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Synthesis and properties of siRNAs containing 5′-amino-2′,5′-dideoxy-2′α-fluororibonucleosides

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ABSTRACT

Short interfering RNAs (siRNAs) containing P3′→N5′ phosphoramidate linkages were successfully synthesized by introducing 2′-deoxy-2′-fluororibonucleoside and 5′-amino-2′,5′-dideoxy-2′α-fluororibonucleoside in succession. It was found that the introduction of 5′-amino-2′,5′-dideoxy-2′α-fluororibonucleosides into siRNAs improved the nuclease-resistant properties of the siRNAs without loss of their silencing efficacy.

1. Introduction

RNA interference (RNAi) induced by small interfering RNA (siRNA) has emerged as a powerful technique to silence gene expression post-transcriptionally.[1,2] Several research groups have demonstrated the efficacy of siRNA-mediated inhibition of clinically relevant genes not only in vitro but also in vivo.[2–4] An improvement in the nuclease stability of siRNA is an important issue in the therapeutic application of synthetic siRNA. Thus far, several types of siRNAs modified at the base, sugar, or phosphate moieties have been synthesized, and their nuclease-resistant properties and RNAi-inducing activities have been studied.[5–26] Among the abovementioned methods, the introduction of 2′-deoxy-2′α-fluororibonucleosides instead of ribonucleosides into siRNAs is an attractive modification because it enhances both the chemical and biological stabilities of siRNAs without largely reducing their RNAi-inducing activities.[11,17,22]

On the other hand, it has been reported that DNAs possessing phosphoramidate linkages are more nuclease-resistant than the unmodified DNAs. Till date, two types of DNAs containing phosphoramidate linkages have been reported (Fig. 1):[27–36] DNA possessing a P3′→N5′ phosphoramidate[27–29] and DNA containing an N3′→P5′ phosphoramidate.[30–37] The DNA possessing the N3′→P5′ phosphoramidate increases the thermal stability of duplexes with DNA and RNA complements. On the other hand, the DNA containing the P3′→N5′ phosphoramidate largely decreases the thermal stability of duplexes with DNA and RNA complements. Thus, the DNA containing the N3′→P5′ phosphoramidate has been extensively used for antisense studies.[38,39]

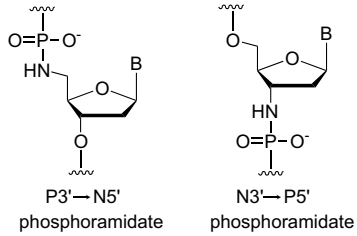

Figure 1. Structures of phosphoramidates.

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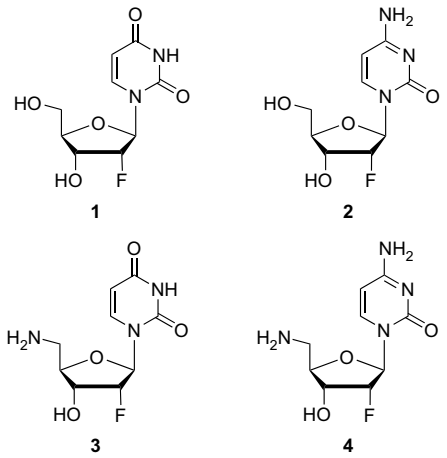
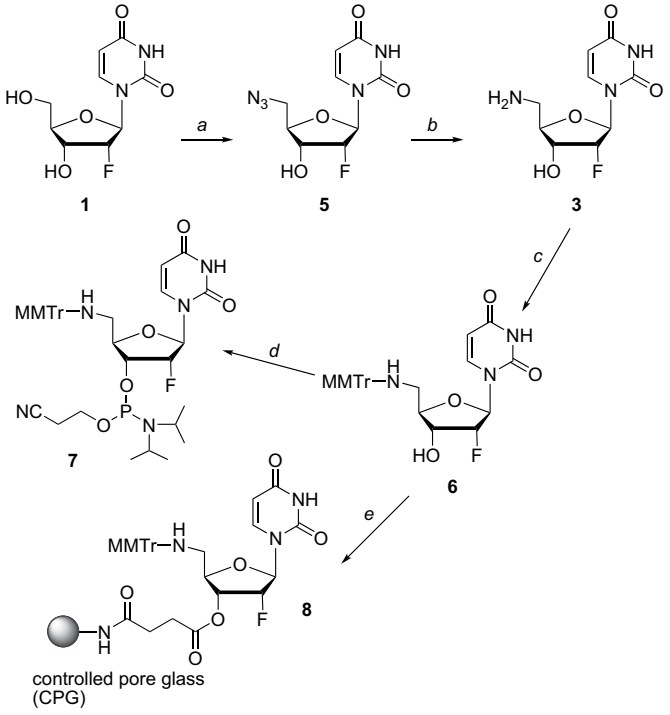


Figure 2. Structures of modified nucleosides.

However, from the synthetic point of view, the introduction of the P3′→N5′ phosphoramidate linkage in the DNA moiety is an attractive modification because the DNA containing the same can be easily synthesized by using 3′-*O*-phosphoramidites of 5′-amino-2′,5′-dideoxyribonucleosides and the normal commercially available 3′-*O*-phosphoramidite units.

From these background information, we planned to synthesize siRNAs containing 5′-amino-2′,5′-dideoxy-2′α-fluororibonucleosides (Fig. 2). We envisioned that the siRNAs would be more nuclease-resistant after the introduction of the phosphoramidate linkage, and the introduction of a fluorine atom into the 2′α-position of the analogs would enhance the thermal stability of the siRNAs. In this paper, we report the synthesis and properties of siRNAs containing 5′-amino-2′,5′-dideoxy-2′α-fluorouridine (**3**) and 5′-amino-2′,5′-dideoxy-2′α-fluorocytidine (**4**).



