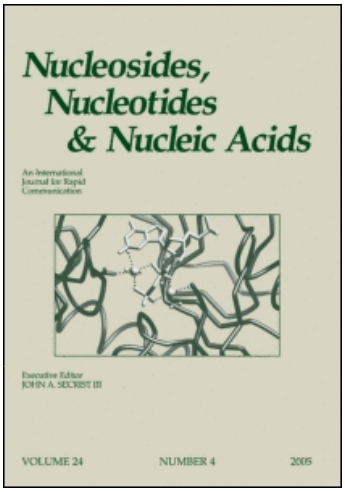

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Synthesis and Some Properties of Modified Oligonucleotides. II. Oligonucleotides Containing 2'-Deoxy-2'-fluoro-β-D-arabinofuranosyl Pyrimidine Nucleosides

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To cite this Article Kois, Pavol , Tocik, Zdenek , Spassova, Maria , Ren, Wu-Yun , Rosenberg, Ivan , Soler, Jaume Farras and Watanabe, Kyoichi A.(1993) 'Synthesis and Some Properties of Modified Oligonucleotides. II. Oligonucleotides Containing 2'-Deoxy-2'-fluoro-β-D-arabinofuranosyl Pyrimidine Nucleosides', Nucleosides, Nucleotides and Nucleic Acids, 12: 10, 1093 — 1109
To link to this Article: DOI: 10.1080/07328319308016207
URL: http://dx.doi.org/10.1080/07328319308016207

NUCLEOSIDES & NUCLEOTIDES, 12(10), 1093-1109 (1993)

SYNTHESIS AND SOME PROPERTIES OF MODIFIED OLIGONUCLEOTIDES. 2. OLIGONUCLEOTIDES CONTAINING 2'-DEOXY-2'-FLUORO-β-D-ARABINOFURANOSYL PYRIMIDINE NUCLEOSIDES.[1]

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ABSTRACT. In order to find the effects of unnatural nucleosides on the stability of duplex, several oligonucleotides containing 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-uracil (FAU), -cytosine (FAC) and -thymine (FMAU) were synthesized by two alternative approaches: phosphoramidite method on an ABI 392 synthesizer and H-phosphonate procedure on our GeneSyn I universal module synthesizer. It was shown from the melting profiles that the presence of FMAU has a large stabilizing effect on the duplex. Replacement of thymidine with FAU, or deoxycytidine with FAC resulted in the formation of less stable duplexes. Temperature-dependent CD spectroscopy demonstrated that the structures of the fluorine containing oligomers are very similar to those of unmodified oligomers.

INTRODUCTION

Many antisense oligonucleotides have been prepared and studied targeting at oncogenic retroviral genes and cellular oncogenes (or proto-oncogenes).[2,3] The results showed that inhibition of the expression for single proteins by oligodeoxynucleotides complementary to their mRNA's is possible. For example, *ras* oncogenes have been implicated in the initiation and progression of many neoplasms,[4] and expression of *ras* mRNA can be regulated both in cell-free and cell culture systems by selective hybridization of complementary antisense oligodeoxynucleotides to *ras* mRNA.[5-7] Recently, Wickstrom *et al.*[8] showed that antisense "DNA *methylphosphonate*", targeted against *c-myc* mRNA, inhibited

This paper is dedicated to Professor Leroy B. Townsend on the occasion of his 60th Birthday.

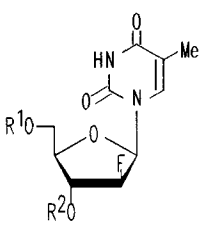
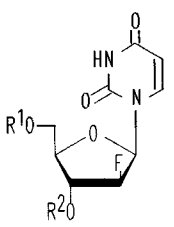
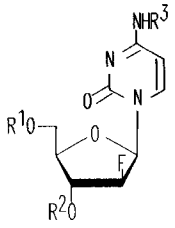
production of cMYC protein in peripheral lymphocytes in Eμ-*c-myc* transgenic mice. The dose used for the experiments was 300nM (0.3 μM) of pentadecamer. A highly sequence-specific efficacy *in vivo* and possible therapeutic uses of fully modified antisense artery model[9] (18-mer) and in a SCID mouse model (24-mer).[10] One of the reasons for the *phosphorothioates* (complementary to *c-myb* RNA) have been demonstrated in a rat carotid 1very high doses required for these most extensively studied oligomers (methylphosphonates and phosphorothioates) may be due to the production of large amounts of mRNA molecules (R: single stranded RNA) is so rapid that large amounts of antisense oligomers (D: single stranded DNA) are required to destroy the R chain of the RD hybrid effectively by RNAse H. Also, their inability to form stable duplexes may be responsible for the necessity of large amounts of antisense oligomers. If an RD hybrid is very unstable, it would dissociate more efficiently than RNAse H can recognize it to form the enzyme-substrate complex. The stability of duplex is decreased when the phosphodiester backbone is replaced by phosphorothioate as indicated by T_m.[11,12] "DNA methylphosphonates"-involving duplexes are also less stable than the duplexes of unmodified oligomers.[13] Therefore, studies to find means to construct oligomers to form stable duplex would be important.

Fluorine with the unique characteristics, which is more lipophilic and electronegative than hydrogen, makes changes in the hydrodynamics of the oligomer as to hydration and ionic environment. This effect is non-specific but may be important in recognition by DNA targeted agents such as intercalators, dyes, RNAs and proteins.

We already found[1] that the FMAU containing oligomers (especially homo-oligomers of FMAU) considerably increase the stability of duplexes with complementary sequence of natural oligomers. A very similar phenomenon is observed with 2'-F-*ribo*-T ($_f$T) by Eckstein *et al.*[14] and Cook *et al.*[15] However, we also found that FAU appears to destabilize the duplex.[1] Therefore, more systematic studies using all the 2'(β)-fluoro-nucleosides to find favorable combinations for forming stable duplexes are warranted.

