Uniformly Modified 2′-Deoxy-2′-fluoro Phosphorothioate Oligonucleotides as Nuclease-Resistant Antisense Compounds with High Affinity and Specificity for RNA Targets†

Andrew M. Kawasaki,* Martin D. Casper, Susan M. Freier, Elena A. Lesnik, Maryann C. Zounes, Lendell L. Cummins, Carolyn Gonzalez, and P. Dan Cook

ISIS Pharmaceuticals, 2280 Faraday Avenue, Carlsbad, California 92008

Received September 8, 1992

"Uniformly" modified phosphodiester or phosphorothioate oligonucleotides incorporating 2′-deoxy-2′-fluoroadenosine, -guanosine, -uridine, and -cytidine, reported herein for the first time, when hybridized with RNA afforded consistent additive enhancement of duplex stability without compromising base-pair specificity. CD spectra of the 2′-deoxy-2′-fluoro-modified oligonucleotides hybridized with RNA indicated that the duplex adopts a fully A-form conformation. The 2′-deoxy-2′-fluoro-modified oligonucleotides in phosphodiester form were not resistant to nucleases; however, the modified phosphorothioate oligonucleotides were highly nuclease resistant and retained exceptional binding affinity to the RNA targets. The stabilizing effects of the 2′-deoxy-2′-fluoro modifications on RNA–DNA duplexes were shown to be superior to those of the 2′-*O*-methylribo substitutions. RNA hybrid duplexes with uniformly 2′-deoxy-2′-fluoro-modified oligonucleotides did not support HeLa RNase H activity; however, incorporation of the modifications into "chimeric" oligonucleotides has been shown to activate mammalian RNase H. "Uniformly" modified 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides afforded antisense molecules with (1) high binding affinity and selectivity for the RNA target and (2) stability toward nucleases.

Introduction

Recently the application of oligonucleotides as antisense inhibitors of gene expression has generated much interest as the potential impact of this technology upon fundamental research, chemotheraphy, and agriculture could be far-reaching.[1,2] Several of the requirements for the successful application of antisense technology are that the oligonucleotide: (1) will hybridize to the target RNA sequence with specificity and affinity; (2) will have sufficient cellular penetration and distribution; and (3) will have sufficient resistance to extra- and intracellular nucleases.[1] The necessity for an optimization of binding affinity of the antisense oligonucleotide can become crucial when phosphate modifications such as phosphorothioates or methylphosphonates, are incorporated to impart stability to nucleases. These modifications result in marked decreases in the stability of the resulting duplexes.[2,3] Finally as an RNase H mediated mechanism is thought to be a major effector of antisense activity,[1] it would be advantageous if the antisense oligonucleotide–RNA duplex could activate RNase H activity.

The formation of a duplex between an antisense oligonucleotide and the nucleic acid sequence either directly provides the antisense mode of action or results in a heteroduplex that activates RNase H mediated cleavage of the RNA. Thus, the type and stability of the duplex is an important design feature. DNA–DNA helical duplexes are known to exist in several families of conformations including the B form which predominates under physiological conditions where the pucker of the sugar is C2′-endo. RNA–RNA duplexes generally adopt only an A or A′ type which is characterized by a C3′-endo sugar pucker.[4,5] It appears that with RNA–DNA hybrid duplexes there can be some flexibility inherent in the DNA strand. Dependent upon the conditions or sequence, the DNA strand may adopt the A,[6,7] B,[8,9] or intermediate[10,11] forms. In general RNA (A-form) duplexes are more thermodynamically stable than DNA (B-form) duplexes.[2] Currently most antisense oligonucleotides that have been employed have been of the deoxy type with or without a modified backbone.[1,2] The use of oligoribonucleotide phosphodiesters is precluded due to their instability under physiological conditions.

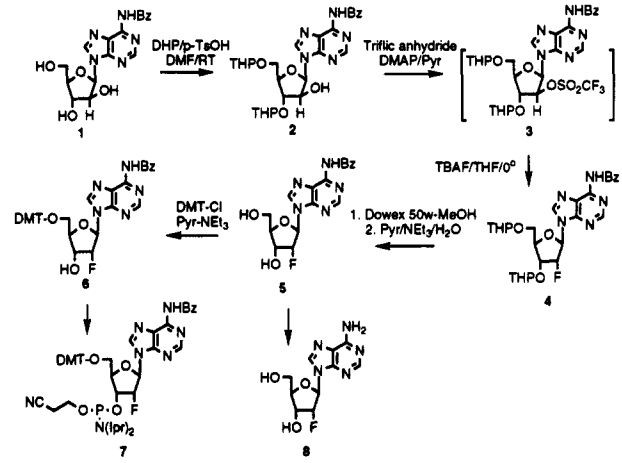
Since RNA–RNA duplexes are generally more stable than DNA–DNA or RNA–DNA,[2] a strategy for optimization of the thermodynamic stability of the RNA–antisense duplex could be to design an antisense oligonucleotide which would bias the conformation of the duplex toward the A type. As the 3′-endo conformation of the sugar appears to be a major determinant in the A form of RNA duplexes,[4,5] we considered modified nucleosides in which the sugar moiety exists preferentially in the 3′-endo conformation, i.e., RNA mimetics. The 2′-deoxy-2′-fluoro-substituted oligonucleotides may be useful antisense molecules as it has been shown that the sugar moiety of 2′-deoxy-2′-fluoro nucleosides exists predominantly in the 3′-endo form[12-14] and exceeds the 3′-endo distribution of the ribonucleosides. It has been proposed that this effect results from the high electronegativity of the fluorine atom acting in conjunction with the gauche effect.[15] We reasoned that an RNA-like (high fractional 3′-endo population) antisense oligonucleotide could, upon hybridization with target RNA, result in an A-form duplex with greater "conformational purity" as opposed to an RNA–DNA duplex with possible variant A/B forms.[10,11] The result could be a more stable antisense–RNA duplex. In fact the 2′-deoxy-2′-fluoro-substituted oligonucleotides have been prepared enzymatically as the homopolymers of 2′-deoxy-2′-fluorocytidine,[16,17] -uridine,[18] -inosine,[17] and -adenosine.[19] These 2′-deoxy-2′-fluoro-substituted homopolymers possess thermal stabilities similar to or higher than those of the corresponding RNA duplexes,[16,18] and

† This article is dedicated to Professor Leroy B. Townsend on the occasion of his 60th birthday.

0022-2623/93/1836-0831$04.00/0 © 1993 American Chemical Society

CHEMGENES EXHIBIT 2025
Shanghai Hongene v. ChemGenes
Case IPR2023-00490

Scheme I



the CD spectra of these 2-'deoxy-2'-fluoro homopolymers show characteristics more closely related to RNA than to DNA.[16,17]

Other RNA-mimetic oligonucleotides have been utilized in biophysical and biochemical investigations, e.g., 2'-*O*-methylribo-substituted oligonucleotides were found to form duplexes with RNA which showed greater stability than the corresponding DNA–RNA duplexes.[20,21] Recently 2'-deoxy-2'-fluoroadenosine, -uridine, -cytidine, and -guanosine were chemically incorporated into a hammerhead ribozyme RNA to study the influence of specific 2'-OH groups upon the kinetics of the catalytic activity.[22–24] Also, to investigate regiospecific hydrolysis of RNA by RNase H from *Escherichia coli*, 2'-deoxy-2'-fluorouridine or -cytidine were incorporated chemically into oligonucleotides.[25]

Thus we have prepared a series of 2'-deoxy-2'-fluoro-substituted oligonucleotides which were incorporated with a phosphodiester or phosphorothioate backbone and compared their melting temperatures, stabilities toward nucleases, and capacities to support RNase H activity. The conformational properties of the modified oligonucleotides were also investigated by circular dichroism.

Results

Synthesis of Protected 2'-Deoxy-2'-fluoro-β-D-ribonucleosides. The synthesis of 2'-deoxy-2'-fluoroadenosine (8)[26,27] (Scheme I) and a 9-phenylxanthene-protected derivative of 8[22] have been reported. We elected to synthesize the protected nucleoside N^6-benzoyl-2'-deoxy-2'-fluoroadenosine 5 by utilizing commercially available 9-β-D-arabinofuranosyladenine as starting material and by modifying literature procedures wherein the 2'-α-fluoro atom is introduced by a S_N2-displacement of a 2'-β-*O*-triflyl group.[27] Thus N^6-benzoyl-9-β-D-arabinofuranosyladenine (1) was selectively protected in moderate yield (50–83%) as the 3',5'-ditetrahydropyranyl (THP) intermediate 2.[28] Triflation of 2 afforded a crude product 3 which was not purified but treated with fluoride to give the 2'-deoxy-2'-fluoro-β-D-ribonucleoside 4[27] in 76% yield from 2. Deprotection of the THP groups provided 5 in 90% yield. Deprotection of the N^6-benzoyl group with methanolic ammonia afforded compound 8, the ^{1}H- and ^{13}C-NMR spectra of which were in agreement with the literature values.[27] Standard methodologies were employed to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates 6 and 7.[29]

The synthesis of 2'-deoxy-2'-fluoroguanosine[30] (17) (Scheme II) has been reported. We elected to prepare the

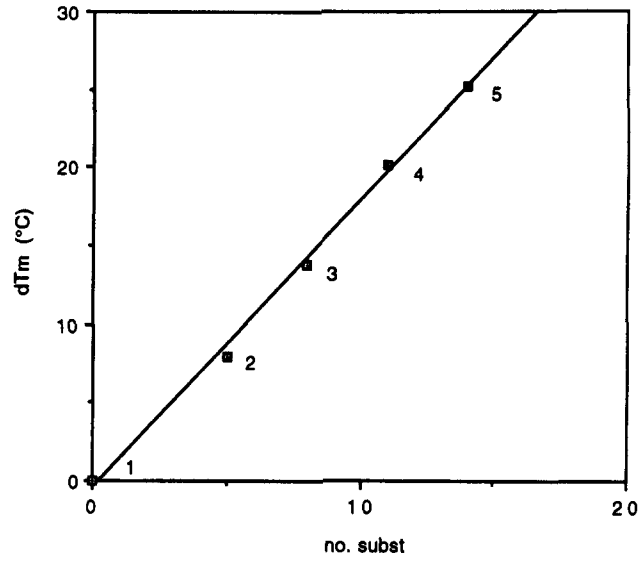
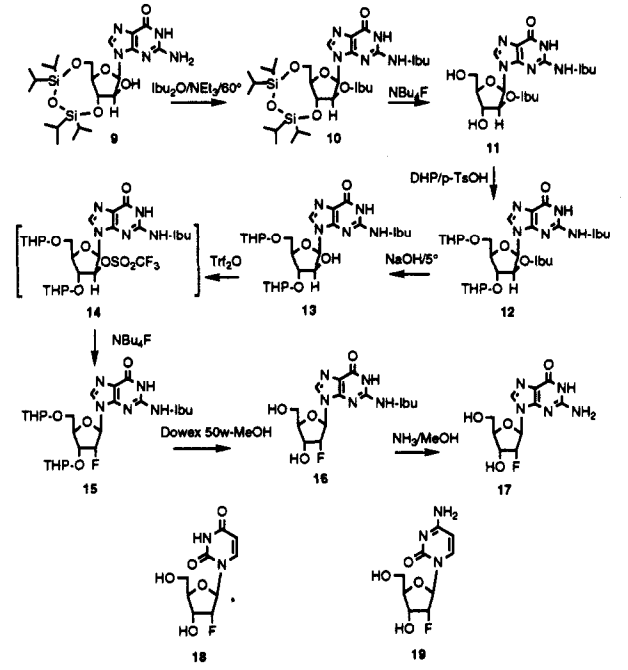


Figure 1. Variation of ΔT_m vs number of 2'-deoxy-2'-fluoro substitutions for pentadecamers of identical sequence. Numbered points refer to entries in Table I.

