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Effects of 2′-*O*-(trifluoromethyl)adenosine on oligodeoxynucleotide hybridization and nuclease stability

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ABSTRACT

The synthesis and properties of oligodeoxynucleotides (ODNs) containing 2′-*O*-(trifluoromethyl)adenosine (2) are described. 2′-*O*-(Trifluoromethyl)adenosine (2) or *N*^{6}-(benzoyl)-2′-*O*-(trifluoromethyl)adenosine (6) was obtained in 22 or 32% yield by treating 2′-*O*-[(methylthio)thiocarbonyl]-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)(TIPDS)adenosine (4) or *N*^{6},*N*^{6}-(dibenzoyl)-2′-*O*-[(methylthio)thiocarbonyl]-3′,5′-*O*-(TIPDS)-adenosine (5), respectively, with pyridinium poly(hydrogen fluoride) in the presence of 1,3-dibromo-5,5-dimethylhydantoin. Nucleoside 2 was incorporated into DNA hexadecamers. ODNs that contained 2 reduced the thermal stability of duplexes with their complementary DNAs but increased the thermal stability of duplexes with their complementary RNAs. Furthermore, ODNs containing 2 were slightly more resistant to snake venom phosphodiesterase than an unmodified ODN.

INTRODUCTION

Many types of oligodeoxynucleotides (ODNs) that have been modified at their base, sugar or phosphodiester moieties have been synthesized (1,2) and used for biological and biophysical studies, such as antisense studies (1–4). In particular, since we can easily introduce various substituents into the 2′ position of a nucleoside, ODN analogs containing various 2′-modified nucleosides have been synthesized and their biochemical and biophysical properties have been studied (5–22). Based on these studies, it is apparent that the affinity of ODNs for their complementary DNA or RNA strands and the stability of ODNs against nucleolytic hydrolysis by nucleases, which are important factors for antisense molecules, are highly dependent on the properties of the 2′-substituents. For instance, it has been reported that 2′-*O*-alkyl modifications such as 2′-*O*-methyl and 2′-*O*-allyl increase the thermal stability of DNA/RNA hybrids as well as the stability of ODNs against nucleolytic degradation by nucleases (5–7,11–17). However, 2′-*C*-alkyl substituents such as 2′-α-methyl, 2′-α-ethyl, 2′-α-allyl and 2′-β-methyl groups increase the stability of ODNs against nucleolytic degradation by 3′-exonuclease, but reduce the thermal stability of DNA/RNA hybrids (10,18). On the other hand, Kawasaki *et al*. reported that the 2′-α-fluoro substituent dramatically increases the thermal stability of DNA/RNA duplexes although it does not increase stability against nucleolytic hydrolysis by nucleases (9). The stability of the duplex with a 2′-α-fluoro substitution could be due to the high electronegativity of the fluorine atom, which shifts the conformational equilibrium of the sugar to 3′-*endo*. Shifting the conformation of the DNA strand to 3′-*endo* will put it in a more RNA-like conformation and will cause the hybridization properties to be more like those of an RNA/RNA geometry.

Based on these findings, we envisioned that ODNs containing 2′-*O*-(trifluoromethyl)adenosine (**2**) instead of 2′-*O*-(methyl)-adenosine (**1**) may increase the thermal stability of duplexes with their complementary DNAs and RNAs without reducing nuclease stability (Fig. 1).

In this paper, we demonstrate the synthesis of ODNs containing 2′-*O*-(trifluoromethyl)adenosine (**2**). The thermal stability of the duplexes between these ODNs and their complementary DNA and RNA strands and the stability of ODNs containing **2** against nucleolytic digestion were also studied.

RESULTS AND DISCUSSION

Synthesis

To synthesize ODNs containing **2**, a 2′-*O*-(trifluoromethyl)-adenosine phosphoramidite unit **8** is needed. However, a method for preparing **2** has not been reported. Recently, Hiyama *et al*. reported that methyl xanthates R-OC(S)SMe are easily converted into trifluoromethyl ethers R-OCF$_3$ by treatment with pyridinium poly(hydrogen fluoride) (HF/pyridine) in the presence of 1,3-dibromo-5,5-dimethylhydantoin (DBH) (23). Therefore, we planned to synthesize **2** using Hiyama's method.

