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A General Method for the Synthesis of 2′-O-Cyanoethylated Oligoribonucleotides Having Promising Hybridization Affinity for DNA and RNA and Enhanced Nuclease Resistance

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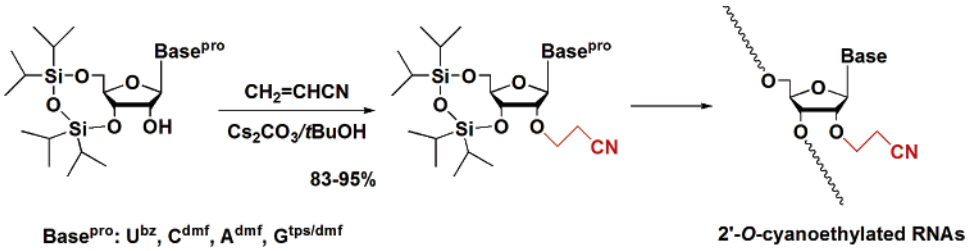

An effective method for the synthesis of 2′-O-cyanoethylated oligoribonucleotides as a new class of 2′-O-modified RNAs was developed. The reaction of appropriately protected ribonucleoside derivatives with acrylonitrile in *t*-BuOH in the presence of Cs_2CO_3 gave 2′-O-cyanoethylated ribonucleoside derivatives in excellent yields, which were converted by a successive selective deprotection/protection strategy to 2′-O-cyanoethylated 5′-*O*-dimethoxytritylribonucleoside 3′-phosphoramidite derivatives in high yields. Fully 2′-O-cyanoethylated oligoribonucleotides, $(Uce)_{12}$ and $(GceAceCceUce)_3$, were successfully synthesized in the phosphoramidite approach by use of the phosphoramidite building blocks. It was also found that oligoribonucleotides having a 2′-O-cyanoethylated ribonucleoside (Uce, Cce, Ace, or Gce) could be obtained by the selective removal of the TBDMS group from fully protected oligoribonucleotide intermediates without loss of the cyanoethyl group by use of $NEt_3 \cdot 3HF$ as a desilylating reagent. The detailed T_m experiments revealed that oligoribonucleotides containing 2′-O-cyanoethylated ribonucleosides have higher hybridization affinity for both DNA and RNA than the corresponding unmodified and 2′-O-methylated oligoribonucleotides. In addition, introduction of a cyanoethyl group into the 2′-position of RNA resulted in significant increase of nuclease resistance toward snake venom and bovine spleen phosphodiesterases compared with that of the methyl group.

Introduction

The chemical modification of natural RNAs has proved to be of great importance to improve their original physicochemical and biochemical properties such as hybridization affinity and nuclease resistance. These modified RNAs have been applied to the gene regulation in antisense, antigene, and RNA interference (RNAi) strategies.[1−4] Particularly, RNAi mediated by small interfering RNAs (siRNAs) has been recognized as a new powerful tool to control gene functions and has been used for gene therapy targeting mRNAs.[3,4] However, RNAs are well-known to be rapidly degraded by cellular enzymes. To mitigate this inherent disadvantage of RNAs as drugs for clinical application, a large number of oligoribonucleotide derivatives having an ether-type skeleton at the 2′ position have been reported to date.[5−21] In 1987, Inoue and Ohtsuka reported that, among them, 2′-O-methyl

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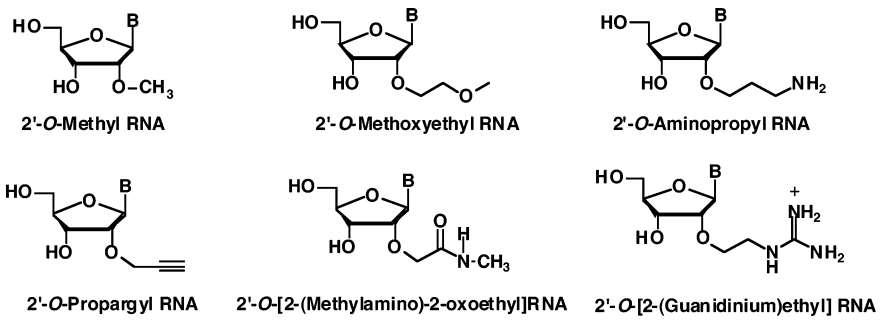

FIGURE 1. Modified ribonucleosides at the 2′-*O*-hydroxyl group.

RNAs exhibited enhanced chemical stability and affinity for RNAs.[9] Thereafter, it turned out that 2′-O-alkylated RNAs having modified groups such as 2′-*O*-methoxyethyl,[11,12] 2′-*O*-propargyl,[15] 2′-*O*-aminopropyl,[16] 2′-*O*-dimethylaminooxyethyl,[17a] 2′-*O*-[2-(methylamino)-2-oxoethyl],[19c] and 2′-*O*-[2-(guanidinium)ethyl][21] have excellent nuclease resistance and affinity for the complementary oligonucleotides (Figure 1).