EXPERIMENTAL PART

Material and Methods. All solvents were of analytical grade. Triethylamine was dried over CaH_2. For preparation of solid supports, LCAA-CPG 500A (Sigma) was used. Modified nucleosides, FMAU (Ia),[16,17] FAU (IIa)[18] and FAC ((IIIa)[18,19] (Figure 1) were prepared by the procedures developed in our laboratory. Dimethoxytrityl chloride and CPG-linked regular 2'-deoxy-nucleosides were obtained from Chemgenes. Bis(diisopropylamino)-2-

Ia $R^1 = R^2 = H$ (FMAU) **IIa** $R^1 = R^2 = H$ (FAU) **IIIa** $R^1 = R^2 = H$ (FAC)

b Series: R^1 = DMTr, R^2 = H, R^3 = Bz

c Series: R^1 = DMTr, R^2 = succinyl-LCAA-CPG, R^3 = Bz

d Series: R^1 = DMTr, R^2 = $-P(N(iPr)_2)-OCH_2CH_2CN$, R^3 = Bz

e Series: R^1 = DMTr, R^2 = $-P(H)(=O)-O^- NEt_3^+$, R^3 = Bz

Figure 1. Modified nucleosides incorporated into oligomers

cyanoethoxy-phosphane, tetrazole, anhydrous pyridine and acetonitrile were purchased from Aldrich. All moisture-sensitive reactions were performed under an atmosphere of dry argon. Nuclease P1 and bacterial alkaline phosphatase were purchased from Sigma.

Silica TLC plates (Merck DC-Alufolien Kieselgel 60 F_{245} sheets) were developed in solvent system A ($CHCl_3$-MeOH 9:1 v/v) or B ($CHCl_3$-EtOAc-Et_3N 45:45:10 v/v/v). Compounds were visualized under UV light or exposure to trifluoroacetic acid vapor for compounds containing a dimethoxytrityl group. Silica gel G60 (EM Science) was used for flash chromatography.

Analytical reversed phase (RP) HPLC was performed on a Rainin Microsorb C18 column (0.46 x 25 cm, 5 μm, flow rate 1 mL/min) using ISCO 2004i HPLC bio-inert instrument equipped with a dual-pump gradient system, V^4 variable UV-VIS detector and ChemResearch PC software. Preparative HPLC for purification of oligonucleotides was performed on a Rainin Dynamax 300A C18 column (1 x 25 cm, 5 μm, 3 mL/min). Two systems were used as mobile phases. A: 3% CH_3CN in 0.1 M Et_3NHCO_3, B: 70% CH_3CN.

For analytical measurements, linear gradient of B to 30% during 25 minutes was used, and for preparative chromatography, linear gradient of B to 45% in 40 minutes was used.

^1H and ^{31}P NMR spectra were recorded on a Bruker AMX-400 spectrometer with Me_4Si as the internal standard. Chemical shifts are reported in ppm (δ), and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet) and (dd) (double doublet). Values given for coupling constants are first order.

Synthesis of nucleoside analogues: Dimethoxytritylation, silylation and benzoylation were performed according to the well-established procedures.[20]

N^4-Benzoyl-1-(2-deoxy-2-fluoro-5-O-[4,4'-dimethoxy]trityl-β-D-arabinofuranosyl)cytosine (IIIb, DMTrO-FAC-Bz) was obtained in 80 % yield: ^1H NMR (Me_2SO-d_6) δ 3.70-3.80 (m, 2H, H5',5"), 3.75 (s, 6H, 2 x OMe), 4.09-4.13 (m, 1H, H4'), 4.29 (d, 1H, H3', $J_{2',3'} = J_{3',4'} = 0$, $J_{3',F} = 19.0$ Hz), 5.14 (ddd, H2', $J_{1',2'} = 4.0$, $J_{2',3'} = 2.9$, $J_{2',F} = 52.3$ Hz), 6.04 (s, 1H, 3'-OH, exchangeable), 6.23 (dd, 1H, H1', $J_{1',2'} = 4.0$, $J_{1',F} = 16.1$, Hz), 6.92 (d, 4H, DMTr), 7.24-7.63 (m, 14H, DMTr, H5, H6 and Bz), 8.00 (d, 2H, Bz), 11.2 (bs, 1H, NH, exchangeable).

1-(2-deoxy-2-fluoro-5-O-[4,4'-dimethoxy]trityl-β-D-arabinofuranosyl)uracil (IIb, DMTrO-FAU) was obtained in 80 % yield: ^1H NMR (Me_2SO-d_6) δ 3.27 (m, 2H, H5',5"), 3.74 (s, 6H, 2 x OMe), 3.97 (m, 1H, H4'), 4.25 (d, 1H, H3', $J_{2',3'} = J_{3',4'} = 0$, $J_{3',F} = 20.0$ Hz), 5.05 (ddd, 1H, H2', $J_{1',2'} = 4.1$, $J_{2',3'} = 3.4$, $J_{2',F} = 52.8$ Hz), 5.48 (d, 1H, H5, $J_{5,6} = 8.1$ Hz), 5.99 (s, 1H, 4'OH, exchangeable), 6.18 (dd, 1H, H1', $J_{1',2'} = 4.1$, $J_{1',F} = 15.6$ Hz), 6.90 (d, 4H, DMTr), 7.21-7.39 (m, 9H, DMTr), 11.5 (bs, 1H, NH, exchangeable).

1-(2-deoxy-2-fluoro-5-O-[4,4'-dimethoxy]trityl-β-D-arabinofuranosyl)thymine (Ib, DMTrO-FMAU) was obtained in 75 % yield: ^1H NMR (Me_2SO-d_6) δ 1.59 (s, 3H, 5Me), 3.28 (m, 2H, H5',5"), 3.74 (s, 6H, 2 x OMe), 3.96 (m, 1H, H4'), 4.30 (d, 1H, H3', $J_{2',3'} = J_{3',4'} = 0$, $J_{3',F} = 21.3$ Hz), 5.06 (ddd, 1H, H2', $J_{1',2'} = 4.2$, $J_{2',3'} = 3.4$, $J_{2',F} = 52.8$ Hz), 6.18 (dd, 1H, H1', $J_{1',2'} = 4.2$, $J_{1',F} = 16.0$ Hz), 6.90 (d, 4H, DMTr), 7.21-7.39 (m, 9H, DMTr), 7.42 (d, 1H, H6, $J_{6,1'} = 0.7$ Hz), 11.5 (bs, 1H, NH, exchangeable).