2′-*O*-[(Methylthio)thiocarbonyl]adenosine derivative **4**, which was a precursor of **2**, was obtained in 73% yield by treating 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine [3′,5′-*O*-(TIPDS)adenosine] (**3**) with *n*-BuLi, CS_2 and MeI in THF at –78°C (Scheme 1). The methyl xanthate **4** was then treated with HF/pyridine in the presence of DBH. The desired 2′-*O*-trifluoromethyl derivative **2** was obtained in 22% yield along with 2′,3′-*O*-difluoromethylidene derivative **9**, which would be derived via a 2′,3′-cyclic thiocarbonate intermediate, in 28% yield. On the other hand, when *N*^{6},*N*^{6}-dibenzoyl derivative **5**, which was prepared by the reaction of **4** with BzCl in pyridine,

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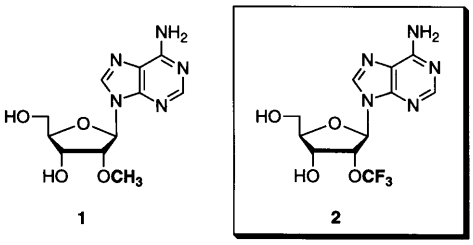
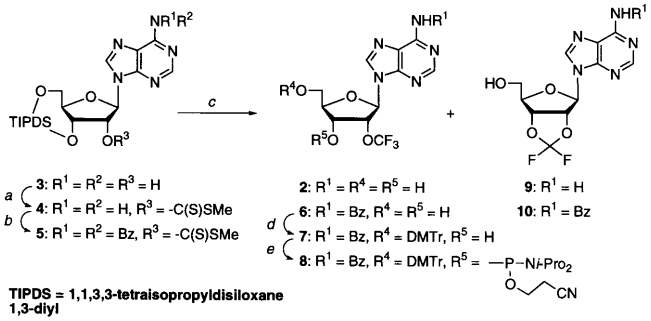


Figure 1. Structures of modified nucleoside analogs.

was treated with HF/pyridine in the presence of DBH, 2′-*O*-trifluoromethyl derivative **6** was obtained in 32% yield along with 2′,3′-*O*-difluoromethylidene derivative **10** in 36% yield. N^6-Benzoyl derivative **6** was used for the next reaction. To introduce **2** into ODNs, the 5′-OH group of **6** was protected with a DMTr group and the resulting **7** was phosphitylated by a standard procedure (24) to give the nucleoside 3′-phosphoramidite **8** in 81% yield.



Scheme 1. (a) (1) *n*-BuLi, CS_2, THF, –78°C, (2) MeI, –78°C, then room temperature, 73%; (b) BzCl, pyridine, 0°C, then room temperature, 93%; (c) HF/pyridine, DBH, CH_2Cl_2, –78°C, then 0°C, 22 (**2**) and 28% (**9**) and 32 (**6**) and 36% (**10**); (d) DMTrCl, pyridine, room temperature, 83%; (e) chloro-(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, *N*,*N*-diisopropylethylamine, CH_2Cl_2, 81%.

Before **2** was introduced into ODNs, the conformation of the sugar moiety of **2** was studied by ^{1}H NMR. The fractional population of the N-conformer of **2** was calculated by the formula, $\%N = J_{3',4'}/(J_{1',2'} + J_{3',4'})$ (25). The $J_{1',2'}$ and $J_{3',4'}$ values of **2** were 6.1 and 2.7 Hz, respectively. Thus, the %N value was estimated to be 31%. On the other hand, the %N values of 2′-deoxyadenosine (dAdo), adenosine (Ado) and 2′-*O*-(methyl)adenosine (**1**) have been reported to be 19, 36 and 42%, respectively, based on an NMR study (26). Uesugi *et al.* reported that there is a relatively good correlation between %N and the group electronegativity of the 2′-α-substituent (26). These results imply that the group electronegativity of OCF_3 is not very large. Quite recently, we determined the group electronegativity of the 3′-OCF_3 substituent in 3′-*O*-(trifluoromethyl)thymidine (27) on the scales of Marriott (28), Inamoto (29–32) and Mullay (33) from calibration graphs which correlated the group electronegativity of various 3′-substituents in 2′,3′-dideoxythymidine derivatives. The group electronegativities of OCF_3 were calculated to be 0.38 (Marriott's scale), 2.64 (Inamoto's scale) and 3.66 (Mullay's scale), respectively, and were smaller than those of OH [0.42 (Marriott's scale), 2.79 (Inamoto's scale) and 3.97 (Mullay's scale)] and OCH_3 [0.43 (Marriott's scale), 2.82 (Inamoto's scale) and 4.03 (Mullay's scale)] (27). These results were consistent with our results regarding the relative %N values of **1**, **2** and Ado.