Scheme II



isobutyryl-protected derivative 16 via an alternative route utilizing the tetraisopropyldisiloxanyl (TPDS) protected 9-β-D-arabinofuranosylguanine nucleoside 9[31] as starting material. For the preparation of 9 on a large scale (10 g), careful manipulation of the temperature was required during the addition of sodium borohydride to avoid extensive degradation. The diisobutyrylarabinofuranosylguanine intermediate 10 was obtained in an 80% yield. Deprotection of the TPDS group afforded 11 followed by protection of the hydroxyl groups with THP to afford the diisobutyryl di-THP protected arabinofuranosylguanine 12 as a diastereomeric mixture. Selective O-deacylation[32] provided 13 in good yields, and triflation of 13 followed by treatment of the crude product 14 with fluoride provided the fluorinated compound 15 as a crude product. Deprotection of the THP groups afforded the desired product 16 in 42% overall yield from 13. Deacylation of 16 provided the unprotected nucleoside 17 the ^{1}H- and ^{13}C-NMR spectra of which confirmed the assigned structure.[30] Standard methodologies afforded the 5'-DMT- and 5'-

Table I. Effects of 2′-Deoxy-2′-fluoro Modifications on DNA(Antisense)–RNA(Sense) Duplex Stability

no.	antisense sequence[a]	no. of subst	T_m (°C)	ΔT_m (°C)	ΔT_m (°C)/subst
1	CGA CTA TGC AAG TAC		45.1 ± 0.01		
2	CGA$_f$ CTA$_f$ TGC A$_f$A$_f$G TA$_f$C	5	53.0 ± 0.1	+7.9 ± 0.09	+1.6 ± 0.02
3	CGA$_f$ CU$_f$A$_f$ U$_f$GC A$_f$A$_f$G U$_f$A$_f$C	8	58.9 ± 0.1	+13.8 ± 0.09	+1.7 ± 0.01
4	C$_f$GA$_f$ C$_f$U$_f$A$_f$ U$_f$GC$_f$ A$_f$A$_f$G U$_f$A$_f$C	11	65.2 ± 0.1	+20.1 ± 0.09	+1.8 ± 0.01
5	C$_f$G$_f$A$_f$ C$_f$U$_f$A$_f$ U$_f$G$_f$C$_f$ A$_f$A$_f$G$_f$ U$_f$A$_f$C	14	70.3 ± 0.0	+25.2 ± 0.06	+1.8 ± 0.0
6	rCrGrA rCrUrA rUrGrC rArArG rUrAC	14	59.2 ± 0.4	+14.1 ± 0.4	+1.0 ± 0.02
7	C$_m$G$_m$A$_m$ C$_m$U$_m$A$_m$ U$_m$G$_m$C$_m$ A$_m$A$_m$G$_m$ U$_m$A$_m$C	14	62.8 ± 0.4	+17.7 ± 0.4	+1.3 ± 0.03
8	ps(CGA CTA TGC AAG TAC)	14	33.9 ± 0.5	−11.2 ± 0.5	−0.8 ± 0.04
9	ps(C$_f$GA$_f$ C$_f$U$_f$A$_f$ U$_f$GC$_f$ A$_f$A$_f$G U$_f$A$_f$C)	11	60.8 ± 0.2	+15.8 ± 0.2	+1.4 ± 0.01
10	ps(C$_f$G$_f$A$_f$ C$_f$U$_f$A$_f$ U$_f$G$_f$C$_f$ A$_f$A$_f$G$_f$ U$_f$A$_f$C)	14	65.8 ± 0.2	+20.7 ± 0.2	+1.5 ± 0.01
11	ps(rCrGrA rCrUrA rUrGrC rArArG rUrAC)	14	51.2 ± 0.2	+6.1 ± 0.2	+0.4 ± 0.01
12	ps(C$_m$G$_m$A$_m$ C$_m$U$_m$A$_m$ U$_m$G$_m$C$_m$ A$_m$A$_m$G$_m$ U$_m$A$_m$C)	14	57.7 ± 0.2	+12.6 ± 0.2	+0.9 ± 0.01

[a] A$_f$, G$_f$, U$_f$, C$_f$ = 2′-deoxy-2′-fluoro nucleoside. rA, rG, rU, rC = ribonucleoside. A$_m$, G$_m$, U$_m$, C$_m$ = 2′-*O*-methylribo nucleoside. ps = phosphorothioate backbone.

DMT-3′-phosphoramidite compounds of **16**. Recently the synthesis of a 9-phenylxanthene-protected derivative of **17** via an alternative route was reported.[33]

Synthesis of 2′-deoxy-2′-fluorouridine **18** (Scheme II) was conveniently effected in 60–75% yields on a large scale (10 g) by the modification of a literature procedure[34] in which 2,2′-anhydro-1-β-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride–pyridine.[35] For the synthesis of 2′-deoxy-2′-fluorocytidine (**19**)[36] (Scheme II), amination of 2′-deoxy-2′-fluorouridine was effected via the C4-triazolo intermediate,[37] then 2′-deoxy-2′-fluorocytidine was selectively protected[38] to give N^4-benzoyl-2′-deoxy-2′-fluorocytidine (**20**). Standard methodologies gave the 5′-DMT- and 5′-DMT-3′-phosphoramidite compounds of **18** and **20**. The ^{1}H-NMR spectra of both 2′-deoxy-2′-fluorouridine (**18**)[14] and -cytidine (**19**)[39] were in agreement with the literature values.

Synthesis of 2′-Deoxy-2′-fluoro-Containing Oligonucleotides. The 2′-deoxy-2′-fluoro-modified oligonucleotides were prepared in moderate yields. As it has been reported that the 2′-deoxy-2′-fluoro pyrimidines are susceptible to elimination of fluoride under basic or neutral thermal conditions,[36,40] we chose to deprotect the modified oligonucleotides using methanolic ammonia at room temperature. Under the usual oligonucleotide deprotection conditions (concentrated NH_4OH, 55 °C), we in fact observed significant degradation of the 2′-deoxy-2′-fluoropyrimidine nucleosides while the nucleosides were stable to methanolic ammonia at room temperature. It was demonstrated by ^{1}H NMR (400 MHz) with nucleosides and trinucleotides that protecting groups were cleanly removed by methanolic ammonia from cytidine, adenosine, and guanosine bases. Further proof of base deprotection was provided by the compositional analyses of the synthetic oligonucleotides.

^{1}H-NMR Analysis of Oligonucleotide Trimers Containing 2′-Deoxy-2′-fluoro Nucleosides. Trimers with a 2′-deoxy-2′-fluoro nucleoside incorporated at the 2nd position, i.e., TA$_f$C, TG$_f$C, AU$_f$C, and TC$_f$C, were synthesized, and their ^{1}H-NMR (400 MHz) spectra were examined to verify the presence of the modified nucleosides. For all of the trimers examined the aromatic and sugar protons were accounted for. Especially evident was the appearance of the 2′-proton, in the 5.0–5.5 ppm range, as a strongly coupled doublet due to the presence of the 2′-fluorine atom ($J_{2',F} \approx 50$ Hz). In addition the spectrum of TG$_f$C showed an absence of a methyl signal for the *N*-isobutyryl group at about 1 ppm which confirmed the deprotection of the N^2-protecting group.

Modified Oligonucleotide Compositional Analysis. Four oligonucleotides which contained three or five 2′-deoxy-2′-fluoro nucleotides were digested with snake venom phosphodiesterase to determine the base compositions:

TCC AGG TGT CCG U$_f$U$_f$U$_f$ C:	dC: 4.8, U$_f$: 2.9, dG: 4.0, T: 3.0, dA: 1.0.
TC$_f$C$_f$ AGG TGT C$_f$C$_f$G C$_f$AT C:	dC: 0.9, C$_f$: 5.0, dG: 4.1, T: 4.0, dA: 2.0.
CGA CTA TGC AAA$_f$ A$_f$A$_f$C:	dC: 3.8, dG: 2.1, T: 2.0, dA: 4.0, A$_f$: 3.0.
CGA CTA TGC AAG$_f$ G$_f$G$_f$C:	dC: 4.0, dG: 2.1, T: 2.0, G$_f$: 3.1, dA: 4.1.

Ratios were determined relative to thymidine, which was set to its expected value. In all cases the expected ratios of modified to unmodified nucleosides were observed. In addition these results corroborated the removal of the protecting groups under the mild deprotection conditions employed.

Hybridization Thermodynamics. To investigate the effects of 2′-deoxy-2′-fluoro modifications on hybridization affinity, a pentadecamer derived from the human papilloma virus genome was modified with 2′-deoxy-2′-fluoro, 2′-*O*-methylribo, ribo (2′-hydroxyl), and phosphorothioate substitutions (Table I, 1–12), hybridized with their RNA complements, and the melting temperatures (T_m) of the heteroduplexes were compared to those of the heteroduplex formed with unmodified oligodeoxynucleotide **1**. Inspection of the results indicates that serial substitutions with 2′-deoxy-2′-fluoro nucleosides (A$_f$, U$_f$, C$_f$, G$_f$) led to consistently additive increases in the melting temperatures relative to the unmodified oligonucleotide **1**. Substitutions incorporating 5 A$_f$'s (**2**), 8 A$_f$'s and U$_f$'s (**3**), 11 A$_f$'s, U$_f$'s, and C$_f$'s (**4**), or 14 A$_f$'s, U$_f$'s, C$_f$'s, and G$_f$'s ("uniformly" modified oligonucleotide) (**5**) afforded an average increase in T_m of +1.8 °C/substitution. Inspection of a plot of the increase in T_m as a function of the number of substitutions (Figure 1) for the modified phosphodiester oligonucleotides (**2–5**) indicates a linear correlation ($R^2 = 0.997$) between the two parameters. As a comparison the oligoribonucleotide **6** and the "uniformly" modified 2′-*O*-methyloligoribonucleotide **7** exhibited duplex stabilities which were less than that of the "uniformly" 2′-deoxy-2′-fluoro-modified oligonucleotide **5**, i.e., increases in T_m of +14.1 °C and +17.7 °C, respectively, vs +25.2 °C (Table I). All three modifications (2′-deoxy-2′-fluoro, ribo, and 2′-*O*-methylribo) appear to enhance the stability of the duplex relative to the unmodified DNA (**1**) in the rank order: 2′-deoxy-2′-fluoro > 2′-*O*-methylribo > ribo.

The stabilizing effects of substitutions with 2′-deoxy-2′-fluoro nucleosides within a phosphorothioate backbone were even more pronounced. Incorporation of 11 A$_f$'s, U$_f$'s, and C$_f$'s (**9**) or 14 A$_f$'s, U$_f$'s, C$_f$'s, and G$_f$'s ("uniformly" modified) (**10**) resulted in increases in T_m's of +15.8 °C and +20.7 °C, respectively, when compared to (**1**) (Table I). Furthermore the 2′-deoxy-2′-fluoro-modified phosphorothioate oligonucleotide **10** showed greater duplex

Table II. Effects of 2′-Deoxy-2′-fluoro Modifications on DNA(Antisense)–RNA(Sense) Duplex Stability

no.	antisense sequence[a]	no. of subst	T_m (°C)	ΔT_m (°C)	ΔT_m (°C)/subst
13	GGA CCG GAA GGT ACG AG		57.0 0.3		
14	GGA$_f$ CCG GA$_f$A$_f$ GGT A$_f$CG A$_f$G	5	65.8 ± 0.1	+8.8 ± 0.3	+1.8 ± 0.06
15	G$_f$G$_f$A$_f$ C$_f$C$_f$G$_f$ G$_f$A$_f$A$_f$ G$_f$G$_f$U$_f$ A$_f$C$_f$G$_f$ A$_f$G	16	89.1 ± 0.1	+32.1 ± 0.3	+2.0 ± 0.02
16	rGrGrA rCrCrG rGrArA rGrGrU rArCrG rAG	16	74.5 ± 0.1	+17.5 ± 0.4	+1.1 ± 0.02
17	G$_m$G$_m$A$_m$ C$_m$C$_m$G$_m$ G$_m$A$_m$A$_m$ G$_m$G$_m$T$_m$ A$_m$C$_m$G$_m$ A$_m$G	16	79.6 ± 0.3	+22.6 ± 0.5	+1.5 ± 0.03

[a] A$_f$, G$_f$, U$_f$, C$_f$ = 2′-deoxy-2′-fluoro nucleoside. rA, rG, rU, rC = ribonucleoside. A$_m$, G$_m$, U$_m$, C$_m$ = 2′-*O*-methylribo nucleoside.

Table III. Effects of 2′-Deoxy-2′-fluoro Modifications on DNA(Antisense)–RNA(Sense) Duplex Stability

no.	antisense sequence[a]	no. of subst	fractional subst	T_m (°C)	ΔT_m (°C)	ΔT_m (°C)/subst
18	CTC GTA CCA$_f$ TTC CGG TCC	1	6	64.1 ± 0.1	+0.4 ± 0.3	+0.4 ± 0.3
19	CTC GTA CCC$_f$ TTC CGG TCC	1	6	69.9 ± 0.0	+1.7 ± 0.5	+1.7 ± 0.5
20	GA$_f$G CTC CCA$_f$ GGC	2	17	64.6 ± 0.1	+4.7 ± 0.0	+2.4 ± 0.0
21	TCC AGG TGT CCG U$_f$U$_f$U$_f$ C	3	19	60.5 ± 0.1	+1.4 ± 0.1	+0.5 ± 0.04
22	U$_f$CC AGG U$_f$GU$_f$ CCG CAU$_f$ C	4	25	64.8 ± 0.1	+2.5 ± 0.4	+0.6 ± 0.1
23	A$_f$CC GA$_f$G GA$_f$T CA$_f$T GTC GTA$_f$ CGC	5	24	67.7 ± 0.1	+4.5 ± 0.2	+0.9 ± 0.04
2	CGA$_f$ CTA$_f$ TGC A$_f$A$_f$G TA$_f$C	5	33	53.0 ± 0.1	+7.9 ± 0.1	+1.6 ± 0.02
14	GGA$_f$ CCG GA$_f$A$_f$ GGT A$_f$CG A$_f$G	5	29	65.8 ± 0.1	+8.9 ± 0.3	+1.8 ± 0.06
24	CU$_f$C GU$_f$A CCU$_f$ U$_f$CC GGU$_f$ CC	5	29	68.5 ± 0.1	+3.4 ± 0.14	+0.7 ± 0.03
25	TC$_f$C$_f$ AGG TGT C$_f$C$_f$G C$_f$AT C	5	31	71.8 ± 0.9	+9.6 ± 1.0	+1.9 ± 0.2
3	CGA$_f$ CU$_f$A$_f$ U$_f$GC A$_f$A$_f$G U$_f$A$_f$C	8	53	58.9 ± 0.1	+13.8 ± 0.1	+1.7 ± 0.01
4	C$_f$GA$_f$ C$_f$U$_f$A$_f$ U$_f$GC$_f$ A$_f$A$_f$G U$_f$A$_f$C	11	73	65.2 ± 0.1	+20.1 ± 0.1	+1.8 ± 0.01
26	C$_f$U$_f$C$_f$ GU$_f$A$_f$ C$_f$C$_f$U$_f$ U$_f$U$_f$C$_f$ C$_f$GG U$_f$C$_f$C	14	78	81.6 ± 0.1	+18.3 ± 0.4	+1.3 ± 0.03
5	C$_f$G$_f$A$_f$ C$_f$U$_f$A$_f$ U$_f$G$_f$C$_f$ A$_f$A$_f$G U$_f$A$_f$C	14	93	70.3 ± 0.0	+25.2 ± 0.06	+1.8 ± 0.0
15	G$_f$G$_f$A$_f$ C$_f$C$_f$G$_f$ G$_f$A$_f$A$_f$ G$_f$G$_f$U$_f$ A$_f$C$_f$G$_f$ A$_f$G	16	94	89.1 ± 0.1	+32.1 ± 0.3	+2.0 ± 0.02
27	C$_f$U$_f$C$_f$ G$_f$U$_f$A$_f$ C$_f$C$_f$A$_f$ U$_f$U$_f$C$_f$ C$_f$G$_f$G$_f$ U$_f$C$_f$C	17	94	88.6 ± 0.1	+24.9 ± 0.4	1.5 ± 0.02