However, most of the articles related to 2′-O-modified ribonucleosides were limited to pyrimidine ribonucleosides.[13,17,18,20−23] Different approaches were often required for the synthesis of pyrimidine and purine ribonucleoside building blocks.[19a,b] As a typical procedure for the synthesis of ribonucleoside building blocks, direct 2′-O-alkylation of unprotected or partially protected ribonucleosides has been widely applied by use of alkylating reagents in the presence of NaH to give 2′-O-modified ribonucleosides.[11a,13,15−19,21,23] This type of modification is practically useful but often resulted in low yield and required tedious separation of the desired 2′-O-alkylated products and was not always the best choice for all four canonical nucleosides.[19a,b] Particularly, 2′-*O*-methoxyethyl ribonucleoside derivatives, which have proved to be one of the most useful 2′-O-modified ribonucleosides, have been synthesized from ribose via a multistep reaction involving glycosylation and alkylation of the once-generated 2′-free appropriately protected ribonucleoside derivatives with methoxyethyl bromide.[11a] Although a convenient route to 2′-*O*-methoxyethylribonucleoside derivatives by the ring opening-mediated etherification of 2,2′-cyclouridine derivatives nucleosides was reported, this process is limited to a pyrimidine series.[24] More seriously, few articles are available for full details of the synthetic procedure of four kinds of 2′-O-modified ribonucleoside 3′-phosphoramidite building blocks.[11a,15,19b] Among them, only 2′-O-methylated oligoribonucleotides are commercially available, and thus they have been widely used as chemically stable RNA derivatives for various studies.[1−8]

In this article, we report a general and promising method for the synthesis of 2′-O-cyanoethylated oligoribonucleotide derivatives as a new class of 2′-O-modified RNAs as well as a convenient and widely applicable method for the 2′-O-cyanoethylation of ribonucleoside derivatives by use of acrylonitrile in *t*-BuOH in the presence of Cs_2CO_3.

Results and Discussion

Strategies for the Synthesis of 2′-O-Cyanoethylated RNAs. Cyanoethylation of tRNAs with acrylonitrile was reported in the early 1960s by Yoshida, Ukita, and Ofengand.[25] However, these reactions always gave

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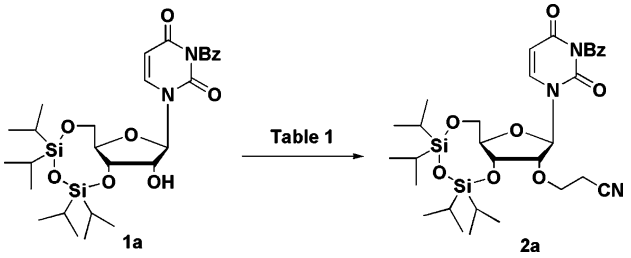
SCHEME 1. Cyanoethylation of the 2′-Hydroxyl Group of Uridine Derivative



base-modified products. Particularly, they used the Michael reaction of acrylonitrile to identify modified bases such as pseudouridine, 4-thiouridine, and inosine in tRNAs. No studies of cyanoethylation of hydroxyl groups of nucleosides, nucleotides, and nucleic acid derivatives have been reported to date. Our interest was focused on the 2-cyanoethyl group as a new type of 2′-substituent since it has a simple structure with less steric hindrance and a polarized cyano group that seems to stabilize water bridge structures around the 2′-position.

In general, however, the Michael reaction of alcohols with acrylonitrile giving rise to 1:1 adducts was sluggish, requiring use of strong bases[26] such as sodium hydroxide, alcoholates, and benzyltrimethylammonium hydroxide (Triton B). Apparently, these basic conditions could not be applied to the synthesis of 2′-O-cyanoethylated ribonucleoside derivatives as the key synthetic intermediates required for the synthesis of 2′-O-cyanoethylated oligoribonucleotides.

To overcome this problem, we have extensively searched for mild conditions for the 2′-O-cyanoethylation of an appropriately protected uridine derivative **1a** (Scheme 1).[27] These results are summarized in Table 1.

The 2′-O-cyanoethylation of N^3-benzoyl-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine (**1a**) was studied by use of various base catalysts and acrylonitrile as a solvent. As shown in Table 1, triethylamine did not give the Michael reaction product **2a**. As expected, the use of strong organic bases such as DBU and Triton B gave **2a**, but the reaction was not completed. In these reactions, considerable polymerization of acrylonitrile occurred with formation of significant amounts of orange materials. DABCO, a strong organic base, as well as *N*-methylimidazole and 4-(dimethylamino)pyridine, well-known nucleophilic catalysts, did not give the Michael adduct. It was found that, among the metal carbonates and oxides tested, only Cs_2CO_3 gave the Michael reaction adduct **2a** in 54% yield. In this case, the undesirable polymerization of acrylonitrile was also observed. To avoid this serious side reaction, the mechanism of the polymerization was considered. It is likely that the first step of the polymerization is the addition of the 2′-hydroxyl group to acrylonitrile that gives a carbanion on the α-carbon atom next to the cyano group, and the second step is the reaction of another acrylonitrile molecule on this carbanion. On the other hand, the formation of **2a** requires the protonation of the carbanion. Therefore, addition of suitable proton sources to the carbanion should be effective to prevent the undesirable polymerization.

