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Research paper

Synthesis of small interfering RNAs containing acetal-type nucleoside analogs at their 3′-ends and analysis of their silencing activity and their ability to bind to the Argonaute2 PAZ domain

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ABSTRACT

In this study, we aimed to create small interfering RNAs (siRNAs) with increased silencing activities and nuclease resistance properties. Therefore, we designed and synthesized five types of siRNA containing acetal-type nucleoside analogs at their 3′-dangling ends. We found that the siRNA containing 1-*O*-(2,2,2-trifluoroethyl)-β-D-ribofuranose at the 3′-dangling end was the most potent among the synthesized siRNAs and showed more resistance to nucleolytic degradation by a 3′ exonuclease than a natural RNA did. Thus, modification of siRNAs by addition of 1-*O*-(2,2,2-trifluoroethyl)-β-D-ribofuranose may hold promise as a means of improving the silencing activity and nuclease resistance of siRNAs.

1. Introduction

Small interfering RNAs (siRNAs), which are composed of 21 or 22 nucleotides and contain a 2-nucleotide overhang at the 3′-end, are a type of small RNA. siRNAs are key molecules in RNA interference (RNAi), a cellular pathway for the control of gene expression [1–3]. During RNAi, a guide strand (an antisense strand) of siRNA forms an RNA-induced silencing complex (RISC) with the Argonaute (Ago) protein. The guide strand hybridizes with a target mRNA in the RISC, and then the mRNA is cleaved by the slicer activity of the RISC, leading to inhibition of protein expression of the target mRNA. If the sequences of disease-causing genes are known, the siRNA can be rationally designed and synthesized [2,4]. Thus, siRNAs have attracted a great deal of attention as promising candidates for use as therapeutic agents for "unmet medical needs." To date, a variety of chemically modified siRNAs have been synthesized to improve nuclease resistance and silencing abilities [5–10].

Ago proteins are composed of four domains: the N-terminal (N), Piwi/Argonaute/Zwille (PAZ), middle (MID), and P-element-induced wimpy testis (PIWI) domains [11–13]. It is known that the 5′-OH of the guide strand is phosphorylated by cellular kinases in cells [14], and in the RISC, the 5′-phosphate of the guide strand is recognized by the MID domain and tightly anchored in the Ago protein [15,16]. It has also been reported that the 3′-end of the guide strand is recognized by the PAZ domain and that the nucleotides of the 3′-dangling end are accommodated in the hydrophobic pocket in the domain [17–20]. However, it was suggested by recent studies that the nucleotides are not always anchored in the PAZ domain and released from the domain when the guide strand forms a base pair with the target mRNA [11–13,21]. The nucleotides of the 3′-dangling end of the guide strand repeat binding to and dissociation from the PAZ domain during RNAi.

Recently, we reported the synthesis of a photo-cross-linking microRNA (miRNA) probe consisting of the nucleoside analog 1-*O*-[4-(3-trifluoromethyl-3H-diazirin-3-yl)]benzyl-β-D-ribofuranose (**1**), in which the natural base was replaced with an aryl residue via an acetal linkage (Fig. 1) [22]. We considered that substituents of different sizes could be β-selectively introduced at the 1-position of D-ribofuranose via an acetal linkage by using 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose as a starting material. Based on these reports and results, we designed and synthesized

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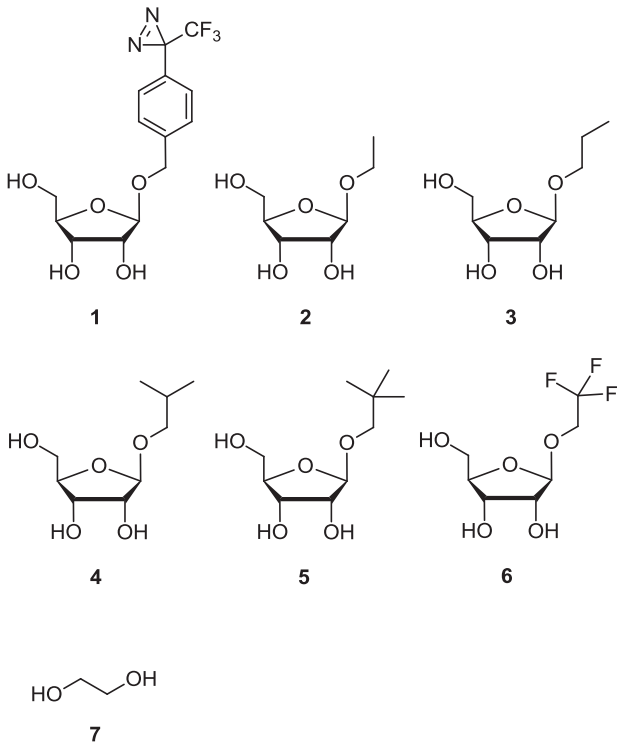


Fig. 1. Structures of nucleoside analogs.

five types of siRNA containing acetal-type nucleoside analogs at their 3′-dangling ends to investigate the influence of the 3′-dangling-end modifications on recognition of the siRNAs by the human Argonaute2 PAZ domain protein.

In this paper, we report the synthesis of five types of siRNA containing the acetal-type nucleoside analogs **2**–**6** at their 3′-dangling ends (Fig. 1). We assessed the properties of the modified siRNAs by a thermal denaturation study, a dual-luciferase reporter assay, an electrophoretic mobility shift assay (EMSA), an enzyme-linked immunosorbent assay (ELISA), and a partial digestion using a 3′ exonuclease.

2. Results

2.1. Synthesis

The synthetic route used to synthesize phosphoramidites **34**–**38** of analogs **2**–**6** is shown in Scheme 1. We considered that various substituents could be β-selectively introduced at the 1-position of D-ribofuranose via neighboring group participation of an acyl-type protective group at the 2-O-position. Glycosylation of ethanol (**8**) with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**13**), which is commercially available, in the presence of TMSOTf at −30 °C in CH_2Cl_2 quantitatively afforded the β-anomer **14**. Stereochemistry of the anomeric carbon was determined from the coupling constant between the protons at the 1-position and 2-position. Subsequently, this compound was debenzoylated in the presence of a catalytic amount of $NaOCH_3$ in CH_3OH, and the primary hydroxy group of **2** was protected by a 4,4′-dimethoxytrityl (DMTr) group to produce the 5-*O*-DMTr derivative **19** with a 77% yield. Compound **19** was treated with TBDMSCl to afford the 3-*O*-TBDMS and 2-*O*-TBDMS derivatives **24** and **25** with 42% and 33% yields, respectively. The 2-*O*-TBDMS derivative **25** was phosphitylated by a standard procedure to give the corresponding phosphoramidite **24** with a 64% yield. In a similar manner, the phosphoramidites **35**–**38**, which had propyl, 2-methylpropyl, 2,2-dimethylpropyl, and 2,2,2-trifluoroethyl groups, respectively, were synthesized from the protected D-ribofuranose derivative **13** with final yields of 21%, 17%, 20%, and 13%, respectively.

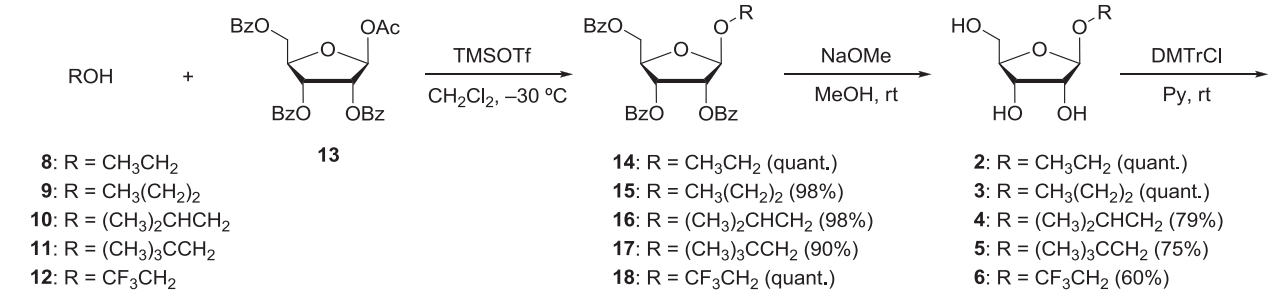
To introduce the analog **6** compound at the 3′-end of an RNA strand, the 5-*O*-DMTr–3-*O*-TBDMS derivative **32** was converted to the corresponding succinate, which was then linked to controlled pore glass (CPG) to generate the solid support **39** linked to compound **32** (40 μmol/g) (Scheme 2). In a similar manner, the solid support **40** linked to ethylene glycol was also synthesized.

Oligonucleotide (ON) sequences used in this study are listed in Tables 1 and 2. In order to synthesize siRNAs with analogs at their 3′-dangling ends, ONs **5**–**16**, which had analogs **2**–**6** at their 3′-end moieties corresponding to the 3′-dangling ends in the siRNAs, were synthesized using solid support **40** as a universal solid support. All ONs containing the analogs were synthesized using the phosphoramidites **34**–**38** and the solid support **39** or **40**. After purification via polyacrylamide gel electrophoresis (PAGE), the ONs were analyzed by a reversed-phase C-18 high-performance liquid chromatography (HPLC). As shown in Fig. S1, a single peak was observed in the profiles for ONs **11**–**16**, while several peaks thought to be attributable to the hydrolysis of the glycosidic bonds of the analogs were observed in the profiles for ONs **7**–**10**. These results suggested that under the acidic conditions used in the ON synthesis, the glycosidic bonds of the analogs with small substituents (e.g., ethyl or propyl groups) were more unstable than the analogs with bulky substituents (e.g., 2,2-dimethylpropyl group) or electron withdrawing groups (e.g., 2,2,2-trifluoroethyl group). ONs **7**–**10** were further purified by the HPLC.

The ONs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and the observed molecular weights from these analyses were in agreement with the structures of the ONs.

2.2. Thermal stability of siRNAs

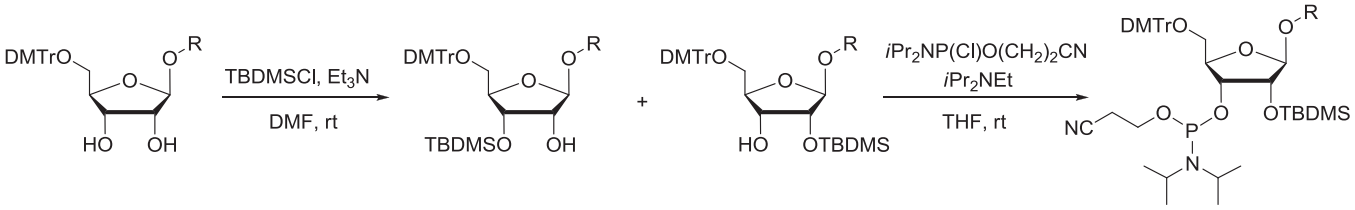
First, the stabilities of the RNA duplexes containing the analogs at the 3′-dangling ends were evaluated by thermal denaturation in a buffer comprising 10 mM sodium phosphate (pH 7.0) and 100 mM NaCl (Fig. S2). Melting temperatures (Tms) and ΔTms (Tm of each duplex − Tm of siRNA **1**) are listed in Table 1. It was reported that a base at a dangling end enhances a thermal stability of an RNA–RNA duplex [23]. The Tm of the unmodified RNA duplex (siRNA **1**) was 78.3 °C. The T*m* of siRNA **2**, which had thymidines instead of uridines at the 3′-dangling ends, was slightly lower than that of siRNA **1** (ΔTm = −0.5 °C). Addition of the 2-hydroxylethylphosphate moiety at the 3′-ends of the duplex (siRNA **3**) barely affected the thermal stability of the duplex. On the other hand, incorporation of analog **2**, which has an ethyl group at the 3′-dangling end, reduced the thermal stability of the duplex (ΔTm = −1.4 °C). However, we found that the duplexes became more thermally stable as the size of the side chains of the analogs increased. The Tm value (78.2 °C) of siRNA **7**, incorporating analog **5** (with the 2,2-dimethypropyl group), was almost the same as that of siRNA **1** (with uridines) (Tm = 78.3 °C). Intriguingly, the Tm (77.9 °C) of siRNA **8**, which had a 2,2,2-trifluoroethyl group, was greater than those of siRNA **4** (Tm = 76.9 °C), siRNA **5** (Tm = 77.1 °C), and siRNA **6** (Tm = 77.0 °C), which had ethyl, propyl, and 2-methylpropyl groups, respectively. These results suggest that dipole–dipole interactions as well as hydrophobic interactions among the alkyl side chains and the adjacent bases in the dangling-end positions are important for enhancing the thermal stability of the duplexes.