ODNs containing **2** were synthesized on a DNA synthesizer by the phosphoramidite method (34). In this study, **2** was incorporated into a heptadecamer. The sequences of the ODN analogs synthesized are shown in Table 1. The average coupling yield of **8** was 96% using a 0.12 M solution of the amidite in CH_3CN with a coupling time of 600 s. The fully protected ODNs (1 µmol) linked to the solid supports were treated with concentrated NH_4OH at 55°C for 16 h and then subjected to C-18 column chromatography; detritylation gave ODNs (**17–21**) in 20–77 OD_{260} units. Each of the resulting ODN analogs showed a single peak on reversed phase HPLC. Furthermore, ODNs **17** and **19** were analyzed by electrospray ionization (ESI) mass spectrometry and the observed molecular weights supported their structures (Materials and Methods).

Thermal stability

Stable duplex formation with mRNA is one of the most important factors in antisense research. Thus, duplex formation by the heptadecamers **11–21** with the complementary RNA, 5′-r[UUG UAU UUG UUU UUC U]-3′ (**22**), was studied by thermal denaturation in a buffer of 0.01 M sodium cacodylate (pH 7.0) containing 0.01 M NaCl. One transition was observed in the melting profile of each duplex. Melting temperatures (T_m values) are listed in Table 1. The stability of duplexes containing **1** or **2** was dependent on the position and number of **1** or **2**. Compared with the control ODN **11**, ODNs **12** and **17**, which contained two molecules of **1** or **2** at the 5′-end and near the 3′-end of the strands, slightly reduced the thermal stability of ODN/RNA duplexes. On the other hand, ODNs **13–16** and **18–21**, which contained three or four molecules of **1** or **2**, greatly increased the thermal stability of ODN/RNA duplexes. The duplexes became more stable as the number of **1** or **2** increased. The ΔT_m^1 [T_m(each ODN) – T_m(control ODN **11**)] values for ODNs containing three or four molecules of **1** or **2** were 3.8–7.7°C. Among the ODNs that contained **1**, ODN **14**, which contained four molecules of **1** at intervals of three or four natural nucleosides, increased the thermal stability of ODN/RNA duplexes the most (ΔT_m^1 = 6.2°C). On the other hand, among the ODNs that contained **2**, ODN **20**, which contained four molecules of **2** near the 5′-end of the strand, increased the thermal stability of ODN/RNA duplexes the most (ΔT_m^1 = 7.7°C). The ΔT_m^2 [T_m(each ODN containing **2**) – T_m(each ODN containing the same number of **1**)] values were calculated to compare the abilities of **1** and **2** to stabilize duplex formation (Table 1). Except for ODN **19**, the ΔT_m^2 values increased as the number of **2** increased. Therefore, nucleoside **2** was found to more efficiently stabilize duplex formation with the RNA than **1**. Nucleoside **2** most efficiently stabilized duplex formation when four molecules of **2** were incorporated near the 5′-end of the strand (ODN **20**, ΔT_m^2 = 1.7°C).

The thermal stabilities of DNA/RNA duplexes containing nucleoside analogs with electronegative substituents at the 2′-α positions are often discussed on the basis of the *gauche* effect between the electronegative substituents and $O^{4'}$ of the furanose ring (35–38). The electronegative substituent at the 2′-α position shifts the conformational equilibrium of the sugar to 3′-*endo*. Shifting the conformation of the DNA strand to 3′-*endo* will put

Table 1. Synthesized ODNs and hybridization data[a]

	ODNs[b]	ODN–RNA[c]			ODN–DNA[d]		
		T_m (°C)	ΔT_m^1 (°C)[e]	ΔT_m^2 (°C)[f]	T_m (°C)	ΔT_m^1 (°C)[e]	ΔT_m^2 (°C)[f]
11	5'-AGA AAA ACA AAT ACA A-3'	59.8	–	–	42.6	–	–
12	5'-**1**GA AAA ACA AAT AC**1** A-3'	57.2	-2.6	–	41.9	-0.7	–
13	5'-**1**GA AAA AC**1** AAT AC**1** A-3'	63.6	3.8	–	39.3	-3.3	–
14	5'-**1**GA AA**1** ACA A**1**T AC**1** A-3'	66.0	6.2	–	37.2	-5.4	–
15	5'-**1**GA **111** ACA AAT ACA A-3'	65.8	6.0	–	40.1	-2.5	–
16	5'-AGA AAA **1**C**1** **11**T ACA A-3'	64.4	4.6	–	38.0	-4.6	–
17	5'-**2**GA AAA ACA AAT AC**2** A-3'	57.4	-2.4	0.2	41.8	-0.8	-0.1
18	5'-**2**GA AAA AC**2** AAT AC**2** A-3'	64.2	4.4	0.6	39.1	-3.5	-0.2
19	5'-**2**GA AA**2** ACA A**2**T AC**2** A-3'	66.3	6.5	0.3	33.0	-9.6	-4.2
20	5'-**2**GA **222** ACA AAT ACA A-3'	67.5	7.7	1.7	33.9	-8.7	-6.2
21	5'-AGA AAA **2**C**2** **22**T ACA A-3'	65.4	5.6	1.0	30.0	-12.6	-8.0