CPG derivatization: Succinylation of the protected nucleosides (**Ib - IIIb**), followed by activation as the *p*-nitrophenyl esters and coupling to long chain-aminoalkyl CPG, afforded the polymer-linked modified nucleosides **Ic** (DMTrO-FMAU-CPG; 36 μmol/g), **IIc** (DMTrO-FAU-CPG; 30 μmol/g), and **IIIc** (DMTrO-FAC(Bz)-CPG; 30 μmol/g).

5'-O-Dimethoxytrityl-FMAU-3'-O-(N,N-diisopropylamino)cyanoethylphosphoramidites (Id, DMTrO-FMAU-(CEPA): Compound **Ib** (695 mg, 1.2 mmol) and diisopropylammonium tetrazolide (103 mg, 0.6 mmol) were dried overnight in high vacuum at room temperature, and then dissolved in anhydrous acetonitrile (6-7 mL). To the solution was added bis(diisopropylamino)-2-cyanoethoxyphosphane (500 mg, 1.3 mmol, commercial product of approx. 80% purity according to ^{31}P NMR). The mixture was allowed to react for 1 hour at room temperature, and then concentrated *in vacuo*. The residue was dissolved in ethyl acetate (saturated with argon, 50 mL), extracted with saturated sodium bicarbonate solution (2 x 25 mL), saturated sodium chloride solution (3 x 25 mL), dried (sodium sulfate), and then concentrated *in vacuo* to a foam. The foam was dissolved in dry dichloromethane (5 mL), and then precipitated by addition of pentane (800 mL). The precipitates were collected by filtration, washed with cold pentane, and dried in a vacuum desiccator. The precipitates contained a small amount of inorganic phosphate according to ^{31}P NMR. The crude product was chromatographed over a silica gel column using a gradient of methanol (0 - 5%) in dichloromethane:triethylamine (99:1 v/v). Appropriate fractions (checked by TLC) were combined and concentrated *in vacuo* to give a diastereomeric mixture of **Id** (790 mg, 85% yield, 100% purity according to RP HPLC (85% acetonitrile/0.1 M TEAB). Traces of phosphorus-containing impurity were detected by ^{31}P NMR. One of the isomers, separated by column chromatography, had the following ^1H NMR characteristic: (Me_2SO-d_6) δ 0.93, 0.96, 1.07, 1.10 (4s, 12H, 2 x CH*Me*$_2$), 1.59 (s, 3H, 5Me), 2.62 and 2.74 (2t, 2H, OCH$_2$*CH*$_2$CN), 3.31 (m, 2H, H5',5"), 3.50 (m, 2H, PO*CH*$_2$CH$_2$CN), 3.73 (s, 6H, 2 x OMe), 4.05 (m, 1H, H4'), 4.55 (m, 1H, H3'), 5.27 (ddd, 1H, H2', $J_{1',2'}$ = 4.8, $J_{2',3'}$ = 3.6, $J_{2',F}$ = 53.5 Hz), 6.23 (dd, 1H, H1', $J_{1',2'}$ = 4.8, $J_{1',F}$ = 15.1 Hz), 6.86 (d, 4H, DMTr), 7.22-7.43 (m, 10H, H6 and DMTr), 11.35 (bs, 1H, NH, exchangeable), ^{31}P NMR (Me_2SO-d_6) δ 137.01 and 137.46 (2 x s).

In a similar manner the following derivatives were prepared. **DMTrO-FAU-(CEPA) (IId)** as a distereomeric mixture: ^1H NMR (Me_2SO-d_6) δ 0.92 (2s, 2H, C*H*Me$_2$), 1.10-1.21 (m, 12H, CH*Me*$_2$ overlapping), 2.75 and 2.89 (2t, 2H, CH$_2$*CH*$_2$CN), 3.32 (m, 2H, H5',5"), 3.49-3.61 (m, 2H, PO*CH*$_2$CH$_2$CN), 3.74 (s, 6H, 2 x OMe), 4.08 (m, 1H, H4'), 4.54 (m, 1H, H3'), 5.19 and 5.32 (2m, 1H, H2', $J_{2',F}$ = 51 Hz), 5.45 and 5.50 (2d, 1H, H5, $J_{5,6}$ = 8.1 Hz), 6.21 (m, 1H, H1', $J_{1',F}$ = 18.6 Hz), 6.88-7.65 (m, 14H, H6 and DMTr), ^{31}P NMR (Me_2SO-d_6) δ 137.29 and 137.54 (2 x s), and **DMTrO-FAC(Bz)-(CEPA) (IIId)** also as a diastereomeric mixture: ^1H NMR (Me_2SO-d_6) δ 1.01-1.35 (8 peaks, 12H, 2 x iPr), 2.44 and 2.63 (2t, 2H,

$OCH_2\underline{CH_2}CN$), 3.35-3.90 (m, 4H, H5',5" and $PO\underline{CH_2}CH_2CN$)), 3.74 (s, 6H, 2 x OMe), 4.27 (m, 1H, H4'), 4.55 (m, 1H, H3'), 5.29 (2dm, 1H, H2', $J_{2',F}$ = 52.0 Hz), 6.32 (dd, 1H, H1', $J_{1',2'}$ = 3.1, $J_{1',F}$ = 19.0 Hz), 6.88 (m, 4H, DMTr), 7.22-8.00 (m, 16H, H5,6, Bz and DMTr), 8.75 (bs, 1H, NHBz), ^{31}P NMR ($CDCl_3$) δ 134.29 and 134.34 (2 x s).

