Synthesis of 5′-O-(4,4′-Dimethoxytrityl)-2′-O-(*tert*-butyldimethylsilyl)-5-fluorouridine 3′-(Cyanoethyl *N,N*-diisopropylphosphoramidite) and Its Use in the Synthesis of RNA

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5-Fluorouridine (FUrd) has been successfully converted to 5′-O-(4,4′-dimethoxytrityl)-2′-O-(*tert*-butyldimethylsilyl)-5-fluorouridine 3′-(cyanoethyl *N,N*-diisopropyl(phosphoramidite) by methods similar to those employed for the preparation of other ribonucleoside phosphoramidites. The desired product, as well as its precursors and the incorrect regioisomers, are prepared and fully characterized by ^{1}H NMR, ^{19}F NMR, ^{31}P NMR, FAB-MS, UV absorbance, elemental analysis, and melting points. The purified material is used for the production of RNA by solid-phase methods. Coupling yields of 94% are obtained with this material. The resulting RNA is readily purified and used for biophysical studies. The self-complemetary RNA decamer 5′-rGCGAAU(FU)CGC is prepared and purified. The material adopts an A-form duplex in solution at neutral pH as characterized by CD spectroscopy. The thermal stability of the duplex is similar to the parent duplex oligoribonucleotide prepared with uridine instead of 5-fluorouridine. The control duplex has a T_m of 53.8 and the FUrd substituted duplex has a T_m of 56.6 ^{xb0}°C as determined by UV hyperchromicity at 260 nm. The CD spectra and UV hyperchromicity data for the duplex oligoribonucleotide containing FUrd have a slight pH dependence indicating that ionization of FUrd has a slight but measurable impact on RNA duplex stability. 1D NMR spectroscopy of the imino hydrogens for this duplex in H_2O solution confirms the formation of Watson–Crick base pairs. The imino hydrogen resonance for the FUrd–A base pair is moved downfield and broadened compared with a control duplex oligoribonucleotide as a consequence of the electron-withdrawing inductive effect of fluorine.

Introduction

5-Fluorouracil (5-FU) is a nucleoside analogue in clinical use for the treatment of solid tumors.[1] 5-FU undergoes many of the same steps of metabolic activation undertaken by the parent nucleoside uracil.[2] Uracil and 5-FU differ chiefly in their susceptibility to methylation at C5. After conversion to dUMP, U is readily methylated to yield dTMP while FdUMP is a potent and nearly irreversible inhibitor for thymidylate synthase (TS), the enzyme that catalyzes this reaction.[3] Treatment of cells with 5-FU depletes intracellular stores of dTMP and inhibits DNA replication and cell division.[4] The effectiveness of 5-FU in cancer chemotherapy is widely believed to result directly from its conversion to FdUMP and the inhibition of TS.[5] Apart from its activity as a monomer 5-FU is known to have RNA-mediated effects.[6] 5-FU is readily converted to FUTP and this is a good substrate for many RNA polymerases.[7] Cells treated with 5-FU incorporate it into all species of RNA. Changes in the efficiency of pre-mRNA splicing and in the assembly of rRNA are among the effects that have been attributed to the incorporation of 5-FU into cellular RNA.[8] The structural basis for these RNA-mediated effects may be investigated by the production of RNA molecules containing 5-FU and by a thorough analysis of their biophysical properties. It has not been determined whether 5-FU acts by substitution at specific site(s) or whether statistical population of all sites at certain threshold levels is responsible for its RNA-mediated effects. We have investigated the thermodynamic consequences of base pair formation involving 5-FU as a neutral monomer and found that it forms base pairs of increased stability and with greater propensity to base pair by using the C4 carbonyl than does uridine.[9] NMR investigations of 5-FU incorporation in DNA have found it has little effect on the global structure of duplex oligonucleotides[10] although it has a large local electronic effect.

To study the effects of 5-FU on RNA struture by NMR spectroscopy and other analytical techniques requires production of milligram quantities of pure RNA. Over the last several years a number of studies have utilized RNA produced by solid-phase synthesis.[11,12] The incorporation of 5-fluorouridine into RNA by these methods