[a]Experimental conditions are described in Materials and Methods.
[b]**1** and **2** (Fig. 1).
[c]The complementary RNA: 5′-r[UUG UAU UUG UUU UUC U]-3′ (**22**).
[d]The complementary DNA: 5′-d[TTG TAT TTG TTT TTC T]-3′(**23**).
[e]$\Delta T_m^1 = T_m$(each ODN) – T_m(control ODN **11**).
[f]$\Delta T_m^2 = T_m$(each ODN containing **2**) – T_m(each ODN containing the same number of **1**).

it in a more RNA-like conformation and will cause the hybridization properties to be more like those of an RNA/RNA geometry. The ODNs that contained **2** stabilized duplex formation with the complementary RNA more than those that contained **1**, although the group electronegativity of OCF_3 is smaller than that of OCH_3. Accordingly, factors other than the *gauche* effect also seemed to contribute to the thermal stability of duplexes containing **2**.

Duplex formation by heptadecamers **11–21** with the complementary DNA, 5′-d[TTG TAT TTG TTT TTC T]-3′ (**23**), was next studied by thermal denaturation in a buffer of 5 mM sodium phosphate (pH 7.0) containing 140 mM KCl and 1 mM $MgCl_2$. One transition was observed in the melting profile of each duplex. T_m values are listed in Table 1. All of the T_m values for duplexes containing **1** or **2** were smaller than that of the control duplex (42.6°C). Therefore, ODNs containing **1** and **2** decreased the thermal stability of duplexes with the DNA. The stability of duplexes was dependent on the number of modified nucleosides. The duplexes became less stable as the numbers of **1** and **2** increased. The ΔT_m^1 values for ODNs **14** and **21** were –5.4 and –12.6°C, respectively. ΔT_m^2 values also became smaller as the number of **2** increased. Therefore, nucleoside **2** was found to destabilize ODN/DNA duplex more than nucleoside **1**.

When an ODN analog containing **1** or **2** forms a duplex with a complementary DNA, methoxy and trifluoromethoxy groups of **1** and **2** should be accommodated in the minor groove, since the 2′ position of a nucleoside is in the minor groove. The minor groove of a DNA/DNA duplex is known to be narrow and deep (39). Therefore, methoxy and trifluoromethoxy groups of **1** and **2**, which are partially incorporated into the ODNs instead of dAdo, may not be well accommodated in the minor groove of the ODN/DNA duplex. Furthermore, since a trifluoromethoxy group is larger than a methoxy group, ODNs containing **2** might reduce the thermal stability of the ODN/DNA duplex more than ODNs containing **1**.

Circular dichroism

To study the global conformation of duplexes, CD spectra of duplexes composed of ODNs containing **1** or **2** and either the complementary DNA (**23**) or RNA (**22**) were measured at 15°C. With DNA (**23**) as a complementary strand, the spectra of the duplexes (**11–23**, **15–23**, and **20–23**) showed positive CD bands at ~282 nm and negative CD bands at ~248 nm, which were attributable to B-like DNA conformation (Fig. 2a). Although the shapes of the spectra were similar to each other, the intensity of the positive and negative CD bands in the spectrum of the duplex containing **2** was slightly less than that of the unmodified duplex (**11–23**) and the intensity of the positive CD band at ~282 nm in the spectrum of the duplex containing **1** was slightly greater than that of the unmodified duplex.

When RNA (**22**) was used as a complementary strand, the spectra of duplexes (**11–22**, **15–22** and **20–22**) showed positive CD bands at ~220 and 268 nm and negative CD bands at ~207 and 246 nm (Fig. 2b). Although the intensity of CD bands in the spectra of the duplex containing **2** was slightly less than that in the duplex containing **1** and the unmodified duplex, the shapes of the spectra were similar to each other. Based on these results, nucleoside **2** did not dramatically change the global conformation of ODN/DNA and ODN/RNA duplexes.

