often indicative of the molecular weight of the complex and heteronuclear ^{19}F–^{1}H NOE experiments[15] are also useful in probing the nature of the complexes formed. ^{19}F is perhaps the most useful nucleus to insert as a probe for intracellular studies because of the high sensitivity and low background concentration of the nucleus.[16] ^{19}F chemical shifts are also sensitive indicators of chemical environment. A number of studies of fluorinated nucleosides have been described, particularly on 5-fluorouridine,[17] but the principal interest to date has been on the pharmacology of these compounds. The incorporation of ^{19}F as an analytical probe in tRNA,[18] and other large naturally occurring DNA and RNA sequences, has been well described.[19] These studies all utilized 5-fluorouridine as a probe for magnetic resonance studies. To our knowledge the use of trifluorothymidine for similar studies has not been described hitherto despite the several advantages it offers compared with, e.g., 5-fluorouridine.

(14) Kremsky, J. N.; Wooters, J. S.; Dougherty, J. P.; Meyers, R. E.; Collins, M.; Brown, E. L. *Nucl. Acids Res.* **1987**, *15*, 2891–2909.
(15) Hso, V. L.; Proto, C.; Handschumacher, R. E.; Armitage, I. M. *Exp. Nucl. Conf. Abs.* **1990**.
(16) American Association for Cancer Research Annual Meeting, Washington, DC, May 1990, Abstract no. 2273.
(17) Young, C. W.; Woodcock, T. M.; Martin, D. S. *Cancer Treat. Rep.* **1981**.
(18) Hills, D. C.; Cotten, M. L.; Horowitz, J. *Biochemistry* **1983**, *22*, 1113–1122.
(19) Gerig, J. T. In *Biological Magnetic Resonance*, Vol. 1; Berliner, L. G., Reubens, J., Eds.; Plenum: New York, 1978; Chapter 4.

The trifluoromethyl group contains three resonances degenerate in chemical shift, making the experiment more sensitive. The micromolar concentrations at which antisense DNA sequences are effective are detectable by ^{19}F NMR in reasonable acquisition times. A 10-μM sample of a 21 mer could be detected in 4 h with a 10:1 signal-to-noise ratio. For intracellular and in vivo studies this is crucial. The resonance is also a sharp singlet as a consequence of unhindered rotation about the trifluoromethyl group and the small four-bond coupling to H3 and H6.

The potency of 5-fluorouridine in antiviral and antitumor screening procedures has been explained in terms of a difference in the pK_A of the imino proton of 5-fU relative to uridine or thymidine, which results in base-pair mismatches during transcription.[20] In contrast the antiviral and antitumor activity of trifluorothymidine[21] is thought to result from inactivation of thymidilate synthetase via covalent complex formation to the α-methyl group, which is activated by fluorine substitution, and not at C6. There is no evidence that trifluorothymidine exerts toxicity by being incorporated into DNA and altering structure or duplex stability, and the studies described here argue against a structural role in the toxicity process. In fact, a number of points of evidence in the present study indicate that normal helix stability and geometry are conserved upon substitution of thymidine by trifluorothymidine. The base pairing study shows a similar change in chemical shift upon base pairing of trifluorothymidine to adenosine as in a normal A-T base pair, indicating that the base pairs are of similar stability. The detection of imino protons at appropriate chemical shifts for G-C, A-T, and A-T_f base pairs indicates that a stable duplex exists in solution although a broadening and lack of dispersion in this region indicates that the structural details of the helix are different from the control oligonucleotide. The similar thermal melting profiles and circular dichroism spectra for trifluorothymidine-containing duplexes as normal duplexes indicates that they are similar in stability and geometry. Finally, molecular modeling studies indicate that duplexes which potentially form intracellularly upon administration of antisense DNA are not disrupted by trifluoromethyl substitution of thymidine residues.