[a] A$_f$, G$_f$, U$_f$, C$_f$ = 2′-deoxy-2′-fluoro nucleoside.

stability than the *phosphodiester* oligoribonucleotide **6** and *phosphodiester* 2′-*O*-methyloligoribonucleotide **7**. In comparison the phosphorothioate oligonucleotide with unmodified nucleosides (**8**) displayed a decrease in T_m of −11.2 °C relative to **1**. The phosphorothioate oligoribonucleotide (**11**) and the phosphorothioate with uniform 2′-*O*-methylribo substitutions (**12**) resulted in an increased T_m of +6.1 °C and +12.6 °C, respectively, relative to **1**. These results indicated that the relative stabilization effects of the three types of substitutions exhibited the same trend as in the case with the phosphodiester backbone, i.e., 2′-deoxy-2′-fluoro > 2′-*O*-methylribo > ribo.

To further compare the stabilizing effects of the 2′-deoxy-2′-fluoro, ribo, and 2′-*O*-methylribo substitutions upon DNA–RNA heteroduplexes, the stabilities of modified septedecamer phosphodiester oligonucleotides of identical arbitrary sequence were examined (Table II). Incorporation of 5 A$_f$'s (**14**) and 16 A$_f$'s, U$_f$'s, C$_f$'s, and G$_f$'s (**15**) resulted in increases of T_m's of +8.8 °C and +32.1 °C, respectively, relative to the unmodified phosphodiester oligonucleotide **13**. The "uniformly" modified oligoribonucleotide **16** and the "uniformly" modified 2′-*O*-methyloligoribonucleotide **17** also showed increases in T_m of +17.5 °C and +22.6 °C, respectively, relative to **13**. The stabilizing effects of the three modifications upon DNA–RNA heteroduplexes were similar to those observed for the pentadecamer series previously discussed (Table I), i.e., in the rank order: 2′-deoxy-2′-fluoro > 2′-*O*-methylribo > ribo.

To address the possibility that the observed stabilizing effects of 2′-deoxy-2′-fluoro substitutions upon RNA–DNA heteroduplexes may be sequence dependent, a total of 16 phosphodiester oligonucleotides comprising six different sequences were prepared with incorporation of 1–17 2′-deoxy-2′-fluoro nucleosides (Table III) (modified oligonucleotides previously discussed are included for completeness). For all of the sequences studied only positive increases of T_m were observed. When the change of T_m was plotted as a function of the fractional substitution within the oligonucleotide (Figure 2), the stabilization effect appeared to be additive or slightly cooperative. The

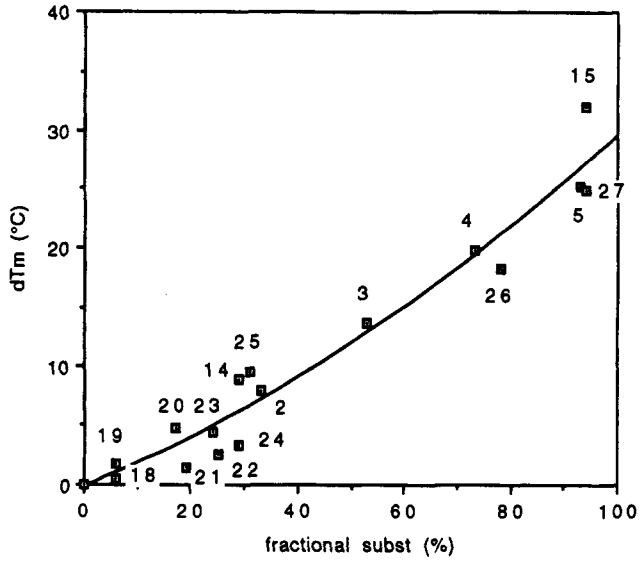


Figure 2. Variation of ΔT_m vs fractional 2′-deoxy-2′-fluoro substitutions for oligonucleotides of various sequences. Numbered points refer to entries in Table III.

maximal increases in T_m's were observed for the "uniformly" modified oligonucleotides **15**, **5**, and **27**.

To further explore the stabilizing effects of the 2′-deoxy-2′-fluoro modifications upon RNA–DNA heteroduplexes within the context of a phosphorothioate backbone, additional sequences of uniformly modified oligonucleotides, **29** and **31**, were prepared (Table IV, previously discussed entry **10** is included for completeness). The two modified oligonucleotides resulted in increases in T_m of 1.2 °C/substitution and +1.7 °C/substitution, respectively, relative to the T_m's of the corresponding phosphorothioate oligonucleotides containing unmodified nucleosides (**28** and **30**). The T_m's of **29** and **31** were not increased as much as that of **10**; nevertheless, significant increases were observed.

Base-Pair Specificity. A measure of base-pair specificity is considered to be reflected in the difference between the ΔG and T_m values of the duplex with Watson–

Table IV. Effects of 2′-Deoxy-2′-fluoro Modifications on Phosphorothioate–DNA(Antisense)–RNA(Sense) Duplex Stability

no.	antisense sequence[a]	no. of subst	T_m (°C)	ΔT_m (°C)	ΔT_m (°C)/subst
8	ps(CGA CTA TGC AAG TAC)		33.9 ± 0.5		
10	ps($C_fG_fA_f$ $C_fU_fA_f$ $U_fG_fC_f$ $A_fA_fG_f$ U_fA_fC)	14	65.8 ± 0.2	+31.9 ± 0.6	+2.3 ± 0.04
28	ps(CCA CAC CGA CGG CGC CC)		64.2 ± 0.1		
29	ps($C_fC_fA_f$ $C_fA_fC_f$ $C_fG_fA_f$ $C_fG_fG_f$ $C_fG_fC_f$ C_fC)	16	83.1 ± 0.1	+18.9 ± 0.2	+1.2 ± 0.01
30	ps(CTC GTA CCA TTC CGG TCC)		52.7 ± 0.6		
31	ps($C_fU_fC_f$ $G_fU_fA_f$ $C_fC_fA_f$ $U_fU_fC_f$ $C_fG_fG_f$ U_fC_fC)	17	80.9 ± 0.1	28.2 ± 0.6	1.7 ± 0.04

[a] A_f, G_f, U_f, C_f = 2′-deoxy-2′-fluoro nucleoside. A_m, G_m, U_m, C_m = 2′-*O*-methylribo nucleoside. ps = phosphorothioate backbone.

Table V. Effects of Single Base Mismatches within 2′-Deoxy-2′-fluoro Phosphodiester and Phosphorothioate Modified Oligonucleotides of Identical Sequence on DNA–RNA Duplex Stability

X strand: deoxy($C_xU_xC_xG_xU_xA_xC_xC_xA_xU_xU_xC_xC_xG_xG_xU_xC_xC$)

Y strand: ribo(GGA CCG GAA YGG UAC GAG)

no.	x strand[a]	T_m match (°C)	av ΔT_m/mismatch (°C)
32	CTC GTA CCA TTC CGG TCC	63.7 ± 0.3	−6.8 ± 0.2
27	$C_fU_fC_f$ $G_fU_fA_f$ $C_fC_fA_f$ $U_fU_fC_f$ $C_fG_fG_f$ U_fC_fC	88.6 ± 0.1	−7.7 ± 0.8
30	ps(CTC GTA CCA TTC CGG TCC)	52.7 ± 0.6	−9.2 ± 0.9
31	ps($C_fU_fC_f$ $G_fU_fA_f$ $C_fC_fA_f$ $U_fU_fC_f$ $C_fG_fG_f$ U_fC_fC)	80.9 ± 0.1	−8.8 ± 0.06

[a] A_f, G_f, U_f, C_f = 2′-deoxy-2′-fluoro nucleoside. ps = phosphorothioate. Y = A, C, U, G, none. ΔT_m = T_m (mismatch or bulge at position 10 of Y strand) − T_m (match at position 10 of Y strand). x = f or none.

Crick base pairs and the corresponding duplex with a single mismatch or bulge.[41] The base-pair specificities (Table V) of "uniformly" modified phosphodiester (**27**) and phosphorothioate (**31**) 2′-deoxy-2′-fluoro octadecamers ("X-strand"), with the RNA complement ("Y-strand") were investigated by effecting single base-pair mismatches or a bulge (Y = A, C, G, none) at the 10-position within the Y-strand. A comparison of the base-pair specificities of the unmodified phosphodiester (**32**) and phosphorothioate with unmodified nucleosides (**30**) of the same sequence showed that the base-pair specificity of "uniformly" modified phosphodiester (**27**) is slightly greater than that of **32**, i.e., the ΔT_m of **27** is more negative than that of **32**, and "uniformly" modified phosphorothioate (**31**) shows a specificity comparable to but slightly less than that of **30**.

Circular Dichroism. The CD spectra of the 2′-deoxy-2′-fluoro-, ribo, and 2′-*O*-methylribo phosphodiester oligonucleotides, **5**, **6**, and **7** (Table I), respectively, when hybridized with RNA exhibit the characteristic A-form pattern, i.e., a nonconservative π–π^* positive band at 260 nm, a negative π–π^* band at 235 nm, and a negative n–π^* band at 210 nm (Figure 3).[42,43] In contrast the unmodified deoxyoligonucleotide **1** forms a duplex with RNA which gives rise to a spectrum which indicates a form intermediate between A and B.

The 2′-deoxy-2′-fluoro, ribo, and 2′-*O*-methylribo phosphorothioate oligonucleotides, **10**, **11**, and **12** (Table I), respectively, when hybridized with RNA also present typical A-form CD patterns (Figure 4) while the phosphorothioate oligonucleotide with unmodified nucleosides **8** appears to assume an intermediate A–B form as does the unmodified phosphodiester oligonucleotide **1**.

Nuclease Degradation Studies. The stability of a uniformly modified 2′-deoxy-2′-fluoro phosphodiester pentadecamer oligonucleotide (**5**, Table I) was investigated at a 10 μM concentration using heat-inactivated 10% fetal calf serum (Figure 5). Degradation of the full-length oligonucleotide **5** to the $n-2$ (the 3′-terminal nucleotide was unmodified) and smaller fragments was monitored by PAGE and autoradiography. The half-life for degradation of the full-length modified oligonucleotide **5**, 45 min, was about equal to that of the unmodified oligonucleotide **1** of identical sequence (Figure 6). After 5 h the modified oligonucleotide **5** was >95% degraded to the $n-2$ and smaller fragments. The modified oligonucleotide

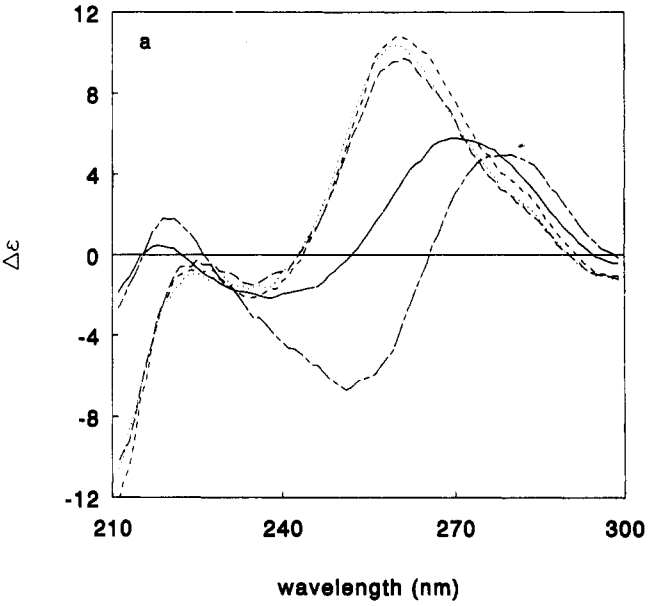


Figure 3. CD spectra of five duplexes formed by phosphodiester pentadecamers and RNA or DNA targets at 20 °C in 0.1 M NaCl, 10 mM sodium phosphate (pH 7.2), 0.5 mM EDTA. (—) **1**-RNA; (- - - -) **1**-DNA; (····) **5**-RNA; (- · - · -) **6**-RNA; (– – –) **7**-RNA. Numbered compounds refer to entries in Table I.