Scheme 1. Reagents and conditions: (a) NaN_3, Ph_3P, CBr_4, DMF, rt, 28 h, 40%; (b) H_2, Pd/C, MeOH, rt, 12 h, 95%; (c) MMTrCl, DMAP, pyridine, rt, 18 h, 61%; (d) chloro-(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, *i*-Pr_2NEt, THF, rt, 1 h, 54%; and (e) (1) succinic anhydride, DMAP, pyridine, rt, 20 h; (2) CPG, WSCI, DMF, rt, 72 h, 20 μmol/g.

2. Results

2.1. Synthesis of phosphoramidite units

In order to synthesize oligoribonucleotides (ONs) containing **3** and **4** using a DNA/RNA synthesizer, phosphoramidites of **3** and **4** were prepared according to the routes shown in Schemes 1 and 2. 2′-Deoxy-2′-α-fluorouridine (**1**)[40] was treated with NaN_3, Ph_3P, and CBr_4 in DMF to afford a 5′-azide derivative **5** in 40% yield. Catalytic hydrogenation of **5** in MeOH and subsequent protection of the amino group with a 4-monomethoxytrityl (MMTr) group afforded an $N^{5'}$-MMTr derivative **6** in 61% yield. Compound **6** was phosphitylated by the standard procedure[41] to afford the corresponding phosphoramidite **7** in 54% yield, which was used as the nucleotide unit for a DNA/RNA synthesizer. To incorporate **3** into the 3′-ends of the ONs, **6** was further modified to afford the corresponding 3′-succinate, which was then reacted with controlled pore glass (CPG) to afford a solid support containing **3** (20 μmol/g).

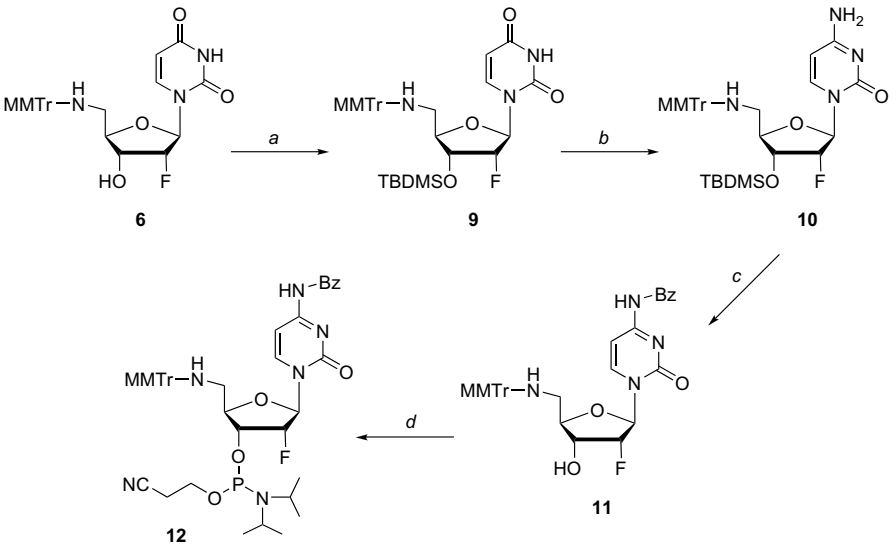
The cytidine derivative **10** was derived from the uridine derivative **6**. After protection of the 3′-hydroxyl function of **6** with a *tert*-butyldimethylsilyl (TBDMS) group, the *O*-TBDMS derivative **9** was treated with 2,4,6-triisopropylbenzenesulfonylchloride (TPSCl) in the presence of DMAP and Et_3N, followed by treatment with NH_4OH to afford the cytidine derivative **10** in 96% yield. After protection of the *exo*-amino function of **10** with a benzoyl (Bz) group, the silyl group was removed by treating with TBAF to afford N^5-MMTr derivative **11** in 65% yield. Compound **11** was phosphitylated by the standard procedure[41] to produce the corresponding phosphoramidite **12** in 46% yield.

2.2. Sugar conformation

Before **3** and **4** were introduced into RNAs, the conformation of the sugar moiety of **3** was studied by ^1H NMR. The fractional populations of the *N*-conformers of **3**, thymidine, and 5′-amino-5′-deoxythymidine were calculated by the formulae, $\%S=(J_{1',2'}-1)/6.9\times100$ and $\%N=100-\%S$.[42] The $J_{1',2'}$ values of **3**, thymidine, and 5′-amino-5′-deoxythymidine were 2.6 Hz, 6.9 Hz, and 6.9 Hz, respectively. Thus, the %*N* values of **3**, thymidine, and 5′-amino-5′-deoxythymidine were estimated to be 77%, 14%, and 14%, respectively. From these results, it was found that the introduction of a fluorine atom to the 2′α-position of 5′-amino-5′-deoxypyrimidine shifts the conformational equilibrium of the sugar moiety to a C3′-*endo* conformation.

2.3. Synthesis of ONs

All ONs were synthesized using a DNA/RNA synthesizer. It is reported that the P3′→N5′ phosphoramidate linkage in RNA is very unstable and is easily hydrolyzed.[43] The lability of the linkage has been interpreted by the neighboring participation effect of the 2′-hydroxyl group adjacent to the *N*-protonated phosphoramidate. To avoid this effect, 2′-fluoro-2′-deoxynucleoside and 5′-amino-2′,5′-dideoxy-2′α-fluoronucleoside were introduced into the ONs in succession (Table 1). The fully protected ONs (1.0 μmol each) linked to solid supports were treated with concentrated NH_4OH/EtOH (3:1, v/v) at room temperature for 12 h and then with 1.0 M TBAF/THF at room temperature for 12 h. Released ONs were purified by denaturing 20% polyacrylamide gel electrophoresis (20% PAGE) to afford deprotected ONs **23**–**28**. These ONs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and their observed molecular weights were in agreement with their structures.



Scheme 2. Reagents and conditions: (a) TBDMSCl, imidazole, DMF, rt, 12 h, 92%; (b) (1) 2,4,6,-triisopropylbenzenesulfonyl chloride, Et_3N, DMAP, CH_3CN, rt, 1 h, then concd NH_4OH, 0 °C to rt, 2 h, 96%; (c) (1) BzCl, pyridine, rt, 30 min, (2) TBAF, THF, rt, 19 h, 65%; and (d) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, *i*-Pr_2NEt, THF, rt, 1 h, 46%.