Nuclease resistance

Several studies have demonstrated that 3′-exonuclease activities were the major cause of the degradation of unmodified ODNs in serum. We examined the stability of ODNs containing **2** against

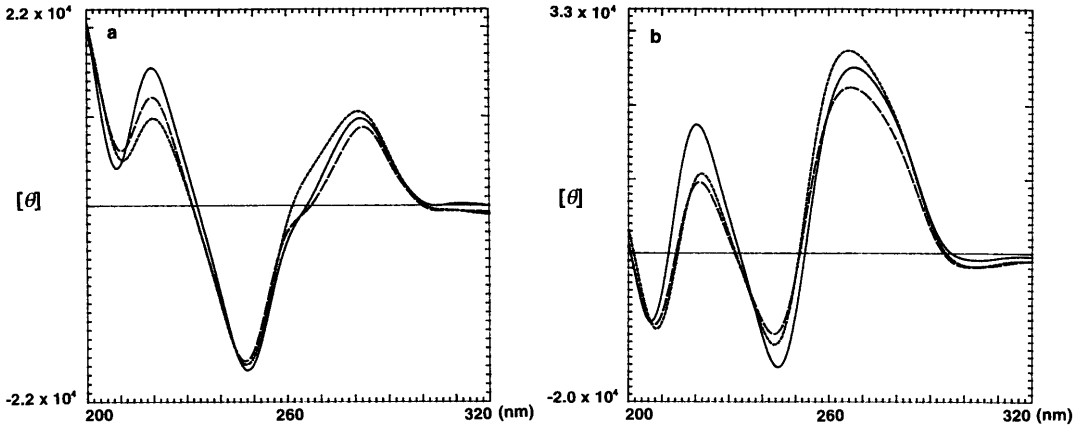


Figure 2. CD spectra of ODN/DNA and ODN/RNA duplexes at 15°C in 5 mM sodium phosphate (pH 7.0) containing 140 mM KCl and 1 mM $MgCl_2$ for ODN/DNA duplexes and 10 mM sodium phosphate (pH 7.0) containing 10 mM NaCl for ODN/RNA duplexes. (**a**) ODN/DNA duplexes: (—) **11–23**; (- - -) **15–23**; (– –) **20–23**. (**b**) ODN/RNA duplexes: (—) **11–22**; (- - -) **15–22**; (– –) **20–22**.

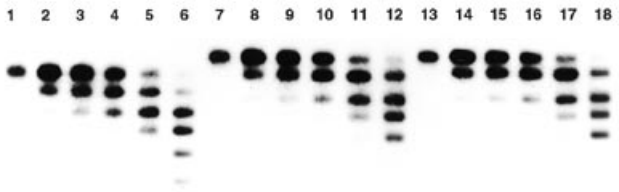


Figure 3. Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled ODNs hydrolyzed by snake venom phosphodiesterase: **11** (lanes 1–6), **14** (lanes 7–12) and **19** (lanes 13–18). ODNs were incubated with snake venom phosphodiesterase for 0 (lanes 1, 7 and 13), 10 (lanes 2, 8 and 14), 20 (lanes 3, 9 and 15), 30 (lanes 4, 10 and 16), 60 (lanes 5, 11 and 17) and 120 min (lanes 6, 12 and 18). Experimental conditions are described in Materials and Methods.

snake venom phosphodiesterase, a 3′-exonuclease. ODNs **11**, **14**, and **19** were labeled at the 5′-end with ^{32}P and incubated with the nuclease and the reactions were analyzed by polyacrylamide gel electrophoresis under denaturing conditions (40) (Fig. 3). In ODNs **14** and **19**, the second nucleosides from the 3′-ends of the strands are **1** and **2**, respectively. The phosphodiester linkages at the 5′-sides of **1** (5,6) and **2** were slightly more resistant to hydrolysis by the enzyme than that of dAdo (Fig. 3, lanes 5, 6, 11, 12, 17 and 18).

Conclusions

In this paper, we described the synthesis and properties of ODNs containing 2′-*O*-(trifluoromethyl)adenosine (**2**). 2′-*O*-(Trifluoromethyl)adenosine (**2**) or N^6-(benzoyl)-2′-*O*-(trifluoromethyl)adenosine (**6**) was obtained in 22 or 32% yield by treating 2′-*O*-[(methylthio)thiocarbonyl]-3′,5′-*O*,-(TIPDS)adenosine (**4**) or N^6,N^6-(dibenzoyl)-2′-*O*-[(methylthio)thiocarbonyl]-3′,5′-*O*-(TIPDS)adenosine (**5**), respectively, with HF/pyridine in the presence of DBH. The nucleoside **2** was incorporated into DNA hexadecamers. ODNs containing **2** decreased the thermal stability of duplexes with their complementary DNAs but increased the thermal stability of duplexes with their complementary RNAs. Furthermore, ODNs containing **2** were slightly more resistant to snake venom phosphodiesterase than an unmodified ODN. These properties seem to make ODN containing **2** a candidate as a novel antisense molecule.