8: R = CH_3CH_2
9: R = $CH_3(CH_2)_2$
10: R = $(CH_3)_2CHCH_2$
11: R = $(CH_3)_3CCH_2$
12: R = CF_3CH_2

14: R = CH_3CH_2 (quant.)
15: R = $CH_3(CH_2)_2$ (98%)
16: R = $(CH_3)_2CHCH_2$ (98%)
17: R = $(CH_3)_3CCH_2$ (90%)
18: R = CF_3CH_2 (quant.)

2: R = CH_3CH_2 (quant.)
3: R = $CH_3(CH_2)_2$ (quant.)
4: R = $(CH_3)_2CHCH_2$ (79%)
5: R = $(CH_3)_3CCH_2$ (75%)
6: R = CF_3CH_2 (60%)



19: R = CH_3CH_2 (77%)
20: R = $CH_3(CH_2)_2$ (83%)
21: R = $(CH_3)_2CHCH_2$ (84%)
22: R = $(CH_3)_3CCH_2$ (99%)
23: R = CF_3CH_2 (93%)

24: R = CH_3CH_2 (42%)
26: R = $CH_3(CH_2)_2$ (43%)
28: R = $(CH_3)_2CHCH_2$ (34%)
30: R = $(CH_3)_3CCH_2$ (29%)
32: R = CF_3CH_2 (40%)

25: R = CH_3CH_2 (33%)
27: R = $CH_3(CH_2)_2$ (37%)
29: R = $(CH_3)_2CHCH_2$ (34%)
31: R = $(CH_3)_3CCH_2$ (36%)
33: R = CF_3CH_2 (29%)

34: R = CH_3CH_2 (64%)
35: R = $CH_3(CH_2)_2$ (71%)
36: R = $(CH_3)_2CHCH_2$ (78%)
37: R = $(CH_3)_3CCH_2$ (83%)
38: R = CF_3CH_2 (83%)

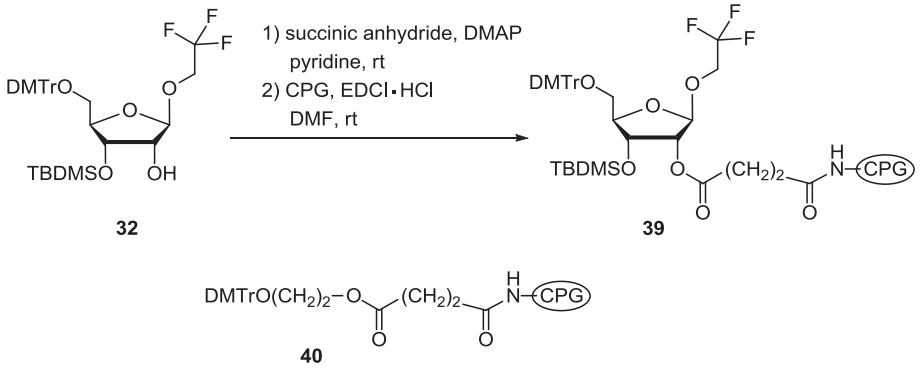
Scheme 1. The experimental procedure is described in Experimental Section.

2.3. Silencing activities of siRNAs

Next, we examined the ability of the siRNAs containing the analogs to suppress gene expression by using a dual-luciferase reporter assay. The psiCHECK-2 vector, which encodes Renilla luciferase and firefly luciferase, was used as the reporter. All siRNAs were designed to target the Renilla luciferase gene. The percent Renilla: firefly luciferase activities compared to transfection without siRNA are shown in Table 1. The luciferase activity ratios for an unmodified siRNA (siRNA **1**) with uridines at the dangling ends were 24.1 and 53.4. Replacement of the uridines at the dangling ends by thymidines slightly reduced the silencing activity of the siRNA (siRNA **2**; 28.9 and 68.0 at 1.0 and 0.1 nM, respectively). Addition of the 2-hydroxylethylphosphate at the 3′-ends of the siRNA also slightly decreased the silencing activity of the siRNA (siRNA **3**; 34.3 and 69.1 at 1.0 and 0.1 nM, respectively). Although the silencing activities of siRNAs **4**–**7**, which had analogs at the dangling ends, were not largely different from each other, the siRNAs, except for siRNAs **5** and **8,** tended to become more potent as the size of the side chains of the analogs increased. Intriguingly, the silencing activity of siRNA **8** (21.3 and 57.4 at 1.0 and 0.1 nM, respectively), which had a 2,2,2-trifluoroethyl group, was greater than that of siRNA **3**, which had an ethyl group, and comparable to that of siRNA **1**, which had uridines at the dangling ends, despite the size of the 2,2,2-trifluoroethyl group being similar to that of the ethyl group.

2.4. Ability of the human Ago2 PAZ domain to bind to modified siRNAs

Next, we evaluated the ability of a recombinant human Ago2 PAZ domain protein to bind to the modified siRNAs by performing an EMSA. The sequences of the siRNAs used in the EMSA are listed in Table 2. All*siRNA*shad dangling endsat the 3′-ends of the sense strands, whereas the 3′-ends of the antisense strands had blunt-end structures. The 5′-OH of the sense strand (ON **17**) of siRNA **9** was labeled with fluorescein. We inferred that the PAZ domain protein would recognize and bind to the dangling-end moieties of



32
39
40

Scheme 2. The experimental procedure is described in Experimental Section.

Table 1
Sequences of siRNAs, their T_m values and their abilities to suppress gene expression.

Abbreviation of siRNA	Abbreviation of ON	Sequence[a]	T_m (°C)	ΔT_m (°C)[b]	Upper: 1 nM[c] lower:0.1 nM
Control	(Buffer)	–	–	–	100 ± 1.2
siRNA **1**	ON **1**	Sense (passenger) strand	78.3	–	24.1 ± 1.6
		5′-GGCCUUUCACUACUCCUAC**UU**-3′			53.4 ± 4.6
	ON **2**	3′-**UU**CCGGAAAGUGAUGAGGAUG-5′			
		Antisense (guide) strand			
siRNA **2**	ON **3**	5′-GGCCUUUCACUACUCCUAC**tt**-3′	77.8	−0.5	28.9 ± 3.3
	ON **4**	3′-**tt**CCGGAAAGUGAUGAGGAUG-5′			68.0 ± 1.7
siRNA **3**	ON **5**	5′-GGCCUUUCACUACUCCUAC**tt7**-3′	77.8	−0.5	34.3 ± 1.9
	ON **6**	3′-**7tt**CCGGAAAGUGAUGAGGAUG-5′			69.1 ± 1.8
siRNA **4**	ON **7**	5′-GGCCUUUCACUACUCCUAC**227**-3′	76.9	−1.4	35.5 ± 2.9
	ON **8**	3′-**722**CCGGAAAGUGAUGAGGAUG-5′			71.4 ± 3.1
siRNA **5**	ON **9**	5′-GGCCUUUCACUACUCCUAC**337**-3′	77.1	−1.2	24.4 ± 1.2
	ON **10**	3′-**733**CCGGAAAGUGAUGAGGAUG-5′			60.0 ± 5.5
siRNA **6**	ON **11**	5′-GGCCUUUCACUACUCCUAC**447**-3′	77.0	−1.3	31.7 ± 3.5
	ON **12**	3′-**744**CCGGAAAGUGAUGAGGAUG-5′			61.9 ± 1.0
siRNA **7**	ON **13**	5′-GGCCUUUCACUACUCCUAC**557**-3′	78.2	−0.1	27.8 ± 0.7
	ON **14**	3′-**755**CCGGAAAGUGAUGAGGAUG-5′			59.4 ± 3.7
siRNA **8**	ON **15**	5′-GGCCUUUCACUACUCCUAC**667**-3′	77.9	−0.4	21.3 ± 1.9
	ON **16**	3′-**766**CCGGAAAGUGAUGAGGAUG-5′			57.4 ± 0.9

[a] Small letters indicate 2′-deoxyribonucleosides.
[b] $\Delta T_m = T_m$ (each siRNA) − T_m (siRNA **1**).
[c] 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.

the siRNAs. First, the labeled siRNA **9** was incubated with various concentrations of the recombinant human Ago2 PAZ domain protein at 37 °C in a buffer of 20 mM Tris–HCl (pH 8.0), 0.1 M NaCl, and 1 mM dithiothreitol (DTT), and the mixtures were analyzed by nondenaturing polyacrylamide gel electrophoresis (PAGE) at 4 °C. As shown in Fig. 2, lower-mobility bands corresponding to the siRNA-protein complexes were observed. The dissociation constant (K_d) was estimated to be about 20 μM.

Next, we carried out the EMSA in the presence of siRNAs **10**–**17** as competitors. As shown in Fig. 3, formation of the siRNA-protein complex between the labeled siRNA **9** and the PAZ domain protein was severely inhibited by adding the unlabeled siRNA **10**. The ratio of the siRNA-protein complex between the labeled siRNA **9** and the PAZ domain protein decreased from 53% to 28% or 14% in the presence of a 10- or 100-fold molar excess, respectively, of the unlabeled siRNA **10**. In contrast, the inhibitory effect of siRNA **11**, which had thymidines at the 3′-dangling end, on the formation of the labeled siRNA-protein complex was significantly weaker than that of siRNA **10**, which had uridines at the 3′-dangling end. This result is consistent with previous reports [20,24] and implies that the 2′-OH groups or the sugar conformation of the nucleosides at the dangling end is important for recognition of the siRNA by the PAZ domain protein. The inhibitory ability of siRNA **12**, which had 2-hydroxylethylphosphate at the 3′-OH of the 3′-dangling end, was not largely different from that of siRNA **11**. This result indicates that the 2-hydroxylethylphosphate moiety does not significantly influence the binding of the PAZ domain protein to the siRNA. The inhibitory effects of siRNAs **13**–**16**, with their respective analogs, on the formation of the labeled siRNA-protein complex were similar to that of siRNA **12**, which had thymidines at the 3′-dangling end. Although the inhibitory activities of siRNAs **13**–**16** were not largely different from each other, the siRNAs tended to become more

Table 2
Sequences of siRNAs used for EMSA.