TABLE 1. Results of 2′-O-Cyanoethylation of 1a under Various Conditions[a]

entry	base (1.0 equiv)	solvent (reagent or cosolvent)	time (h)	yield of **2a** (%)
1	Et_3N	CH_2=CHCN	24	no reaction
2	Triton B	CH_2=CHCN	2	24
3	DBU	CH_2=CHCN	24	50
4	MgO	CH_2=CHCN	24	no reaction
5	CaO	CH_2=CHCN	24	no reaction
6	*N*-MeIm	CH_2=CHCN	24	no reaction
7	DMAP	CH_2=CHCN	24	0
8	DABCO	CH_2=CHCN	24	no reaction
9	Na_2CO_3	CH_2=CHCN	24	no reaction
10	K_2CO_3	CH_2=CHCN	24	no reaction
11	Cs_2CO_3	CH_2=CHCN	24	54
12	Cs_2CO_3	MeOH−CH_2=CHCN (20 equiv)	24	complex mixture
13	Cs_2CO_3	THF−CH_2=CHCN (20 equiv)	24	34
14	Cs_2CO_3	CH_2=CHCN−*t*-BuOH (10 equiv)	12	72
15	Cs_2CO_3	CH_2=CHCN−*t*-BuOH (40 equiv)	2.5	93
16	Cs_2CO_3	*t*-BuOH−CH_2=CHCN (20 equiv)	1	95

[a] A 0.2 M solution of **1a** was used under these conditions.

In consideration of this mechanism, we selected methanol as not only a proton source but also the cosolvent. In this case, unfortunately, debenzoylation of **2a** was observed to give methyl benzoate. This result indicated that a kind of methoxide anion generated by Cs_2CO_3 worked as a nucleophile to attack the benzoyl group. Therefore, we next selected *tert*-butyl alcohol as a proton source. As shown in entry 16 of Table 1, the combined use of Cs_2CO_3 and *tert*-butyl alcohol gave the desired product **2a** in an excellent yield of 95%. Moreover, orange precipitates were not observed under these conditions. The effectiveness of the *tert*-butyl alcohol could be attributed to its efficient proton-donor property capable of protonating the once-generated carbanion. Since the acidity of *tert*-butyl alcohol ($pK_a = 18$) is rather lower than that of methanol ($pK_a = 15.5$), it is more difficult to generate a basic species such as the *t*-butoxide ion directly by the action of Cs_2CO_3. Even if such an alchoxide-like species is generated, the benzoyl group would remain stable because of the poor nucleophilicity of the sterically hindered *tert*-butoxide ion. Furthermore, Cs_2CO_3 is scarcely soluble in *tert*-butyl alcohol so that the supernatant of the resulting heterogeneous reaction mixture can be kept as a nonbasic medium, which is favorable for the 2′-O-cyanoethylation of **1a** without damaging the base labile-protecting groups.

Our new method for the O-cyanoethylation was applied to the synthesis of 2′-O-cyanoethylated cytidine, adenosine, and guanosine derivatives (**2b**−**d**). In the case of **2b** and **2c**, the amino groups on the base moieties were protected with the dimethylaminomethylene group.[28] The

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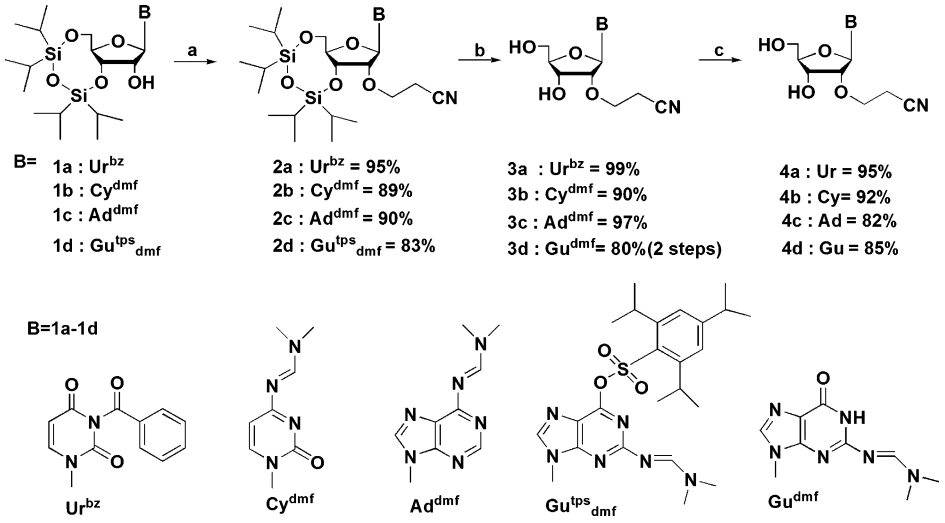
SCHEME 2. Cyanoethylation of Ribonucleosides and Their Nucleoside Derivatives[a]



[a] Reagents and conditions: (a) acrylonitrile, *tert*-butyl alcohol, rt; (b) $Et_3N{\cdot}3HF$, Et_3N, THF, rt; (c) deprotection of the base moieties.