Triethylammonium salts of modified nucleoside 3'-hydrogenphosphonates were prepared as described previously.[1]

Triethylammonium 5'-O-dimethoxytrityl-FMAU-3'-O-hydrogenphosphonate (Ie, DMTrO-FMAU-HP-Et$_3$N), ^1H NMR ($CDCl_3$) δ 1.26 (t, 9H, 3 x $NCH_2\underline{CH_3}$), 1.70 (s, 3H, 5Me), 2.96 (q, 6H, 3 x $N\underline{CH_2}CH_3$), 3.35-3.43 (m, 2H, H5',5"), 3.77 (s, 3H, OMe), 4.24 (m, 1H, H4'), 4.84 (ddd, 1H, H3', $J_{2',3'}$ = 0, $J_{3',4'}$ = 3.2 $J_{3',F}$ = 27.6, $J_{3',P}$ = 10.1 Hz), 5.24 (dd, 1H, H2', $J_{2',F}$ = 51, $J_{1',2'}$ = 3.1, $J_{2',3'}$ = 0 Hz), 6.26 (dd, 1H, H1', $J_{1',2'}$ = 3.1, $J_{1',F}$ = 20.9 Hz), 6.89 (d, 1H, PH, $J_{P,H}$ = 627 Hz), 6,79 - 7,47 (m, 13H, DMTr), 7.34 (s, 1H, H6), ^{31}P NMR ($CDCl_3$) δ -3.33 ($J_{P,H}$ = 626.9, $J_{3',P}$ = 10.1 Hz); **triethylammonium 5'-O-dimethoxytrityl-FAU-3'-O-hydrogenphosphonate (IIe, DMTrO-FAU-HP-Et$_3$N)**, ^1H NMR ($CDCl_3$) δ 1.29 -1.33 (3 x t overlapped, 9H, 3 x $NCH_2\underline{CH_3}$), 3.05 (3 x q overlapped, 6H, 3 x $N\underline{CH_2}CH_3$), 3.37 (m, 2H, H5',5"), 3.78 (s, 6H, 2 x OMe), 4.29 (m, 1H, H4'), 4.81 (m, 1H, H3'), 5.24 (dd, 1H, H2', $J_{2',F}$ = 51.2, $J_{1',2'}$ = 2.4, $J_{2',3'}$ = 0 Hz), 5.54 (d, 1H, H5, $J_{5,6}$ = 8.2 Hz), 6.25 (dd, 1H, H1', $J_{1',2'}$ = 2.4, $J_{1',F}$ = 20.3 Hz), 6.92 (d, 1H, PH, $J_{P,H}$ = 632 Hz), 6.82 (d, H6, $J_{5,6}$ = 8.2 Hz), 6,79 - 7,47 (m, 13H, DMTr), ^{31}P NMR ($CDCl_3$) δ -3.23 ($J_{P,H}$ = 631.5, $J_{3',P}$ = 10.0 Hz); and **triethylammonium 5'-O-dimethoxytrityl-FAC-N^4-benzoyl-3'-O-hydrogenphosphonate (IIIe, DMTrO-FAC-Bz-HP-Et$_3$N)** ^1H NMR ($CDCl_3$) δ 1.25 -1.33 (3 x t overlapped, 9H, 3 x $NCH_2\underline{CH_3}$), 2.96 - 3.03 (3 x q overlapped, 6H, 3 x $N\underline{CH_2}CH_3$), 3.41 (m, 2H, H5',5"), 3.80 (s, 6H, 2 x OMe), 4.40 (m, 1H, H4'), 4.79 (m, 1H, H3'), 5.38 (dd, 1H, H2', $J_{2',F}$ = 50.7, $J_{1',2'}$ = 2.8, $J_{2',3'}$ = 0 Hz), 6.31 (dd, 1H, H1', $J_{1',2'}$ = 2.8, $J_{1',F}$ = 20.1 Hz), 6.92 (d, 1H, PH, $J_{P,H}$ = 632 Hz), 6,83 - 7,89 (m, 20H, DMTr, Bz, H5,6), 8.75 (bs, 1H, NH), ^{31}P NMR ($CDCl_3$) δ -3.86 ($J_{P,H}$ = 625, $J_{3',P}$ = 9.89 Hz).

Synthesis of oligonucleotides: Oligomers **(2-6)**, containing larger numbers of 2'-fluorinated nucleotides, were synthesized in a 0.5 μmol scale on the GeneSyn synthesizer[1] using the H-phosphonate procedure. Homo-oligomers dT_{11} **(1)** dA_{11} and the Dickerson's sequence **(7)**, as well as those oligonucleotides **(8-13)**, containing mainly natural nucleotides, were synthesized on an Applied Biosystems Model 392 DNA/RNA 2-column synthesizer by the standard CE-phosphoramidite method in 1 μmol scale.

Cleavage of the oligonucleotides from the polymer support and removal of the protecting groups were accomplished by treatment with concentrated aqueous ammonia (1.5

mL) in a screw-capped glass vial. After 48 hours at room temperature the suspension was filtered and concentrated to about 0.7-0.8 mL by Speed-Vac. A part of this crude oligonucleotide solution (100 μL) was mixed with *n*-butanol (1 mL) in an Eppendorf tube, and the mixture was shaken for 5 minutes. The tube was centrifuged, the supernatant decanted, and the residue dissolved in water (100 μL). The solution was again extracted with *n*-butanol, and the precipitates were dried in an exicator *in vacuo* to give crude oligomer (about 10 OD units per tube). After purification by RP HPLC, the A_{260}/A_{235} ratio of the oligomers increased from 1.3 to 2.9. The oligonucleotides were stored in the lyophilized form at -30 °C.

Enzymic hydrolysis: The oligonucleotides (2 A_{260} units) were incubated in 100 μL of a buffer solution, made of 20mM NaOAc (pH 5.5) and 5mM $ZnCl_2$, containing 2 U of nuclease P_1 from *Penicillium citrinum* for 3 hours at 37 °C. The rate of phosphodiester bond cleavage was analyzed at different intervals of time by an RP HPLC.

For estimation of nucleoside composition, the pH of the reaction mixture was raised by addition of 10 μL of 1 M sodium glycinate buffer to pH 8, and then 2 U of alkaline phosphatase were added for dephosphorylation of the deoxynucleotides in DNA digest. After 2 hours of incubation at 37 °C, aliquots were examined by analytical RP HPLC.