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(1) Heidelberger, C.; Chaudhuri, N. K.; Dannenberg, P.; Mooren, D.; Griesbach, L.; Duschinsky, R.; Schnitzer, R. J.; Pleven, E.; Scheiner, J. *Nature* **1957**, *179*, 663–666.
(2) Parker, W. B.; Cheng, Y. C. *Pharmac. Ther.* **1990**, *48*, 381–395.
(3) Santi, D. V.; McHenry, C. S.; Sommer, H. *Biochemistry* **1974**, *13*, 471–480.
(4) Weckbecker, G. *Pharmacol. Ther.* **1991** *50*, 367–424.
(5) Hansen, R. M. *Cancer Invest.* **1991**, *9*, 637–642.
(6) Spiegelman, S.; Nayak, R.; Sawyer, R.; Stolfi, R.; Martin, D. *Cancer* **1980**, *45*, 1129–1134.
(7) Weckbecker, G.; Keppler, D. *Biochem. Pharmacol.* **1984**, *33*, 2291–2298.
(8) Dolnick, B. J.; Pink, J. J. *J. Biol. Chem.* **1985**, *260*, 3006–3014.
(9) Gmeiner, W. H.; Sahasrabudhe, P.; Anderson, J., submitted for publication.
(10) Stolarski, R.; Egan, W.; James, T. L. *Biochemistry* **1992**, *31*, 7027–7042.
(11) Chou, S-H.; Flynn, P.; Reid, B. *Biochemistry* **1989**, *28*, 2422–2435.

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requires its conversion into a suitably protected nucleoside phosphoramidite (Scheme 1). The most common protecting groups are the 4,4′-dimethoxytrityl for the 5′-hydroxyl and the *tert*-butyldimethylsilyl for the 2′-hydroxyl.[13] The chemical stability of the C−F bond indicates that precautionary measures to protect the site of F substitution from hydrolysis or chemical modification are unwarranted. We have adapted the methods described by Usman et al.[14] for the preparation of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-(cyanoethyl *N*,*N*-diisopropyl(phosphoramidite) to prepare the analogous 5-fluorouridine compound. The lack of regiospecificity in the silylation of the 2′-hydroxyl remains problematic although the desired compound is readily obtained in pure form. The phosphoramidite of FUrd is amenable to use in the large-scale (15 μmol) synthesis of RNA by solid-phase methods. RNA containing FUrd at specific site(s) produced by solid-phase synthesis is useful for both structural studies by NMR spectroscopy and other methods and for biological evaluations on the effects of 5-FU on RNA function.

Experimental Section

General Materials and Methods. All synthetic reactions were carried out in oven-dried glassware under an argon atmosphere. Pyridine was distilled from CaH_2 and stored over activated 4 Å molecular sieves. THF was omnisolve grade and used without further purification. All liquid reagents and solutions were transferred or added via syringe. All chemicals were from Sigma except 2-cyanoethyl *N*,*N*′-diisopropylphosphonamidic chloride that was from Aldrich. All synthetic reactions were followed by an aqueous workup, which consisted of washing the organic solution with three 25 mL aliquots of saturated $NaHCO_3$ and NaCl solutions. The organic layers were then separated and dried over anhydrous Na_2SO_4, and the solvent was removed under vacuum using a rotary evaporator. ^{1}H and ^{31}P NMR spectra were obtained on a Varian UNITY 500 spectrometer. ^{1}H NMR spectra were referenced to the internal C_6H_6 signal in the samples (δ 7.15 ppm). ^{31}P NMR spectra were referenced relative to 85% H_3PO_4 as an external standard (δ 0.0 ppm). ^{19}F NMR spectra were acquired on a Varian XL-300 spectrometer. ^{19}F spectra were referenced relative to external trifluorotoluene in C_6D_6 (δ 0.0 ppm). Mass spectrometry data was collected by FAB. Thin layer chromatographic data (R_f values) were obtained with Whatman Al Sil G/UV analytical sheets developed with $CHCl_3/CH_3OH$ (9/1, solvent A), $C_6H_{14}/CH_3COOC_2H_5$ (65/35, solvent B) or $CHCl_3/(C_2N_5)_3N$ (95/5, solvent C). Preparative column chromatography was performed with Baker analyzed reagent silica gel (60−200 mesh) by using a 4 × 30 cm column and the same solvents as for analytical TLC. Elemental analyses were conducted by Galbraith Laboratories.

Synthesis of 5′-*O*-(4,4′-Dimethoxytrityl)-5-fluorouridine (1). 5-Fluorouridine (5.24 g, 20.0 mmol, 1 equiv) was dissolved in 20 mL of anhydrous pyridine under an Ar atmosphere to which was added 8.13 g (24 mmol, 1.2 equiv) of 4,4′-dimethoxytrityl chloride in four equal fractions over 2 h. The reaction mixture was stirred at room temperature overnight then diluted with 25 mL of dichloromethane followed by aqueous workup as described in the general methods. The product, which was a yellow foam, then was purified by column chromatography on silica gel using solvent A to yield 10.11 g of compound **1** (90%). **1**: R_f (solvent A) 0.52; ^{1}H NMR (C_6D_6) 10.36 (s, 1H), 7.88 (s, 1H), 7.46−6.75 (m, 13H) 5.87 (s, 1H), 4.40 (s, 2H), 4.19 (s, 1H), 3.71 (s, 6H), 3.41 (s, 2H); ^{19}F NMR (C_6D_6) 5.10 (bs); MS (FAB) 303.1 (dimethoxytrityl), 587.1 (M + Na$^+$), 609.1 (M + 2Na$^+$); UV λ_{max} = 272 nm; mp 197 °C (dec).