cytidine and adenosine derivatives **1b**[29] and **1c**[30] underwent smooth 2′-O-cyanoethylation with acrylonitrile under conditions similar to those described for the synthesis of **2a** to give **2b** and **2c** in 89 and 90% yields, respectively. For the synthesis of the guanosine derivative **2d**, the 6-O-position was protected with a 2,4,6-triisopropylbenzenesulfonyl group[31] and the 2-amino group was masked with the dimethylaminomethylene group. Thus, a similar Michael reaction of **1d** with acrylonitrile gave **2d** in 83% yield, as shown in Scheme 2. It should be noted that during the reaction the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl (TIPS) group and the other base-protecting groups remained intact. Particularly, it was found that the TPS group could be conveniently used as the 6-O-protecting group of the guanine residue. The TIPS groups of **2a**−**d** were selectively deprotected by treatment with $Et_3N{\cdot}3HF$ to give the 3′,5′-O-unprotected ribonucleoside derivatives (**3a**−**3d**) in high yields. In the case of **3d**, the TPS group was selectively removed by an oximate reagent of *o*-nitrobenzaldoxime and tetramethylguanidine before the $Et_3N{\cdot}3HF$ treatment. Finally, treatment of **3a** and **3b** with NH_4OH−EtOH (1:1 and 3:1, respectively, v/v) at room temperature for 1 h gave **4a** and **4b** in 95 and 92% yields, respectively, as shown in Scheme 2. Treatment of **3c** with hydrazine monohydrate in CH_3CN at room temperature for 3 h gave **4c** in 82% yield. The reaction of **3d** with cyclohexylamine in THF gave the desired product **4d** in 85% yield.

Next, the sugar puckering modes of the new modified ribonucleosides **4a**−**4d** were studied by use of ^1H NMR. The $J_{1',2'}$ and $J_{3',4'}$ values and the percentage (%N) of the C3′-*endo* form are summarized, as shown in Table 2, and compared with those of the corresponding 2′-O-methylated derivatives. The %N values were calculated according to the equation of %N(C3′-*endo*) = $\{J_{3',4'}\,(\mathrm{Hz})/J_{1',2'}\,(\mathrm{Hz}) + J_{3',4'}\,(\mathrm{Hz})\} \times 100$.[32]

TABLE 2. Sugar Puckering Modes of 2′-O-Cyanoethylated Ribonucleosides and 2′-O-Methylated Ribonucleosides (25 °C, 500 MHz NMR in D_2O)

	U_{OMe}	U_{OCE} **4a**	C_{OMe}	C_{OCE} **4b**	A_{OMe}	A_{OCE} **4c**	G_{OMe}	G_{OCE} **4d**
$J_{1',2'}$	3.9	3.7	3.4	3.2	6.4	6.4	6.1	6.1
$J_{3',4'}$	5.8	6.1	6.6	7.1	3.2	3.2	3.9	3.7
$J_{1',2'} + J_{3',4'}$	9.7	9.8	10.0	10.3	9.6	9.6	10.0	9.8
%N (C3′-*endo*)	60	63	66	69	33	33	39	38

[a] These values were calculated according to the equation %N(C3′-*endo*) = $\{J_{3',4'}\,(\mathrm{Hz})/J_{1',2'}\,(\mathrm{Hz}) + J_{3',4'}\,(\mathrm{Hz})\} \times 100$.[32]

As a result, it was found that the %N values of the 2′-O-cyanomethylated purine ribonucleosides **4c**,**d** were essentially similar to those of 2′-O-methylated ribonucleosides, but in the 2′-O-cyanoethylated pyrimidine ribonucleosides **4a**,**b** the C3′-*endo* form was increased slightly to a degree of 3%. It is interesting that, although the 2-cyanoethyl group has two methylene groups and a cyano group, the 2′-O-cyanoethylated ribonucleosides maintain the ratio of the N- and S-type conformers sugar puckering mode in the same manner as 2′-O-methylated ribonucleosides.

Oligoribonucleotides Synthesis. For the synthesis of 2′-O-cyanoethylated oligoribonucleotides, the acetyl group was chosen as the protecting group for the cytosine base. 4-*N*-Acetyl-2′-*O*-(2-cyanoethyl)cytidine (**4b′**) was obtained by reaction of **4b** with acetic anhydride in the presence of EtOH. For the adenine and guanine bases, the dimethylaminomethylene group was used.[28] The uracil base was not protected, as reported usually.[33] Compounds **4a**, **4b′**, **3c**, and **3d** thus obtained were converted to the phosphoramidite derivatives **6a**−**d** via the 5′-O-dimethoxytritylated products **5a**−**d** according to