UV measurement: Spectral analyses and melting temperature experiments were performed using a Gilford Response spectrophotometer equipped with a six-position Peltier thermocell. Sample solutions (equimolar mixture of the complementary oligonucleotides) were placed in 360 μL quartz cuvettes (10mm path length) with teflon stoppers. The composition of buffers were: A: 10 mM HEPES pH 6.9, 100 mM NaCl, B: 10 mM Tris buffer pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl (medium salt), and C: 10 mM Tris buffer, pH 7.5, 10 mM $MgCl_2$, 1 M NaCl (high salt). The absorbance at 260 nm *vs* temperature was measured at 0.5°C intervals and T_m determined as the maximum of the first derivative of the melting curve. The extinction coefficient (ϵ) of the oligonucleotide was estimated as sum of the ϵ values of the constituent monomeric nucleosides multiplied by 0.9 or in the case of self-complementary oligomer by a factor of 0.8.[21]

The CD spectra of oligonucleotides were measured on a JASCO J-710 spectropolarimeter in a 1 mm thermostatted 200 μL cell in the same buffer as in the measuring of T_m. The spectra are average of 3 scans, and are corrected with a spectrum of buffer alone and then smoothed.

RESULTS AND DISCUSSION

The aim of this study was to examine the effect of the 2'-β-fluoro substituent on the stability of a duplex. Thus, we synthesized several oligonucleotides containing 2'-fluoroarabinosyl nucleotides, and compared their basic characteristics with unmodified oligomers or with those containing the 2'-α-fluoro-(ribo)-nucleotides, which had been studied earlier by Eckstein *et al.*[22,23]

Two types of models were analyzed: *i.e.*, dT_{11}/dA_{11} duplex with variable number of dT modification in the pyrimidine strand (**1-6**, Table **1**), and the self-complementary Drew-Dickerson's dodecamer,[24] d(CGCGAATTCGCG), with varied degree of modification at dC or dT in the sequence (**8-13**). The latter forms a duplex in which both strands contain the same number of modified nucleotides.

Synthesis, Purification and Characterization of Oligonucleotides: Nucleosides **Ia, IIa** and N^4-benzoylated **IIIa** (Figure 1) were protected at the 5'-position by reaction with 4,4'-dimethoxytrityl chloride in pyridine. The products **Ib** and **IIIb** were then treated with bis(diisopropylamino)-2-cyanoethoxyphosphane[25] to give the corresponding phosphoramidites **Id** and **IIId**. Reaction of **Ib - IIIb** with tris(1,1,1,3,3,3-hexafluoro-2-propyl)-phosphite[26] afforded the corresponding H-phosphonates, **Ie - IIIe**.

The 2'-fluorinated monomers were found to be less reactive than the unmodified counterparts. Oligomers containing modified nucleotides at separate positions were synthesized effectively by the standard protocol. For the effective synthesis of oligomers containing modified units at contiguous positions, however, it was necessary to change the concentration of the monomers up to 0.12 M and to increase the time of condensation step.

Cleavage of the oligonucleotide from the solid support and removal of N- and P-protecting groups were accomplished with concentrated aqueous ammonia within 48 hours at room temperature without significant degradation of modified oligonucleotides. An even prolonged ammonia treatment (72 hours) caused no apparent increase of degradation. Figure 2 shows examples of two oligonucleotides synthesized by different procedures.

It was found that extraction of benzamide and other organic impurities with *n*-butanol prior to HPLC purification was very useful as the A_{260}/A_{235} ratio of the crude oligomer increased from 1.3 to 2.9. This ratio did not change after purification by preparative RP HPLC.

Incorporation of modified units into the synthesized oligomers was verified by enzymatic hydrolysis with nuclease P1, followed by alkaline phosphatase and reverse phase

Table 1. Melting characteristics and thermal hypochromicities of the oligonucleotides containing 2'-fluoro-pyrimidines as substitute for thymidine or 2'-deoxycytidine.

Oligomer[a]	Modified nucleoside	T_m (°C)	System[b]	ΔT_m[c]	[h]therm[d] [%]
1 dT_{11}/dA_{11}		23.0	A	-	-
		31.0	B	-	3
		35.3	C	-	-
2 dT_5NT_5/dA_{11}	N = FMAU	33.1	B	+ 2.1	1.3
3 $dT_4N_3T_4/dA_{11}$	N = FMAU	35.1	B	+ 4.1	1.1
4 dN_5T_6/dA_{11}	N = FMAU	34.8	B	+ 3.8	2.3
		41.0	C	+10.0	-
5 dN_{11}/dA_{11}	N = FMAU	33.5	A	+10.0	-
		43.2	B	+12.2	1.7
		52.0	C	+16.7	-
6 dN_{11}/dA_{11}	N = FAU	25.1	B	- 4.9	-
		34.5	C	- 0.8	-
7 dCGCGAATTCGCG		57.2	A	-	13
		61.0	B	-	15
		65.8	C	-	15.3
8 dNGNGAATTNGNG	N = FAC	52.1	A	- 5.1	16
		57.5	B	- 3.5	15.8
		63.8	C	- 2.0	16
9 dNGNGAATTCGCG	N = FAC	56.6	A	- 0.6	11.6
		58.4	B	- 2.6	11.4
		65.2	C	- 0.6	15
10 dCGNGAATTNGCG	N = FAC	56.2	A	- 1.0	12.5
		59.4	B	- 1.6	15
11 dCGCGAANNCGCG	N = FMAU	63.3	A	+ 5.1	12.5
		64.2	B	+ 3.2	14
12 dCGCGAANTCGCG	N = FMAU	59.1	A	+ 1.9	12.5
		63.3	B	+ 2.3	14
13 dCGCGAATNCGCG	N = FMAU	59.6	A	+ 2.4	12.5
		62.8	B	+ 1.8	14.3

[a] Oligomers 1 - 6 were hybridized with equimolar amount of dA_{11}; concentration of oligomers was 5 μM for one strand.

[b] Final concentration of buffers in 360 μL cuvette
A: 10 mM HEPES pH 6.9, 100 mM NaCl
B: 10 mM Tris buffer pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl (medium salt)
C: 10 mM Tris buffer, pH 7.5, 10 mM $MgCl_2$, 1 M NaCl (high salt)

[c] ΔT_m: difference in T_m between the modified system and unmodified duplex 1 or 7 in the same buffer; estimated T_m accuracy is $\pm$ 0.5 °C.