MATERIALS AND METHODS

NMR spectra were recorded at 270 or 500 (^{1}H), at 125 (^{13}C) and at 202 MHz (^{31}P) and are reported in p.p.m. downfield from TMS or 85% H_3PO_4. *J* values are given in Hertz. Mass spectra were obtained by the fast atom bombardment (FAB) method. Thin layer chromatography was done on Merck coated plates $60F_{254}$. The silica gel or the neutralized silica gel used for column chromatography were Merck silica gel 5715 or ICN silica 60A, respectively.

2′-*O*-[(Methylthio)thiocarbonyl]-3′,5′-*O*-(TIPDS)adenosine (4)

n-BuLi (1.68 M in hexane, 0.11 ml, 1.8 mmol) was added to a solution of 3,5-*O*-(TIPDS)adenosine (200 mg, 0.4 mmol) in THF (5 ml) at –78°C and the resulting mixture was stirred at –78°C. After 10 min, CS_2 (0.23 ml, 3.9 mmol) was added to the solution and the resulting mixture was sirred at –78°C. After 1 h, MeI (73 µl, 1.2 mmol) was added to the solution and the resulting mixture was stirred at room temperature for 15 h. The mixture was diluted with AcOEt and washed with H_2O and then brine. The separated organic layer was dried (Na_2SO_4) and evaporated. The residue was purified by column chromatography (SiO_2, hexane:AcOEt = 1:1) to give **4** (171 mg, 73% as a white solid). ^{1}H NMR ($CDCl_3$) δ 8.28 (s, 1H), 7.94 (s, 1H), 6.53 (dd, 1H, *J* = 2.3 and 5.4 Hz), 6.08 (d, 1H, *J* = 2.3 Hz), 5.87 (br s, 2H, NH_2), 5.37 (dd, 1H, *J* = 5.4 and 8.6 Hz), 4.20–4.03 (m, 3H), 2.61 (s, 3H), 1.13–0.94 (m, 28H); ^{13}C NMR ($CDCl_3$) δ 215.1, 155.5, 153.2, 149.3, 139.7, 120.3, 87.5, 87.4, 83.3, 82.2, 69.9, 60.9, 19.1, 17.4, 17.3, 17.1, 16.9, 13.2, 13.0, 12.8; EI-LRMS *m/z* 599 (M^+).

2′-*O*-(Trifluoromethyl)adenosine (2) and 2′,3′-*O*-(difluoromethylidene)adenosine (9)

A solution of **4** (780 mg, 1.3 mmol) and HF/pyridine complex (12 ml, Aldrich) in CH_2Cl_2 (10 ml) was added to a suspension of DBH (1.5 g, 5.2 mmol) in CH_2Cl_2 (10 ml) at –78°C and the

resulting mixture was stirred at 0°C for 3 h. The reaction mixture was poured into an aqueous saturated solution of $NaHCO_3$ (50 ml) and the mixture was concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, 12% EtOH in $CHCl_3$) to give **2** (96 mg, 22% as a white solid) and **9** (115 mg, 28% as a white solid). The physical data of **2**: ^{1}H NMR (DMSO-d_6) δ 8.41 (s, 1H), 8.15 (s, 1H), 7.47 (br s, 2H, NH_2), 6.23 (d, 1H, *J* = 6.1 Hz), 5.99 (d, 1H, OH, *J* = 5.3 Hz), 5.53–5.48 (m, 2H), 4.42 (m, 1H), 4.06 (m, 1H), 3.71 (ddd, 1H, *J* = 3.6, 4.6 and 12.3 Hz), 3.59 (ddd, 1H, *J* = 3.6, 7.0 and 12.3 Hz); FAB-LRMS *m/z* 336 (MH^+); FAB-HRMS calculated for $C_{11}H_{13}F_3N_5O_4$ 336.0919, found 336.0904. The physical data of **9**: ^{1}H NMR (DMSO-d_6) δ 8.33 (s, 1H), 8.17 (s, 1H), 7.39 (br s, 2H, NH_2), 6.40 (d, 1H, *J* = 2.4 Hz), 6.06 (m, 1H), 5.60 (m, 1H), 5.27 (t, 1H, OH, *J* = 5.4 Hz), 4.38 (m, 1H), 3.56–3.52 (m, 2H); FAB-LRMS *m/z* 316 (MH^+); FAB-HRMS calculated for $C_{11}H_{12}F_2N_5O_4$ 316.0857, found 316.0837.