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-5-fluorouridine (2a). Compound **1** (10 g, 18 mmol, 1 equiv) was dissolved in 40 mL of anhydrous pyridine under an Ar atmosphere to which was added imidazole (5.0 g, 72 mmol, 4 equiv). The solution was stirred for one hour under an Ar atmosphere at which time *tert*-butyldimethylsilyl chloride (3 g, 20 mmol, 1.1 equiv) was added as a single portion. The reaction proceeded for 5 h at room temperature. The reaction mixture was diluted with 25 mL of CH_2Cl_2 followed by an aqueous workup as described in the general methods. Following evaporation of solvent the crude product was purified by column chromatography on silica gel with solvent B to yield 2.1 g of compound **2a** as a white foam (20%). **2a:** R_f (solvent B) 0.29; ^{1}H NMR (C_6D_6) 9.38 (s, 1H), 7.87 (d, J = 6.2 Hz, 1H), 7.60−6.76 (m, 13H), 5.75 (t, J = 2.0 Hz,1H), 4.19 (m, 1H), 4.08 (dd, J = 4.7, 2.0 Hz, 1H), 3.94 (m, 1H), 3.51−3.42 (m, 2H), 3.26 (s, 6H), 0.87 (s, 9H), 0.24 (s, 3H), 0.12 (s, 3H); ^{19}F NMR (C_6D_6) 4.33 (d, J = 6.2Hz); MS (FAB) 701.0 (M + Na$^+$); UV λ_{max} = 272 nm; mp: 89 °C. Anal. Calcd for $C_{36}H_{43}N_2O_8F_1Si_1$: C, 63.69; H, 6.40; N, 4.13. Found: C, 63.01; H, 6.71; N, 3.57.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-5-fluorouridine (2b). In the same reaction used to prepare compound **2a** 2.0 g of **2b** is produced (18% yield). **2b:** R_f (solvent B) 0.20; ^{1}H NMR (C_6D_6) 10.88 (s, 1H), 8.03 (d, J = 5.0 Hz, 1H), 7.65−6.79 (m, 13H), 6.18 (d, J = 2.5Hz, 1H), 4.68 (bs, 1H), 4.66 (m, 1H), 4.27 (m, 1H), 3.61 (dd, J = 11.0 Hz, 2.0 Hz, 1H), 3.38−3.31 (m, 2H), 3.27 (s, 6H), 0.96 (s, 9H), 0.30 (s, 3H), 0.16 (s, 3H); ^{19}F NMR (C_6D_6) 4.49 (dd, J = 5.0 Hz, 2.5 Hz); MS (FAB) 701.0 (M + Na$^+$); UV λ_{max} = 272 nm; mp 90 °C. Anal. Calcd for $C_{36}H_{43}N_2O_8F_1Si_1$: C, 63.69; H, 6.40; N, 4.13. Found: C, 63.45; H, 6.59; N, 3.84.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-2′-*O*-(*tert*-butyldimethylsilyl)-5-fluorouridine (2c). In the same reaction used to prepare compound **2a**, 0.8 g of **2c** is produced (13% yield). **2c**: R_f (solvent B) 0.46; ^{1}H NMR (C_6D_6) 8.90 (bs, 1H), 8.15 (d, J = 6.0 Hz, 1H), 7.63−6.71 (m, 13H), 5.92 (d, J = 2.4 Hz, 1H), 4.33 (t, J = 3.4 Hz, 1H), 4.22 (m, 1H), 3.67 (dd, J = 11.0 Hz, 2.2 Hz, 1H), 3.54−3.34 (m, 2H), 3.27 (s, 6H), 1.01 (s, 9H), 0.80 (s, 9H), 0.31 (s, 3H), 0.21 (s, 3H), 0.00 (s, 3H), −0.087 (s, 3H); ^{19}F NMR (C_6D_6) 4.07 (d, J = 6.0 Hz); MS (FAB) 815.5 (M + Na$^+$); UV λ_{max} = 273 nm; mp 104 °C. Anal. Calcd for $C_{42}H_{57}B_2O_8F_1Si_2$: C, 63.59; H, 7.26; N, 3.53. Found: C, 64.29; H, 7.37; N, 3.13.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-5-fluorouridine 3′-(Cyanoethyl *N*,*N*-Diisopropylphosphoramidite) (3a). To a stirred THF (10 mL) solution of 1.5 mL (11.6 mmol, 4 equiv) of diisopropylethylamine, 1.03 mL (4.4 mmol, 1.5 equiv) of cyanoethyl *N*,*N*-diisopropylphosphonamidic chloride, and a catalytic amount (0.09 g, 0.7 mmol, 0.25 equiv) of DMAP under an Ar atmosphere was added, dropwise, a solution of the protected ribonucleoside **2a** (2 g, 2.9 mmol, 1 equiv) in 5 mL of THF at room temperature with stirring. After being stirred for 3 h, the reaction was worked up by diluting with 20 mL of $CH_3COOC_2H_5$ and an aqueous workup using five aliquots of saturated NaCl. The crude material was purified on silica gel using solvent C to yield 0.9 g of **3a** as a white foam (55%). The material exists as a 5:4 ratio of stereoisomers about phosphorus and two chemical shifts are observed for many of the positions. The secondary shifts are indicated in parentheses. **3a**: R_f (solvent C) 0.45; ^{1}H NMR (C_6D_6) 8.11 (8.07) (d, J = 6.1 Hz, 1H), 7.69−6.80 (m, 13H), 6.09 (5.99) (d, J = 3.8 Hz, 1H), 4.76 (4.72) (m, 1H), 4.54 (m,1H), 3.72 (m, 1H), 3.63−3.46 (m, 2H), 3.34, 3.32 (d, 6H), 1.06 (1.05) (s, 9H), 0.39 (0.37) (s, 3H), 0.33 (0.31) (s, 3H); ^{19}F NMR (C_6D_6) 3.86 (d, J = 6.1 Hz), 3.54 (d, J = 6.1 Hz); ^{31}P NMR (C_6D_6) 167.45, 166.84; MS (FAB) 901.3 (M + Na$^+$); UV λ_{max} = 272 nm; mp 78 °C. Anal. Calcd for $C_{45}H_{60}N_4O_9F_1Si_1P_1$: C, 61.47; H, 6.89; N, 6.37. Found: C, 61.59; H, 7.61; N, 6.60.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-5-fluorouridine 2′-(Cyanoethyl *N*,*N*-diisopropyl(phosphoramidite) (3b). To characterize the unwanted phosphoramidite compound **3b**, **2b** was converted to its