[d] [h]therm: 10 - 80 °C; 260 nm; the [h]therm of 1 - 5 measured without complementary dA_{11}

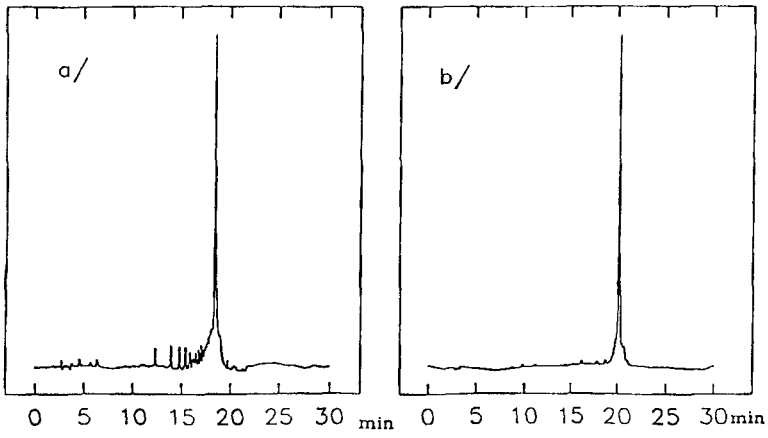


Figure 2. HPLC profiles of crude oligonucleotides. a) FAC_{20} synthesized by the phosphoramidite method, b) dT_4-$FMAU_3$-dT_4 prepared by the H-phosphonate method.

HPLC analysis of the mixture of nucleosides, which showed that the oligomers contained nucleosides in the expected relative ratio. Modified nucleosides identified in products have the same retention times as standard samples indicating that no modification of bases occurred during the synthesis (data not shown).

Stability of Oligonucleotide Duplexes: In the previous studies of this series,[1] we found that divalent cation remarkably stabilized the modified duplex. We also found that rather strong interaction was found in the case of duplex $FMAU_{11}/dA_{11}$. Similar results were observed by Eckstein *et al.*[14] with 2'-fluorothymidine (2'-F-ribo analogue) containing duplex d_fT_{11}/dA_{12}. In this paper, we report the thermal melting (T_m) characteristics of a series of duplexes containing 2'-fluoro-arabino-pyrimidine nucleosides in comparison with the corresponding unmodified duplexes. The normalized melting curves of the FMAU, FAU and FAC containing oligomers are compared in Figures 3 and 4. In all cases, a biphasic melting profile was observed. The melting curves were also recorded upon cooling the samples to check the reversibility of the process, which proved to be good in all cases. The data were analyzed and plotted by the Sigma Plot program to give the melting parameters which are compiled in Table 1. Melting curves were measured in buffered systems at various ionic strengths given in Table 1. These experiments confirmed that the presence of FMAU has a large stabilizing effect in all cases, and magnesium cations remarkably stabilize duplex.

It was found that incorporation of one FAU unit into an oligonucleotide induced little effects on the T_m while two isolated FAU units caused distinct destabilization.[1] We now

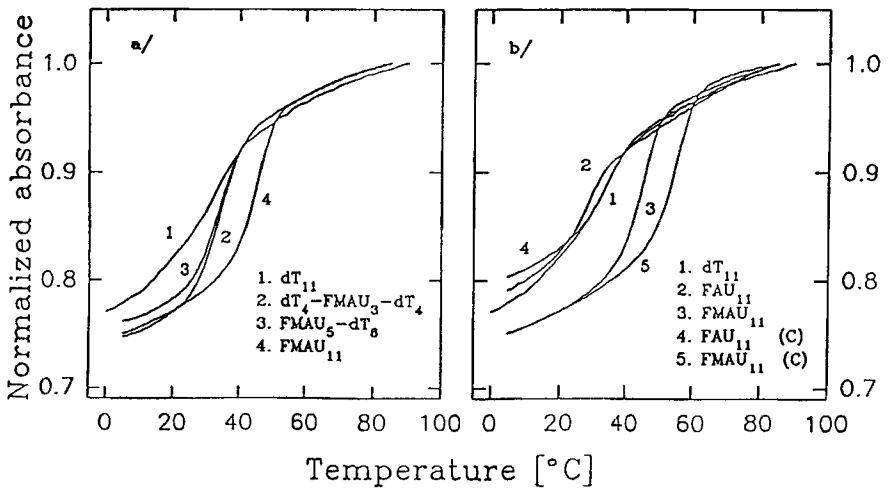


Figure 3. **Melting profiles of duplexes of FMAU and FAU containing oligomers hybridized with dA_{11} [5 μM concentration of each strand in buffer B unless specified buffer (C)].**

found that homo-oligomer FAU_{11} destabilized duplex by 4.9 °C in medium salt buffer B (0.1 M NaCl), but only 0.8 °C in high salt buffer C (1 M NaCl) (Figure 3). This is in agreement with earlier observations with FMAU-containing oligomers, that the duplexes are more stable in a high salt buffer.

It should be noted from this as well as previous experiments[1] that both 5-substituted nucleosides, FIAU[1] and FMAU, have a duplex stabilizing effect, whereas the 5-unsubstituted nucleosides, FAU and FAC, caused destabilization of the duplexes. Thus, stability of duplexes is apparently influenced by both the 2'-fluorine group and 5-substituent.

Thermodynamic parameters of double-helix formation were obtained from plots of T_m^{-1} *vs* logarithm of oligonucleotide concentration[27] (Figure 5), and the enthalpy and entropy changes yielded are listed in Table 2. It is also apparent that self-complementary oligomers (8 - 13) are in the form of duplex rather than in the hairpin form[28] (Figure 5).

All oligomers containing FAC in place of dC in the sequence (8 - 10) formed slightly *less* stable duplex (Figure 4b) and FMAU in place of dT (11 - 13) form slightly *more* stable duplex (Figure 4a) than unmodified oligomer. It is obvious that Mg^{++} influences the stability of duplexes. However, the ion does not convert the tendency of oligomers containing modified nucleotides toward their stabilizing effect, namely, FMAU always stabilize and FAC always destabilize regardless the presence or absence of Mg^{++} (Table 1). The fully substituted oligomer 8 was found to form the least stable duplex. It is also interesting to

Table 2. Thermodynamic parameters for duplex formation of oligonucleotides.