Abbreviation of siRNA	Abbreviation of ON	Sequence[a]
siRNA **9**	ON **17**	Sense (passenger) strand
		F-5′-GGCCUUUCACUACUCCUAC**UU**-3′
	ON **18**	3′-CCGGAAAGUGAUGAGGAUG-5′
		Antisense (Guide) strand
siRNA **10**	ON **1**	5′-GGCCUUUCACUACUCCUAC**UU**-3′
	ON **18**	3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **11**	ON **3**	5′-GGCCUUUCACUACUCCUAC**tt**-3′
	ON **18**	3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **12**	ON **5**	5′-GGCCUUUCACUACUCCUAC**tt7**-3′
	ON **18**	3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **13**	ON **7**	5′-GGCCUUUCACUACUCCUAC**227**-3′
	ON **18**	3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **14**	ON **9**	5′-GGCCUUUCACUACUCCUAC**337**-3′
	ON **18**	3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **15**	ON **11**	5′-GGCCUUUCACUACUCCUAC**447**-3′
	ON **18**	3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **16**	ON **13**	5′-GGCCUUUCACUACUCCUAC**557**-3′
	ON **18**	3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **17**	ON **15**	5′-GGCCUUUCACUACUCCUAC**667**-3′
	ON **18**	3′-CCGGAAAGUGAUGAGGAUG-5′

[a] Small letters indicate 2′-deoxyribonucleosides. F denotes a fluorescin residue.

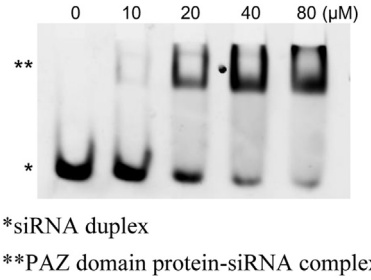


Fig. 2. EMSA to detect binding of siRNA **9** to various concentrations of the recombinant human Ago2 PAZ domain protein. Experimental conditions are described in the Experimental Section.

potent as the size of the side chains of the analogs increased. The inhibitory activity of siRNA **17**, which had the 2,2,2-trifluoroethyl group, was similar to that of siRNA **13**, which had an ethyl group.

2.5. Enzyme-linked immunosorbent assay

Furthermore, we performed ELISA to examine the ability of the human Ago2 PAZ domain to bind to modified siRNAs, the sequences of which are listed in Table 3. All siRNAs had biotin residues at the 3′-end of the antisense strands, and they were immobilized on the streptavidin-coated 96-well microplate. The human Ago2 PAZ domain protein was then added to each well. The protein, which bound to the siRNAs, was detected by an anti-His tag monoclonal antibody conjugated with horseradish peroxidase (HRP). The activity of HRP was evaluated by an oxidation reaction using 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate. The absorbance of each well at 450 nm is shown in Fig. 4. A trend similar to that noted in the EMSA was observed. The binding ability of the human Ago2 PAZ domain protein to siRNA **19**, which had thymidines at the 3′-dangling end of the sense strand, was weaker than that to siRNA **18**, which had uridines at the 3′-dangling end. Binding ability of the protein to siRNA **21**, which had an ethyl group, was slightly weaker than that to siRNA **24**, which had a 2,2-dimethylpropyl group, and was similar to that to siRNA **25**, which had a 2,2,2-trifluoroethyl group.

2.6. Nuclease resistance properties of modified RNAs

As siRNA is readily hydrolyzed by ubiquitous nucleases existing inside and outside cells, improving the nuclease resistance of siRNA is important for therapeutic applications of synthetic siRNA. Therefore, we next investigated the nuclease resistance properties of the modified ONs by using snake venom phosphodiesterase (SVPD), a 3′ exonuclease. Unmodified ONs **17** and **20** and modified ONs **21**–**24**, which were labeled with fluorescein at the 5′-ends, were incubated with SVPD. The reactions were analyzed using PAGE under denaturing conditions. As shown in Fig. 5, unmodified ONs **17** and **20** were randomly hydrolyzed after 1 min of incubation, whereas modified ONs **21**–**24** were resistant to the enzyme. The half-lives (t1/2) of unmodified ONs **17** and **20** were <1 min, while those of modified ONs **21**–**24** were 14, 12, 12, and 11 min, respectively. On the other hand, the stability of the siRNA modified with analog **5** at the 3′-dangling end in 40% bovine serum was the same as that of the unmodified siRNA (data not shown).

3. Discussion

In order to create novel siRNAs that are more potent and nuclease resistant than natural unmodified siRNAs, we designed and synthesized five types of siRNA containing the acetal-type nucleoside analogs **2**–**6** at their 3′-dangling ends. We assessed the properties of the modified siRNAs by T_m measurement, a dual-luciferase reporter assay, an EMSA, an ELISA, and a partial digestion using SVPD. The binding affinities of the human Ago2 PAZ domain protein for the modified siRNAs tended to increase with increase in the size of the side chains of the analogs, although they were apparently weaker than that for the siRNA with uridines at its 3′-

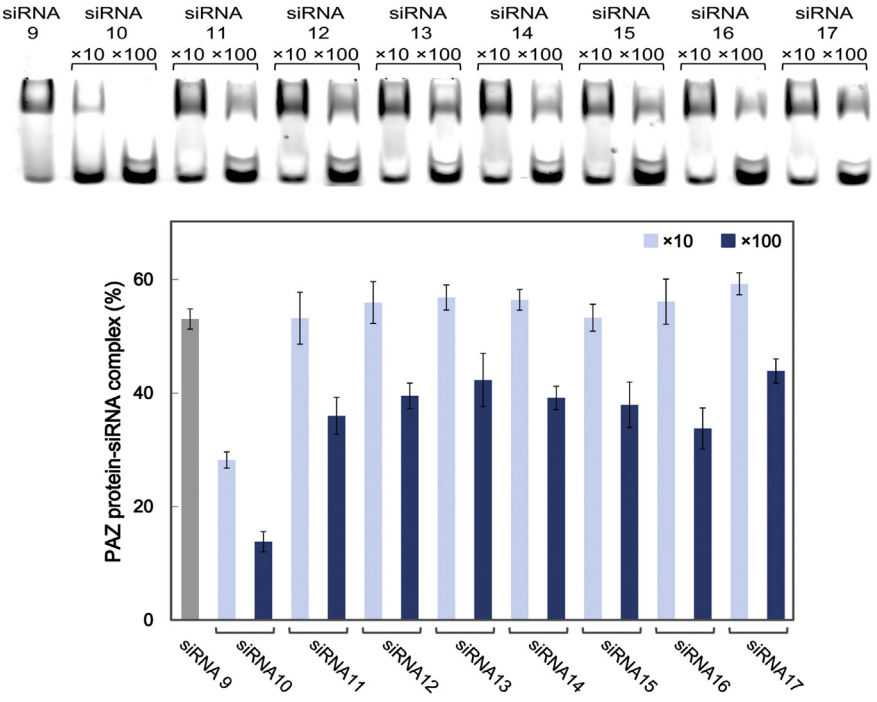


Fig. 3. EMSA in the presence of competitors (siRNAs **10**–**17**). Experimental conditions are described in the Experimental Section.

Table 3
Sequences of siRNAs used for ELISA.

Abbreviation of siRNA	Abbreviation of ON	Sequence[a]
siRNA **18**	ON **1**	Sense (passenger) strand 5′-GGCCUUUCACUACUCCUAC**UU**-3′
	ON **19**	**Bio**-3′-CCGGAAAGUGAUGAGGAUG-5′ Antisense (Guide) strand
siRNA **19**	ON **3**	5′-GGCCUUUCACUACUCCUAC**tt**-3′
	ON **19**	**Bio**-3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **20**	ON **5**	5′-GGCCUUUCACUACUCCUAC**tt7**-3′
	ON **19**	**Bio**-3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **21**	ON **7**	5′-GGCCUUUCACUACUCCUAC**227**-3′
	ON **19**	**Bio**-3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **22**	ON **9**	5′-GGCCUUUCACUACUCCUAC**337**-3′
	ON **19**	**Bio**-3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **23**	ON **11**	5′-GGCCUUUCACUACUCCUAC**447**-3′
	ON **19**	**Bio**-3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **24**	ON **13**	5′-GGCCUUUCACUACUCCUAC**557**-3′
	ON **19**	**Bio**-3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA **25**	ON **15**	5′-GGCCUUUCACUACUCCUAC**667**-3′
	ON **19**	**Bio**-3′-CCGGAAAGUGAUGAGGAUG-5′

[a] Bio denotes a biotin residue.

dangling end. On the other hand, for siRNAs **4**, **6**, and **7**, the silencing activities of the siRNAs increased with increase in the size of the side chains of the analogs, but siRNAs **5** and **8** were more potent than siRNAs **4**, **6**, and **7**, despite the sizes of the side chains of analogs **3** and **6** being smaller than those of analogs **4** and **5**. These results suggest that although the 3′-dangling-end moiety is important for enhancing the silencing activity of the siRNA, a higher binding affinity of the 3′-dangling-end moiety for the PAZ domain protein is not always positively correlated with a higher silencing efficacy of the siRNA. It was suggested by recent studies that the nucleotides of the 3′-end of the guide strand are not always anchored in the PAZ domain in the Ago protein and released from the domain when the guide strand forms a base pair with the target mRNA [11–13,21]. Thus, enhancing the dissociation of the 3′-dangling-end nucleosides from the PAZ domain might also be important for improving the silencing activity of siRNA.

We found that the silencing activity of siRNA **8**, which had a 2,2,2-trifluoroethyl group, was greater than that of siRNA **3**, which had an ethyl group, and comparable to that of siRNA **1**, which had uridines at the dangling ends, despite the size of the 2,2,2-trifluoroethyl group being similar to that of the ethyl group. It was previously reported that thermodynamic features of siRNAs influence the silencing activity of siRNAs [25–29]. The T_m value of siRNA **8**, which had a 2,2,2-trifluoroethyl group, was greater than those of siRNAs **4**–**6**, which had ethyl, propyl, and 2-methylpropyl groups, respectively. Thus, this high thermal stability of siRNA **8** might be advantageous for eliciting a high silencing activity. Furthermore, ON **24**, which had an analog containing the 2,2,2-trifluoroethyl group, was more resistant to SVPD, a 3′ exonuclease, than unmodified ONs.

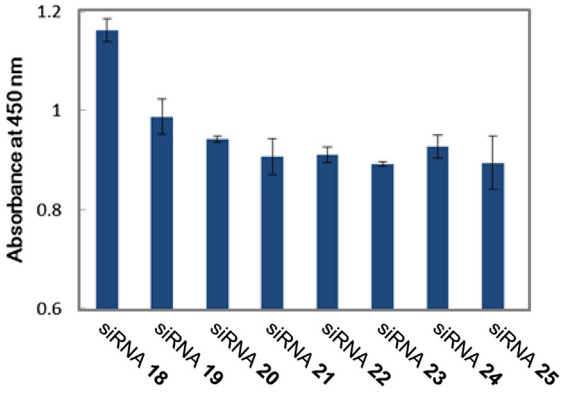


Fig. 4. ELISA to detect binding of siRNAs to the recombinant human Ago2 PAZ domain protein. Bio denotes a biotin residue. Experimental conditions are described in the Experimental Section.

4. Conclusion

We have demonstrated the synthesis of siRNAs containing acetal-type nucleoside analogs at their 3′-ends. Several groups including our group have previously reported the synthesis of siRNAs containing aromatic compounds at their 3′-ends [7,8,30–32]. Bulky compounds such as pyrene and phenanthrene decreased the silencing activities of the siRNAs owing to steric hindrance or a strong hydrophobic interaction with the PAZ domain [7,30]. Introduction of fluorine atoms into the aromatic compounds enhanced the silencing activities of the siRNAs [30,32]. The results indicate that the hydrogen bonds existing between the fluorine atoms and the amino acids in the hydrophobic pocket of the PAZ domain are favorable for eliciting RNA interference. In this study, we also found that the silencing activity of siRNA with an analog containing the 2,2,2-trifluoroethyl group was greater than that of siRNAs with analogs containing other groups and comparable to that of siRNAs with natural uridines at their dangling ends, although a significant increase in the binding affinity of siRNA containing the 2,2,2-trifluoroethyl-modified nucleoside analog to the PAZ domain was not observed by EMSA and ELISA. Furthermore, siRNA with an analog containing the 2,2,2-trifluoroethyl group at its 3′-end was more resistant to hydrolytic degradation by a 3′ exonuclease than a natural RNA was. Thus, modification of siRNAs by a nucleoside analog containing a 2,2,2-trifluoroethyl group may hold promise as a means of improving the silencing activity and nuclease resistance of siRNAs.