(12) Wang, A. C.; Kim, S. G.; Flynn, P. F.; Chou, S.-H.; Orban, J.; Reid, B. R. *Biochemistry* **1992**, *31*, 3940−3946.

(13) Pon, R. T. Solid-phase supports in oligonucleotide synthesis. In *Oligonucleotide Synthesis Protocols*; Agrawal, S., Ed.; 1991; Chapter 26.

(14) Usman, N.; Ogilvie, K. K.; Jinag, M.-Y.; Cedergren, R. J. *J. Am. Chem. Soc.* **1987**, *109*, 7845−7854.

phosphoramidite by the same procedure as the conversion of **2a** to **3a**. The material exists as a 1:1 ratio of stereoisomers about phosphorus and two chemical shifts are observed for many of the positions. The secondary shifts are indicated in parentheses. **3b**: R_f (solvent C) 0.45; ^{1}H NMR (C_6D_6) 8.16, (8.09) (d, J = 6.1 Hz, 1H), 7.69–6.80 (m, 13H), 6.40, (6.30) (d, J = 4.0 Hz, 1H), 4.70–4.64 (m, 1H), 4.53–4.47 (m, 1H), 4.27–4.21 (m, 1H), 3.71–3.64 (m, 2H), 3.31 (s, 6H), 0.90 (0.89) (s, 9H), 0.23 (0.16) (s, 3H), 0.08 (0.04) (s, 3H); ^{19}F NMR (C_6D_6) 4.32 (4.14) (d, J = 6.1 Hz); ^{31}P NMR (C_6D_6) 167.61, (167.11); MS (FAB) 901.3 (M + Na$^+$); UV λ_{max} = 272 nm; mp 76 °C. Anal. Calcd for $C_{45}H_{60}N_4O_9F_1Si_1P_1$: C, 61.47; H, 6.89; N, 6.37. Found: C, 61.11; H, 7.37; N, 6.41.

Synthesis and Purification of Oligoribonucleotides. Synthesis[15] was performed on an Applied Biosystems 380B DNA on a 15 μmol scale. After synthesis and deprotection, the crude material was desalted on Sephadex G-25 and lyophilized. Aliquots of 200–250 ODU were then purified by HPLC on a Waters Protein-Pak DEAE-5PW anion exchange column (22.5 mm × 150 mm). A gradient from 0–0.15 M sodium perchlorate (90 min) at 5 mL/min was used to elute the product. Pure fractions were combined, lyophilized, and desalted on Sephadex G-25. Trityl analysis showed that coupling yields averaged 93.7%. The CPG was removed from the column and heated (50 °C) overnight in anhydrous ammonia/methanol. The CPG was washed with anhydrous methanol and the combined methanol fractions evaporated to dryness. The dried product (18 mg) was suspended in neat triethylamine trihydrofluoride (1.5 mL) and stirred overnight, during which time a thick white precipitate formed. Water (2.5 mL) was added and the resulting solution was evaporated to dryness. The yield of crude completely deprotected material was 584 ODU. The crude was then purified by HPLC using a Waters Protein-Pak DEAE-5PW anion-exchange column (22.5 mm × 150 mm). A gradient from 10% CH_3CN/H_2O to 10% CH_3CN/0.18 M aqueous sodium perchlorate (90 min) at 5 mL/min was used to elute the product. Pure fractions were combined, lyophilized, and desalted on an Econo-pak PD-10 column to yield 140 ODU of purified oligoribonucleotide.