10 containing a phosphorothioate backbone was stable (less than 10% degradation to $n-1$) under the experimental conditions over a period of 24 h.

RNase H Assay. The effect of uniformly modified 2′-deoxy-2′-fluoro phosphodiester oligonucleotide **5** (Table I) on the activity of HeLa RNase H was assessed using an in vitro assay (Figure 7). The modified oligonucleotide **5** was compared with an unmodified control phosphodiester **1** and a phosphorothioate deoxyoligonucleotide **8** of the same sequence for the ability to support RNase H activity when in a heteroduplex with the complementary RNA strand.

Cleavage of RNA was observed for the phosphodiester **1** and the phosphorothioate **8** DNA oligonucleotides while the 2′-deoxy-2′-fluoro oligonucleotide **5** exhibited an absence of RNA cleavage. At decreased extract concentration the phosphorothioate oligonucleotide **8** resulted in slightly less cleavage of RNA relative to the phosphodiester oligonucleotide **1**. Based on the presence of full-

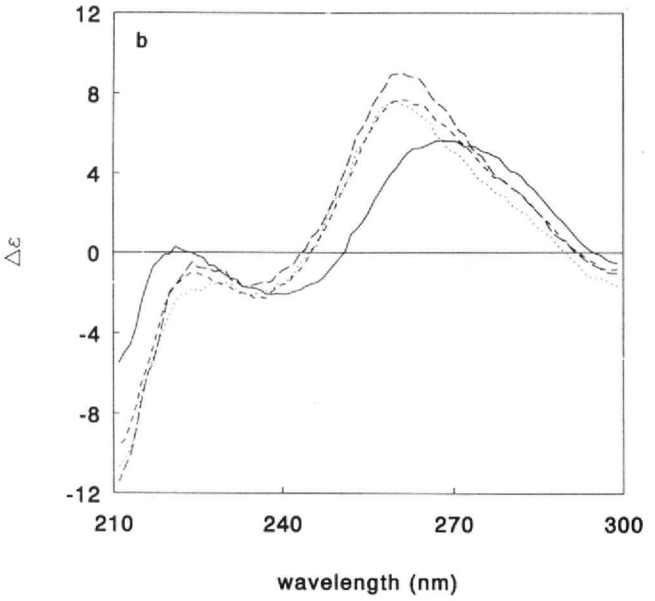


Figure 4. CD spectra of four duplexes formed by phosphorothioate pentadecamers and RNA targets at 20 °C in 0.1 M NaCl, 10 mM sodium phosphate (pH 7.2), 0.5 mM EDTA. (—) **8**–RNA; (····) **10**–RNA; (- - - -) **11**–RNA; (– – –) **12**–RNA. Numbered compounds refer to entries in Table I.

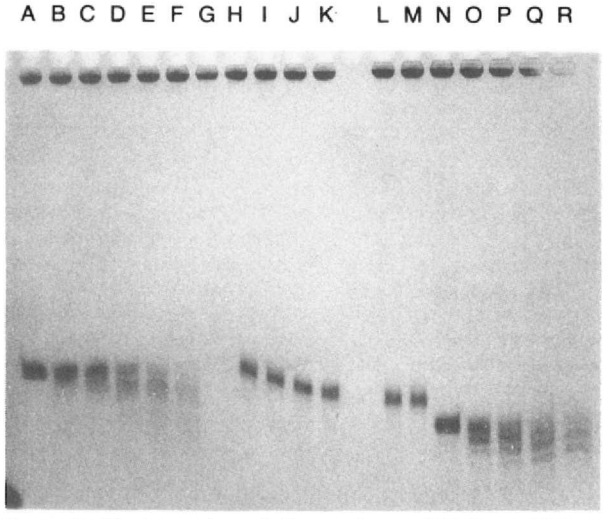


Figure 5. Nuclease degradation analyses of 2′-deoxy-2′-fluoro-modified pentadecamer oligonucleotides using heat inactivated 10% fetal calf serum. Lanes A–G: oligonucleotide **5** at 0, 0.5, 1, 2, 3, 4.5, and 24 h of incubation, respectively. Lanes H–K: oligonucleotide **10** at 2, 3, 4.5, and 24 h, respectively. Lanes L–R: oligonucleotide **1** at 0, 0.5, 1, 2, 3, 4.5, and 24 h, respectively. Numbered compounds refer to entries in Table I.

length RNA there was virtually complete cleavage of the phosphodiester **1** DNA–RNA duplex, approximately 80–90% cleavage of the phosphorothioate **8** DNA–RNA duplex, and virtually no cleavage of the 2′-deoxy-2′-fluoro-modified oligonucleotide **5**–RNA duplex (Figures 7 and 8).

Discussion

Antisense oligonucleotides incorporating 2′-deoxy-2′-fluoro nucleosides, when hybridized with RNA, afford consistent additive enhancement of duplex stability (Tables I–IV) without compromising base-pair specificity (Table V). This effect is observed within a phosphodiester or phosphorothioate backbone, and it appears that the phenomena is not sequence-dependent based on the multiple number of sequences in which the stabilization

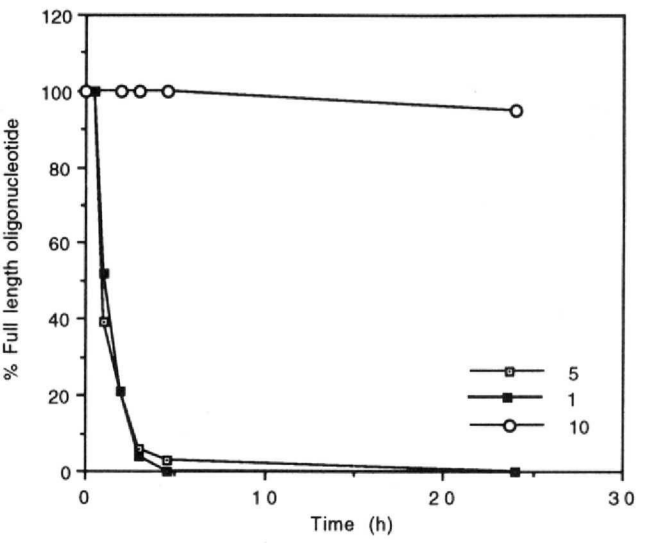


Figure 6. Nuclease degradation analyses of 2′-deoxy-2′-fluoro-modified pentadecamer oligonucleotides as a function of incubation with heat inactivated 10% fetal calf serum.

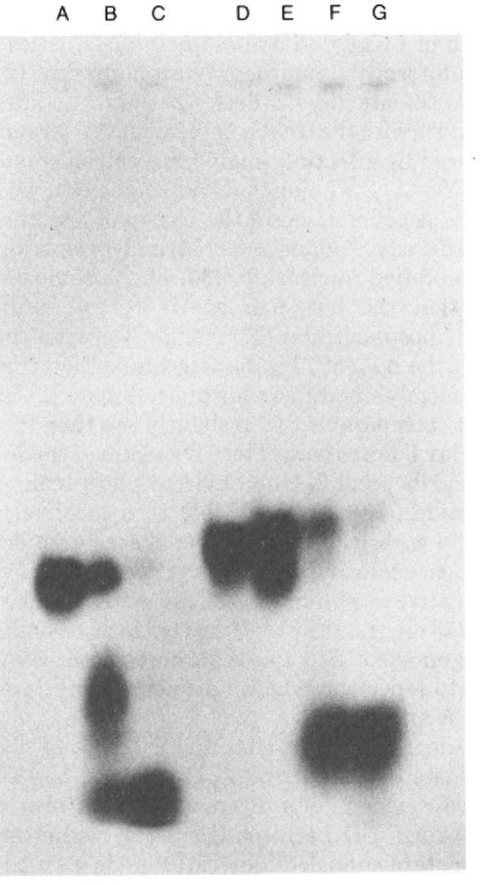


Figure 7. Effect of 2′-deoxy-2′-fluoro-modified pentadecamer oligonucleotides on RNase H mediated hydrolysis of RNA using purified HeLa nuclear extract. Lanes A–C: oligonucleotides **5**, **8**, and **1**, respectively, using 0.2 μg/reaction purified HeLa nuclear extract. Lanes D–G: control (no DNA present), oligonucleotides **5**, **8**, and **1**, respectively, using 2 μg/reaction purified HeLa nuclear extract. Numbered compounds refer to entries in Table I.

was observed. We propose that the resultant duplex stabilization could be due to a high fractional population of 3′-endo sugar pucker within the antisense oligonucleotide which in turn impacts upon other parameters of the duplex. Upon hybridization with RNA the 2′-deoxy-2′-fluoro substituent may bias the modified oligonucleotide toward the A form and the ensuing heteroduplex may adopt a more "conformationally pure" A-type duplex which

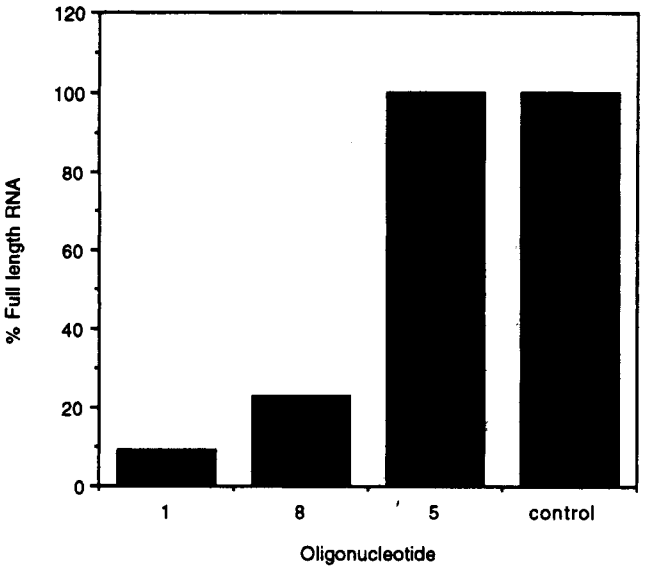


Figure 8. Effect of 2′-deoxy-2′-fluoro-modified pentadecamer oligonucleotide on RNase H mediated hydrolysis of RNA using purified HeLa nuclear extract (2 μg/reaction). Numbered compounds refer to entries in Table I. Control = no DNA present.

provides an enhanced thermodynamic stability characteristic of RNA–RNA helices. Indeed the CD spectra of the 2′-deoxy-2′-fluoro-modified oligonucleotides hybridized with RNA (Figures 3 and 4) indicate that the duplex adopts a fully A-form conformation as opposed to a variant A/B form.[10,11]

It was demonstrated within two phosphodiester and one phosphorothioate sequences that the stabilizing effects of the three types of 2′-functionalities investigated displayed the rank order: 2′-deoxy-2′-fluoro > 2′-*O*-methylribo > ribo (Tables I and II). This trend may also be related to the propensity of the different sugar moieties to adopt the 3′-endo conformation as discussed.

Apparently the 2′-deoxy-2′-fluoro modification does not alter the overall structure of the oligonucleotide in such a way as to prevent recognition and cleavage by nucleases (Figures 5 and 6). Thus it is crucial that the stabilizing effects upon the heteroduplex afforded by the 2′-deoxy-2′-fluoro modifications have remained operative within the context of a nuclease-resistant phosphorothioate backbone (Figures 5 and 6). Moreover the resultant T_m increases more than compensate for the known depression of duplex melting temperatures resulting from the phosphorothioate substitutions.[1,3] In fact the "uniformly" 2′-deoxy-2′-fluoro-modified phosphorothioate oligonucleotide **10** showed higher duplex stability than the analogous ribo or 2′-*O*-methylribo phosphodiesters (**6** and **7**) or phosphorothioates (**11** and **12**) (Table I). This is highly significant as a majority of antisense oligonucleotides are of the phosphorothioate class. Antisense oligonucleotides containing the 2′-*O*-methylribo modification are also attractive due to enhanced binding affinity but this class of oligonucleotides may not possess sufficient nuclease resistance.[1,44] Thus, the doubly modified 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides provide a higher binding affinity than the 2′-*O*-methylribo phosphodiester or 2′-*O*-methylribo phosphorothioate oligonucleotides, and moreover, the 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides are highly nuclease resistant.