4.1. Experimental Section

4.1.1. General remarks

$CDCl_3$ (CIL) or DMSO-d6 (CIL) was used as a solvent for obtaining NMR spectra. Chemical shifts (δ) are given in parts per million (ppm) downfield from $(CH_3)_4Si$ (δ 0.00 for ^1H NMR in $CDCl_3$), or a solvent (for ^{13}C NMR and ^1H NMR in DMSO-d6) as an internal reference with coupling constants (J) in Hz. The abbreviations s, d,

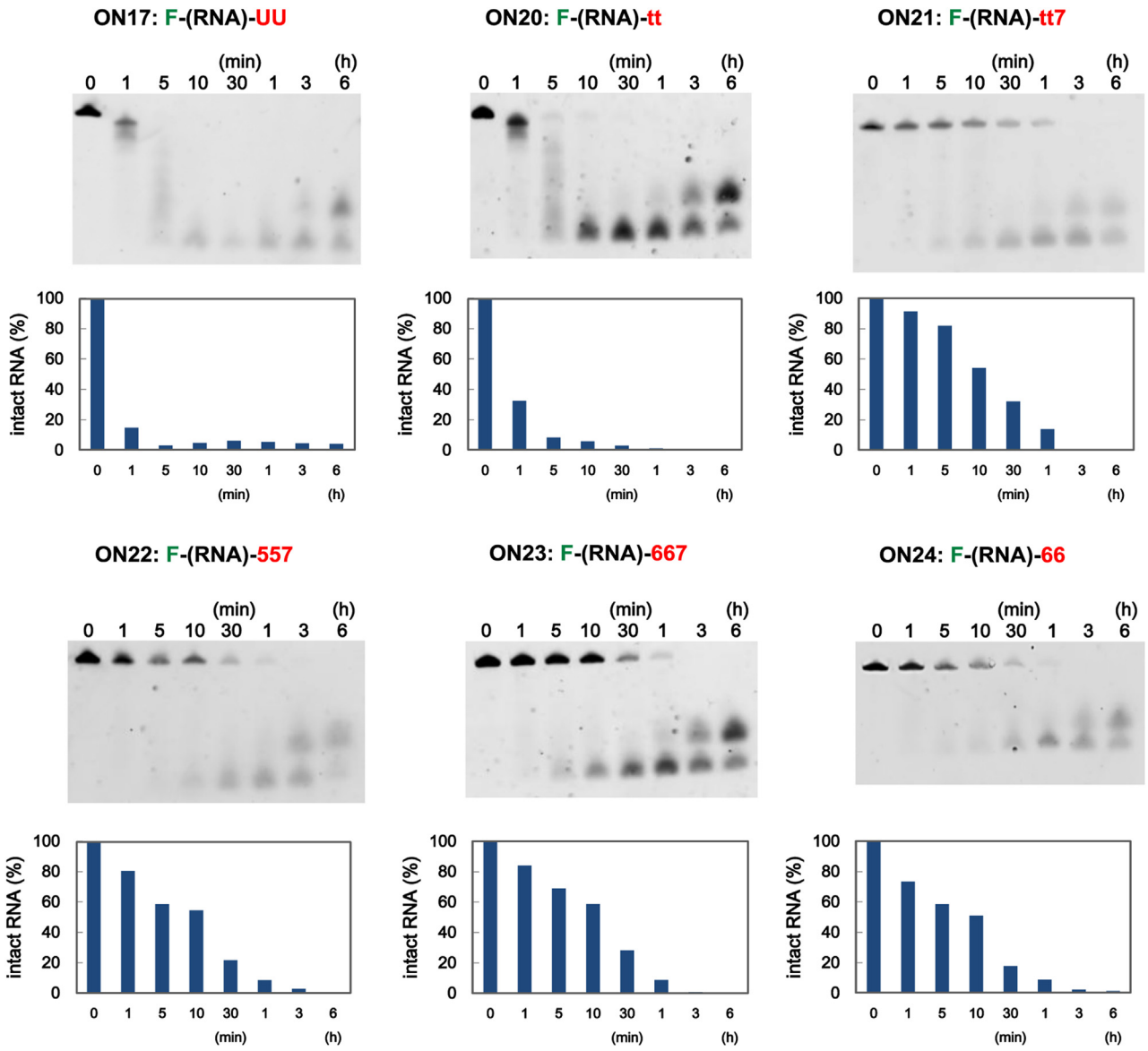


Fig. 5. 20% PAGE of ONs hydrolyzed by SVPD. F denotes a fluorescin. The sequence of the RNA part is 5′-GGCCUUUCACUACUCCUAC-3′. Experimental conditions are described in the Experimental Section.

and q signify singlet, doublet, and quartet, respectively.

*4.1.2. 2,3,5-O-Tribenzoyl-1-O-ethyl-β-D-ribofuranose (**14**)*

To a solution of 1-*O*-acethyl-2,3,5-*O*-tribenzoyl-β-D-ribofuranose (3.00 g, 5.95 mmol) in CH_2Cl_2 (30 mL) was added dropwise TMSOTf (1.29 mL, 7.14 mmol) and ethanol (0.35 mL, 5.95 mmol) at −30 °C. The mixture was stirred at −30 °C for 1.5 h and quenched by using aqueous $NaHCO_3$ (saturated). The mixture was partitioned between $CHCl_3$ and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 20% EtOAc in hexane) to give **14** (2.91 g, 5.94 mmol, 100%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.18 (t, 3H, J = 7.1), 3.54 (dq, 1H, J = 6.8 and 8.5), 3.84 (dq, 1H, J = 6.9 and 11.9), 4.52 (dd, 1H, J = 6.4 and 12.8), 4.66–4.74 (m, 2H), 5.26 (s, 1H), 5.67 (d, 1H, J = 4.6), 5.88 (dd, 1H, J = 5.0 and 6.4), 7.25–8.11 (m, 15H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 15.0, 64.0, 65.0, 72.7, 75.8, 78.9, 105.4, 128.4, 128.6, 129.1, 129.4, 129.8, 129.9, 133.2, 133.4, 133.5, 165.4, 165.5, 166.3. Anal. Calcd for $C_{28}H_{26}O_8 \cdot 7/10H_2O$: C, 66.84; H, 5.49. Found: C, 66.86; H, 5.19.

*4.1.3. 1-O-Ethyl-β-D-ribofuranose (**2**)*

To a stirred solution of **14** (3.05 g, 6.22 mmol) in MeOH (30 mL) was added a catalytic amount of a 28% MeOH solution of sodium methoxide at room temperature. The mixture was stirred at room temperature for 12 h and quenched by using aqueous NH_4Cl (saturated) (1 mL). The solvent was evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, 9% MeOH in $CHCl_3$) to give **2** (1.11 g, 6.22 mmol, 100%): ^{1}H NMR (400 MHz, $CDCl_3$) 1.20 (t, 3H, J = 7.1), 2.31 (s, 1H), 3.12 (s, 1H), 3.52 (dq, 1H, J = 7.4 and 8.3), 3.67 (dd, 1H, J = 5.0 and 11.4), 3.73–3.79 (m, 3H), 4.03–4.06 (m, 2H), 4.30 (d, 1H, J = 5.5) 4.95 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 15.2, 63.3, 64.3, 71.4, 75.7, 84.3, 107.4. Anal. Calcd for $C_7H_{14}O_5 \cdot 3/5H_2O$: C, 44.49; H, 8.11. Found: C, 44.56; H, 8.02.

*4.1.4. 5-O-(4,4′-Dimethoxytrityl)-1-O-ethyl-β-D-ribofuranose (**19**)*

To a solution of **2** (0.97 g, 5.43 mmol) in pyridine (10 mL) was added DMTrCl (2.21 g, 6.52 mmol) at room temperature. The mixture was stirred at room temperature for 5 h and 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% EtOAc in hexane) to give **19** (2.01 g, 4.19 mmol, 77%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.11 (t, 3H, J = 6.9), 2.26 (d, 1H, J = 5.5), 2.48 (d, 1H, J = 3.2), 3.27 (dd, 2H, J = 3.2 and 5.5), 3.44 (dq, 1H, J = 6.9 and 8.3), 3.73 (dq, 1H, J = 7.3 and 8.2), 3.80 (s, 6H), 4.04–4.08 (m, 2H), 4.27 (dd, 1H, *J* = 5.28 and 11.2), 4.96 (s, 1H), 6.81–7.48 (m, 13H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 15.0, 55.2, 63.4, 65.0, 72.9, 75.4, 81.9, 86.1, 106.9, 113.1, 126.8, 127.8, 128.2, 130.0, 136.1, 144.8, 158.5. Anal. Calcd for $C_{28}H_{32}O_7 \cdot 9/10H_2O$: C, 67.70; H, 6.86. Found: C, 67.50; H, 6.56.

4.1.5. 3-O-(Tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-ethyl-β-D-ribofuranose (**24**) and 2-O-(tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-ethyl-β-D-ribofuranose (**25**)

To a stirred solution of **19** (1.39 g, 2.89 mmol) and Et_3N (1.21 mL, 8.67 mmol) in DMF (8 mL) was added dropwise TBDMSCl (0.87 g, 5.78 mmol) in DMF (6 mL) at room temperature. The mixture was stirred at room temperature for 12 h and 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, 20% EtOAc in hexane) to give **24** (0.73 g, 1.22 mmol, 42%) and **25** (0.56 g, 0.94 mmol, 33%). Physical data of **24**: ^{1}H NMR (400 MHz, $CDCl_3$) δ −0.13 (s, 3H), −0.00 (s, 3H), 0.81 (s, 9H), 1.15 (t, 3H, J = 7.1), 2.73 (d, 1H, J = 2.3), 3.03 (dd, 1H, J = 5.3 and 10.3), 3.31 (d, 1H, J = 10.1), 3.53 (dd, 1H, J = 7.4 and 16.5), 3.78–3.89 (m, 7H), 3.89 (s, 1H), 4.05 (s, 1H), 4.33 (dd, 1H, J = 6.4 and 11.0), 5.03 (s, 1H), 6.80–7.51 (m, 13H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −4.85, −4.82, 15.2, 18.0, 25.7, 55.3, 63.6, 64.0, 72.5, 75.5, 82.6, 85.9, 107.0, 113.1, 126.7, 127.8, 128.4, 130.2, 136.3, 136.4, 145.0, 158.5. Anal. Calcd for $C_{34}H_{46}O_7Si \cdot 1/2H_2O$: C, 67.63; H, 7.85. Found: C, 67.43; H, 7.72. Physical data of **25**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.14 (t, 6H, J = 2.3), 0.93 (s, 9H), 1.15 (t, 3H, *J* = 7.1), 2.49 (d, 1H, J = 7.4), 3.10 (dd, 1H, J = 5.0 and 9.9), 3.31 (dd, 1H, J = 3.2 and 10.1), 3.48 (dq, 1H, J = 6.8 and 11.9), 3.78–3.86 (m, 7H), 4.07–4.10 (m, 2H), 4.13 (d, 1H, J = 1.8), 4.90 (s, 1H), 6.80–7.51 (m, 13H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −4.9, −4.5, 0.1, 15.2, 18.2, 25.8, 55.3, 63.8, 64.9, 72.4, 76.7, 83.5, 86.0, 107.4, 113.1, 126.7, 127.8, 128.4, 130.2, 136.3, 136.4, 145.1, 158.5. Anal. Calcd for $C_{34}H_{46}O_7Si \cdot 1.0H_2O$: C, 66.64; H, 7.90. Found: C, 66.61; H, 7.61.