2.4. Thermal stabilities of siRNAs

The thermal stability of the siRNAs was studied by thermal denaturation in a buffer of 0.1 M sodium phosphate (pH 7.0) containing 0.01 M NaCl. The melting temperatures (T_ms) of siRNAs **13**, **14**, and **15** were 76.0 °C, 78.5 °C, and 77.0 °C, respectively. From these results, it was found that although the thermal stability of siRNA **15** containing **1** and **4** is lower than that of siRNA **14** containing **1** and **2**, the introduction of the analogs **1** and **4** slightly enhances the thermal stability of the duplex as compared to that of normal siRNA **13**.

2.5. Circular dichroism (CD)

To study the global conformation of the siRNAs containing the analogs, the CD spectra of the siRNAs were measured in a buffer of 0.1 M sodium phosphate (pH 7.0) containing 0.01 M NaCl. As shown in Figure 3, negative and positive CD bands at ~209 nm and ~263 nm, respectively, which were attributable to A-type duplexes, were observed. Although the intensity of the positive band of siRNA **14** containing analog **4** was slightly smaller than that of normal siRNA **13**, the shapes of their spectra were similar. These results imply that the global conformation of siRNA **14** containing analog **4** is not significantly different from that of the normal siRNA **13**.

Table 1
Sequences of oligonucleotides (ONs) used in this study

No. of siRNA	No. of ON	sequence
siRNA**13**	ON **18**	5′-CUUCUUCGUCGAGACCAUG*tt*-3′
	ON **19**	3′-*tt*GAAGAAGCAGCUCUGGUAC-5′
siRNA**14**	ON **20**	5′-CUUCU**1** **2**GUCGAGACCAUG*tt*-3′
	ON **21**	3′-*tt*GAAGAAGCAG**2** **1**CUGGUAC-5′
siRNA**15**	ON **22**	5′-CUUCU**1** **4**GUCGAGACCAUG*tt*-3′
	ON **23**	3′-*tt*GAAGAAGCAG**4** **1**CUGGUAC-5′
siRNA**16**	ON **22**	5′-CUUCUUCGUCGAGACCAUG*tt*-3′
	ON **19**	3′-*tt*GAAGAAGCAG**4** **1**CUGGUAC-5′
siRNA**17**	ON **18**	5′-CUUCU**1** **4**GUCGAGACCAUG*tt*-3′
	ON **23**	3′-*tt*GAAGAAGCAGCUCUGGUAC-5′
—	ON **24**	**F**-5′-AAAAAAAAAAAAAAAUU-3′
—	ON **25**	**F**-5′-AAAAAAAAAAAAAAA**1** **1**-3′
—	ON **26**	**F**-5′-AAAAAAAAAAAAAAA**1** **3**-3′
—	ON **27**	**F**-5′-AAAAAAAAUUAAAAAAA-3′
—	ON **28**	**F**-5′-AAAAAAAA**1** **1**AAAAAAA-3′
—	ON **29**	**F**-5′-AAAAAAAA**1** **3**AAAAAAA-3′

The uppercased letters indicate ribonucleosides and lowercased italicized letters represent 2′-deoxyribonucleosides. **F** indicates fluorescein.

2.6. Dual-luciferase assay

The ability of the modified siRNAs to suppress gene expression was studied by a dual-luciferase assay using a psiCHECK-2 vector, which contained *Renilla* and firefly luciferase genes. The siRNA sequences were designed to target *Renilla* luciferase. HeLa cells were co-transfected with the vector and indicated the amounts of siRNAs. The signals of *Renilla* luciferase were normalized to those of the firefly luciferase.

As shown in Figure 4, the silencing activity of siRNA **15** containing the phosphoramidate linkage was slightly weaker than that of siRNA **13** at each concentration. However, the RNAi-inducing ability of siRNA **15** containing the phosphoramidate linkage was almost equal to that of siRNA **14**.

Next, in order to examine the difference in the effects of modifications between passenger strands (sense strands) and guide strands (antisense strands), the silencing activities of siRNAs **16** and **17** were tested. siRNA **16** contains the modified nucleoside **4** in the guide strand, whereas siRNA **17** involves the modified nucleoside **4** in the passenger strand. As shown in Figure 5, the silencing activity of siRNA **16** containing the analog in the guide strand was slightly

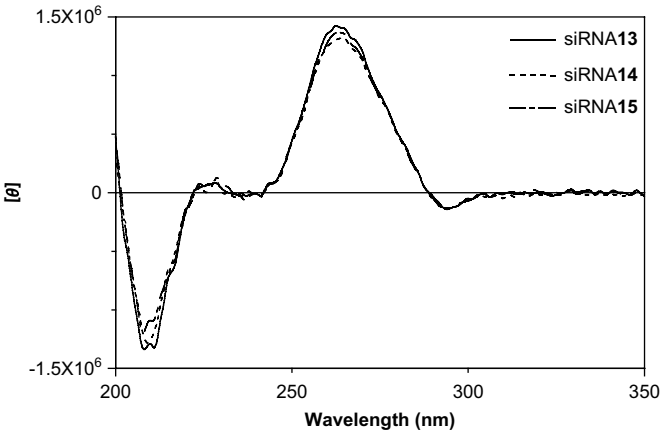


Figure 3. Circular dichroism (CD) spectra.

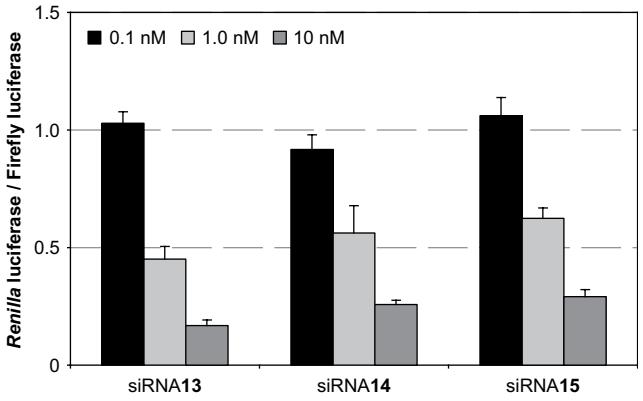


Figure 4. Dual-luciferase assay (1). Experimental conditions are described in Section 4.

weaker than that of siRNA **17** containing the analog in the passenger strand at each concentration. From these results, it was suggested that the modification at the guide strand influences the silencing activities of siRNAs more significantly than the modification at the passenger strand dose.