UV Hyperchromicity Studies. Thermal melt analyses[16,17] of the self-complementary duplex oligoribonucleotides were done on a Cary 1 UV spectrophotometer. Solutions of the oligoribonucleotides in 2 mM cacodylate, 100 mM NaCl, 2 mM EDTA, 5 mM $MgCl_2$ at pH 7.3 and 8.1 were placed in a 1 cm pathlength quartz cuvette. Initial OD readings were 0.8. The oligoribonucleotides were heated and slowly cooled prior to thermal melt analyses. A temperature ramp from 10 to 80 °C was run at 0.5 °C/min. Ten measurements were made for each sample and each pH. The melting temperature was calculated from the UV hyperchromicity data by using the Cary thermal melt analysis software which calculates the first derivative of the hyperchromicity data.

Circular Dichroism Spectra. Spectra were recorded on a Jasco J-710 spectropolarimeter. Spectra were recorded from 230 to 330 nm at ambient temperature using a cylindrical cuvette with a pathlength of 2 mm. Ellipticity was reported as $A_L - A_R$ rather than as molar ellipticity due to uncertainty in the decamer concentrations.

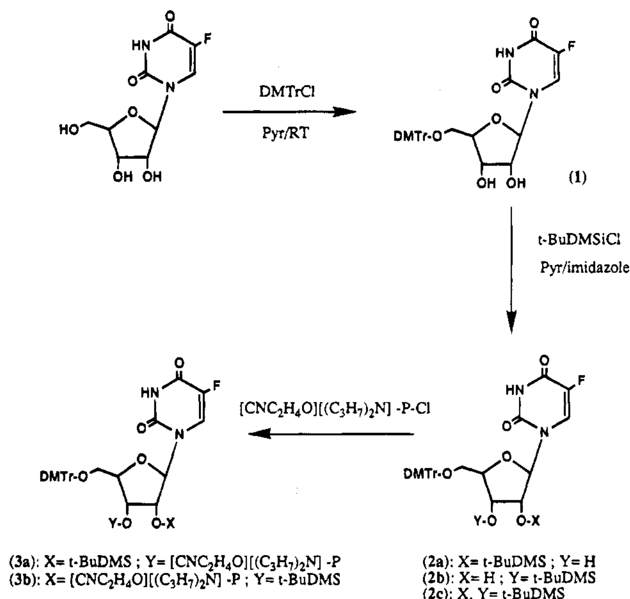
NMR Experiments. 1D NMR experiments on the duplex oligoribonucleotides were done on a Varian UNITY 500 NMR spectrometer. Samples were approximately 2.5 mM in duplex dissolved in 2 mM sodium cacodylate, 100 mM NaCl, and 2 mM EDTA, at 15 °C. Solvent suppression was achieved by using a binomial 1-3-3-1 pulse sequence. NOE difference spectra were obtained by subtracting the FIDs from experiments in which either the imino hydrogen was selectively irradiated for 1.5 s or irradiation was applied 10 kHz off resonance.

(15) Gait, M. J., Ed. *Oligonucleotide Synthesis: A Practical Approach IRL*; Oxford Press: Oxford, 1984.

(16) LeBlanc, D. A.; Morden, K. M. *Biochemistry* **1991**, *30*, 4042–4047.

(17) Hall, K. B.; McLaughlin, L. W. *Biochemistry* **1991**, *30*, 10606–10613.

Scheme 1. Preparation of Synthetic Intermediates for 5-FU Incorporation in RNA Oligonucleotides