Oligonucleotides containing trifluorothymidine can be prepared by using the procedures applicable to the standard nucleosides and their physical and chemical properties are closely comparable. Trifluorothymidine does not disrupt the helix-forming properties of oligonucleotides containing it as demonstrated by imino proton studies and thermal melting and CD studies. The ^{19}F nucleus is easily detected, even at low concentrations, and the chemical shifts and relaxation times are indicative of molecular structure. The description of the chemical synthesis and physical properties of oligonucleotides containing trifluorothymidine contained here is the first of its kind and it should facilitate future studies of the mechanism of action of antisense oligonucleotides intracellularly. Work to incorporate trifluorothymidine into sequences of α-DNA, which are not degraded intracellularly by nuclease action, is in progress in an effort to detect antisense oligonucleotides by ^{19}F NMR intracellularly.

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Registry No. 5'-dGAGGAT*CT*T*CAT*CT*T*CCCCGG, 133227-72-2; 5'-dCCGGGGAAGATGAAGATCCTC, 133227-73-3; 5'-*O*-(4,4'-dimethoxytrityl)-α,α,α-trifluorothymidine, 133128-06-0; trifluorothymidine, 70-00-8; 5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-(methyl phosphoramidite)-α,α,α-trifluorothymidine, 133100-90-0; *N*,*N*-diisopropylmethylphosphonamidic chloride, 110972-27-5; 3',5'-*O*-TPDS-α,α,α-trifluorothymidine, 133100-91-1; 3',5'-*O*-TPDS-2'-deoxyadenosine, 84828-84-2; 2-deoxyadenosine, 958-09-8.

Supplementary Material Available: 1D-^1H NMR spectrum of the hybrid duplex (1 page). Ordering information is given on any current masthead page.

(20) Kremer, A. B.; Mikita, T.; Beardsley, G. P. *Biochemistry* **1987**, *26*, 391-397.
(21) Wataya, Y.; Sonobe, Y.; Maeda, M.; Yamaizumi, Z.; Aida, M.; Santi, D. V. *J. Chem. Soc., Perkin Trans. 1* **1987**, 2141-2147.

Pyridine Coenzyme Analogues. 3. Synthesis of Three NAD$^+$ Analogues Containing a 2'-Deoxy-2'-substituted Nicotinamide Arabinofuranosyl Moiety

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A general method for the preparation of 2'-deoxy-2'-substituted arabino-nicotinamide-adenine dinucleotide (NAD) analogues is described. Starting from 1,2:5,6-di-*O*-isopropylidine-α-D-allofuranose, the 2'-amino-, 2'-azido-, and 2'-fluoro-arabino-NAD analogues have been prepared. We report an improved phosphorylation procedure for nicotinamide nucleosides using pyrophosphoryl chloride in *m*-cresol. The selective reduction of azido substituents by aqueous dithiothreitol (DTT) in the presence of the readily reducible nicotinamide moiety is also reported. With both the 2'-azido and the 2'-fluoro substituents the cis configuration predominates for the incoming nicotinamide, thus allowing the stereoselective formation of the β anomer in high yield.

Introduction

The nicotinamide-adenine dinucleotide coenzyme, NAD$^+$, has two distinct physiological roles: first, as a redox coenzyme, it mediates aerobic and anaerobic energy metabolism, and second, it serves as a donor of the ADP ribose moiety in the ADP-ribosylation of proteins and the synthesis of poly ADP ribose.[1] The role of pyridine nu-

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(1) (a) Pekala, P. H.; Anderson, B. M. In *The Pyridine Nucleotide Coenzymes*; Everse, J., Anderson, B. M., You, K. S., Eds.; Academic Press: New York, 1982; p 325. (b) Althaus, F. R.; Richter, C. In *ADP-Ribosylation of Proteins: Enzymology and Biological Significance*; Althaus, F. R., Richter, C., Eds.; Springer-Verlag: Berlin, 1987; p 50. (c) Ueda, K.; Hayaishi, O. *Ann. Rev. Biochem.* **1985**, *54*, 73.

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