2.7. Nuclease resistance

We examined the susceptibility of the ONs containing the analogs to nucleolytic digestion. In this study, we used two types of nucleases, snake venom phosphodiesterase (SVPD) and RNase A, as models for a 3′-exonuclease and an endonuclease, respectively. The stability of the ONs in bovine serum was also examined.

ONs **24**, **25**, and **26** labeled at their 5′-ends with fluorescein were incubated with SVPD. ONs **25** and **26** contain the modified nucleosides at their 3′-ends. The reactions were analyzed by PAGE under denaturing conditions. As shown in Figure 6, after 1 h of incubation, all the ONs were completely hydrolyzed. Thus, it was found that the ON containing the phosphoramidate linkage was not resistant to 3′-exonuclease.

Next, the susceptibility of ONs **27**, **28**, and **29** to nucleolytic digestion by RNase A was studied. RNase A preferentially cleaves ONs on the 3′-side of the pyrimidine nucleosides. ONs **28** and **29** contain the modified nucleosides at the middle of polyadenylate sequences. As shown in Figure 7a, ONs **28** and **29** were highly resistant to the nuclease under the conditions where the unmodified ON was completely digested. In order to compare the nuclease-resistant property of ON **29** containing the phosphoramidate linkage with that of ON **28**, the ONs were incubated with the enzyme at a high concentration. As shown in Figure 7b, though the band corresponding to the ON **28** disappeared after 3 h incubation (lane 10),

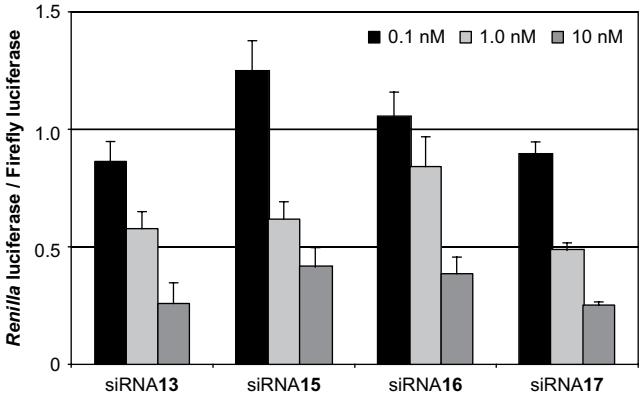


Figure 5. Dual-luciferase assay (2). Experimental conditions are described in Section 4.

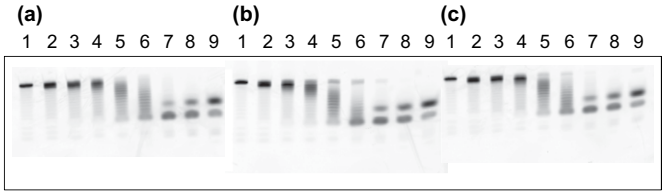


Figure 6. 20% PAGE of ONs hydrolyzed by SVPD. (a) ON **24**; (b) ON **25**; and (c) ON **26**. ONs were incubated with SVPD for 0 min (lane 1), 1 min (lane 2), 5 min (lane 3), 10 min (lane 4), 30 min (lane 5), 1 h (lane 6), 3 h (lane 7), 6 h (lane 8), and 15 h (lane 9). Experimental conditions are described in Section 4.

the band corresponding to the ON **29** remained stable under the same conditions (lane 16). Thus, it was found that ON **29** containing the phosphoramidate linkage was more resistant to RNase A than ON **28**.

Finally, the stability of the ONs in bovine serum was examined. ONs **24**, **25**, and **26** were incubated in PBS containing 5% bovine serum and analyzed by 20% PAGE under denaturing conditions (Fig. 8). The unmodified ON **27** was completely digested after 1 min incubation, and a band attributable to the cleavage around the uridine residues was also observed. On the other hand, ONs **28** and **29** were gradually digested from the 3′-ends of the strands. The results imply that the ONs containing analogs **1** and **3** are highly resistant to the activity of endonuclease in bovine serum.

3. Discussion

We have designed and synthesized siRNA possessing P3′→N5′ phosphoramidate linkages. It has been reported that P3′→N5′ phosphoramidate linkages in RNA are very unstable and easily hydrolyzed.[43] The lability of the linkages has been explained on the basis of the neighboring group participation effect of the 2′-hydroxyl group adjacent to the *N*-protonated phosphoramidate. We have succeeded in synthesizing RNA possessing phosphoramidate linkages by the successive introduction of 2′-deoxy-2′-fluororibonucleoside and 5′-amino-2′,5′-dideoxy-2′α-fluororibonucleoside into the RNA.