4.1.6. 2-O-(Tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-ethyl-3-O-[(2-cyanoethoxy)(N,N-diisopropyamino)]phosphanyl-β-D-ribofuranose (**34**)

To a solution of **25** (0.74 g, 1.25 mmol) in THF (8 mL) was added N,N-diisopropylethylamine (1.09 mL, 6.25 mmol) and chloro(2-cyanoethoxy)(*N*,N-diisopropylamino)phosphine (0.56 mL, 2.50 mmol) at room temperature. The mixture was stirred at room temperature for 1 h and 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, 33% EtOAc in hexane) to give **34** (0.64 g, 0.80 mmol, 64%): ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.1, 149.7.

4.1.7. 2,3,5-O-Tribenzoyl-1-O-propyl-β-D-ribofuranose (**15**)

To a solution of 1-*O*-acethyl-2,3,5-*O*-tribenzoyl-β-D-ribofuranose (3.00 g, 5.95 mmol) in CH_2Cl_2 (30 mL) was added dropwise TMSOTf (1.29 mL, 7.14 mmol) and 1-propanol (0.53 mL, 7.14 mmol) at −30 °C. The mixture was stirred at −30 °C for 30 min and quenched by using aqueous $NaHCO_3$ (saturated). The mixture was partitioned between $CHCl_3$ and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 20% EtOAc in hexane) to give **15** (2.94 g, 5.83 mmol, 98%): ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.90 (t, 3H, *J* = 7.6), 1.55–1.59 (m, 2H), 3.42 (dd, 1H, J = 6.9 and 15.8), 3.74 (dd, 1H, J = 6.8 and 14.4), 4.52–4.54 (m, 1H), 4.69–4.72 (m, 2H), 5.25 (s, 1H), 5.68 (d, 1H, J = 4.8), 5.87 (dd, 1H, J = 2.8 and 5.0), 7.31–8.07 (m, 15H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 10.5, 14.2, 21.0, 22.6, 60.4, 65.1, 70.1, 72.7, 75.6, 78.7, 105.5, 128.3, 128.5, 129.0, 129.3, 129.7, 129.8, 129.8, 133.1, 133.3, 133.4. Anal. Calcd for $C_{29}H_{28}O_8$: C, 69.04; H, 5.59. Found: C, 68.80; H, 5.58.

4.1.8. 1-O-Propyl-β-D-ribofuranose (**3**)

To a stirred solution of **15** (2.87 g, 5.69 mmol) in MeOH (29 mL) was added a catalytic amount of a 28% MeOH solution of sodium methoxide at room temperature. The mixture was stirred at room temperature for 12 h and quenched by using aqueous NH_4Cl (saturated) (1 mL). The solvent was evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, 9% MeOH in $CHCl_3$) to give **3** (1.09 g, 5.69 mmol, 100%): ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.92 (t, 3H, J = 7.6), 1.57–1.63 (m, 2H), 2.42 (dd, 1H, *J* = 3.8 and 8.6), 2.77 (d, 1H, J = 6.2), 2.91 (d, 1H, J = 3.4), 3.42 (dt, 1H, J = 6.9 and 9.6), 3.65–3.70 (m, 2H), 3.81 (dd, 1H, J = 8.2 and 11.7), 4.07–4.09 (m, 2H), 4.39 (d, 1H, J = 5.5), 4.96 (s, 1H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 10.5, 22.8, 63.5, 70.2, 71.6, 75.5, 83.8, 107.4. Anal. Calcd for $C_8H_{16}O_5 \cdot 1/2H_2O$: C, 47.75; H, 8.52. Found: C, 47.66; H, 8.33.

4.1.9. 5-O-(4,4′-Dimethoxy)trityl-1-O-propyl-β-D-ribofuranose (**20**)

To a solution of **3** (0.65 g, 3.38 mmol) in pyridine (7 mL) was added DMTrCl (1.58 g, 4.06 mmol) at room temperature. The mixture was stirred at room temperature for 1 h and 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, 33% EtOAc in hexane) to give **20** (1.35 g, 2.80 mmol, 83%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.73 (t, 3H, *J* = 7.5), 1.37–1.44 (m, 2H), 2.31 (d, 1H, J = 5.7), 2.56 (d, 1H, J = 3.5), 3.15 (dd, 1H, J = 5.7 and 9.8), 3.19–3.26 (m, 2H), 3.53 (dt, 1H, J = 6.9 and 9.8), 3.70 (s, 6H), 3.95–4.03 (m, 2H), 4.16 (dd, 1H, J = 5.7 and 10.9), 4.86 (s, 1H), 6.73–7.39 (m, 13H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 10.6, 22.8, 55.3, 65.3, 69.7, 73.2, 75.4, 82.0, 86.2, 107.2, 113.2, 126.9, 127.9, 128.2, 130.1, 130.2, 136.1, 136.2, 144.9, 158.6. Anal. Calcd for $C2_8H_{32}O_7 \cdot 4/5H_2O$: C, 68.43; H, 7.05. Found: C, 68.14; H, 6.83.

4.1.10. 3-O-(Tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-propyl-β-D-ribofuranose (**26**) and 2-O-(tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-propyl-β-D-ribofuranose (**27**)

To a stirred solution of **20** (1.70 g, 3.44 mmol) and Et_3N (1.40 mL, 10.32 mmol) in DMF (20 mL) was added dropwise TBDMSCl (1.04 g, 6.88 mmol) in DMF (15 mL) at room temperature. The mixture was stirred at room temperature for 12 h and 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, 20–25% EtOAc in hexane) to give **26** (0.90 g, 1.48 mmol, 43%) and **27** (0.77 g, 1.27 mmol, 37%). Physical data of **26**: ^{1}H NMR (400 MHz, $CDCl_3$) δ −0.13 (s, 3H), −0.00 (s, 3H), 0.81 (s, 9H), 2.74 (d, 1H, J = 2.3), 3.03 (dd, 1H, J = 5.0 and 10.1), 3.29 (dd, 1H, J = 3.2 and 10.1), 3.40 (dt, 1H, *J* = 6.4 and 9.2), 3.73 (dt, 1H, J = 6.9 and 9.6), 3.78 (s, 6H), 3.90 (s, 1H), 4.06–4.07 (m, 1H), 4.31 (dd, 1H, J = 4.6 and 6.4), 5.02 (s, 1H), 6.80–7.51 (m, 13H); ^{13}C NMR (151 MHz, $CDCl_3$) δ −5.0, −4.9, 10.5, 17.9, 22.7, 25.6, 55.2, 64.1, 69.8, 72.5, 75.4, 82.6, 85.8, 107.2, 113.0, 126.6, 127.7, 128.3, 130.1, 136.2, 136.2, 144.9, 158.4. Anal. Calcd for $C_{35}H_{48}O_7Si \cdot 1/2H_2O$: C, 68.04; H, 7.99. Found: C, 67.82; H, 7.76. Physical data of **27**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.12 (t, 6H, J = 7.2), 0.85 (t, 3H, J = 7.6), 0.91 (s, 9H), 1.49–1.55 (m, 2H), 2.50 (d, 1H, J = 6.9), 3.07 (dd, 1H, J = 4.8 and 10.3), 3.28 (dd, 1H, J = 3.4 and 10.3), 3.34 (dt, 1H, J = 6.8 and 9.6), 3.69 (dt, 1H, J = 6.2 and 8.9), 3.76 (s,

6H), 4.01–4.07 (m, 2H), 4.13 (dd, 1H, J = 2.0 and 4.8), 4.88 (s, 1H), 6.78–7.49 (m, 13H); ^{13}C NMR (151 MHz, $CDCl_3$) δ −0.5, −0.1, 15.1, 22.6, 27.3, 30.3, 59.7, 69.4, 74.5, 77.0, 81.1, 88.0, 90.5, 112.1, 117.5, 131.1, 132.2, 132.8, 134.6, 134.7, 140.7, 140.8, 149.5, 162.9. Anal. Calcd for $C_{35}H_{48}O_7Si \cdot 4/5H_2O$: C, 67.45; H, 8.02. Found: C, 67.33; H, 7.80.

4.1.11. 2-O-(Tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-propyl-3-O-[(2-cyanoethoxy)(N,N-diisopropyamino)] phosphanyl-β-D-ribofuranose (**35**)

To a solution of **27** (0.62 g, 1.03 mmol) in THF (6.5 mL) was added N,N-diisopropylethylamine (0.90 mL, 5.15 mmol) and chloro(2-cyanoethoxy)(*N*,N-diisopropylamino)phosphine (0.46 mL, 2.06 mmol) at room temperature. The mixture was stirred at room temperature for 1 h and 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, 25% EtOAc in hexane) to give **35** (0.59 g, 0.73 mmol, 71%): ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.4, 149.8.

4.1.12. 2,3,5-O-Tribenzoyl-1-O-(2-methylpropyl)-β-D-ribofuranose (**16**)

To a solution of 1-*O*-acethyl-2,3,5-*O*-tribenzoyl-β-D-ribofuranose (2.00 g, 3.97 mmol) in CH_2Cl_2 (20 mL) was added dropwise TMSOTf (0.86 mL, 4.76 mmol) and 2-methyl-1-propanol (0.37 mL, 3.97 mmol) at −30 °C. The mixture was stirred at −30 °C for 2.5 h and quenched by using aqueous $NaHCO_3$ (saturated). The mixture was partitioned between $CHCl_3$ and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 20% EtOAc in hexane) to give **16** (2.02 g, 3.90 mmol, 98%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87–0.92 (m, 6H), 1.80–1.87 (m, 1H), 3.21 (dd, 1H, J = 6.9 and 9.2), 3.58 (dd, 1H, J = 6.4 and 9.2), 4.53 (dd, 1H, J = 5.3 and 11.2), 4.66–4.73 (m, 2H), 5.24 (s, 1H), 5.69 (d, 1H, J = 4.6), 5.85 (dd, 1H, J = 4.6 and 6.9), 7.25–8.12 (m, 15H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 19.2, 19.3, 28.3, 64.1, 65.2, 70.9, 71.9, 72.8, 75.1, 75.6, 78.7, 101.0, 105.7, 128.3, 128.3, 128.5, 129.0, 129.3, 129.7, 129.8, 133.1, 133.3, 133.4, 165.3, 165.4, 166.2. Anal. Calcd for $C_{30}H_{30}O_8 \cdot 7/10H_2O$: C, 67.84; H, 5.96. Found: C, 67.59; H, 5.73.

4.1.13. 1-O-(2-Methylpropyl)-β-D-ribofuranose (**4**)

To a stirred solution of **16** (2.02 g, 3.90 mmol) in MeOH (15 mL) was added a catalytic amount of a 28% MeOH solution of sodium methoxide at room temperature. The mixture was stirred at room temperature for 12 h and quenched by using aqueous NH_4Cl (saturated) (1 mL). The solvent was evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, 9% MeOH in $CHCl_3$) to give **4** (0.63 g, 3.06 mmol, 79%): ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.89 (d, 6H, J = 8.0), 1.79–1.90 (m, 1H), 2.24 (d, 1H, J = 3.7), 2.48 (d, 1H, J = 6.4), 2.67 (d, 1H, *J* = 3.2), 3.21 (dd, 1H, J = 6.4 and 9.2), 3.51 (dd, 1H, J = 6.8 and 9.2), 3.64–3.70 (m, 1H), 3.79–3.84 (m, 1H), 4.07–4.11 (m, 2H), 4.42 (d, 1H, J = 5.5), 4.95 (s, 1H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 19.2, 28.4, 62.9, 71.2, 75.5, 75.8, 84.4, 107.8. Anal. Calcd for $C_9H_{18}O_5$: C, 52.41; H, 8.80. Found: C, 52.12; H, 8.78.