As an RNase H mediated mechanism is generally regarded as a major effector of antisense activity,[1] it was a disappointment to observe that the RNA hybrid duplex with uniformly 2′-deoxy-2′-fluoro-modified oligonucleotides did not serve as substrate for HeLa RNase H (Figures 7 and 8). Given that RNase H does not recognize double stranded RNA–RNA but only RNA–DNA as substrate,[45] these results imply that the duplex formed by RNA and the modified oligonucleotide has assumed a structure which is distinct from that of an RNA–DNA duplex. The CD studies (Figures 3 and 4) coincide with these biological findings. Such a helix may not provide enough flexibility within the minor groove for RNase H to react as recently proposed.[46] As we desire to retain RNase H activity in the application of some 2′-deoxy-2′-fluoro-modified oligonucleotides, we have incorporated the modifications into "chimeric" oligonucleotides composed of a region of RNase H-resistant 2′-deoxy-2′-fluoro nucleotides linked to an unmodified region capable of activating RNase H. We have found that these chimeric oligonucleotides are capable of activating mammalian RNase H in vitro upon hybridization with RNA and display potent antisense activity against RNA targets in cells.[47,48] Similar "chimeric" oligonucleotides incorporating 2′-*O*-methylribo and 2′-deoxy-2′-fluoro nucleosides have been utilized for regiospecific cleavage of RNA by RNase H.[25,49,50]

"Uniformly" modified 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides, reported herein for the first time, afforded antisense molecules with (1) high binding affinity and selectivity for the RNA target and (2) stability toward nucleases. Applications of these types of antisense oligonucleotides against certain biological targets are in progress.

Experimental Section

General. NMR spectra were obtained with the following instruments. ^{1}H NMR: Varian Gemini-200 (199.975 MHz) or Varian Unity 400 (399.952 MHz). ^{13}C NMR: Varian Gemini-200 (50.289 MHz). ^{31}P NMR: Varian Gemini-200 (79.990 MHz) or Varian Unity 400 (159.981 MHz). NMR spectra were recorded using either deuteriochloroform (tetramethylsilane or phosphoric acid internal standard), dimethyl sulfoxide-d_6, or deuterium oxide (2,2-dimethyl-2-silapentane-5-sulfonate internal standard) as solvent. The following abbrevations were used to designate the multiplicity of individual signals: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, br s = broad singlet. Mass spectra were acquired on a VG 70-SEQ instrument (VG Analytical (Fisons)), using fast atom bombardment ionization (7-kV Xe atoms).

Tetrabutylammonium fluoride hydrate was dried by coevaporation with anhydrous pyridine three times. Solvent ratios for column chromatography are given as volume/volume. Evaporations of solvents were performed in vacuo (60 Torr) at 30 °C unless otherwise specified. 9-β-D-Arabinofuranosyladenine was purchased from Sigma Chemical Co.

Synthesis of 2′-Deoxy-2′-fluoro Nucleosides. N^6-Benzoyl-9-(3,5-di-*O*-tetrahydropyran-2-yl-β-D-arabinofuranosyl)adenine (2). The title compound was prepared by modifications of a reported procedure.[28] N^6-Benzoyl-9-β-D-arabinofuranosyladenine (1) (2.62 g, 7.06 mmol), prepared via routine peracylation procedures,[32] was dissolved in anhydrous dimethylformamide (150 mL) under an argon atmosphere, and *p*-toluenesulfonic acid monohydrate (1.32 g, 6.92 mmol) was added. This solution was cooled to 5 °C, and 3,4-dihydro-2*H*-pyran (1.26 mL, 13.8 mmol) was added. The reaction mixture was allowed to warm to 23 °C. Over a period of 5 h a total of 10 equiv of 3,4-dihydro-2*H*-pyran was added in 2-equiv portions in the fashion described. The reaction mixture was cooled to 5 °C, saturated aqueous sodium bicarbonate was added slowly to a pH of 8, and then water (600 mL) was added. The aqueous mixture was extracted with CH_2Cl_2 (4 × 200 mL), and the organic phases were combined and dried over magnesium sulfate. The solvent was evaporated in vacuo to give an oil which was evaporated with *p*-xylene in vacuo (1 Torr) at 40 °C to give an oil which was dissolved in CH_2Cl_2 (100 mL). Hexanes (200 mL) were added to the solution, and

the lower-boiling solvent was evaporated in vacuo to leave a white suspension in hexanes. This solid was filtered and washed with hexanes (3 × 10 mL) and then purified by column chromatography using silica and CH_2Cl_2–methanol (93:7) as eluent to give the title compound **2** as a white foam (3.19 g, 83%) which consisted of a diastereomeric mixture. ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 11.2 (br s, 1, NH), 8.75 (s, 1 H8), 8.5–7.6 (m, 6, Bz), 6.41 (m, 1, H1′), 5.91 (m, 1, 2′-OH), 5.0–3.5 (m, ribose, THP), 1.8–1.5 (m, 12, H3-, H4-, H5-THP).

N^6-Benzoyl-2′-deoxy-2′-fluoro-3′,5′-di-*O*-tetrahydropyran-2-yladenosine (4). The title compound was prepared by modifications of a reported procedure.[27] Thus compound **2** (2.65 g, 4.91 mmol) was dissolved in anhydrous CH_2Cl_2 (130 mL) under an argon atmosphere, and anhydrous pyridine (3.34 mL, 41.3 mmol) and (*N*,*N*-dimethylamino)pyridine (1.95 g, 16.0 mmol) were added. The reaction mixture was cooled to 5 °C, and trifluoromethanesulfonic anhydride (1.36 mL, 8.05 mmol) was added slowly. After the reaction mixture was stirred at 5 °C for 1 h, it was poured into cold saturated aqueous sodium bicarbonate (140 mL). The mixture was shaken, and the organic phase was separated and kept at 5 °C. The aqueous phase was extracted with CH_2Cl_2 (2 × 140 mL), and the organic extracts were combined and dried over magnesium sulfate. The solvent was evaporated in vacuo to give an oil which was evaporated with anhydrous CH_3CN twice to give N^6-benzoyl-9-[2-*O*-(trifluoromethylsulfonyl)-3,5-di-*O*-tetrahydropyran-2-yl-β-D-arabinofuranosyl]adenine (**3**) as a crude oil which was not further purified. This crude oil was dissolved in anhydrous tetrahydrofuran (120 mL), and this solution was cooled to 5 °C under an argon atmosphere. Dried tetrabutylammonium fluoride (12.8 g, 49.1 mmol) was dissolved in anhydrous tetrahydrofuran (50 mL), and half of this volume was slowly added via syringe to the cold reaction mixture. After stirring at 5 °C for 1 h, the remainder of the reagent was added slowly. The reaction mixture was stirred at 5 °C for an additional 4 h, and then the solvent was evaporated in vacuo to give an oil. This oil was dissolved in CH_2Cl_2 (250 mL) and washed with brine three times. The organic phases was separated and dried over magnesium sulfate, and the solvent was evaporated to give an oil. The crude product was purified by column chromatography using silica with ethyl acetate as eluent to yield the title compound **4** as an oil (2.03 g, 76%) which consisted of a diastereomeric mixture.

N^6-Benzoyl-2′-deoxy-2′-fluoroadenosine (5). Compound **4** (1.31 g, 2.42 mmol) was dissolved in methanol (50 mL), and Dowex 50W × 2-100 (4 cm^3, 2.4 mequiv) was added to the reaction mixture. The reaction mixture was stirred at 23 °C for 1 h, the resin was filtered, and the filtrate was evaporated in vacuo to give a residue. The Dowex resin was then washed with pyridine–triethylamine–water (1:3:3) (3 × 25 mL), toluene (50 mL) was added to the washes, and the solvent was evaporated separately in vacuo (1 Torr) at 24 °C to give a residue. The crude products were combined, dissolved in hot (60 °C) water (30 mL), and washed with CH_2Cl_2 (2 × 10 mL). The aqueous phase was evaporated in vacuo (1 Torr) at 40 °C to give an oil which was evaporated with anhydrous pyridine (2 × 10 mL) and dried in vacuo (1 Torr) at 23 °C in the presence of phosphorous pentoxide for 16 h to give the title compound **5** as a yellow foam (1.05 g) which contained minor impurities. ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 8.75 (s, 1, H8), 8.69 (s, 1, H2), 8.1–7.5 (m, 5, H-Ar), 6.37 (dd, 1, H1′, $J_{1',2'}$ = 2.3 Hz, $J_{1',F}$ = 17.2 Hz), 5.77 (d, 1, 3′-OH), 5.50 (dt, 1, H2′, $J_{2',F}$ = 53.9 Hz), 5.18 (m, 1, 5′-OH), 4.50 (m, 1, H3′), 3.99 (m, 1, H4′), 3.68 (m, 2, H5′a, H5′b).

N^6-Benzoyl-2′-deoxy-2′-fluoro-5′-*O*-(4,4′-dimethoxytrityl)-adenosine (6). Compound **5** (1.08 g, 2.89 mmol) was dissolved in anhydrous pyridine (20 mL) under an argon atmosphere, and triethylamine (0.52 mL, 3.76 mmol) was added followed by addition of 4,4′-dimethoxytrityl chloride (1.13 g, 3.32 mmol). After 4 h of stirring at 23 °C, diethyl ether (40 mL) was added and this mixture was washed with water (2 × 10 mL). The organic phase was separated and dried over magnesium sulfate. Triethylamine (0.1 mL) was added to the solution, and the solvent was evaporated in vacuo to give an oil which was evaporated with toluene (20 mL) containing triethylamine (0.1 mL). This crude product was purified by column chromatography using silica and ethyl acetate–triethylamine (99:1) followed by ethyl acetate–methanol–triethylamine (80:19:1) to give the title compound **6** in two fractions as a foam (1.02 g, 63% from **4**). ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.02 (br s, 1, NH), 8.77 (s, 1, H8), 8.22 (s, 1, H2), 8.0–6.7 (m, 18, H-Ar), 6.32 (dd, 1, H1′, $J_{1',2'}$ = 2.4 Hz), 5.69 (ddd, 1, H2′, $J_{2',F}$ = 52.9 Hz), 4.83 (m, 1, H3′), 4.24 (m, 1, H4′), 3.78 (s, 6, OCH_3), 3.50 (m, 2, H5′a, H5′b). FAB-MS: 676 [M + H]$^+$.

N^6-Benzoyl-2′-deoxy-2′-fluoro-5′-*O*-(4,4′-dimethoxytrityl)-adenosine 3′-[β-Cyanoethyl *N*,*N*-diisopropylphosphoramidite] (7). Compound **6** (5.67 g, 8.39 mmol) and diisopropylamine hydrotetrazolide (722 mg, 4.20 mmol) were dissolved in anhydrous CH_2Cl_2 (60 mL) followed by addition of bis(diisopropylamino)(β-cyanoethoxy)phosphine (2.92 mL, 9.23 mmol). After the reaction mixture was stirred at 23 °C under an argon atmosphere for 8 h, CH_2Cl_2–NEt_3 (99:1, 200 mL) was added and the mixture was washed with saturated $NaHCO_3$ followed by washings with brine (2 × 50 mL). The organic phase was dried with $MgSO_4$, and the solvent was evaporated in vacuo to give a foam which was evaporated in vacuo (1 Torr) with anhydrous CH_3CN three times to give a foam. After further drying in vacuo (1 Torr) overnight, the product was dissolved in anhydrous CH_2Cl_2 (5 mL) and added dropwise to anhydrous hexanes (500 mL) to afford a white precipitate which was filtered, washed with hexanes, and dried in vacuo (1 Torr) at 23 °C to afford the title compound **7** as a white solid (5.73 g, 78%). ^{31}P NMR (161.9 MHz, $CDCl_3$): δ 153.0 (d, 1, $J_{P,F}$ = 8.1 Hz), 152.3 (d, 1, $J_{P,F}$ = 11.3 Hz). FAB-MS: 876 [M + H]$^+$.

2′-Deoxy-2′-fluoroadenosine (8). Compound **5** (200 mg, 0.54 mmol) was dissolved in methanolic ammonia (5 mL, saturated at 0 °C) at 5 °C. The reaction flask was sealed, and the mixture was allowed to warm to 20°. After 16 h the solvent was evaporated in vacuo to a small volume to give a white suspension which was filtered and washed with cold methanol to yield a white solid (104 mg). ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 8.35 (s, 1, H8), 8.14 (s, 1, H2), 7.36 (br s, 2, NH_2), 6.20 (dd, 1, H1′, $J_{1',2'}$ = 2.82 Hz, $J_{1',F}$ = 16.2 Hz), 5.40 (ddd, 1, H2′, $J_{2',3'}$ = 4.34 Hz, $J_{2',F}$ = 52.7 Hz), 5.70 (d, 1, 3′-OH), 5.25 (m, 1, 5′-OH), 4.47 (m, 1, H3′), 3.97 (m, 1, H4′), 3.65 (m, 2, H5′, H5′b). ^{13}C NMR (50 MHz, Me_2SO-d_6): δ 156.12 (C6), 152.88 (C2), 148.90 (C4), 139.55 (C8), 119.12 (C5), 93.46 (C2′, $J_{2',F}$ = 186.9 Hz), 85.93 (C1′, $J_{1',F}$ = 33.0 Hz), 84.20 (C4′), 68.37 (C3′, $J_{3',F}$ = 15.6 Hz), 60.46 (C5′).