The thermal stability of siRNA possessing phosphoramidate linkages was studied by thermal denaturation. It has been reported that DNA containing P3′→N5′ phosphoramidate significantly

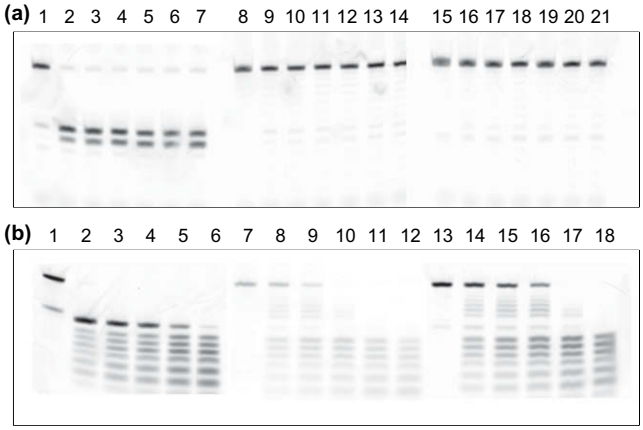


Figure 7. 20% PAGE of ONs hydrolyzed by RNase A. (a) ON **27** (lanes 1–7); ON **28** (lanes 8–14); ON **29** (lanes 15–21). ONs were incubated with RNase A (10 ng) for 0 min (lanes 1, 8, and 15), 5 min (lanes 2, 9, and 16), 10 min (lanes 3, 10, and 17), 20 min (lanes 4, 11, and 18), 30 min (lanes 5, 12, and 19), 60 min (lanes 6, 13, and 20), and 90 min (lanes 7, 14, and 21). (b) ON **27** (lanes 1–6); ON **28** (lanes 7–12); ON **29** (lanes 13–18). ONs were incubated with RNase A (100 ng) for 0 min (lanes 1, 7, and 13), 1 h (lanes 2, 8, and 14), 2 h (lanes 3, 9, and 15), 3 h (lanes 4, 10, and 16), 5 h (lanes 5, 11, and 17), and 7 h (lanes 6, 12, and 18). Experimental conditions are described in Section 4.

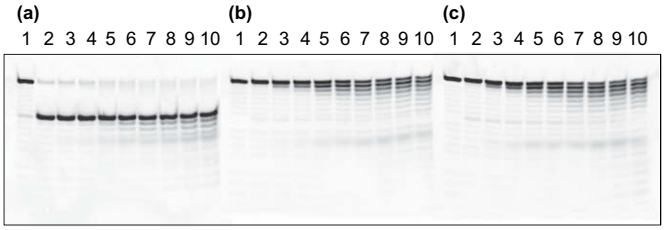


Figure 8. 20% PAGE of ONs incubated in PBS containing 5% bovine serum. (a) ON **24**; (b) ON **25**; (c) ON **26**. ONs were incubated for 0 min (lane 1), 1 min (lane 2), 5 min (lane 3), 10 min (lane 4), 30 min (lane 5), 1 h (lane 6), 3 h (lane 7), 6 h (lane 8), 13 h (lane 9), and 24 h (lane 10). Experimental conditions are described in Section 4.

decreases the thermal stability of duplexes with DNA and RNA complements.[27–29] The T_m values of normal siRNA **13**, siRNA **14** containing 2′-deoxy-2′-fluororibonucleosides **1** and **2**, and siRNA **15** containing **1** and the 5′-amino-2′,5′-dideoxy-2′α-fluororibonucleoside **4** were 76.0 °C, 78.5 °C, and 77.0 °C, respectively. Although the thermal stability of siRNA **15**, containing **1** and **4**, was lower than that of siRNA **14**, containing **1** and **2**, the introduction of analogs **1** and **4** to siRNA **15** did result in the enhancement of the thermal stability of the duplex to a level slightly above that of siRNA **13**. The %*N* values of 5′-amino-2′,5′-dideoxy-2′α-fluorouridine (**3**) was estimated to be 77%, which was similar to that of natural ribonucleosides. Therefore, it was suggested that the introduction of a fluorine atom to the 2′α-position of 5′-amino-5′-deoxypyrimidine shifted the conformational equilibrium of the sugar moiety to a C3′-*endo* conformation, thus enhancing the thermal stability of the A-type duplex even though it contained the phosphoramidate linkage.

The RNAi-inducing ability of siRNA containing phosphoramidates was studied using a dual-luciferase assay. Although the introduction of phosphoramidate linkages into siRNA marginally reduced its silencing activity, the RNAi-inducing ability of siRNA with phosphoramidates was almost equal to that of siRNA containing the corresponding 2′-deoxy-2′-fluororibonucleosides. CD spectroscopy revealed that the global conformation of siRNA **14** containing 5′-amino-2′,5′-dideoxy-2′α-fluorocytidine (**4**) was not significantly different from that of siRNA **13**. Thus, it was shown that the introduction of **4** into siRNA influences neither the global conformation nor the RNAi-inducing ability of siRNA to a significant extent.

The susceptibility to nucleolytic digestion of the RNA containing the analogs was also studied. It was found that although RNA that contains phosphoramidate linkages was not resistant to snake venom phosphodiesterase (3′-exonuclease), it was more resistant to nucleolytic hydrolysis by RNase A (an endonuclease) than normal RNA or RNA containing the corresponding 2′-deoxy-2′-fluororibonucleosides. These results suggest that the introduction of these phosphoramidate linkages into siRNA would be a useful modification for improving the nuclease-resistant property of siRNA.

4. Experimental section

4.1. General remarks

The NMR spectra were recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C), and were reported in parts per million downfield from tetramethylsilane. The coupling constants (*J*) are expressed in hertz. The mass spectra were obtained by fast atom bombardment (FAB). Thin-layer chromatography was performed on Merck coated plates $60F_{254}$. Silica gel column chromatography was carried out on Wakogel C-300.

4.2. 5′-Azide-2′,5′-dideoxy-2′-fluorouridine (5)