Results and Discussion

Synthesis of Protected 5-FUrd Phosphoramidite. The procedure for the preparation of the protected 2′-O-silylated 5-fluorouridine 3′-O-phosphoramidite is shown in Scheme 1. The procedure is an adaptation of the method of Usman et al.[14] for the preparation of 2′-O-silylated uridine 3′-O-phosphoramidite. The 5-fluorouridine compound is well-behaved through all steps. Tritylation of 5-fluorouridine occurs regiospecifically and in good yield at the 5′-hydroxyl to yield compound **1**. Silylation with *tert*-butyldimethylsilyl chloride yields a mixture of all three possible products **2a**, **2b**, and **2c**. The desired product, 5′-O-(4,4′-dimethoxytrityl)-2′-O-(*tert*-butyldimethylsilyl)-5-fluorouridine (**2a**), can be chromatographically purified on silica gel in about 20% yield. The remaining two products, the 3′-O-(*tert*-butyldimethylsilyl) isomer **2b** and the bis-silylated product **2c**, are produced in 18 and 13% yield. These materials may be recycled by removal of the silyl protecting group(s) with tetrabutylammonium fluoride in THF. Use of pyridine as solvent rather than THF reduced the amount of bis-silylated material produced. There is a slight preference for formation of the desired isomer **2a**. The purification of **2a** on silica gel requires use of a weakly eluting mobile phase in order to cleanly resolve compounds **2a** and **2b**. Compounds **2a** and **2b** in addition to having clearly different mobilities by analytical TLC have distinct ^{1}H NMR spectra, particularly at H1′, H2′, and H3′. ^{1}H NMR confirmed the regioisomers were cleanly separated from one another. Isolated yields of pure **2a** of 20% based on **1** could be obtained by this method. The remaining material could be recovered and converted back to compound **1**. The desired product **2a** is readily converted to the 5-fluorouridine phosphoramidite by using the procedure of Usman et al.[14] The correct nucleoside phosphoramidite, compound **3a**, showed a single set of peaks in the {^{1}H}^{31}P NMR spectrum. The peaks, in a 5:4 ratio, correspond to the stereoisomers about phosphorus. Conversion of compound **2b** to its phosphoramidite **3b** produced a single set of peaks in the {^{1}H}^{31}P NMR spectrum at a different chemical shift from compound **3a**. The ^{31}P NMR spectra for compound **3a** and

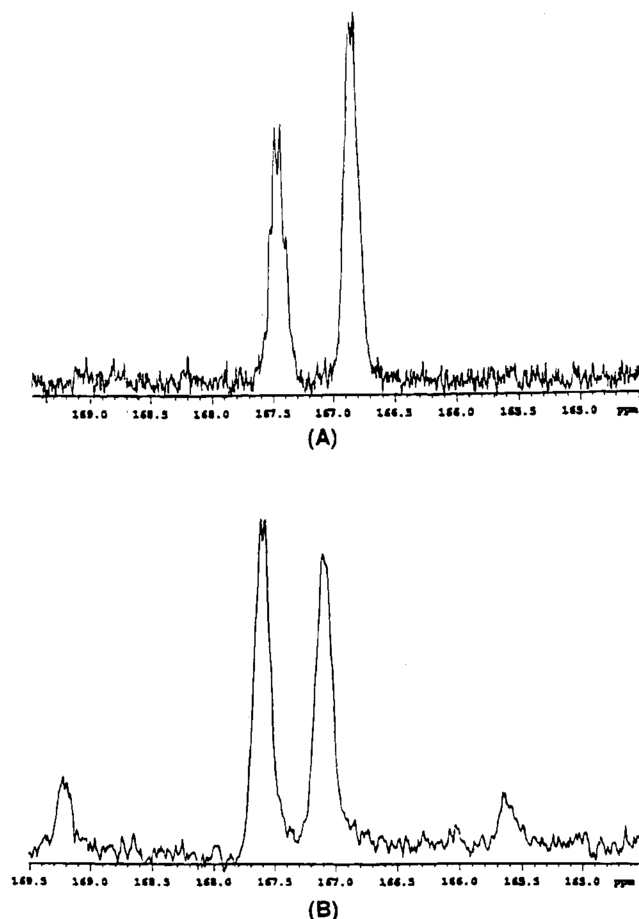


Figure 1. The ^{31}P NMR spectrum of (A) the desired phosphoramidite compound **3a** and (B) the undesired 2′-*O*-phosphoramidite compound **3b**. Both compounds exist as a pair of stereoisomers about phosphorus. The peaks from **3a** are shifted upfield approximately 0.2 ppm compared with **3b**.

for **3b** are shown in Figure 1. Compound **3a** was analyzed by ^{1}H NMR, ^{19}F NMR, and FAB-MS and found to analyze correctly.

Incorporation of (3a) into RNA. For incorporation of 2′-*O*-*tert*-butyldimethylsilyl 5-fluorouridine into RNA by solid-phase synthesis, compound **3a** is dissolved in dry CH_3CN at a concentration of 0.1 M. The normal cycles of detritylation, nucleoside addition, and capping recommended by ABI were performed without modification for compound **3a** along with the other 2′-*O*-*tert*-butyldimethylsilyl nucleoside phosphoramidites that were purchased commercially. Coupling yields based on trityl recoveries were typically >93%. The oligoribonucleotide is deprotected from the CPG support in the usual manner and purification by anion-exchange HPLC also proceeds as for oligoribonucleotides prepared solely from commercial nucleoside phosphoramidites.