N^2-Isobutyryl-9-(2-*O*-isobutyryl-3,5-*O*-(tetraisopropyldisiloxanylene)-β-D-arabinofuranosyl)guanine (10). 9-(3,5-*O*-(Tetraisopropyldisiloxanylene)-β-D-arabinofuranosyl)-guanine[31] (**9**) was prepared with modifications to the reported procedure. Thus 3′,5′-*O*-(tetraisopropyldisiloxanylene)guanosine (21.0 g, 40.0 mmol) was oxidized with DMSO–acetic anhydride, and the mixture was cooled to −78 °C. While the mixture was mechanically stirred, sodium borohydride (2.0 g, 52.8 mmol) was added at −78 °C in one portion, the reaction mixture was allowed to slowly warm to 0 °C and stirred at 0 °C for 30 min. The described addition of sodium borohydride at −78 °C was repeated twice again, and then the mixture was allowed to warm to 23 °C and stirred for 1 h. EtOAc (1 L) was added to the mixture, and it was washed with brine (2 × 2 L). The organic phase was dried over $MgSO_4$, and the solvent was evaporated in vacuo to give a residue which was evaporated with toluene twice. The crude product was purified by column chromatography using silica and CH_2Cl_2–EtOAc–MeOH (45:45:10) as eluent to give the compound **9** as a white solid (10.5 g, 50%). Compound **9** (1.71 g, 3.25 mmol) was dissolved in anhydrous pyridine (40 mL), NEt_3 (2.0 mL, 14.6 mmol) and isobutyric anhydride (5.39 mL, 32.5 mmol) were added, and the reaction mixture was heated at 60 °C for 20 h under an argon atmosphere. The reaction mixture was cooled to 23 °C, MeOH (3 mL) was added, and the mixture was stirred for 30 min. The solvent was evaporated in vacuo to give a residue which was dissolved in EtOAc (50 mL). This solution was washed with saturated $NaHCO_3$, the organic phase was dried with $MgSO_4$, and the solvent was evaporated in vacuo to give an oil which was evaporated with toluene and purified by column chromatography using CH_2Cl_2–acetone (90:10) to give the title compound **10** as an oil (1.72 g, 80%). ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 12.10 (br s, 1, NH), 11.78 (br s, 1, NH), 7.99 (s, 1, H8), 6.26 (d, 1, H1′), 5.62 (m, 1, H2′), 4.56 (m, 1, H3′), 4.04 (m, 2, H5′, H5′b), 3.98 (m, 1, H4′), 2.76 (m, 1, $HCMe_2$), 2.21 (m, 1, $HCMe_2$), 1.0 (m, 28, TPDS), 0.78 (d, 6, CH_3), 0.66 (d, 6, CH_3). FAB-MS: 666 [M + H]$^+$.

N^2-Isobutyryl-9-(2-*O*-isobutyryl-β-D-arabinofuranosyl)-guanine (11). Deprotection of compound **10** (9.83 g, 14.8 mmol) was performed as usual with 1 M *n*-Bu_4NF (29.5 mL, 29.5 mmol). The crude product was purified by column chromatography using

silica and EtOAc–MeOH (85:15) as eluent to give the title compound as a white solid (4.98 g, 80%). ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 12.09 (br s, 1, NH), 11.72 (br s, 1, NH), 8.14 (s, 1, H8), 6.27 (d, 1, H1′), 5.86 (d, 1, 3′-OH), 5.27 (m, 1, H2′), 5.09 (m, 1, 5′-OH), 4.37 (m, 1, H3′), 3.87 (m, 1, H4′), 3.66 (m, 2, H5′a, H5′b), 2.76 (m, 1, $HCMe_2$), 2.30 (m, 1, $HCMe_2$), 0.84 (d, 6, CH_3), 0.68 (d, 6, CH_3). FAB-MS: 424 [M + H]$^+$.

N^2-Isobutyryl-9-(3,5-di-*O*-tetrahydropyran-2-yl-β-D-arabinofuranosyl)guanine (12). Compound 11 (4.90 g, 11.6 mmol) was dissolved in anhydrous 1,4-dioxane (98 mL), *p*-toluenesulfonic acid monohydrate (0.97 g, 5.10 mmol) and 3,4-dihydro-2*H*-pyran (9.34 mL, 102 mmol) were added, and then the reaction mixture was stirred at 23 °C for 2 h. The mixture was cooled to 5 °C, saturated $NaHCO_3$ (125 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 × 125 mL). The organic extracts were combined and dried with $MgSO_4$, and the solvent was evaporated to give a residue which was dissolved in a minimal amount of CH_2Cl_2. This solution was added dropwise to an excess (100-fold) of hexanes to afford a white precipitate which was filtered and washed with hexanes to give the title compound 12 as a white solid (5.59 g, 82%) which consisted of a diastereomeric mixture. ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 6.28 (m, 1, H1′), 4.8–4.5 (m, 3, H3′, H2-THP), 4.0–3.3 (m, 6, H5′, H5′b, H6-THP), 1.8–1.3 (m, 12 H, H3-, H4-, H5-THP).

N^2-Isobutyryl-9-(3,5-di-*O*-tetrahydropyran-2-yl-β-D-arabinofuranosyl)guanine (13). Deacylation was performed in the usual fashion[32] using pyridine–MeOH–water (65:30:5, 52 mL) as solvent. The product was purified by column chromatography using silica and EtOAc–MeOH (95:5) as the eluent to afford the title compound 13 as an oil (3.85 g, 78%) which consisted of a diastereomeric mixture. ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 6.08 (m, 1, H1′), 5.9 (br s, 1, 2′-OH), 1.8–1.3 (m, 12 H, THP). FAB-MS: 522 [M + H]$^+$.

N^2-Isobutyryl-9-(2-fluoro-3,5-di-*O*-tetrahydropyran-2-yl-β-D-arabinofuranosyl)guanine (15). The same methodology as for the synthesis of 4 was utilized. Thus compound 13 (3.84 g, 7.39 mmol) was reacted to obtain a product which was subjected to column chromatography using silica and EtOAc–MeOH (95:5) as eluent to afford the title compound 15 as an oil (5.25 g) which consisted of a diastereomeric mixture contaminated with tetrabutylammonium salts. ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 6.16 (d, 1, H1′, $J_{1',F}$ = 15.9 Hz).

N^2-Isobutyryl-2′-deoxy-2′-fluoroguanosine (16). A procedure which was similar to that used for the synthesis of 5 was used. Thus compound 15 (3.8 g) as a crude product was hydrolyzed to afford a product which was purified by column chromatography using EtOAc–MeOH (80:20) as eluent to yield 16 as a white solid (1.09 g, 41% from 13). ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 12.11 (br s, 1, NH), 11.69 (br s, 1, NH), 8.26 (s, 1, H8), 6.10 (dd, 1, H1′, $J_{1',2'}$ = 2.0 Hz, $J_{1',F}$ = 16.2 Hz), 5.70 (d, 1, 3′-OH), 5.27 (dd, 1, H2′, $J_{2',F}$ = 52.8 Hz), 5.18 (m, 1, 5′-OH), 4.37 (m, 1, H3′), 3.96 (m, 1, H4′), 3.67 (m, 2, H5′, H5′b), 2.77 (m, 1, $HCMe_2$), 1.11 (d, 6, CH_3).

2′-Deoxy-2′-fluoroguanosine (17). The reaction conditions used were similar to those used for the synthesis of 8. Thus compound 16 (100 mg, 0.28 mmol) was reacted to give a residue which was triturated with hot MeOH twice to yield the title compound 17 as a white solid (63 mg, 80%). ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 10.69 (br s, 1, NH), 7.96 (s, 1, H8), 6.57 (br s, 2, NH_2), 6.02 (dd, 1, H1′, $J_{1',2'}$ = 2.9 Hz, $J_{1',F}$ = 16.2 Hz), 5.67 (d, 1, 3′-OH), 5.22 (ddd, 1, H2′, $J_{2',3'}$ = 4.3 Hz, $J_{2',F}$ = 52.8), 5.15 (m, 1, 5′-OH), 4.38 (m, 1, H3′), 3.94 (m, 1, H4′), 3.67 (m, 2, H5′a, H5′b). ^{13}C NMR (50 MHz, Me_2SO-d_6): δ 157.35 (C6), 154.30 (C2), 150.96 (C4), 135.34 (C8), 116.79 (C5), 93.81 (C2′, $J_{2',F}$ = 185.8 Hz), 85.14 (C1′, $J_{1',F}$ = 32.6 Hz), 83.86 (C4′), 68.19 (C3′, $J_{3',F}$ = 15.4 Hz), 60.28 (C5′).

N^2-Isobutyryl-2′-deoxy-2′-fluoro-5′-*O*-(4,4′-dimethoxytrityl)guanosine. The reaction conditions utilized were similar to those used for the synthesis of 6. Thus compound 16 (0.92 g, 2.60 mmol) was reacted to yield a crude product which was purified by column chromatography using EtOAc–NEt_3 (99:1), followed by EtOAc–MeOH–NEt_3 (94:5:1) to give the title compound as a foam (1.49 g, 87%). ^{1}H NMR (200 MHz, Me_2SO-d_6): δ 12.11 (br s, 1, NH), 11.61 (br s, 1, NH), 8.12 (s, 1, H8), 7.4–6.7 (m, 13, H-Ar), 6.20 (d, 1, H1′, $J_{1',F}$ = 18.3 Hz), 5.65 (d, 1, 3′-OH), 5.42 (dd, 1, H2′, $J_{2',F}$ = 52.3 Hz), 4.59 (m, 1, H3′), 4.11 (m, 1, H4′), 3.71 (s, 6, OCH_3), 2.76 (m, 1, $HCMe_2$), 1.10 (d, 6, CH_3). FAB-MS: 658 [M + H]$^+$.

N^2-Isobutyryl-2′-deoxy-2′-fluoro-5′-*O*-(4,4′-dimethoxytrityl)guanosine 3′-[β-Cyanoethyl *N,N*-diisopropylphosphoramidite]. The reaction conditions utilized were similar to those used for the synthesis of 7. Thus N^2-isobutyryl-9-[2′-fluoro-5′-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]guanine (4.15 g, 6.33 mmol) was reacted to yield the title compound as a white solid (4.76 g, 89%). ^{31}P NMR (80 MHz, $CDCl_3$): δ 151.32 (d, 1, $J_{P,F}$ = 17.1 Hz), 150.41 (d, 1, $J_{P,F}$ = 9.2 Hz). FAB-MS: 858 [M + H]$^+$.

2′-Deoxy-2′-fluorouridine (18). The title compound was prepared by a modification of a reported procedure[34] wherein 70% hydrogen fluoride–pyridine was used in place of anhydrous hydrogen fluoride. Thus 2,2′-anhydro-1-β-D-arabinofuranosyluracil (10.15 g, 50.0 mmol) was reacted to afford the title compound 18 as a hygroscopic foam (6.58 g, 60%). ^{1}H NMR (200 MHz, D_2O): δ 7.82 (d, 1, H6, $J_{5,6}$ = 7.9 Hz), 6.00 (d, 1, H1′, $J_{1',F}$ = 19.7 Hz), 5.86 (d, 1, H5), 5.21 (dd, 1, H2′, $J_{2',3'}$ = 4.87 Hz, $J_{2',F}$ = 52.7 Hz), 4.38 (m, 1, H3′), 4.11 (m, 1, H4′), 3.91 (m, 2, H5′, H5′b). ^{13}C NMR (50 MHz, Me_2SO-d_6): δ 163.21 (C4), 150.24 (C2), 140.45 (C6), 101.61 (C5), 93.51 (d, C2′, $J_{2',F}$ = 184.6 Hz), 87.20 (d, C1′, $J_{1',F}$ = 34.0 Hz), 83.16 (C4′), 67.30 (d, C3′, $J_{3',F}$ = 15.8 Hz), 59.23 (C5′).

2′-Deoxy-2′-fluoro-5′-*O*-(4,4′-dimethoxytrityl)uridine. The procedure utilized was similar to that used for the synthesis of compound 6. Thus compound 18 (1.31 g, 5.36 mmol) was reacted to give a crude product which was purified by column chromatography using silica and hexanes–EtOAc–NEt_3 (25:74:1) as eluent to give the title compound as a foam (2.38 g, 81%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.91 (d, 1, H6, $J_{5,6}$ = 8.2 Hz), 7.4–6.8 (m, 13, H-Ar), 6.07 (d, 1, H1′, $J_{1',F}$ = 15.2 Hz), 5.34 (d, 1, H5), 5.03 (dd, 1, H2′, $J_{2',F}$ = 52.8 Hz), 4.54 (m, 1, H3′), 4.11 (m, 1, H4′), 3.58 (m, 2, H5′, H5′b). FAB-MS: 548 [M]$^+$.

2′-Deoxy-2′-fluoro-5′-*O*-(4,4′-dimethoxytrityl)uridine 3′-[β-Cyanoethyl *N,N*-diisopropylphosphoramidite]. The procedure utilized was similar to that used for the synthesis of 7. Thus 1-[2-fluoro-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]uracil (2.38 g, 4.35 mmol) was reacted to yield the title compound as a white solid (2.92 g, 90%). ^{31}P NMR (80 MHz, $CDCl_3$): δ 151.84 (d, 1, $J_{P,F}$ = 9.3 Hz), 151.65 (d, 1, $J_{P,F}$ = 8.9 Hz). FAB-MS: 748 [M + H]$^+$.