A mixture of 2′,5′-dideoxy-2′-fluorouridine (**1**) (1.96 g, 7.96 mmol), NaN_3 (2.59 g, 39.8 mmol), Ph_3P (2.71 g, 10.3 mmol), and CBr_4 (3.96 g, 11.9 mmol) in DMF (40 mL) was stirred at room temperature. After 4 h, Ph_3P (1.04 g, 4.00 mmol) and CBr_4 (2.64 g, 7.96 mmol) were added to the mixture, and then the entire mixture was stirred at room temperature. After 24 h, the solvent was evaporated in vacuo, and the residue was partitioned between EtOAc and H_2O. The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 5–6% MeOH in $CHCl_3$) to afford **5** (0.86 g, 3.17 mmol) in 40% yield: ^{1}H NMR (DMSO-d_6) δ 3.52 (dd, 1H, *J*=5.6 and 13.7, 5′-H), 3.73 (dd, 1H, *J*=2.8 and 13.7, 5′-H), 3.93 (m, 1H, 4′-H), 4.23 (m, 1H, 3′-H), 5.19 (ddd, 1H, *J*=1.2, 4.7 and 52.4, 2′-H), 5.65 (d, 1H, *J*=8.0, 5-H), 5.74 (d, 1H, *J*=6.5, 3′-OH), 5.85 (dd, 1H, *J*=1.2 and 21.7, 1′-H), 7.66 (d, 1H, *J*=8.0, 6-H), 11.44 (br s, 1H, 3-NH); ^{13}C NMR (DMSO-d_6) δ 50.61, 68.81 (d, *J*=16.4), 80.56, 89.42 (d, *J*=36.0), 92.79 (d, *J*=184.2), 102.02, 141.82, 150.15, 163.15; LRMS (FAB) *m*/*z* 272 (MH^+); HRMS (FAB) calcd for $C_9H_{11}N_5FO_4$ (MH^+) 272.0795, found 272.0804. Anal. Calcd for $C_9H_{10}N_5FO_5$: C, 39.86; H, 3.72; N, 25.82. Found: C, 39.81; H, 3.70; N, 25.89.

4.3. 5′-Amino-2′,5′-dideoxy-2′-fluorouridine (3)

A mixture **5** (0.21 g, 0.77 mmol) and Pd–C (10%, 51 mg) in MeOH (3.8 mL) was stirred under atmospheric pressure of H_2 at room temperature. After 12 h, the catalyst was filtered off with Celite. The filtrate was evaporated under reduced pressure and dried in vacuo to give **3** (0.18 g, 0.73 mmol) in 95% yield: ^{1}H NMR (DMSO-d_6) δ 2.75 (dd, 1H, *J*=4.6 and 13.9, 5′-H), 2.88 (dd, 1H, *J*=3.0 and 13.9, 5′-H), 3.76 (m, 1H, 4′-H), 4.11 (m, 1H, 3′-H), 5.07 (dd, 1H, *J*=2.6 and 53.3, 2′-H), 5.60 (d, 1H, *J*=8.0, 5-H), 5.86 (dd, 1H, *J*=2.6 and 18.4, 1′-H), 7.94 (d, 1H, *J*=8.0, 6-H); LRMS (FAB) *m*/*z* 246 (MH^+); HRMS (FAB) calcd for $C_9H_{13}N_3FO_4$ (MH^+) 246.0890, found 246.0884.

4.4. 5′-Amino-2′,5′-dideoxy-2′-fluoro-5′-*N*-(4′-monomethoxytrityl)uridine (6)

A mixture of **3** (0.18 g, 0.73 mmol), MMTrCl (0.30 g, 0.97 mmol), and DMAP (9 mg, 74 μmol) in pyridine (3.7 mL) was stirred at room temperature. After 6 h, MMTrCl (0.23 g, 0.74 mmol) and DMAP (9 mg, 74 μmol) were added to the mixture, and the whole mixture was stirred at room temperature. After 12 h, the mixture was partitioned between EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 50–100% EtOAc in hexane) to give **6** (0.23 g, 0.45 mmol) in 61% yield: ^{1}H NMR ($CDCl_3$) δ 2.46 (dd, 1H, *J*=6.0 and 13.0, 5′-H), 2.71 (dd, 1H, *J*=3.4 and 13.0, 5′-H), 3.79 (s, 3H, OCH_3), 4.06 (m, 1H, 4′-H), 4.34 (m, 1H, 3′-H), 5.10 (dd, 1H, *J*=4.6 and 53.3, 2′-H), 5.69 (d, 1H, *J*=8.0, 5-H), 5.79 (d, 1H, *J*=19.1, 1′-H), 6.81–7.46 (m, 18H, 6-H and MMTr); ^{13}C NMR ($CDCl_3$) δ 44.66, 55.21, 70.24, 70.79 (d, *J*=17.2), 82.42, 90.29 (d, *J*=36.0), 93.50 (d, *J*=185.0), 94.43, 102.83, 113.26, 126.49, 127.97, 128.45, 129.75, 137.54, 140.64, 145.75, 149.52, 158.03, 162.67; LRMS (FAB) *m*/*z* 518 (MH^+); HRMS (FAB) calcd for $C_{29}H_{29}N_3FO_5$ (MH^+) 518.2091, found 518.2082. Anal. Calcd for $C_{29}H_{28}N_3FO_5 \cdot 1/4H_2O$: C, 66.72; H, 5.50; N, 8.05. Found: C, 66.77; H, 5.50; N, 7.95.

4.5. 5′-Amino-3′-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)-phosphinyl]-2′,5′-dideoxy-2′-fluoro-5′-*N*-(4′-monomethoxytrityl)uridine (7)

A mixture of **6** (0.49 g, 0.94 mmol), *N*,*N*-diisopropylethylamine (0.96 mL, 5.63 mmol), and chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine (0.42 mL, 1.88 mmol) in THF (4.7 mL) was stirred

at room temperature for 1 h. The mixture was partitioned between $CHCl_3$ and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (a neutralized SiO_2, 50–100% EtOAc in hexane) to give **7** (0.36 g, 0.51 mmol) in 54% yield: ^{31}P NMR ($CDCl_3$) δ 151.2, 151.6.

4.6. Solid support synthesis

A mixture of **7** (0.20 g, 0.39 mmol), succinic anhydride (0.12 g, 1.20 mmol), and DMAP (12 mg, 0.10 mmol) in pyridine (4 mL) was stirred at room temperature. After 20 h, the solution was partitioned between $CHCl_3$ and H_2O, and the organic layer was washed with H_2O and brine. The separated organic phase was dried (Na_2SO_4) and concentrated to give a succinate. Aminopropyl controlled pore glass (0.49 g, 96 μmol) was added to a solution of the succinate and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (90 mg, 0.47 mmol) in DMF (10 mL), and the mixture was kept for 72 h at room temperature. After the resin was washed with pyridine, a capping solution (10 mL, 0.1 M DMAP in pyridine/Ac_2O=9:1, v/v) was added and the whole mixture was kept for 24 h at room temperature. The resin was washed with MeOH and acetone, and dried in vacuo. Amount of loaded compound **7** to the solid support was 20 μmol/g from calculation of released dimethoxytrityl cation by a solution of 70% $HClO_4$/EtOH (3:2, v/v).