N^6,*N*^{6}-(Dibenzoyl)-2′-*O*-[(methylthio)thiocarbonyl]-3′,5′-*O*-(TIPDS)adenosine (5)

BzCl (72 µl, 0.6 mmol) was added to a solution of **4** (100 mg, 0.2 mmol) in pyridine (5 ml) at 0°C and it was stirred at room temperature for 12 h. Ice–water and AcOEt were added, the resulting mixture was partitioned and the organic layer was washed with H_2O and brine, dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by column chromatography (SiO_2, hexane:AcOEt = 2:1) to give **5** (150 mg, 93% as a white foam). ^{1}H NMR ($CDCl_3$) δ 8.75 (s, 1H), 8.21 (s, 1H), 7.88–7.35 (m, 10H), 6.55 (m, 1H), 6.13 (s, 1H), 5.36 (dd, 1H, *J* = 5.5 and 8.5 Hz), 4.19–4.04 (m, 3H), 2.62 (s, 3H), 1.14–0.92 (m, 28H); FAB-LRMS *m/z* 808 (MH^+); FAB-HRMS calculated for $C_{38}H_{50}N_5O_7S_2Si_2$ 808.2690, found 808.2704.

N^6-(Benzoyl)-2′-*O*-(trifluoromethyl)adenosine (6) and *N*^{6}-(benzoyl)-2′,3′-*O*-(difluoromethylidene)adenosine (10)

A solution of **5** (250 mg, 0.4 mmol) and HF/pyridine complex (5 ml; Aldrich) in CH_2Cl_2 (10 ml) was added to a suspension of DBH (430 mg, 1.5 mmol) in CH_2Cl_2 (5 ml) at –78°C and the resulting mixture was stirred at 0°C for 3 h. The reaction mixture was poured into an aqueous saturated solution of $NaHCO_3$ (100 ml) and the mixture was concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, 5% MeOH in $CHCl_3$) to give **6** (56 mg, 32% as a white foam) and **10** (60 mg, 36% as a white foam). The physical data of **6**: ^{1}H NMR ($CDCl_3$) δ 9.09 (br s, 1H), 8.82 (s, 1H), 8.07 (s, 1H), 8.05–7.53 (m, 5H), 6.13 (d, 1H, *J* = 7.6 Hz), 5.99 (br d, 1H), 5.63 (dd, 1H, *J* = 4.4 and 7.6 Hz), 4.71 (d, 1H, *J* = 4.4 Hz), 4.41 (s, 1H), 4.00 (br d, 1H), 3.79 (br m, 1H), 2.72 (br s, 1H); FAB-LRMS *m/z* 440 (MH^+); FAB-HRMS calculated for $C_{18}H_{17}O_5N_5F_3$ 440.1182, found 440.1189. The physical data of **10**: ^{1}H NMR (DMSO-d_6) δ 11.30 (s, 1H), 8.83 (s, 1H), 8.69 (s, 1H), 8.16–7.58 (m, 5H), 6.59 (d, 1H, *J* = 2.3 Hz), 6.21 (m, 1H), 5.69 (m, 1H), 5.30 (m, 1H), 4.51 (m, 1H), 3.64–3.61 (m, 2H); FAB-LRMS *m/z* 420 (MH^+); FAB-HRMS calculated for $C_{18}H_{16}F_2N_5O_5$ 420.1119, found 420.1120.

N^6-(Benzoyl)-5′-*O*-(dimethoxytrityl)-2′-*O*-(trifluoromethyl)-adenosine (7)

A mixture of **6** (145 mg, 0.4 mmol) and DMTrCl (305 mg, 0.9 mmol) in pyridine (5 ml) was stirred at room temperature. After 12 h, ice–water and AcOEt were added, the resulting mixture was partitioned and the organic layer was washed with H_2O and brine, dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by column chromatography (SiO_2, hexane:AcOEt = 1:1) to give **7** (246 mg, 83% a pale yellow foam). ^{1}H NMR ($CDCl_3$) δ 8.99 (br s, 1H, NH), 8.68 (s, 1H), 8.11 (s, 1H), 8.04–6.78 (m, 18H), 6.31 (d, 1H, *J* = 5.9 Hz), 5.81 (m, 1H), 4.74 (m, 1H), 4.30 (m, 1H), 3.78 (s, 6H), 3.57 (dd, 1H, *J* = 3.6 and 10.8 Hz), 3.42 (dd, 1H, *J* = 3.8 and 10.8 Hz), 2.61 (d, 1H, OH, *J* = 4.3 Hz).