4.1.14. 5-O-(4,4′-Dimethoxytrityl)-1-O-(2-methylpropyl)-β-D-ribofuranose (**21**)

To a solution of **4** (0.23 g, 1.12 mmol) in pyridine (5 mL) was added DMTrCl (0.38 g, 1.12 mmol) at room temperature. The mixture was stirred at room temperature for 1 h and 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, 33% EtOAc in hexane) to give **21** (0.48 g, 0.94 mmol, 84%): ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.77 (d, 3H, J = 6.9), 0.81 (d, 3H, J = 6.4), 1.69–1.76 (m, 1H), 2.29 (d, 1H, *J* = 5.5), 2.49 (d, 1H, J = 3.7), 3.11 (dd, 1H, J = 6.9 and 8.7), 3.20 (dd, 1H, J = 5.5 and 9.2), 3.32 (dd, 1H, *J* = 5.5 and 9.6), 3.41 (dd, 1H, J = 6.9 and 9.2), 3.79 (s, 6H), 4.05–4.10 (m, 2H), 4.23 (dd, 1H, J = 5.48 and 11.0), 4.93 (s, 1H), 6.81–7.47 (m, 13H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 19.2, 28.2, 55.2, 65.4, 73.3, 74.6, 75.3, 81.8, 86.2, 107.2, 113.1, 126.8, 127.8, 128.1, 130.0, 130.1, 136.0, 136.1, 144.8, 158.5. Anal. Calcd for $C_{30}H_{36}O_7 \cdot 1/2H_2O$: C, 69.61; H, 7.21. Found: C, 69.39; H, 7.19.

4.1.15. 3-O-(Tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-(2-methylpropyl)-β-D-ribofuranose (**28**) and 2-O-(tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-(2-methylpropyl)-β-D-ribofuranose (**29**)

To a stirred solution of **21** (1.29 g, 2.54 mmol) and Et_3N (1.10 mL, 7.62 mmol) in DMF (12 mL) was added dropwise TBDMSCl (0.77 g, 5.08 mmol) in DMF (3 mL) at room temperature. The mixture was stirred at room temperature for 12 h and 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, 11% EtOAc in hexane) to give **28** (0.53 g, 0.85 mmol, 34%) and **29** (0.53 g, 0.85 mmol, 34%). Physical data of **28**: ^{1}H NMR (400 MHz, $CDCl_3$) δ −0.12 (s, 3H), 0.00 (s, 3H), 0.82 (s, 9H), 1.74–1.85 (m, 1H), 2.73 (d, 1H, J = 2.3), 3.04 (dd, 1H, J = 5.5 and 10.1), 3.19 (dd, 1H, J = 8.0 and 9.2), 3.27 (dd, 1H, J = 3.2 and 10.1), 3.53 (dd, 1H, *J* = 6.9 and 9.2), 3.78 (s, 6H), 3.90 (dd, 1H, J = 2.7 and 4.6), 4.05–4.09 (m, 1H), 4.26 (dd, 1H, *J* = 4.6 and 6.0), 4.99 (s, 1H), 6.80–7.51 (m, 13H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −4.9, −4.8, 18.0, 19.4, 19.4, 25.7, 28.3, 55.3, 64.4, 72.7, 74.9, 75.5, 82.7, 86.0, 107.5, 113.1, 126.7, 127.8, 128.4, 130.2, 136.3, 145.0, 158.5. Anal. Calcd for $C_{36}H_{50}O_7 \cdot 1/2H_2O$: C, 68.43; H, 8.14. Found: C, 68.46; H, 7.99. Physical data of **29**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.03 (s, 3H), 0.04 (s, 3H), 0.72 (d, 3H, J = 6.4), 0.77 (d, 3H, J = 6.9), 0.82 (s, 9H), 1.68–1.71 (m, 1H), 2.43 (d, 1H, *J* = 6.8), 2.98 (dd, 1H, J = 5.0 and 10.1), 3.04 (dd, 1H, J = 6.9 and 16.0), 3.19 (dd, 1H, J = 3.7 and 10.1), 3.44 (dd, 1H, J = 6.4 and 15.6), 3.67 (s, 6H), 3.91 (dd, 1H, *J* = 4.8 and 11.7), 3.98 (d, 1H, J = 4.1), 4.06 (s, 1H), 4.78 (s, 1H), 6.69–7.40 (m, 13H). ^{13}C NMR (126 MHz, $CDCl_3$) δ −4.9, −4.5, 18.2, 19.4, 19.5, 25.8, 28.5, 55.3, 65.1, 72.7, 75.2, 76.7, 83.6, 86.1, 107.9, 113.1, 126.7, 127.8, 128.4, 130.2, 130.2, 136.3, 136.4, 145.1, 158.5. Anal. Calcd for $C_{36}H_{50}O_7 \cdot 1/2H_2O$: C, 68.43; H, 8.14. Found: C, 68.39; H, 8.29.

4.1.16. 2-O-(Tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-(2-methylpropyl)-3-O-[(2-cyanoethoxy)(N,N-diisopropyamino)] phosphanyl-β-D-ribofuranose (**36**)

To a solution of **29** (0.53 g, 0.85 mmol) in THF (5.5 mL) was added N,N-diisopropylethylamine (0.74 mL, 4.25 mmol) and chloro(2-cyanoethoxy)(*N*,N-diisopropylamino)phosphine (0.38 mL, 1.70 mmol) at room temperature. The mixture was stirred at room temperature for 1 h and 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, 33% EtOAc in hexane) to give **36** (0.54 g, 0.66 mmol, 78%): ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.4, 149.6.

4.1.17. 2,3,5-O-Tribenzoyl-1-O-(2,2-dimethylpropyl)-β-D-ribofuranose (**17**)

To a solution of 1-*O*-acethyl-2,3,5-*O*-tribenzoyl-β-D-ribofuranose (3.00 g, 5.95 mmol) in CH_2Cl_2 (25 mL) was added dropwise TMSOTf (1.29 mL, 7.14 mmol) and 2,2-dimethyl-1-propanol (0.52 g, 5.95 mmol) in CH_2Cl_2 (5 mL) at −30 °C. The mixture was stirred at −30 °C for 3.5 h and quenched by using aqueous $NaHCO_3$ (saturated). The mixture was partitioned between $CHCl_3$ and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated)

and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 20% EtOAc in hexane) to give **17** (2.84 g, 5.33 mmol, 90%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (s, 9H), 3.10 (d, 1H, *J* = 9.2), 3.52 (d, 1H, J = 9.2), 4.55 (dd, 1H, J = 6.0 and 11.4), 4.65 (dd, *J* = 4.6 and 11.4), 4.73 (dd, 1H, J = 6.9 and 11.5), 5.24 (s, 1H), 5.71 (d, 1H, *J* = 5.0), 5.82 (dd, 1H, J = 5.0 and 6.8), 7.28–8.12 (m, 15H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −4.9, −4.8, 18.0, 19.4, 19.4, 25.7, 28.3, 55.3, 64.4, 72.7, 74.9, 75.5, 82.7, 86.0, 107.5, 113.1, 126.7, 127.8, 128.4, 130.2, 136.3, 145.0, 158.5. Anal. Calcd for $C_{31}H_{32}O_8 \cdot 1/2H_2O$: C, 68.75; H, 6.14. Found: C, 68.66; H, 5.89.

4.1.18. 1-O-(2,2-Dimethylpropyl)-β-D-ribofuranose (**5**)

To a stirred solution of **17** (2.25 g, 4.23 mmol) in MeOH (23 mL) was added a catalytic amount of a 28% MeOH solution of sodium methoxide at room temperature. The mixture was stirred at room temperature for 12 h and quenched by using aqueous NH_4Cl (saturated) (1 mL). The solvent was evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, 9% MeOH in $CHCl_3$) to give **5** (0.70 g, 3.18 mmol, 75%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (s, 9H), 2.18 (s, 1H), 2.41 (s, 1H), 2.61 (s, 1H), 3.05 (d, 1H, J = 7.8), 3.44 (d, 1H, J = 8.7), 3.67 (m, 1H), 3.80–3.83 (m, 1H), 4.10 (m, 2H), 4.43 (s, 1H), 4.94 (s, 1H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 26.5, 31.3, 63.6, 71.3, 74.4, 76.7, 83.4, 107.2. Anal. Calcd for $C_{10}H_{20}O_5$: C, 54.53; H, 9.15. Found: C, 54.42; H, 9.14.

4.1.19. 5-O-(4,4′-Dimethoxytrityl)-1-O-(2,2-dimethylpropyl)-β-D-ribofuranose (**22**)

To a solution of **5** (0.38 g, 1.74 mmol) in pyridine (5 mL) was added DMTrCl (0.71 g, 2.09 mmol) at room temperature. The mixture was stirred at room temperature for 1 h and 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% EtOAc in hexane) to give **22** (0.90 g, 1.72 mmol, 99%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.77 (s, 9H), 2.27 (d, 1H, J = 5.5), 2.50 (s, 1H), 2.93 (d, 1H, *J* = 8.7), 3.17 (dd, 1H, J = 6.0 and 9.2), 3.30–3.35 (m, 2H), 3.78 (s, 6H), 4.06–4.12 (m, 2H), 4.19 (t, 1H, *J* = 5.26), 4.91 (s, 1H), 6.81–7.47 (m, 13H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 26.5, 31.5, 55.2, 65.7, 73.7, 75.3, 78.1, 81.7, 86.2, 107.5, 113.1, 126.8, 127.8, 128.1, 130.0, 130.0, 136.0, 136.1, 144.8, 158.5. HRMS (ESI-TOF) *m*/*z*: Calcd for $C_{31}H_{38}O_7$ $[M + Na]^+$ 545.2515; Found 545.2509.

4.1.20. 3-O-(Tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-(2,2-dimethylpropyl)-β-D-ribofuranose (**30**) and 2-O-(tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-(2,2-dimethylpropyl)-β-D-ribofuranose (**31**)

To a stirred solution of **22** (0.92 g, 1.76 mmol) and Et_3N (0.74 mL, 5.28 mmol) in DMF (6 mL) was added dropwise TBDMSCl (0.53 g, 3.52 mmol) in DMF (4 mL) at room temperature. The mixture was stirred at room temperature for 12 h and 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, 17% EtOAc in hexane) to give **30** (0.32 g, 0.51 mmol, 29%) and **31** (0.40 g, 0.63 mmol, 36%). Physical data of **30**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.10 (s, 3H), 0.01 (s, 3H), 0.82 (s, 9H), 0.82 (s, 9H), 2.73 (d, 1H, *J* = 2.3), 3.02–3.06 (m, 2H), 3.23 (dd, 1H, J = 3.2 and 10.1), 3.44 (d, 1H, J = 9.2), 3.78 (s, 6H), 3.90 (s, 1H), 4.08 (d, 1H, *J* = 3.2), 4.24 (dd, 1H, J = 5.0 and 6.4), 4.97 (s, 1H), 6.79–7.50 (m, 13H); ^{13}C NMR (151 MHz, $CDCl_3$) δ −5.0, −4.9, 17.9, 25.6, 26.6, 31.7, 55.2, 64.7, 72.8, 75.4, 78.4, 82.6, 85.9, 108.0, 113.0, 126.6, 127.7, 128.2, 130.1, 136.2, 144.9, 158.4. Anal. Calcd for $C_{37}H_{52}O_7Si \cdot 1/2H_2O$: C, 68.80; H, 8.27. Found: C, 68.93; H, 8.20. Physical data of **31**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.14 (d, 6H, J = 3.2), 0.85 (s, 9H), 0.92 (d, 9H, *J* = 3.2), 2.56 (d, 1H, J = 6.9), 3.01 (d, 1H, J = 9.2), 3.09 (dd, 1H, *J* = 5.0 and 10.1), 3.28 (dd, 1H, J = 3.9 and 9.8), 3.44 (d, 1H, J = 8.7), 3.78 (s, 6H), 3.99 (dd, 1H, J = 4.6 and 11.5), 4.10 (d, 1H, *J* = 4.1), 4.17 (dd, 1H, J = 2.3 and 5.0), 4.88 (s, 1H), 6.80–7.51 (m, 13H); ^{13}C NMR (151 MHz, $CDCl_3$) δ −6.8, −6.4, −5.4, 16.3, 23.8, 23.9, 24.8, 29.9, 53.3, 63.2, 70.9, 74.8, 77.0, 81.7, 84.1, 106.4, 111.2, 124.8, 125.9, 126.4, 128.3, 128.3, 134.4, 134.4, 143.2, 156.5. Anal. Calcd for $C_{37}H_{52}O_7Si \cdot 4/5H_2O$: C, 68.27; H, 8.30. Found: C, 68.09; H, 8.35.