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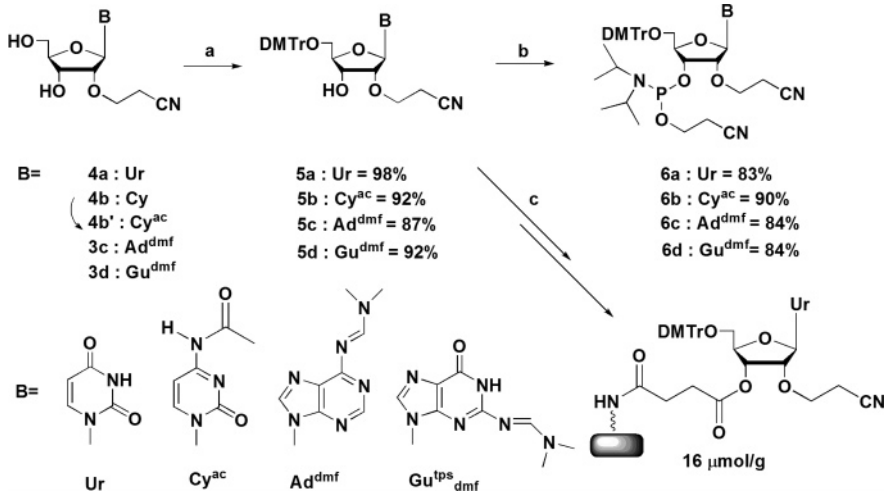
SCHEME 3. Preparation of Phosphoramidite Building Blocks and Polymer Support[a]



[a] Reagents and conditions: (a) DMTrCl, pyridine, rt; (b) Chloro(2-cyanoethyl)(*N, N*-diisopropylamino)phosphine, ethyldiisopropylamine, CH_2Cl_2, rt; (c) succinic anhydride, DMAP, CH_2Cl_2, rt; LCAA-CPG, DCC, CH_2Cl_2, rt.

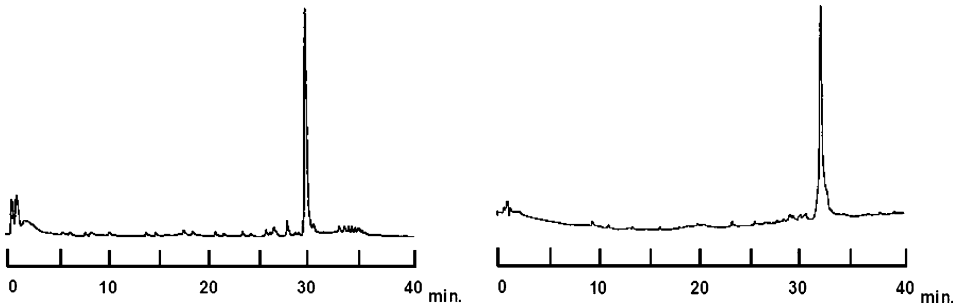


FIGURE 2. Anion exchange HPLC profile of crude cyanoethylated RNAs. (Left) $(Uce)_{12}$. (Right) $(GceAceCceUce)_3$.

the standard procedure (Scheme 3).[33] The 5′-tritylated compound **5a** was attached to long-chain aminoalkyl CPG via a succinyl linker to give the solid support.

The synthesis of fully and partially 2′-O-cyanoethylated oligoribonucleotides was performed by the phosphoramidite approach using the phosphoramidite building blocks **6a**−**d** and the widely used 2′-*O*-TBDMS-ribonucleoside phosphoramidite units by use of an ABI 392 DNA/RNA automated synthesizer. After the chain elongation for the synthesis of the fully 2′-O-cyanoethylated dodecauridylate, simple treatment of the protected oligomer on the resin with concentrated NH_3 gave the desired product $(Uce)_{12}$ as the main peak, as shown in Figure 2. However, in the case of the synthesis of $(GceAceCceUce)_3$ having all four ribonucleosides, the ammonia treatment was somewhat detrimental since the cyanoethyl group was not stable during base deprotection. Therefore, we extensively studied more suitable conditions for the full deprotection of the base- and phosphate-protecting groups. Consequently, it was found that treatment of fully protected oligoribonucleotides on CPG with concentrated NH_4OH-NH_4OAc (10:1, w/w)[28] at room temperature for 90 min was very effective for the selective removal of the dmf and acetyl groups on the base moieties. When only concentrated NH_3 was used, part of the cyanoethyl group was eliminated and the dmf was much more slowly deprotected. Addition of NH_4OAc to concentrated NH_4OH was essential to avoid elimination of the cyanoethyl group and to accelerate removal of the dmf group. Thus, the fully 2′-O-cyanoethylated dodecaoligoribonucleotide $(GceAceCceUce)_3$ was successfully synthesized. It was also found that the use of *n*-propylamine−THF (1:1, v/v) as the deblocking reagent of the Pac (A), isopropyl-pac (G), and acetyl (C) groups resulted in the selective deprotecton of the acyl-type protecting groups [Pac (A), isopropyl-Pac (G), and acetyl (C)] without damage to the cyanoethyl and TBDMS groups at the 2′-position. Moreover, the successive treatment of the resulting N-unprotected RNA species with $Et_3N{\cdot}3HF$ gave the desired RNA oligomers containing a 2′-O-cyanoethylated ribonucleoside. Under these conditions, the 2′-*O*-cyanoethyl group remained intact. Contrary to this result, tetrabutylammonium fluoride could not be used as the desilylating reagent. Surprisingly, in this case, the 2-cyanoethyl ether was considerably deprotected.[34] These products were purified by use of reverse-phase C_{18} cartridge and anion exchange HPLC. The purified products were characterized by MALDI-TOF mass (Table 3).

(34) This result suggests that the cyanoethyl group can be used as a new promising 2′-hydroxyl protecting group for the RNA synthesis. The details of this study will be shortly reported by us.