N^6-(Benzoyl)-5′-*O*-(dimethoxytrityl)-2′-*O*-(trifluoromethyl)-adenosine 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropyl-phosphoramidite) (8)

After successive co-evaporation with pyridine, **7** (463 mg, 0.63 mmol) was dissolved in CH_2Cl_2 (5 ml) containing *N*,*N*-diisopropylethylamine (313 µl, 1.85 mmol). Chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine (280 µl, 1.24 mmol) was added to the solution and the reaction mixture was stirred at room temperature. After 30 min, aqueous saturated $NaHCO_3$ and AcOEt were added to the mixture, the separated organic layer was washed with aqueous saturated $NaHCO_3$ and brine, dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography (neutralized SiO_2, hexane:AcOEt = 1:1) to give **8** (480 mg, 81% as a white foam). ^{31}P NMR ($CDCl_3$) δ 152.5, 152.2; FAB-LRMS *m/z* 942 (MH^+); FAB-HRMS calculated for $C_{48}H_{52}F_3N_7O_8P$ 942.3567, found 942.3569.

Synthesis of ODNs

ODNs were synthesized on a DNA synthesizer (Applied Biosystem Model 392) by the phosphoramidite method (34). The fully protected ODNs were then deblocked and purified by the same procedure as for the purification of normal ODNs (24), i.e. each ODN linked to the resin was treated with concentrated NH_4OH at 55°C for 16 h and the released ODN protected by a DMTr group at the 5′-end was chromatographed on a C-18 silica gel column (1 × 10 cm; Waters) with a linear gradient of MeCN from 0 to 30% in 0.1 M TEAA buffer (pH 7.0). The fractions were concentrated and the residue was treated with aqueous 80% AcOH at room temperature for 20 min, then the solution was concentrated and the residue was co-evaporated with H_2O. The residue was dissolved in H_2O and the solution was washed with Et_2O, then the H_2O layer was concentrated to give the deprotected ODNs **17** (49), **18** (20), **19** (77), **20** (74) and **21** (31) (the yields are indicated in parentheses as OD units at 260 nm starting from the 1 µmol scale).

Electrospray ionization mass spectrometry

Spectra were obtained on a Quattro II (Micromass, Manchester, UK) triple quadrupole mass spectrometer equipped with an ESI source in the negative ion mode. The HPLC-purified ODN samples were dissolved in aqueous 50% 2-propanol containing 1% triethylamine (10 pmol ODN/µl) and introduced into the ion source through a loop injector with a carrier solvent, 33% aqueous MeOH, flowing at 10 ml/min flow rate. About 15 scans were

acquired in an ~1 min period and combined to obtain smoothed spectra. All molecular masses of the ODNs were calculated from the multiple charge negative ion spectra. The observed average molecular masses of **17** and **19** were 5076.00 and 5244.25, respectively, and fit the calculated molecular weights (theoretical average molecular masses) for these compounds, i.e. 5076.31 (for **17**, $C_{160}H_{192}N_{73}O_{85}F_6P_{15}$) and 5244.31 (for **19**, $C_{162}H_{190}N_{73}O_{87}F_{12}P_{15}$) within a commonly accepted error range for ESI MS, 0.01%.

Thermal denaturation and CD spectroscopy

Each solution contains each ODN (3 µM) and the complementary RNA **22** (3 µM) or DNA **23** (3 µM) in an appropriate buffer. The solution containing each ODN was heated at 100°C for 3 min, then cooled gradually to an appropriate temperature and used for the thermal denaturation studies. Thermal-induced transitions of each mixture were monitored at 260 nm on a Perkin Elmer Lambda2S. Sample temperature was increased by 0.5°C/min. Samples for CD spectroscopy were prepared by the same procedure used in the thermal denaturation study and spectra were measured on a JASCO J720 Spectropolarimeter at 15°C. The ellipticities of duplexes were recorded from 200 to 320 nm in a cuvette with a path length of 1 mm. CD data were converted into mdeg/mol strands/cm.

Partial hydrolysis of ODNs with snake venom phosphodiesterase

Each ODN labeled with ^{32}P at the 5′-end (10 pmol) was incubated with snake venom phosphodiesterase (0.4 µg) in the presence of *Torula* RNA (0.15 OD_{260} units) in a buffer containing 37.5 mM Tris–HCl (pH 8.0) and 7.5 mM $MgCl_2$ (total 20 µl) at 37°C. At appropriate periods, aliquots of the reaction mixture were separated and added to a solution of EDTA (5 mM, 10 µl), then the mixtures were heated at 100°C for 5 min. The solutions were analyzed by electrophoresis on 20% polyacrylamide gel containing 7 M urea (40).

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