2′-Deoxy-2′-fluorocytidine (19). The procedure utilized was similar to that reported for the conversion of uridine derivatives to cytidine derivatives.[37] Thus compound 18 (3.64 g, 14.9 mmol) was reacted to afford the title compound 19 as white crystals (2.51 g, 69%). ^{1}H NMR (200 MHz, Me_2SO-d_6): 7.87 (d, 1, H6, $J_{5,6}$ = 7.4 Hz), 7.19 (br s, 2, NH_2), 5.86 (dd, 1, H1′, $J_{1',2'}$ = 1.7 Hz, $J_{1',F}$ = 17.9 Hz), 5.69 (d, 1, H5), 5.55 (d, 1, 3′-OH), 5.16 (m, 1, 5′-OH), 4.85 (ddd, 1, H2′, $J_{2',3'}$ = 4.3 Hz, $J_{2',F}$ = 53.3 Hz), 4.09 (m, 1, H3′), 3.86 (m, 1, H4′), 3.65 (m, 2, H5′a, H5′b). ^{13}C NMR (50 MHz, Me_2SO-d_6): δ 165.76 (C4), 155.02 (C2), 141.21 (C6), 94.01 (C5), 94.10 (C2′, $J_{2',F}$ = 184.2 Hz), 88.16 (C1′, $J_{1',F}$ = 33.4 Hz), 82.77 (C4′), 67.29 (C3′, $J_{3',F}$ = 16.4 Hz), 59.34 (C5′).

N^4-Benzoyl-2′-deoxy-2′-fluorocytidine (20). The procedure utilized was similar to that reported for the selective benzoylation of cytidine.[38] Thus compound 19 was reacted to give an oil which was evaporated with *p*-xylene to give a white solid. Trituration of the product with Et_2O afforded the title compound 20 as a white solid (1.97 g, 92%).

N^4-Benzoyl-2′-deoxy-2′-fluoro-5′-*O*-(4,4′-dimethoxytrityl)cytidine. The procedure utilized was similar to that used for the synthesis of compound 6. Thus compound 20 (2.18 g, 6.30 mmol) was reacted to give a crude product which was purified by column chromatography using silica and hexanes–EtOAc–NEt_3 (9:90:1) as eluent to give the title compound as a foam (3.70 g, 91%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.52 (d, 1, H6, $J_{5,6}$ = 7.5 Hz), 7.9–6.8 (m, 19, H-Ar), 6.14 (d, 1, H1′, $J_{1',F}$ = 15.9 Hz), 5.10 (dd, 1, H2′, $J_{2',F}$ = 52.0 Hz), 4.53 (m, 1, H3′), 4.16 (m, 1, H4′), 3.83 (s, 6, OCH_3), 3.64 (m, 2, H5′a, H5′b). FAB-MS: 652 [M]$^+$.

N^4-Benzoyl-2′-deoxy-2′-fluoro-5′-*O*-(4,4′-dimethoxytrityl)cytidine 3′-[β-Cyanoethyl *N,N*-diisopropylphosphoramidite]. The procedure utilized was similar to that used for the synthesis of 7. Thus 1-[2-fluoro-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]cytosine (3.80 g, 5.84 mmol) was reacted to yield the title compound as a white solid (4.47 g, 90%). ^{31}P NMR

(80 MHz, $CDCl_3$): δ 152.47 (d, 1, $J_{P,F}$ = 7.9 Hz), 152.12 (d, 1, $J_{P,F}$ = 7.0 Hz). FAB-MS: 852 $[M + H]^+$.

Oligonucleotide Synthesis. The modified 2′-deoxy-2′-fluoro oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer. Standard phosphoramidite coupling chemistry[51,52] was utilized except the standard synthesis cycle was modified by increasing the phosphoramidite condensation time to 6 min. The 3′-terminal nucleotide is derived from the solid support (CPG) and is unmodified. The 5′-terminal dimethoxytrityl protection group was retained to allow for reverse-phase LC purification. Oligonucleotides were deprotected by overnight incubation at room temperature in methanolic ammonia (saturated at 0 °C) for 24 h. The 5′-O-dimethoxytritylated oligonucleotides were purified by C-4 reversed-phase HPLC. Analytical gel electrophoresis on a 20% polyacrylamide denaturing gel[53] demonstrated the purity to be greater than 90%. Complementary RNA oligonucleotides were synthesized as the 2′-*O*-(*tert*-butyldimethylsilyl)-protected ribonucleotides.[54]

Phosphorothioate 2′-deoxy-2′-fluoro oligonucleotides were synthesized by replacing the iodine oxidation solution with a 0.2 M solution of 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in acetonitrile. The thiation wait step was increased to 10 min and was followed by the capping step. All standard DNA synthesis reagents were purchased from Applied Biosystems Inc. (Foster City, CA). The thiation reagent was synthesized according to a literature procedure.[55] Purification procedures were performed according to the phosphodiester oligonucleotides protocols.

^{1}H-NMR Analysis of Trimer Oligonucleotides Containing 2′-Deoxy-2′-fluoro Nucleotides. TA_fC. ^{1}H NMR (400 MHz, D_2O): δ 8.39 (s, 1, H8-A_f), 8.13 (s, 1, H6-A_f), 6.23 (dd, 1, H1′-A_f, $J_{1',F}$ = 14.8 Hz), 5.44 (ddd, 1, H2′-A_f, $J_{2',F}$ = 53.3 Hz).

TG_fC. ^{1}H NMR (400 MHz, D_2O): δ 7.98 (s, 1, H8-G_f), 6.13 (dd, 1, H1′-G_f, $J_{1',F}$ = 14.9 Hz), 5.49 (ddd, 1, H2′-G_f, $J_{2',F}$ = 51.2 Hz).

AU_fC. ^{1}H NMR (400 MHz, D_2O): δ 7.39 (d, 1, H6-Uf, $J_{5,6}$ = 8.1 Hz), 5.70 (dd, 1, H2′-U_f, $J_{1',F}$ = 19.0 Hz), 5.39 (d, 1, H5-U_f), 5.07 (ddd, 1, H2′, $J_{2',F}$ = 52.1 Hz).

TC_fC. ^{1}H NMR (400 MHz, D_2O): δ 7.95 (d, 1, H6-C_f or H6-C), 7.80 (d, 1, H6-C_f, or H6-C), 6.06 (d, 1, H5-C_f or H5-C), 5.97 (d, 1, H5-C_f or H5-C), 5.96 (dd, 1, H1′, $J_{1',F}$ = 17.9 Hz), 5.24 (ddd, 1, H2′-C_f, $J_{2',F}$ = 52.1 Hz).

Modified Oligonucleotide Compositional Analysis. The relative nucleotide ratios of oligomers containing 2′-deoxy-2′-fluoro modifications were determined by enzymatic cleavage followed by dephosphorylation to the corresponding nucleosides, followed by HPLC analysis. Enzymatic degradation was carried out using 0.1 units of snake venom phosphodiesterase (Pharmacia, Piscataway, NJ), 23 units nuclease P1 (Gibco BRL, Gaithersburg, MD), 24 units calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, IN), and 10 nmol of oligomer in 50 mM Tris-HCl pH 8.5, 14 mM $MgCl_2$, and 72 mM NaCl in a total volume of 20 mL. Reactions were incubated at 37 °C for 30 min. HPLC analysis was carried out using a Waters Model 715 automatic injector, Model 600E pump, Model 991 detector, and an Alltech (Alltech Associates, Inc., Deerfield, IL) nucleoside/nucleotide column (4.6 × 250 mm). All analyses were performed at room temperature. The solvents used were (A) water and (B) acetonitrile. Separation of U_f and A_f nucleosides was accomplished with the following gradient: 0–5 min, 2% B (isocratic); 5–20 min, 2% B to 10% B (linear); 20–40 min, 10% B to 50% B. Separation of C_f and G_f was accomplished with a gradient of 0–20 min, 3% B (isocratic); 20–30 min, 3% B to 10% B (linear); 30–45 min, 10% B (isocratic). The integrated area per nanomole was determined using nucleoside standards. Relative nucleoside ratios were calculated by converting integrated areas to molar values and comparing all values to thymidine, which was set at its expected value for each oligomer.

Hybridization Thermodynamics. Absorbance vs temperature profiles were measured on a Gilford Response spectrophotometer in buffer containing 0.1 M NaCl, 10 mM sodium phosphate (pH 7.2), 0.1 mM EDTA, and 4 mM oligonucleotides. Extinction coefficients at 260 nm were calculated from nearest neighbor approximation.[56] The heating rate was 1 °C/min. The T_m's and free energies of duplex formation were obtained from fits of the absorbance vs temperature data to a two state model with linear sloping baselines.[57] Reported parameters are averages of at least three experiments.

Circular Dichroism Spectra. CD spectra were recorded in melting buffer on a JASCO J-600 spectropolarimeter. The ellipticities of duplexes were recorded from 210 to 300 nm in a cuvette with a path length of 10 mm. CD data were converted into mdeg·mol of residues^{-1}·cm^{-1}.

Nuclease Degradation Studies. Oligonucleotides (0.1 mg/mL) were incubated at 37 °C in DMEM supplemented with 10% heat-inactivated fetal calf serum. At various times aliquots were removed, mixed in an equal volume of 9 M urea/2× TBE buffer, and frozen at 20 °C. Samples were analyzed by polyacrylamide electrophoresis (20% PA/7 M urea), visualized by "Stains ALL" and subjected to quantitation using a Molecular Dynamics densitometer. Integrations of peaks representing the full length 15-mer and the $n - 1$, $n - 2$, and $n - 3$ oligonucleotides were summed, and the percent degradation calculated for each time point by comparison to the time = 0 sample.

RNase H Assay. Synthetic oligonucleotide RNA complement was 5′-end labeled using T4 polynucleotide kinase (Promega, catalog no. M410A) and γ-[^{32}P]ATP (ICN, catalog no. 3502005). The labeled RNA was then gel purified and precipitated to afford material suitable for use in RNase H reactions. RNase H reactions were performed at 1 nM RNA:10 nM DNA and were hybridized at 37 °C for 15 min in 20 mM Tris-Cl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 10 mg/mL tRNA (BRL, catalog no. 5401SA), 40 U/reaction RNasin (Promega catalog no. N251B). The duplexes were then slow cooled to room temperature, and purified HeLa nuclear extract[58] was added at 2 and 0.2 mg/reaction. The reactions were incubated at 37 °C for 10 min then quenched in 9 M urea/2× TBE. These reactions were loaded directly onto 20% PA/7M urea, and the results were quantitated using a Molecular Dynamics phosphor imager.

Acknowledgment. We thank Professor Ignacio Tinoco, Jr., University of California Berkeley, for the use of CD instrumentation and the Department of Medicinal Chemistry, University of Utah, for mass spectra. We also thank Richard Griffey, Patrick Wheeler, and Joel Martin for NMR spectra of modified oligonucleotides and Henri Sasmor for editing the manuscript.

References

(1) Cook, P. D. Medicinal Chemistry Strategies for Antisense Research. In *Antisense Research and Applications*; Crooke, S. T., Lebleu, B., Eds.; CRC Press, Inc.: Boca Raton, FL, 1992.
(2) Uhlmann, E.; Peyman, A. Antisense Oligonucleotides: A New Therapeutic Principle. *Chem. Rev.* **1990**, *90*, 563–584.
(3) Hoke, G. D.; Draper, K.; Freier, S. M.; Gonzalez, C.; Driver, V. B.; Zounes, M. C.; Ecker, D. J. Effects of Phosphorothioate Capping on Antisense Oligonucleotide Stability, Hybridization and Antiviral Efficacy Versus Herpes Simplex Virus Infection. *Nucleic Acids Res.* **1991**, *19*, 5743–5748.
(4) Saenger, W. Principles of Nucleic Acid Structure. *Springer Advanced Texts in Chemistry*; Cantor, C. R., Ed.; Springer-Verlag: New York, 1984.
(5) Chastain, M.; Tinoco, I., Jr. Structural Elements in RNA. *Prog. Nucleic Acid Res. Mol. Biol.* **1991**, *41*, 131–177.
(6) Wang, A. H.-J.; Fujii, S.; Van Boom, J. H.; Van der Marel, G. A.; Van Boeckel, S. A. A.; Rich, A. Molecular Structure of r(GCG)d(TATACGC): a DNA-RNA Hybrid Helix Joined to Double Helical DNA. *Nature* **1982**, *299*, 601–604.
(7) Egli, M.; Usman, N.; Zhang, S.; Rich, A. Crystal Structure of an Okazaki Fragment at 2-A Resolution. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 534–538.
(8) Zimmerman, S. B.; Pheiffer, B. H. A RNA-DNA Hybrid that Can Adopt Two Conformations: An X-ray Diffraction Study of Poly(rA)-Poly(dT) in Concentrated Solution or in Fibers. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 78–82.
(9) Reid, D. G.; Salisbury, S. A.; Brown, T.; Williams, D. H.; Vasseur, J.-J.; Rayner, B.; Imbach, J.-L. Use of Inter-Proton Nuclear Overhauser Effects to Assign the Nuclear Magnetic Resonance Spectra of Oligodeoxynucleotide and Hybrid Duplexes in Aqueous Solution. *Eur. J. Biochem.* **1983**, *135*, 307–314.
(10) Mellema, J.-R.; Haasnoot, C. A. G.; Van der Marel, G. A.; Wille, G.; Van Boeckel, S. A. A.; Van Boom, J. H.; Altona, C. Proton NMR Studies on the Covalently Linked RNA-DNA Hybrid r(GCG)d(TATACGC). Assignment of Proton Resonances by Application of the Nuclear Overhauser Effect. *Nucleic Acids Res.* **1983**, *16*, 5717–5738.
(11) Chou, S.-H.; Flynn, P.; Reid, B. High-Resolution NMR Study of a Synthetic DNA-RNA Hybrid Dodecamer Containing the Consensus Pribnow Promoter Sequence: d(CGTTATAATGCG)-r(CGCAUUAUAACG). *Biochemistry* **1989**, *28*, 2435–2443.