4.1.21. 2-O-(Tert-butyldimethyl)silyl-5-O-(4,4′-dimethoxy)trityl-1-O-(2,2-dimethyl)propyl-3-O-[(2-cyanoethoxy)(N,N-diisopropyamino)]phosphanyl-β-D-ribofuranose (**37**)

To a solution of **31** (0.57 g, 0.90 mmol) in THF (6.0 mL) was added N,*N*-diisopropylethylamine (0.79 mL, 4.50 mmol) and chloro(2-cyanoethoxy)(N,N-diisopropylamino)phosphine (0.40 mL, 1.80 mmol) at room temperature. The mixture was stirred at room temperature for 1 h and 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, 25% EtOAc in hexane) to give **37** (0.63 g, 0.75 mmol, 83%): ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.2, 149.5.

4.1.22. 2,3,5-Tri-O-benzoyl-1-O-(2,2,2-trifluoroethyl)-β-D-ribofuranose (**18**)

To a solution of 1-*O*-acethyl-2,3,5-*O*-tribenzoyl-β-D-ribofuranose (2.00 g, 3.97 mmol) in CH_2Cl_2 (20 mL) was added dropwise TMSOTf (0.86 mL, 4.76 mmol) and 2,2,2-trifluoroethanol (0.28 mL, 3.97 mmol) at −30 °C. The mixture was stirred at −30 °C for 1.5 h and quenched by using aqueous $NaHCO_3$ (saturated). The mixture was partitioned between $CHCl_3$ and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 20% EtOAc in hexane) to give **18** (2.16 g, 3.97 mmol, 100%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.90–4.15 (m, 2H), 4.56 (dd, 1H, *J* = 5.5 and 11.9), 4.70 (dd, 1H, J = 4.2 and 12.4), 4.78 (dd, 1H, J = 5.5 and 11.0), 5.37 (s, 1H), 5.78 (d, 1H, J = 5.0), 5.88 (dd, 1H, *J* = 5.0 and 7.4), 7.30–8.07 (m, 15H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 64.5, 64.6, 64.9, 71.9, 75.3, 79.9, 105.6, 128.5, 128.5, 128.6, 129.6, 129.8, 129.8, 129.9, 133.4, 133.6, 133.7, 165.2, 165.4, 166.2. Anal. Calcd for $C_{28}H_{23}F_3O_8 \cdot 1/10H_2O$: C, 61.56; H, 4.28. Found: C, 61.38; H, 4.24.

4.1.23. 1-O-(2,2,2-Trifluoroethyl)-β-D-ribofuranose (**6**)

To a stirred solution of **18** (2.00 g, 3.68 mmol) in MeOH (20 mL) was added a catalytic amount of a 28% MeOH solution of sodium methoxide at room temperature. The mixture was stirred at room temperature for 12 h and quenched by using aqueous NH_4Cl (saturated) (1 mL). The solvent was evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, 9% MeOH in $CHCl_3$) to give **6** (0.51 g, 2.19 mmol, 60%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.79 (dd, 1H, *J* = 4.6 and 7.8), 2.30 (d, 1H, J = 6.9), 2.60 (d, 1H, J = 3.7), 3.69–3.75 (m, 1H), 3.81–3.94 (m, 2H), 3.97–4.03 (m, 1H), 4.10–4.18 (m, 1H), 4.18 (d, 1H, *J* = 4.1), 4.40 (dd, 1H, J = 6.4 and 11.4), 5.06 (s, 1H); ^{13}C NMR (126 MHz, DMSO-D6) δ: 63.1, 63.3, 63.5, 71.0, 74.7, 84.7, 107.3. Anal. Calcd for $C_7H_{11}F_3O_5$: C, 36.22; H, 4.78. Found: C, 35.93; H, 4.69.

4.1.24. 5-O-(4,4′-Dimethoxytrityl)-1-O-(2,2,2-trifluoroethyl)-β-D-ribofuranose (**23**)

To a solution of **6** (0.48 g, 2.07 mmol) in pyridine (13 mL) was added DMTrCl (0.84 g, 2.48 mmol) at room temperature. The mixture was stirred at room temperature for 1.5 h and 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% EtOAc in hexane) to give **23** (1.03 g, 1.93 mmol, 93%): ^{1}H NMR (400 MHz,

$CDCl_3$) δ 2.23 (d, 1H, J = 5.5), 2.56 (s, 1H), 3.29 (d, 2H, *J* = 5.0), 3.74–3.89 (m, 8H), 4.10–4.14 (m, 2H), 4.30 (dd, 1H, J = 5.5 and 11.0), 5.04 (s, 1H), 6.82–7.45 (m, 13H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 55.3, 64.2, 64.5, 64.6, 72.4, 75.1, 82.8, 86.4, 107.1, 113.3, 122.7, 124.9, 127.0, 128.0, 128.2, 130.1, 136.0, 144.8, 158.7. Anal. Calcd for $C_{28}H_{23}F_3O_8 \cdot 1/2H_2O$: C, 61.87; H, 5.56. Found: C, 61.72; H, 5.29.

4.1.25. 3-O-(Tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-(2,2,2-trifluoroethyl)-β-D-ribofuranose (**32**) and 2-O-(tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-1-O-(2,2,2-trifluoroethyl)-β-D-ribofuranose (**33**)

To a stirred solution of **23** (1.23 g, 2.30 mmol) and Et_3N (0.96 mL, 6.90 mmol) in DMF (12 mL) was added dropwise TBDMSCl (0.69 g, 4.60 mmol) in DMF (8 mL) at room temperature. The mixture was stirred at room temperature for 12 h and 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, 11% EtOAc in hexane) to give **32** (0.59 g, 0.91 mmol, 40%) and **33** (0.43 g, 0.66 mmol, 29%). Physical data of **32**: ^{1}H NMR (400 MHz, $CDCl_3$) δ −0.13 (s, 3H), −0.00 (s, 3H), 0.81 (s, 9H), 2.77 (d, 1H, *J* = 1.4), 3.03 (dd, 1H, J = 5.0 and 10.1), 3.35 (dd, 1H, J = 2.8 and 10.5), 3.79 (s, 6H), 3.82–3.95 (m, 2H), 3.99 (d, 1H, *J* = 2.8), 4.12 (d, 1H, J = 6.4), 4.32 (dd, 1H, J = 4.6 and 11.4), 5.11 (s, 1H), 6.81–7.48 (m, 13H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −4.9, 18.0, 25.7, 55.3, 63.7, 64.4, 64.6, 72.1, 75.2, 83.5, 86.1, 107.4, 113.2, 126.9, 127.9, 128.3, 130.1, 130.1, 136.0, 136.1, 144.8, 158.6. Anal. Calcd for $C_{34}H_{43}F_3O_7Si \cdot 1/2H_2O$: C, 62.08; H, 6.74. Found: C, 61.80; H, 6.60. Physical data of **33**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.14 (s, 3H), 0.14 (s, 3H), 0.92 (s, 9H), 2.45 (d, 1H, *J* = 7.4), 3.11 (dd, 1H, J = 5.0 and 10.1), 3.34 (dd, 1H, *J* = 3.2 and 10.6), 3.78 (s, 6H), 3.81–3.96 (m, 2H), 4.07–4.13 (m, 2H), 4.23 (dd, 1H, J = 1.8 and 4.6), 4.99 (s, 1H), 6.81–7.48 (m, 13H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −5.0, −4.7, 18.2, 25.8, 55.3, 64.4, 64.5, 64.8, 72.1, 76.4, 84.5, 86.2, 107.7, 113.2, 126.9, 127.9, 128.3, 130.2, 136.0, 136.1, 144.9, 158.6. HRMS (ESI-TOF) *m*/*z*: Calcd for $C_{34}H_{43}F_3O_7Si$ $[M + Na]^+$ 671.2628; Found 671.2631.

4.1.26. 2-O-(Tert-butyldimethylsilyl)-5-O-(4,4′-dimethoxytrityl)-3-O-[(2-cyanoethoxy) (N,N-diisopropyamino)]phosphanyl-1-O-(2,2,2-trifluoroethyl)-β-D-ribofuranose (**38**)

To a solution of **33** (0.25 g, 0.39 mmol) in THF (3 mL) was added *N*,N-diisopropylethylamine (0.34 mL, 1.95 mmol) and chloro(2-cyanoethoxy)(N,*N*-diisopropylamino)phosphine (0.17 mL, 0.78 mmol) at room temperature. The mixture was stirred at room temperature for 45 min and 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, 33% EtOAc in hexane) to give **38** (0.28 g, 0.32 mmol, 83%): ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.7, 150.2.

4.1.27. Solid support synthesis

A mixture of **32** (0.13 g, 0.20 mmol), succinic anhydride (80 mg, 0.8 mmol), and DMAP (50 mg, 0.4 mmol) in pyridine (2.5 mL) was stirred at room temperature for 18.5 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 to give the corresponding succinate. A mixture of the succinate (0.18 g, 0.24 mmol), aminopropyl controlled pore glass (0.45 g, 60 μmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (50 mg, 0.24 mmol) in DMF (2.4 mL) was shaken at room temperature for 4 days. After the resin was washed with pyridine, a capping solution (15 mL, 0.1 M DMAP in pyridine:Ac_2O = 9:1, v/v) was added and the whole mixture was shaken at room temperature for 24 h. The resin was washed with pyridine, EtOH, and MeCN, and dried in vacuo. The amount of the compound **32** loaded on the solid support **39** was 40 μmol/g from the calculation based on absorbance of dimethoxytrityl cation released by treating with a solution of 70% $HClO_4$:EtOH (3:2, v/v). In a similar manner, the solid support **40** was obtained in 35 μmol/g of loading amount.