4.7. 5′-Amino-3′-*O*-(*tert*-butyldimethylsilyl)-2′,5′-dideoxy-2′-fluoro-5′-*N*-(4′-monomethoxytrityl)uridine (9)

A mixture of **6** (3.00 g, 5.80 mmol), TBDMSCl (1.31 g, 8.69 mmol), and imidazole (1.18 g, 17.4 mmol) in DMF (19 mL) was stirred at room temperature. After 12 h, EtOH (8 mL) was added to the mixture, and then the whole mixture was stirred at room temperature. After 30 min, the mixture was partitioned between EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 30–40% EtOAc in hexane) to give **9** (3.37 g, 5.32 mmol) in 92% yield: ^{1}H NMR ($CDCl_3$) δ 0.00–0.06 (m, 6H, Si–CH_3), 0.85 (s, 9H, Si–*t*-Bu), 2.31 (dd, 1H, *J*=6.8 and 12.8, 5′-H), 2.65 (dd, 1H, *J*=2.8 and 12.8, 5′-H), 3.80 (s, 3H, OCH_3), 4.09 (m, 1H, 4′-H), 4.16 (m, 1H, 3′-H), 4.96 (ddd, 1H, *J*=1.8, 4.4, and 53.3, 2′-H), 5.71 (d, 1H, *J*=7.7, 5-H), 5.77 (dd, 1H, *J*=1.8 and 18.6, 1′-H), 6.82–7.48 (m, 18H, 6-H and MMTr), 8.21 (s, 1H, 3-NH); ^{13}C NMR ($CDCl_3$) δ −5.07, −4.71, 18.02, 25.56, 45.27, 55.22, 70.29, 71.40 (d, *J*=16.4), 83.12, 90.97 (d, *J*=35.2), 92.19 (d, *J*=192.3), 102.71, 113.25, 126.46, 127.95, 128.48, 129.76, 137.64, 140.89, 145.77, 149.48, 158.03, 162.54; LRMS (FAB) *m*/*z* 633 (MH^+).

4.8. 5′-Amino-3′-*O*-(*tert*-butyldimethylsilyl)-2′,5′-dideoxy-2′-fluoro-5′-*N*-(4′-monomethoxytrityl)cytidine (10)

A mixture of **9** (3.36 g, 5.32 mmol), Et_3N (1.48 mL, 10.6 mmol), DMAP (1.30 g, 10.6 mmol), and 2,4,6-triisopropylbenzenesulfonylchloride (3.22 g, 10.6 mmol) in CH_3CN (27 mL) was stirred at room temperature for 1 h. The mixture was cooled in an ice-bath. Concentrated NH_4OH (12.5 mL) was added, and the whole mixture was stirred at room temperature for 2 h. The mixture was partitioned between EtOAc and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated), brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 3% MeOH in $CHCl_3$) to give **10** (3.22 g, 5.09 mmol) in 96% yield: ^{1}H NMR ($CDCl_3$) δ 0.00 (s, 3H, Si–CH_3), 0.08 (s, 3H, Si–CH_3), 0.86 (s, 9H, Si–*t*-Bu), 2.37 (dd, 1H, *J*=7.0 and 12.7, 5′-H), 2.71 (dd, 1H, *J*=2.3 and 12.7, 5′-H), 3.84 (s, 3H, OCH_3), 4.05 (ddd, 1H, *J*=4.5, 8.6, and 20.9, 3′-H), 4.24 (m, 1H, 4′-H), 5.03 (dd, 1H, *J*=4.5 and 53.3, 2′-H), 5.67 (d, 1H, *J*=7.2, 5-H), 5.81 (d, 1H, *J*=18.8, 1-H), 6.86–7.54 (m, 18H, 6-H and MMTr); ^{13}C NMR ($CDCl_3$) δ −5.09, −4.70, 17.98, 25.56, 45.36, 55.20, 70.32, 71.31 (d, *J*=15.8), 82.40, 91.92 (d, *J*=36.0), 93.17 (d, *J*=188.0), 99.17, 113.20, 126.34, 127.89, 128.54, 129.83, 137.85, 141.94, 145.89, 155.22, 165.87. Anal. Calcd for $C_{35}H_{43}N_4FO_4Si\cdot 6/5H_2O$: C, 64.43; H, 7.01; N, 8.59. Found: C, 64.08; H, 6.61; N, 8.28.

4.9. 5′-Amino-4-*N*-benzoyl-2′,5′-dideoxy-2′-fluoro-5′-*N*-(4′-monomethoxytrityl)cytidine (11)

A mixture of **10** (77 mg, 0.12 mmol) and BzCl (18 μl, 0.16 mmol) in pyridine (1 mL) was stirred at room temperature. After 30 min, the mixture was partitioned between EtOAc and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated), brine, dried (Na_2SO_4), and concentrated in vacuo. The residue was stirred with TBAF (1 M in THF, 0.17 mL, 0.17 mmol) in THF (1.1 mL) at room temperature. After 19 h, the solvent was evaporated, and the resulting residue was purified with column chromatography (SiO_2, 2% MeOH in $CHCl_3$) to give **11** (45 mg, 73 μmol) in 65% yield: ^{1}H NMR ($CDCl_3$) δ 2.47 (dd, 1H, *J*=6.2 and 13.0, 5′-H), 2.84 (m, 1H, 5′-H), 3.81 (s, 3H, OCH_3), 4.12 (d, 1H, *J*=21.7, 3′-H), 4.21 (m, 1H, 4′-H), 5.12 (dd, 1H, *J*=4.2 and 52.6, 2′-H), 5.96 (d, 1H, *J*=17.8, 1′-H), 6.84–7.90 (m, 18H, 6-H and MMTr), 8.72 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 45.12, 55.23, 70.37, 70.87 (d, *J*=17.2), 82.38, 90.55 (d, *J*=34.4), 93.28 (d, *J*=186.5), 99.44, 113.31, 126.55, 127.59, 128.02, 128.46, 128.49, 129.08, 129.75, 133.34, 137.54, 145.70, 145.72, 158.06. Anal. Calcd for $C_{36}H_{33}N_4FO_5Si\cdot H_2O$: C, 67.70; H, 5.52; N, 8.77. Found: C, 67.60; H, 5.42; N, 8.56.