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TABLE 3. Oligoribonucleotides Synthesized by Use of 2′-O-Cyanoethylated Ribonucleosides

oligoribonucleotides sequence[a]	conditions for deprotection[b]	MALDI-TOF mass calcd	MALDI-TOF mass found
UU	NH_4OH	602.11	602.85
UUUUUUUUUUU	NH_4OH	4236.51	4238.41
GACU	NH_4OH-NH_4OAc	1434.31	1434.12
GACUGACUGACU	NH_4OH-NH_4OAc	4428.86	4428.55
GCUA**G**ACUAUCUA	*n*-$PrNH_2$ and then $Et_3N{\cdot}3HF$	4135.59	4134.27
GCUAG**A**CUAUCUA	*n*-$PrNH_2$ and then $Et_3N{\cdot}3HF$	4135.59	4135.38
GCUAGA**C**UAUCUA	*n*-$PrNH_2$ and then $Et_3N{\cdot}3HF$	4135.59	4133.90
GCUAGAC**U**AUCUA	*n*-$PrNH_2$ and then $Et_3N{\cdot}3HF$	4135.59	4135.37
GAGCC**A**AGCIUCGGCUC	*n*-$PrNH_2$ and then $Et_3N{\cdot}3HF$	5474.80	5473.90

[a] Conditions for release of oligonucleotides from CPG and removal of the protecting groups of the base moieties and the 2′-hydroxyl group. [b] None: Natural ribonucleoside, bold: 2′-*O*-Cyanoethylribonucleoside.

TABLE 4. Hybridization Properties of 2′-O-Cyanoethylated RNA Derivatives

	sequence and complement	vs DNA	ΔT_m	ΔT_m/mod.	vs RNA	ΔT_m	ΔT_m/mod.
1	UUUUUUUUUUUU	8.5			14.1		
2	*UUUUUUUUUUUU*	10.3	+1.8		26.1	+12.0	+1.0
3	**UUUUUUUUUUUU**	17.1	+8.6	+0.6	33.5	+19.4	+1.6
4	GACUGACUGACU	47.9			59.1		
5	*GACUGACUGACU*	46.9	−1.0		63.6	+4.5	+0.4
6	**GACUGACUGACU**	53.8	+5.9	+0.5	68.4	+9.3	+0.8
7	CGUAGACUAUCUA	41.5			54.8		
8	CGUAGAC*U*AUCUA	41.0	−0.5	−0.5	54.9	+0.1	+0.1
9	CGUAGAC**U**AUCUA	42.6	+1.1	+1.1	55.9	+1.1	+1.1
10	CGUAGA*C*UAUCUA	40.3	−1.2	−1.2	54.3	−0.5	−0.5
11	CGUAGA**C**UAUCUA	42.9	+1.4	+1.4	56.9	+2.1	+2.1
12	CGUAG*A*CUAUCUA	37.6	−3.9	−3.9	50.5	−4.3	−4.3
13	CGUAG**A**CUAUCUA	42.3	+0.8	+0.8	55.5	+0.7	+0.7
14	CGUA*G*ACUAUCUA	38.2	−3.3	−3.3	51.3	−3.5	−3.5
15	CGUA**G**ACUAUCUA	42.4	+0.9	+0.9	56.1	+1.3	+1.3

[a] Conditions: 10 mM sodium phosphate buffer (pH 7.0), 100 mM NaCl, 0.1 mM EDTA, and 2.0 μM duplex. [b] None: Natural ribonucleoside, italic: 2′-*O*-methylribonucleoside, bold: 2′-*O*-cyanoethylribonucleoside.

Hybridization Properties of 2′-O-Cyanoethylated Oligoribonucleotides. Hybridization properties of oligoribonucleotides containing 2′-O-cyanoethylated nucleosides with the complementary DNA or RNA strands were studied. 2′-O-Methylated oligoribonucleotides were used as the control. The hybridization properties of 2′-O-cyanoethylated RNA derivatives are summarized in Table 4. The hybridization affinity of the 2′-O-cyanoethylated dodecauridylate $(Uce)_{12}$ for dA_{12} or A_{12} was compared with that of the unmodified U_{12} and 2′-O-methylated dodecauridylate $(Um)_{12}$. As a result, it turned out that the thermodynamic stability of $(Uce)_{12}-dA_{12}$ (T_m = 17.1 °C) was considerably higher by 8.6 °C and 6.8 °C than that of $U_{12}-dA_{12}$ and $(Um)_{12}-dA_{12}$, respectively, as shown in Table 4. Similarly, the RNA−RNA duplex $(Uce)_{12}-A_{12}$ exhibited a T_m value (T_m = 33.5 °C) higher than that of $U_{12}-A_{12}$ (T_m = 14.1 °C) and $(Um)_{12}-A_{12}$ (T_m = 26.1 °C). These results clearly suggested that the duplex stabilizing effect of the 2′-*O*-cyanoethyl modification is stronger than that of the 2′-*O*-methyl modification. The hybridization affinity of a fully cyanoethylated RNA derivative of $(GceAceCceUce)_3$ with a mixed sequence for the complementary DNA oligomer was also much higher (T_m = 53.8 °C) than the unmodified one (T_m = 47.9 °C) and the 2′-*O*-methyl oligomer (T_m = 46.9 °C). The same is true for the hybridization affinity of $(GceAceCceUce)_3$ for the complementary RNA strand, as shown in entries 4−6 of Table 4, but this modified RNA−RNA duplex resulted in a more significant stabilizing effect than the modified RNA−DNA duplex.