4.1.28. Oligonucleotide (ON) synthesis

Synthesis was carried out with a DNA/RNA synthesizer by phosphoramidite method according to the normal protocol. Deprotection of bases and phosphates was performed in concentrated NH_4OH:EtOH (3:1, v/v) at 55 °C for 4 h. 2′-TBDMS groups were removed by $Et_3N \cdot 3HF$ (125 μL, Aldrich) in DMSO (100 μL, Aldrich) at 65 °C for 90 min. The reaction was quenched with 0.1 M TEAA buffer (pH 7.0) and desalted on Sep-Pak C18 cartridge. Deprotected ONs were purified by 20% PAGE containing 7 M urea and/or reversed-phase C-18 HPLC using a gradient of solution A (5% MeCN in 0.1 M TEAA buffer, pH 7.0) and solution B (50% MeCN in 0.1 M TEAA buffer, pH 7.0) to give the purified ON **1** (6), ON **2** (9), ON **3** (6), ON **4** (10), ON **5** (9), ON **6** (16), ON **7** (1), ON **8** (3), ON **9** (2), ON **10** (3), ON **11** (5), ON **12** (5), ON **13** (7), ON **14** (5), ON **15** (10), ON **16** (16), ON **17** (16), ON **18** (15), ON **20** (23), ON **21** (19), ON **22** (12), ON **23** (18), ON **24** (3). The yields are indicated in parentheses as OD units at 260 nm starting from 0.2 or 1.0 μmol scale.

4.1.29. MALDI-TOF/MS analysis of oligonucleotides (ONs)

Spectra were obtained with a SHIMAZU/KRATOS time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm, 3-ns pulse). A solution of 3-hydroxypicolinic acid (3-HPA) and diammonium hydrogen citrate in H_2O was used as the matrix. Data of synthetic ONs: ON **1** *m*/*z* = 6505.86 (calcd for $C_{194}H_{245}N_{65}O_{150}P_{20}$ $[M-H]^-$, 6505.87); ON **2** *m*/*z* = 6815.21 (calcd for $C_{203}H_{248}N_{86}O_{144}P_{20}$ $[M-H]^-$, 6815.15); ON **3** *m*/*z* = 6501.81 (calcd for $C_{196}H_{249}N_{65}O_{148}P_{20}$ $[M-H]^-$, 6501.93); ON **4** *m*/*z* = 6811.59 (calcd for $C_{205}H_{252}N_{86}O_{142}P_{20}$ $[M-H]^-$, 6811.21); ON **5** *m*/*z* = 6625.21 (calcd for $C_{198}H_{254}N_{65}O_{152}P_{21}$ $[M-H]^-$, 6625.96); ON **6** *m*/*z* = 6936.55 (calcd for $C_{207}H_{257}N_{86}O_{146}P_{21}$ $[M-H]^-$, 6935.24); ON **7** *m*/*z* = 6498.06 (calcd for $C_{192}H_{254}N_{61}O_{152}P_{21}$ $[M-H]^-$, 6497.87); ON **8** *m*/*z* = 6807.11 (calcd for $C_{201}H_{257}N_{82}O_{146}P_{21}$ $[M-H]^-$, 6807.14); ON **9** *m*/*z* = 6526.12 (calcd for $C_{194}H_{258}N_{61}O_{152}P_{21}$ $[M-H]^-$, 6525.92); ON **10** *m*/*z* = 6835.36 (calcd for $C_{203}H_{261}N_{82}O_{146}P_{21}$ $[M-H]^-$, 6835.20); ON **11** *m*/*z* = 6553.76 (calcd for $C_{196}H_{262}N_{61}O_{152}P_{21}$ $[M-H]^-$, 6554.98); ON **12** *m*/*z* = 6863.47 (calcd for $C_{205}H_{265}N_{82}O_{146}P_{21}$ $[M-H]^-$, 6863.25); ON **13** *m*/*z* = 6582.34 (calcd for $C_{198}H_{266}N_{61}O_{152}P_{21}$ $[M-H]^-$, 6582.03); ON **14** *m*/*z* = 6891.75 (calcd for $C_{207}H_{269}N_{82}O_{146}P_{21}$ $[M-H]^-$, 6891.31); ON **15** *m*/*z* = 6605.26 (calcd for $C_{192}H_{248}F_6N_{61}O_{152}P_{21}$ $[M-H]^-$, 6605.81); ON **16** *m*/*z* = 6915.77 (calcd for $C_{201}H_{251}F_6N_{82}O_{146}P_{21}$ $[M-H]^-$, 6915.09); ON **17** *m*/*z* = 7043.06 (calcd for $C_{221}H_{269}N_{66}O_{159}P_{21}$ $[M-H]^-$, 7043.34); ON **18** *m*/*z* = 6202.99 (calcd for $C_{185}H_{226}N_{82}O_{128}P_{18}$ $[M-H]^-$, 6202.82); ON **20** *m*/*z* = 7039.43 (calcd for $C_{223}H_{273}N_{66}O_{157}P_{21}$ $[M-H]^-$, 7039.39); ON **21** *m*/*z* = 7163.68 (calcd for $C_{225}H_{278}N_{66}O_{161}P_{22}$ $[M-H]^-$, 7163.42); ON **22** *m*/*z* = 7121.75 (calcd for $C_{225}H_{290}N_{62}O_{161}P_{22}$ $[M-H]^-$, 7119.49); ON **23** *m*/*z* = 7143.60 (calcd for $C_{219}H_{272}F_6N_{62}O_{161}P_{22}$ $[M-H]^-$, 7143.27); ON **24** *m*/*z* = 7018.93 (calcd for $C_{217}H_{267}F_6N_{62}O_{157}P_{21}$ $[M-H]^-$, 7019.24).

4.1.30. Preparation of the human Ago2 PAZ domain

A synthetic DNA encoding the human Ago2 PAZ domain (residues 225–369 in Protein Data Bank entry 4EI1) with an N-terminal His-tag was purchased from Life Technologies and cloned into the pETY_Blue vector [33] by the patch cloning method as described in the literature [34]. The Escherichia coli strain harboring the resulting plasmid, pETY_PAZ, was cultivated at 37 °C in LB-ampicillin medium. The expression of the PAZ domain was

induced by adding isopropylthiogalactoside (IPTG) to a final concentration of 0.5 mM and by overnight cultivation at a lowered temperature, i.e., 20 °C. The cells expressing the PAZ domain were disrupted by sonication in Buffer A (20 mM Tris–HCl (pH 7.6), 1 mM $MgCl_2$, 200 mM KCl, 6 mM β-mercaptoethanol, 5% glycerin, and 400 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)), and the cell extract obtained by centrifugation was loaded onto Ni-nitrilotriacetic acid (Ni-NTA) agarose resin (2 mL) at 4 °C. The resin was then washed with Buffer A containing 10 mM imidazole, and proteins containing the PAZ domain were eluted with Buffer A containing 250 mM imidazole. The buffer of the eluate was exchanged for Buffer B (20 mM HEPES-KOH (pH 7.5), 10 mM $MgCl_2$, and 6 mM β-mercaptoethanol) and applied to a HiTrap Heparin HP column (1 mL). The PAZ domain was eluted with a linear gradient (7 mL) from 0 to 500 mM KCl in Buffer B. Finally, PAZ was concentrated by ultrafiltration and stored at −80 °C in a buffer containing 40 mM Tris–HCl (pH 7.6), 2 mM $MgCl_2$, 80 mM KCl, and 2 mM DTT. The amount of the purified PAZ domain was determined from the absorbance at 280 nm and by using the ProtParam tool (http://web.expasy.org/protrapam/).

4.1.31. Thermal denaturation study

The solution containing the duplex in a buffer comprising 10 mM sodium phosphate (pH 7.0) and 0.1 M NaCl was heated at 95 °C for 3 min, cooled gradually to an appropriate temperature, and then used for the thermal denaturation study. The thermal-induced transition of each mixture was monitored at 260 nm on UV–Vis spectrophotometer fitted with a temperature controller in quartz cuvettes with a path length of 1.0 cm and a 3.0 μM duplex concentration in a buffer of 10 mM sodium phosphate (pH 7.0) and 0.1 M NaCl. The sample temperature was increased by 0.5 °C/min.

4.1.32. Dual-luciferase reporter 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% bovine serum (BS). Twenty-four hours before transfection, HeLa cells (4×10^4/mL) were transferred to a 96-well plate (100 μL/well). They were transfected using TransFast (Promega). Cells in each well were transfected with a solution (35 μL) of psiCHECK-2 vector (20 ng), the indicated amounts of siRNA, and TransFast (0.3 μg) in Opti-MEM I Reduced-Serum Medium (Invitrogen), and incubated at 37 °C. Transfection without siRNA was used as a control. After 1 h, MEM (100 μL) containing 10% BS was added to each well, and the whole was incubated at 37 °C. After 24 h, solution in each well was removed and the plate was left unattended at −80 °C. After more than 4 h, activity of firefly and *Renilla* luciferases in cell 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.

4.1.33. Electrophoretic mobility shift assay (EMSA)

siRNA **9** (final concentration: 10 μM), comprising labeled ON **17** and ON **18**, was incubated with various amounts of the PAZ domain protein (final concentration: 0 μM, 10 μM, 20 μM, 40 μM, or 80 μM) in an EMSA buffer (10 μL) comprising 20 mM Tris–HCl (pH 8.0), 0.1 M NaCl, and 1 mM DTT at 37 °C for 10 min. An aliquot (5 μL) of the reaction mixture was mixed with a loading buffer (an EMSA buffer containing 50% glycerin; 5 μL) on ice. Each sample was analyzed by 8% nondenaturing PAGE in buffer containing 5% glycerin at 10 °C for 1.5 h at 200 V. The gel was visualized by use of a Luminescent Image analyzer LAS-4000 (Fujifilm). The competition assay was carried out as follows: A mixture of siRNA **9** (final concentration: 10 μM) and the PAZ domain protein (final concentration: 40 μM) was incubated in the presence of various amounts of competitor (final concentration: 100 μM or 1000 μM) in the EMSA buffer (5 μL) at 37 °C for 10 min. The reaction mixture (5 μL) was mixed with the loading buffer (5 μL) on ice. Each sample was analyzed by 8% nondenaturing PAGE in buffer containing 5% glycerin at 10 °C for 1.5 h at 200 V. The gel was visualized by use of a Luminescent Image analyzer LAS-4000 (Fujifilm).

4.1.34. Enzyme-linked immunosorbent assay (ELISA)

A solution of dsRNAs (100 nM, 50 μL), which were modified with biotin at the 3′-end of the passenger strand, were added to a streptavidin-coated 96-well plate. After 15 min, each well was washed three times with a solution (200 μL × 3) of 0.05% Tween 20 in PBS (pH 7.4) and then a solution (100 μL) of 3% BSA in PBS buffer (pH 7.4) was added. After 2 h, each well was washed and then a solution of the PAZ domain protein (100 nM, 50 μL) in PBS buffer (pH 7.4) contained 1% BSA was added. The plate was incubated at 37 °C for 10 min. Each well was washed and then a solution of the anti-His tag antibody conjugated horseradish peroxidase (50 μL) in PBS containing 1% BSA was added. After 1 h, each well was washed and then a peroxidase (POD) substrate (50 μL) was added. After 10 min, a stop solution (1 M H_2SO_4, 50 μL) was added and then an absorbance of each well at 450 nm was measured by a microplate reader.

4.1.35. Partial digestion of ONs by SVPD

Each ON (600 pmol) labeled with fluorescein at the 5′-end was incubated with SVPD (0.075 unit) in a buffer (150 μL) comprising 0.1 M Tris–HCl (pH 8.0) and 20 mM $MgCl_2$ at 37 °C. After 0, 1, 5, 10, 30, 60, 180, or 360 min, an aliquot (5 μL) of the reaction mixture was mixed with the loading buffer (15 μL), comprising Tris-borate-EDTA (TBE) buffer and 20% glycerin, on ice. Each sample was analyzed by 20% denaturing PAGE at room temperature for 2 h at 20 mA. The gel was visualized by use of a Luminescent Image analyzer LAS-4000 (Fujifilm).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.09.011.

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