Oligomer	modified unit N	System	T_m [°C]	-ΔH [kJ/mol]	-ΔS [kJ/mol.K]	-ΔG[a] [kJ/mol]
N_{11}	FMAU	B	43.2	305	0.86	48.7
		C	52.0	286	0.78	53.6
$T_4N_3T_4$	FMAU	B	35.1	303	0.88	40.8
N_5T_6	FMAU	B	34.8	265	0.76	38.5
		C	41.0	380	1.11	49.2
CGCGAANNCGCG	FMAU	B	64.2	462	1.25	89.5
CGCGAANTCGCG	FMAU	B	63.3	429	1.17	80.3
CGCGAATNCGCG	FMAU	B	62.8	397	1.08	75.2
NGNGAATTNGNG	FAC	B	57.5	458	1.28	76.6
NGNGAATTCGCG	FAC	B	58.4	381	1.04	71.1
CGNGAATTNGCG	FAC	B	59.4	422	1.16	76.3
CGCGAATTCGCG		B	61.0	335	0.9	66.8

[a] Calculated at 25 °C. As to conditions for measuring T_m, see Table 1.

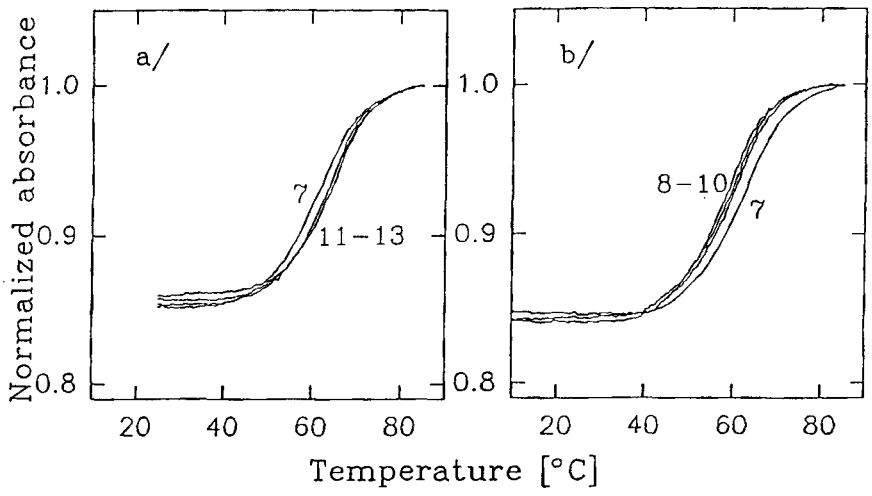
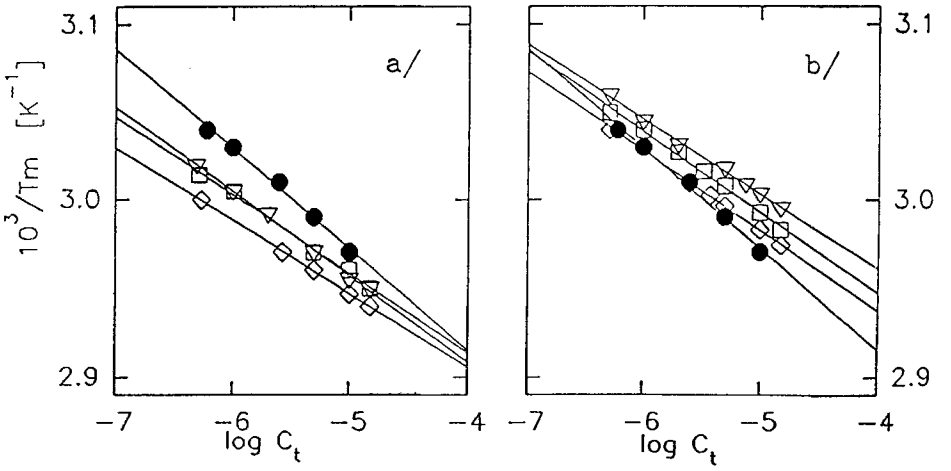


Figure 4. Melting profiles of self-complementary oligonucleotides 7 - 13 (see Table 1). a) FMAU replaced some dT (11 - 13), b) FAC replaced some dC (8 - 10).



(a) FMAU modified oligomers: • **7**, △ **13**, □ **12**, ◇ **11** (see Table 1)
(b) FAC modified oligomers: • **7**, △ **8**, □ **9**, ◇ **10**

Figure 5. Plot of T_m^{-1} vs log concentration of oligonucleotides containing (a) FMAU and (b) FAC.

note that **8** showed the highest *hypochromicity* indicating the highest base stacking. Self-complementary oligomers in Table **1** (**7** - **13**) are 11-16% hypochromic relative to the monomer, while **1** - **6** exhibited very low hypochromicity as expected.

CD Spectral Studies: The conformation of these duplexes were investigated by temperature CD spectroscopy. As shown in Figure **6**, all oligonucleotides exhibit the similar Cotton effect compared to the regular oligomers, which are known to adapt the B DNA-like structure.[29] The intensity of the 245 nm negative band depends on the number and location of fluorinated nucleotides in the sequences. The overall conformation of all complexes appears to be the same. In the known B DNA structures, there is a wide spread of sugar conformation between C2'-*endo* and C3'-*endo*. In the A-type DNA, on the other hand, the sugar pucker is consistently C3'-*endo*.[30] At present, however, due to the lack of sufficient information it is not possible to determine the actual structure of 2'-fluorinated oligonucleotides.