UV Hyperchromicity Studies of 5′ rGCGAAU(FU)CGC. To determine the effects of FUrd incorporation on the thermal stability of RNA duplexes, solutions of rGCGAAU(FU)CGC and rGCGAAUUCGC were prepared in 2 mM cacodylate, 100 mM NaCl, 2 mM EDTA, and 5 mM $MgCl_2$. UV absorbance at 260 nm was measured as a function of temperature for both samples after warming and slowly cooling the samples. Initial absorbances were near 0.80 in 1 cm pathlength quartz cuvettes. The thermal melt data was acquired ten times and also at two pH values, 7.3 and 8.1. The results are summarized in Table 1. The control oligomer had a

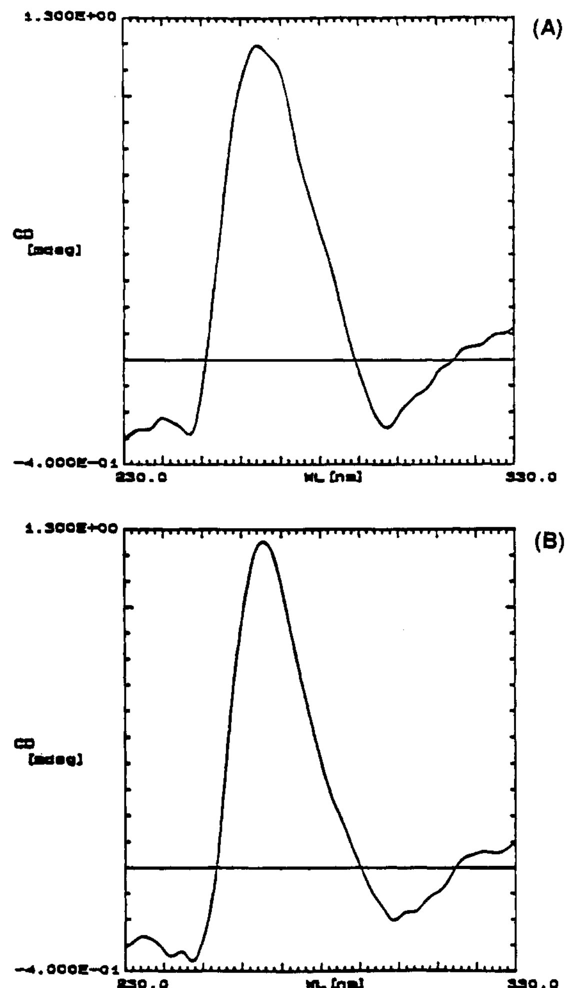


Figure 2. The circular dichroism spectra of rGCGAAUUCGC (A) and rGCGAAU(FU)CGC (B) in 2 mM sodium cacodylate, 100 mM NaCl, 2 mM EDTA, and 5 mM $MgCl_2$ at pH 7.3.

Table 1. Melting Temperature (°C) of FUrd-Substituted RNA

oligonucleotide	pH 7.3	pH 8.1
5′rGCGAAUUCGC	53.78 ± 0.26	55.85 ± 0.26
5′rGCGAAU(FU)CGC	56.58 ± 0.38	57.80 ± 0.18
		$n = 10$

sharp melting transition at 53.78 °C at pH 7.3 with little variance. Increasing the pH to 8.1 increases the T_m to 55.58 °C. A reproducible stabilization is observed for the FUrd substituted oligonucleotide compared with the control oligomer near physiological pH. At pH 7.3 the T_m of the control oligomer is 53.78 and the FUrd substituted oligomer is 56.58 °C. At the higher pH, the stabilization effect by FUrd is slightly less. The results indicate that FUrd has a slightly stabilizing effect on duplex oligonucleotide secondary structure near physiological pH that is less stabilizing at elevated pH values where the imino hydrogen is appreciably ionized.

CD Studies on 5′ rGCGAAU(FU)CGC. The circular dichroism data for 5′ rGCGAAU(FU)CGC and for rGCGAAUUCGC in the same buffer used for the thermal melt analyses are shown in Figure 2. The spectra show large positive ellipticities near 270 nm and a small negative band near 290 nm. The spectra for both oligomers at pH 7.3 are shown in Figure 2. These spectral characterisitics are consistent with the adoption of an A-form duplex for both oligonucleotides. The rGCGAAU(FU)CGC duplex has a slightly narrower posi-