4.10. 5′-Amino-*N*-4-benzoyl-3′-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphinyl]-2′-fluoro-2′,5′-dideoxy-5′-*N*-(4′-monomethoxytrityl)cytidine (12)

A mixture of **11** (0.19 g, 0.31 mmol), *N*,*N*-diisopropylethylamine (0.32 mL, 1.84 mmol), and chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine (0.14 mL, 0.61 mmol) in THF (1.5 mL) was stirred at room temperature for 1 h. The mixture was partitioned between $CHCl_3$ and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (a neutralized SiO_2, 50–100% EtOAc in hexane) to give **12** (0.12 g, 0.51 mmol) in 46% yield: ^{31}P NMR ($CDCl_3$) δ 151.4, 151.7.

4.11. RNA synthesis

Synthesis was carried out with a DNA/RNA synthesizer by phosphoramidite method. Deprotection of bases and phosphates was performed in concentrated NH_4OH/EtOH (3:1, v/v) at room temperature for 12 h. 2′-TBDMS groups were removed by 1.0 M tetrabutylammonium fluoride (TBAF, Aldrich) in THF at room temperature for 12 h. The reaction was quenched with 0.1 M TEAA buffer (pH 7.0) and desalted on a Sep-Pak C18 cartridge. Deprotected ONs were purified by 20% PAGE containing 7 M urea to give the highly purified ON **20** (3), ON **21** (6), ON **22** (7), ON **23** (6), ON **25** (3), ON **26** (9), ON **28** (11), ON **29** (19). The yields are indicated in parentheses as OD units at 260 nm starting from 1.0 μmol scale. Extinction coefficients of the ONs were calculated from those of mononucleotides and dinucleotides according to the nearest-neighbor approximation method.[44]

4.12. MALDI-TOF/MS analysis of RNAs

Spectra were obtained with a time-of-flight mass spectrometer. ON**20**: calculated mass, 6583.9; observed mass, 6582.7. ON **21**: calculated mass, 6733.0; observed mass, 6732.2. ON **22**: calculated mass, 6581.9; observed mass, 6585.1. ON **23**: calculated mass, 6731.0; observed mass, 6736.9. ON **25**: calculated mass, 6056.3;

observed mass, 6053.8. ON **26**: calculated mass, 6055.3; observed mass, 6057.3. ON **28**: calculated mass, 6056.3; observed mass, 6052.3. ON **29**: calculated mass, 6055.3; observed mass, 6057.3.

4.13. Thermal denaturation and CD spectroscopy

Each solution containing each siRNA (3 μM) in a buffer composed of 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0) and 100 mM NaCl was heated at 95 °C for 5 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 with a spectrophotometer. The sample temperature was increased by 0.5 °C/min. CD spectra were measured by a spectropolarimeter. Samples for CD spectroscopy were prepared by the same procedure used in the thermal denaturation study, and spectra were measured at 15 °C. The molar ellipticity was calculated from the equation $[\theta]=\theta/cl$, where θ is the relative intensity, c the sample concentration, and l the cell path length in centimeters.

4.14. Dual-luciferase assay

HeLa cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air in minimum essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Twenty-four hours before transfection, HeLa cells (4×10^4/mL) were transferred to 96-well plates (100 μL/well). They were transfected, using TransFast (Promega), according to instructions for transfection of adherent cell lines. Cells in each well were transfected with a solution (35 μL) of 20 ng of psiCHECK-2 vector (Promega), the indicated amounts of siRNAs, and 0.3 μg of TransFast in Opti-MEM I Reduced-Serum Medium (Invitrogen), and incubated at 37 °C. After 1 h, MEM (100 μL) containing 10% FBS and antibiotics was added to each well, and the whole mixture was further incubated at 37 °C. After 24 h, cell extracts were prepared in Passive Lysis Buffer (Promega). Activities of firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (Promega) according to a manufacturer's protocol. The results were confirmed by at least three independent transfection experiments with two cultures each and are expressed as the average from four experiments as mean±SD.

4.15. Partial hydrolysis of ONs with snake venom phosphodiesterase

Each ON (600 pmol) labeled with fluorescein at 5′-end was incubated with snake venom phosphodiesterase (5×10^{-3} units) in a buffer containing 33 mM Tris–HCl (pH 8.0) and 7 mM $MgCl_2$ (total 300 μL) at 37 °C. At appropriate periods, aliquots (5 μL) of the reaction mixture were separated and added to a solution of 9 M urea (15 μL). The mixtures were analyzed by electrophoresis on 20% polyacrylamide gel containing 7 M urea. The labeled ON in the gel was visualized by a Typhoon system (Amersham Biosciences).

4.16. Partial hydrolysis of ONs with RNase A

Each ON (450 pmol) labeled with fluorescein at 5′-end was incubated with RNase A (10 ng or 100 ng) in a buffer of 10 mM Tris–HCl (pH 8.0) containing 10 mM NaCl (total 300 μL) at 37 °C. At appropriate periods, aliquots (5 μL) of the reaction mixture were separated and added to a solution of 9 M urea (15 μL). The mixtures were analyzed by gel electrophoresis described above.

4.17. Stability of ON in the PBS containing bovine serum

Each ON (600 pmol) labeled with fluorescein at 5′-end was incubated in PBS (300 μL) containing 5% bovine serum at 37 °C. At appropriate periods, aliquots (5 μL) of the reaction mixture were separated and added to a solution of 9 M urea (15 μL). The mixtures were analyzed by gel electrophoresis described above.

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