Stability of Fluorinated Oligonucleotides toward Enzymic Hydrolysis: All the tested oligonucleotides were hydrolyzed to their 5'-mononucleotide units by nuclease P1 and then by alkaline phosphatase to their corresponding nucleosides because of presence of natural

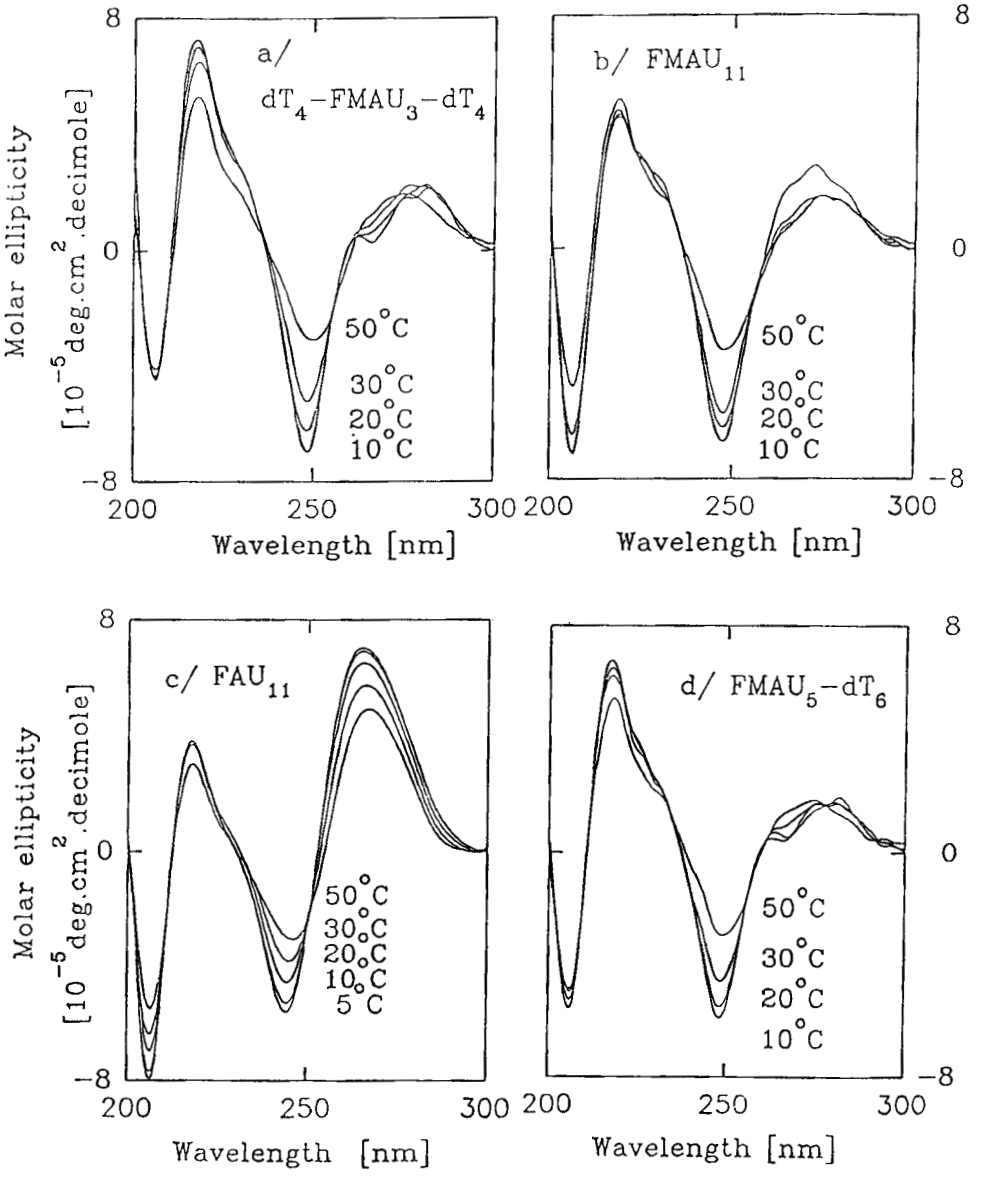


Figure 6. CD spectral changes of duplexes of FMAU and FAU containing oligomers hybridized with dA_{11} (5 μM concentration of each strand in buffer B, except FAU_{11} which is 15 μM).

phosphodiester bonds. Semi-quantitative studies on the stability of homo-oligomers of 2'-F-ara-C (FAC_{20}) and 2'-F-5-methyl-ara-U ($FMAU_{11}$) against nuclease P1 were performed using HPLC. The half-lives ($t_{1/2}$) of $FMAU_{11}$ and FAC_{20} were compared with those of the natural oligomers dA_{11} and dT_{11} under parallel experiments. Under these conditions, $t_{1/2}$ for dA_{11} and dT_{11} was approximately 15 minutes, and for $FMAU_{11}$ and FAC_{20} approximately 1 hour and 2 hours, respectively. This 4-8 fold increase in stability can be significant in drug development.

CONCLUSION:

A series of 2'-fluoro-arabinosyl-pyrimidine H-phosphonates and phosphoramidites were prepared and used in the synthesis of several modified oligonucleotides in order to study the effects of such fluorinated nucleosides on the thermodynamic properties of oligonucleotides.

The stability of duplexes, as indicated by the T_m data so far available, depends upon several factors: the type of nucleosides, number and position of modifications. The type of nucleosides is the determinant of the stability: the presence of FMAU in the oligomers increases the T_m, whereas replacement of dT with FAU or dC with FAC decreases the T_m of the duplexes. The scale of these effects (increasing or decreasing) on the stability depends on the number and positions of the modified units in the sequence. Substitution of a single nucleotide by a modified nucleotide did not cause serious effect on the formation and stability of duplex. Such modification may be useful in biological experiments without compromising the hybridizing properties of oligomers.

The reasons for weaker affinity of FAU_{11}/dA_{11} are not entirely clear. It is apparent that a combination of 2'-fluorine and the substituent of C-5 of pyrimidine influences the overall stability of duplex.

Melting experiments and temperature-dependent CD spectroscopic studies demonstrated that all the oligonucleotides (1 - 13) form a right-handed B-DNA like double helix.

The present findings warrant further studies on the structure and interactions of 2'-fluorinated oligo-ara-nucleotides as potentially important biomolecules. Studies are currently extended to 2'-fluoro-arabinosyl-purine nucleoside containing oligonucleotides.

ACKNOWLEDGEMENT

This investigation was supported in parts by funds from the National Cancer Institute, National Institutes of Health, U. S. Department of Health and Human Services, Grants CA18601 and CA08748.

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Received 6/8/93
Accepted 9/28/93