(12) Uesugi, S.; Miki, H.; Ikehara, M.; Iwahashi, H.; Kyogoku, Y. A Linear Relationship Between Electronegativity of 2'-Substituents and Conformation of Adenine Nucleosides. *Tetrahedron Lett.* **1979**, *42*, 4073–4076.
(13) Ikehara, M. 2'-Substituted 2'-Deoxypurinenucleotides Their Conformation and Properties. *Heterocycles* **1984**, *21*, 75–90.
(14) Guschlbauer, W.; Jankowski, K. Nucleoside Conformation is Determined by the Electronegativity of the Sugar Substituent. *Nucleic Acids Res.* **1980**, *8*, 1421–1433.
(15) Olson, W. K.; Sussman, J. L. How Flexible Is the Furanose Ring? 1. A Comparison of Experimental and Theoretical Studies. *J. Am. Chem. Soc.* **1982**, *104*, 270–278.
(16) Guschlbauer, W.; Blandin, M.; Drocourt, J. L.; Thang, M. N. Poly-2'-Deoxy-2'-Fluoro-Cytidylic Acid: Enzymatic Synthesis, Spectroscopic Characterization and Interaction with Poly-Inosinic Acid. *Nucleic Acids Res.* **1977**, *4*, 1933–1943.
(17) Kakiuchi, N.; Marek, C.; Rousseau, N.; Leng, M.; De Clercq, E.; Guschlbauer, W. Polynucleotide Helix Geometry and Stability. *J. Biol. Chem.* **1982**, *257*, 1924–1928.
(18) Janik, B.; Kotik, M. P.; Kreiser, T. H.; Reverman, L. F.; Sommer, R. G.; Wilson, D. P. Synthesis and Properties of 2'-Fluoro-2'-Deoxyuridylic Acid. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 1153–1160.
(19) Ikehara, M.; Fukui, T.; Kakiuchi, N. Polynucleotides. LII. Synthesis and Properties of Poly(2'-Deoxy-2'-Fluoroadenylic Acid). *Nucleic Acids Res.* **1978**, *5*, 1877–1887.
(20) Inoue, H.; Hayase, Y.; Imura, A.; Iwai, S.; Miura, K.; Ohtsuka, E. Synthesis and Hybridization Studies on Two Complementary Nona-(2'-O-Methyl)Ribonucleotides. *Nucleic Acids Res.* **1987**, *15*, 6131–6148.
(21) Freier, S. Hybridization: Considerations Affecting Antisense Drugs. In *Antisense Research* and *Applications*; Crooke, S. T., Lebleu, B., Eds.; CRC Press, Inc.: Boca Raton, FL, 1992.
(22) Olsen, D. B.; Benseler, F.; Aurup, H.; Pieken, W. A.; Eckstein, F. Study of a Hammerhead Ribozyme Containing 2'-Modified Adenosine Residues. *Biochemistry* **1991**, *30*, 9735–9741.
(23) Pieken, W. A.; Olsen, D. B.; Benseler, F.; Aurup, H.; Eckstein, F. Kinetic Characterization of Ribonuclease-Resistant 2'-Modified Hammerhead Ribozymes. *Science* **1991**, *253*, 314–317.
(24) Williams, D. M.; Pieken, W. A.; Eckstein, F. Function of Specific 2'-Hydroxyl Groups of Guanosines in a Hammerhead Ribozyme Probed by 2' Modifications. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 918–921.
(25) Schmidt, S.; Niemann, A.; Krynetskaya, N. F.; Oretskaya, T. S.; Metelev, V. G.; Sukhomlinov, V. V.; Shabarova, Z. A.; Cech, D. The Use of Oligonucleotide Probes Containing 2'-Deoxy-2'-Fluoronucleosides for Regiospecific Cleavage of RNA by RNase H from *Escherichia Coli*. *Biochim. Biophys. Acta* **1992**, *1130*, 41–46.
(26) Ranganathan, R. Modification of the 2'-Position of Purine Nucleosides: Syntheses of 2'-α-Substituted-2'-Deoxyadenosine Analogs. *Tetrahedron Lett.* **1977**, 1291–1294.
(27) Ikehara, M.; Miki, H. Studies of Nucleosides and Nucleotides. LXXXII. Cyclonucleosides. (39). Synthesis and Properties of 2'-Halogeno-2'-deoxyadenosines. *Chem. Pharm. Bull.* **1978**, *26*, 2449–2453.
(28) Butke, G.; Chladek, S. Facile Synthesis of 2'-Amino-2'-deoxynucleoside from the Coresponding Arabino Derivative. In *Nucleic Acid Chemistry, part 3*; Townsend, L. B., Tipson, R. S., Eds.; J. Wiley and Sons: New York, 1986; pp 149–152.
(29) Barone, A. D.; Tang, J.-Y.; Caruthers, M. H. In Situ Activation of Bis-dialkylaminophosphines-A New Method for Synthesizing Deoxyoligonucleotides on Polymer Supports. *Nucleic Acids Res.* **1984**, *12*, 4051–4061.
(30) Ikehara, M.; Imura, J. Studies on Nucleosides and Nucleotides. LXXXIX. Purine Cyclonucleosides. (43) Synthesis and Properties of 2'-Halogeno-2'-deoxyguanosines. *Chem. Pharm. Bull.* **1981**, *29*, 3281–3285.
(31) Hansske, F.; Madej, D.; Robins, M. J. 2' and 3'-Ketonucleosides and Their Arabino and Xylo Reduction Products. *Tetrahedron* **1984**, *40*, 125–135.
(32) Schaller, H.; Weiman, G.; Lerch, B.; Khorana, H. G. Studies on Polynucleotides. XXIV. The Stepwise Synthesis of Specific Deoxyribopolynucleotides (4). Protected Derivatives of Deoxyribonucleosides and New Syntheses of Deoxyribonucleoside-3'-Phosphates. *J. Am. Chem. Soc.* **1963**, *85*, 3821–3827.
(33) Benseler, F.; Williams, D. M.; Eckstein, F. Synthesis of Suitably-Protected Phosphoramidites of 2'-Fluoro-2'-Deoxyguanosine and 2'-Amino-2'-Deoxyguanosine for Incorporation into Oligoribonucleotides. *Nucleosides Nucleotides* **1992**, *11*, 1333–1351.
(34) Codington, J. F.; Doerr, I. L.; Fox, J. J. Nucleosides. XVIII. Synthesis of 2'-Fluorothymidine, 2'-Fluorodeoxyuridine, and Other 2'-Halogeno-2'-Deoxy Nucleosides. *J. Org. Chem.* **1964**, *29*, 558–564.
(35) Yoneda, N. The Combination of Hydrogen Fluoride with Organic Bases as Fluorination Agents. *Tetrahedron* **1991**, *47*, 5329–5365.
(36) Doerr, I. L.; Fox, J. J. Nucleosides. XXXIX. 2'-Deoxy-2'-fluorocytidine, 1-β-D-Arabinofuranosyl-2-amino-1,4(2H)-4-iminopyrimidine, and Related Derivatives. *J. Org. Chem.* **1967**, *32*, 1462–1471.
(37) Divakar, K. J.; Reese, C. B. 4-(1,2,4-Triazol-1-yl)- and 4-(3-Nitro-1,2,4-triazol-1-yl)-1-(β-D-2,3,5-tri-*O*-acetylarabinofuranosy)pyrimidin-2(1*H*)-ones). Valuable Intermediates in the Synthesis of Derivatives of 1-(β-Arabinofuranosyl)cytosine (Ara-C). *J. Chem. Soc. Perkin Trans. 1* **1982**, 1171–1176.
(38) Bhat, V.; Ugarkar, B. G.; Sayeed, V. A.; Grimm, K.; Kosora, N.; Domenico, P. A.; Stocker, E. A Simple and Convenient Method for the Selective N-Acylations of Cytosine Nucleosides. *Nucleosides Nucleotides* **1989**, *8*, 179–183.
(39) Blandin, M.; Son, T.-D.; Catlin, J. C.; Guschlbauer, W. Nucleoside Conformations 16. Nuclear Magnetic Resonance and Circular Dichroism Studies on Pyrimidine-2'-Fluoro-2'-Deoxyribonucleosides. *Biochim. Biophys. Acta* **1974**, *361*, 249–256.
(40) Krug, A.; Oretskaya, T. S.; Volkov, E. M.; Cech, D.; Shabarova, Z. A.; Rosenthal, A. The Behaviour of 2'-Deoxy-2'-Fluorouridine Incorporated into Oligonucleotides by the Phosphoramidite Approach. *Nucleosides Nucleotides* **1989**, *8*, 1473–1483.
(41) Freier, S. M.; Lima, W. F.; Sanghvi, Y. S.; Vickers, T.; Zounes, M.; Cook, P. D.; Ecker, D. J. Thermodynamics of Antisense Oligonucleotide Hybridization. In *Gene Regulation Biology of Antisense RNA and DNA*; Raven Press: New York, 1992; pp 95–107.
(42) Yang, J. T.; Samejima, T. Effect of Base Tilting on the Optical Activity of Nucleic Acids: Hypothesis. *Biochem. Biophys. Res. Commun.* **1968**, *33*, 739–745.
(43) Maestre, M. F. Circular Dichroism of DNA Films: Reversibility Studies. *J. Mol. Biol.* **1970**, *52*, 543–556.
(44) Imbach, J. L., Universite de Montpellier II, private communication.
(45) Crouch, R. J. Ribonuclease H: From Discovery to 3D Structure. *New Biologist* **1990**, *2*, 771–777.
(46) Nakamura, H.; Oda, Y.; Iwai, S.; Inoue, H.; Ohtsuka, E.; Kanaya, S.; Kimura, S.; Katsuda, C.; Katayanagi, K. How Does RNase H Recognize a DNA-RNA Hybrid? *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11535–11539.
(47) Ecker, D.; Monia, B. Use of Antisense in Studying Human Genes. *Science Innovation '92, AAAS*, San Francisco, 21–25 July, 1992.
(48) Monia, B. B.; Lesnik, E. A.; Gonzalez, C.; Lima, W. F.; McGee, D.; Guinosso, C.; Kawasaki, A.; Freier, S. M.; Cook, P. D. Evaluation of 2'-Modified Oligonucleotides Containing Deoxy Gaps as Antisense Inhibitors of Gene Expression., submitted results.
(49) Metelev, V. G.; Krynetskaya, N. F.; Purmal, A. A.; Shabarova, Z. A.; Tocik, Z.; Arnold, L.; Smrt, J. Regiospecific Cleavage of RNA by RNase H from *E. Coli* in the Presence of a Complementary Oligonucleotide with Inserted Alternating 2'-O-Methylcytidine Residues. *Collect. Czech. Chem. Commun.* **1990**, *55*, 2781–2786.
(50) Shibahara, S.; Mukai, S.; Nishihara, T.; Inoue, H.; Ohtsuka, E.; Morisawa, H. Site-directed Cleavage of RNA. *Nucleic Acids Res.* **1987**, *15*, 4403–4415.
(51) Beaucage, S. L.; Caruthers, M. H. Deoxynucleoside Phosphoramidites-A New Class of Key Intermediates for Deoxypolynucleotide Synthesis. *Tetrahedron Lett.* **1981**, 1859–1862.
(52) Sinha, N. D.; Biernat, J.; McManus, J.; Koster, H. Polymer Support Oligonucleotide Synthesis XVIII: Use of β-cyanoethyl-N,N-dialkylamino-/N-morpholino Phosphoramidite of Deoxynucleosides for the Synthesis of DNA Fragments Simplifying Deprotection and Isolation of the Final Product. *Nucleic Acids Res.* **1984**, *12*, 4539–4557.
(53) Maniatis, T.; Fritch, E. F.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1982.
(54) Wu, T.; Ogilvie, K. K. A Study on the Alkylsilyl Groups in Oligoribonucleotide Synthesis. *J. Org. Chem.* **1990**, *55*, 4717–4724.
(55) Iyer, R. P.; Egan, W.; Regahn, J. B.; Beaucage, S. L. 3*H*-1,2-Benzodithiole-3-one 1,1-Dioxide as an Improved Sulfurizing Reagent in the Solid-Phase Synthesis of Oligodeoxyribonucleoside Phosphorothioates. *J. Am. Chem. Soc.* **1990**, *112*, 1253–1254.
(56) Puglisi, J. D.; Tinoco, I., Jr. Absorbance Melting Curves of RNA. *Methods of Enzymology*, **1989**, *180*, 304–324.
(57) Petersheim, M.; Turner, D. H. Base-stacking and Base-pairing Contributions to Helix Stability: Thermodynamics of Double-Helix Formation with CCGG, CCGGp, CCGGAp, ACCGGp, CCGGUp, and ACCGGUp. *Biochemistry* **1983**, *22*, 256–263.
(58) Dignam, J. D.; Lebovitz, R. M.; Roeder, R. G. Accurate Transcription Initiation by RNA Polymerase II in a Soluble Extract from Isolated Mammalian Nuclei. *Nucleic Acids Res.* **1983**, *11*, 1475–1498.