To examine the relationship between the stabilizing effect and the nucleobase moiety, comparative hybridization experiments were carried out by use of various RNA strands incorporating one of the four 2′-*O*-cyanoethyl or 2′-*O*-methylnucleosides (Table 4, entries 8−15).

Interestingly, the stabilizing effect of the one-point modification was dependent on the structure of the base moieties. Incorporation of a pyrimidine nucleoside Uce in place of U slightly increased the T_m value versus DNA and RNA by +1.1 and +1.1 °C, respectively. One-point replacement of C with Cce also affected the T_m value to a degree of +1.4 and +2.1 °C versus DNA and RNA, respectively. On the other hand, incorporation of Um and Cm into RNA resulted in a significant decrease of the T_m values of the duplexes with the complementary DNA strands by −0.5 and −1.2 °C, respectively, as shown in entries 7, 8, and 10 of Table 4. The RNA oligomers incorporating Um and Cm showed the same level and weaker hybridization affinity (ΔT_m = +0.1 and −0.5 °C, respectively) for the complementary RNA strand compared with those of the unmodified RNA oligomer, as shown in entries 7, 8, and 10 of Table 4. On the other hand, it was found that the incorporation of the purine nucleosides Ace and Gce resulted in a more equivocal increase of the T_m value. The former affected the stability by +0.8 versus DNA and +0.7 °C versus RNA, and the latter showed a similar tendency with the ΔT_m values of +0.9 versus DNA and 1.3 °C versus RNA. Surprisingly, it was also found that incorporation of Am and Gm into RNA caused marked destabilization of the duplexes with

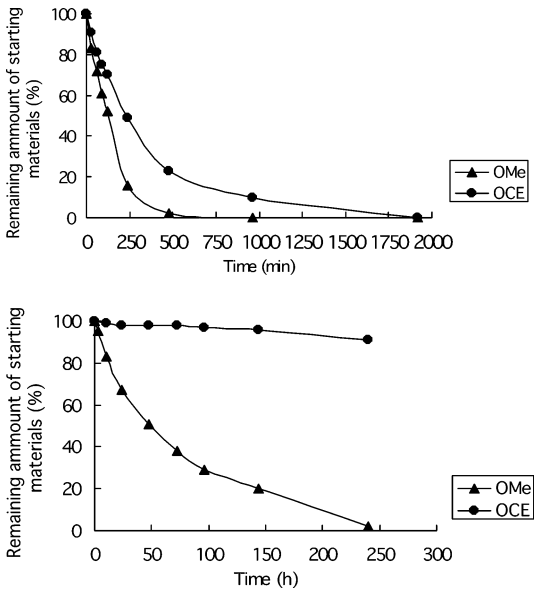

FIGURE 3. Time course of degradation of 2′-O-cyanoethylated diuridylic acids $U_{CE}pU$ and $U_{OMe}pU$ with two kinds of phosphodiesterases. (Top) Snake venom. (Bottom) Bovine spleen.

−3.9 versus DNA and −4.3 °C versus RNA, and −3.3 versus DNA and −3.5 °C versus RNA, respectively.

From these results, it was suggested that the 2′-O-cyanoethylation has a unique effect on the hybridization, which is different from that of 2′-O-methylation. Moreover, the cyanoethylation also proved to contribute to stabilization of duplexes when both pyrimidine and purine nucleosides are incorporated into RNA. The equivocal stabilization effect of the 2′-O-cyanoethylation would provide new insight into the creation of 2′-O-modified RNA derivatives, and its mechanism should be clarified in due course.

Nuclease Stabilities of 2′-O-Cyanoethylated Oligouridylates. Nuclease resistance of 2′-O-cyanoethylated oligoribonucleotides was tested by use of snake venom phosphodiesterase[10b] and bovine spleen phosphodiesterase (Figure 3).[33]

The higher nuclease resistance of $U_{CE}pU$ was clearly observed in the snake venom phosphodiesterase assay compared with that of $U_{OMe}pU$. The half-lives of $U_{CE}pU$ and $U_{OMe}pU$ were determined to be 4 and 2 h, respectively.

It was also found that $U_{CE}pU$ was markedly resistant to bovine spleen phosphodiesterase, a major mammalian phosphodiesterase. More than 90% of $U_{CE}pU$ remained intact after 10 days, while only 2% of $U_{OMe}pU$ remained under the same conditions.