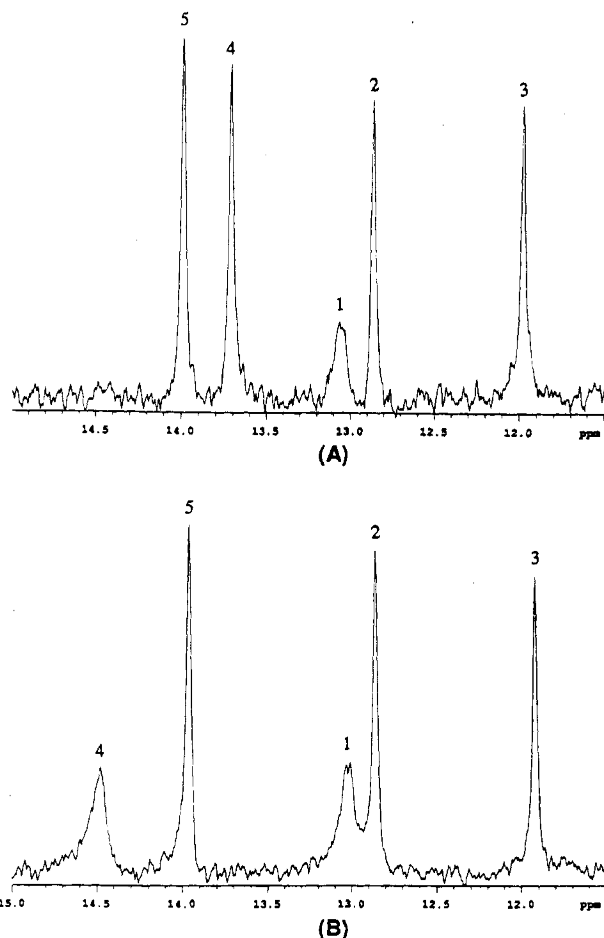


Figure 3. The 1D ^{1}H NMR spectrum of $rG_1CG_3AAU_5U_4CG_2C$ (A) and $rG_1CG_3AAU_5(FU)_4CG_2C$ (B) acquired by using a 1-3-3-1 pulse sequence in H_2O solution. The chemical shifts for the imino hydrogen from each base pair is indicated in the spectra. The resonance from the $FUrd_4$–A base pair is broadened and moved downfield relative to the U_4–A base pair.

tive absorbance at 270 nm but is otherwise nearly indistinguishable from the rGCGAAUUCGC duplex. The CD spectra were also acquired at pH 8.1. There was no apparent pH dependent change in the CD spectra of either oligonucleotide. The CD spectra indicate that FUrd substitution has only a slight effect on RNA duplex secondary structure even at pH values where the FUrd imino hydrogen is appreciably ionized.

Imino Hydrogen Studies. The 1D spectrum of the imino hydrogen region of 5' rGCGAAU(FU)CGC and 5'-rGCGAAUUCGC are shown in Figure 3. The five resonances from imino hydrogens expected from formation of the self-complementary decamer are apparent at 15 °C. The 1D NOE data show transfer of magnetization among imino hydrogens of neighboring base pairs and are used to assign the spectrum. Compared with the resonance from the uridine imino hydrogen, the resonance from the 5-fluorouridine imino hydrogen is markedly reduced in intensity (Figure 3). This is consistent with what has been observed in duplex DNA containing 5-fluorodeoxyuridine. The decrease in peak intensity is due to the rapid exchange of this imino hydrogen with bulk water as a consequence of the decrease in pK_A for 5-FU compared with U (7.3 for 5-FU, 7.9 for U). The decrease in intensity is similar to that observed for the terminal GC base pair in the duplex where exchange of the imino hydrogen is enhanced by fraying at the terminus. The peaks are all well resolved. The imino hydrogen from 5-fluorouridine in addition to being reduced in intensity is shifted downfield compared with an AU base pair. This has also been observed for duplex DNA containing FdU.

The study of FUrd substituted RNA by biophysical methods requires the production of a suitably protected phosphoramidite of this compound. We have shown that the approach taken by Usman[14] and others for the preparation of nucleoside phosphoramidites of the common nucleosides may be applied to FUrd. Tritylation at the 5'-OH is regiospecific and high yielding. Silylation with *tert*-butyl dimethylsilyl chloride occurs without regiospecificity. The desired compound with the 2'-OH silylated may be chromatographically purified and the remaining silylated material recovered and desilylated. Preparation of the phosphoramidite and chromatographic purification is then accomplished in good yield. Analytical data are summarized for all compounds including both the desired and the undesired regioisomers. RNA containing FUrd may be prepared by solid phase methods and purified by HPLC. The self-complementary decamer rGCGAAU(FU)CGC was synthesized on a 15 μmol scale. The duplex rGCGAAU(FU)CGC shows increased stability relative to the control duplex rGCGAAUUCGC near physiological pH (7.3). At basic pH (8.1) the difference in stability is less indicating that ionization of the FUrd residue may destabilize the duplex. Both the FUrd substituted and the control duplex adopt A-form helices as indicated by CD studies. The A-form helix is not effected by an increase in pH from 7.3 to 8.1. The imino hydrogen spectrum of the FUrd substituted duplex is similar to the control duplex except the FUrd imino hydrogen is shifted downfield and noticeably broadened compared with the uridine imino resonances. The results demonstrate the feasibility of preparing milligram quantities of FUrd-substituted RNA for biophysical studies. These and similar studies in the future increase our understanding of the RNA-mediated effects of 5-FU.

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