Conclusion

In this study, we developed a general method for the synthesis of 2′-O-cyanoethylated ribonucleosides and oligoribonucleotides. As a result, the cyanoethyl group could be introduced readily into the 2′-hydroxyl group of the appropriately protected canonical ribonucleosides by use of Michael addition using the acrylonitrile-*t*-BuOH−Cs_2CO_3 system. We succeeded in establishing a deprotection procedure for the synthesis of the fully and partially 2′-O-cyanoethylated RNA oligomers by choosing the conditions suitable for selective deprotection of the base- and phosphate-protecting groups. It was emphasized that the 2′-*O*-cyanoethyl group could remain absolutely intact when a mixture of concentrated NH_4OH−NH_4OAc was employed as the deblocking reagent. Furthermore, it turned out that oligoribonucleotides incorporating 2′-O-cyanoethylated nucleosides enhanced not only the hybridization affinity for DNA and RNA but also nuclease resistance compared with the 2′-*O*-methyl oligoribonucleotides. These promising properties of 2′-O-cyanoethylated RNA oligomers would provide new insight into RNA nanotechnotogy as well as RNAi and antisense strategies since all four common ribonucleoside 3′-phosphoramidite building blocks can be easily obtained by a series of simple reactions as described here. Further applications of this new material are now under study.

Experimental Section

Synthesis 2′-O-Cyanoethylated Nucleoside and Its Phosphoramidite. Example of Uridine Derivatives. *N*^{3}-Benzoyl-2′-*O*-(2-cyanoethyl)-3′,5′-*O*-(1,1,3,3,-tetraisopropyldisiloxane-1,3-diyl)uridine (2a). Compound **1a**[27] (60 mg, 0.102 mmol) was dissolved in *t*-butanol (500 μL). To the solution were added acrylonitrile (131 μL, 2 mmol) and cesium carbonate (35 mg, 0.1 mmol). After being vigorously stirred at room temperature for 1 h, the mixture was filtered by use of Celite. The solvent and excess volatile reagents were evaporated in vacuo. The residue was chromatographed on a silica gel column with hexane−ethyl acetate (3:1, v/v) to give compound **2a** as a white solid (61 mg, 95%): mp 159 °C ($CHCl_3$-*i*-Pr_2O); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.94−1.12 (28 H, m), 2.61−2.63 (2 H, m), 3.91−4.05 (4 H, m), 4.18−4.29 (3 H, m), 5.70 (1 H, s), 5.79 (1 H, d, *J* = 8.30), 7.49−7.94 (5 H, m), 8.00 (1H, d, *J* = 8.3); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 12.5, 12.8, 13.0, 13.4, 16.8, 16.9, 17.0, 17.2, 17.4, 19.1, 59.2, 65.8, 68.1, 81.8, 82.7, 89.0, 101.6, 117.4, 129.2, 130.5, 131.2, 135.3, 138.9, 149.0, 162.1, 168.7. Anal. Calcd for $C_{31}H_{45}N_3O_8Si_2$: C, 57.83; H, 7.04; N, 6.53. Found: C, 58.02; H, 6.78; N, 6.51.

***N*^{3}-Benzoyl-2′-*O*-(2-cyanoethyl)uridine (3a).** Compound **2a** (322 mg, 0.50 mmol) was dissolved in dry THF (5 mL). To the solution were added $Et_3N{\cdot}3HF$ (285 μL, 1.75 mmol) and triethylamine (125 μL, 0.897 mmol). After being stirred at room temperature for 1 h, the reaction mixture was evaporated in vacuo. The residue was chromatographed on a silica gel column with $CHCl_3$−MeOH (98:2−96:4, v/v) to give compound **3a** (199 mg, 99%) as white foam: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.63−2.66 (2H, m), 3.83−3.90 (2H, m), 4.04−4.09 (4H, m), 4.31 (1H, dd, *J* = 5.4, 7.3), 5.81 (1H, d, *J* = 1.7), 5.83 (1H, d, *J* = 8.3), 7.50−7.94 (5H, m), 8.10 (1H, d, *J* = 8.3); ^{13}C NMR ($CDCl_3$) δ 19.0, 60.1, 65.3, 67.8, 82.5, 84.2, 88.7, 102.0, 117.7, 129.3, 130.5, 131.1, 135.5, 140.4, 149.4, 162.3, 168.7; HRMS calcd for $C_{19}H_{19}N_3O_7$ (M + H$^+$) 402.1301, found 402.1315.

2′-*O*-(2-Cyanoethyl)uridine (4a). Compound **3a** (80 mg, 0.20 mmol) was dissolved in EtOH-28% NH_3 aq (2 mL, 1:1, v/v). After being stirred at room temperature for 1 h, the mixture was evaporated in vacuo. The residue was dissolved in MeOH−ether (11 mL, 1:10, v/v), and the solution was extracted three times with H_2O (3 mL). The aqueous extracts were combined and evaporated in vacuo. The residue was chromatographed on a column of silica gel with $CHCl_3$−MeOH (5:1, v/v) to give compound **4a** as a white solid (57 mg, 95%): mp 119 °C (EtOH); ^{1}H NMR (D_2O, 500 MHz) δ 2.70−2.72 (2 H, m), 3.69 (1 H, dd, *J* = 4.2, 12.9), 3.82−3.85 (3 H, m), 4.02−4.04 (1 H, m), 4.08 (1 H, dd, *J* = 3.7, 5.2), 4.19 (1 H, t, *J* = 6.1), 5.77 (1 H, d, *J* = 8.1), 5.87 (1 H, d, *J* = 3.7), 7.80 (1 H, d, 8.06); ^{13}C NMR (D_2O) δ 19.0, 60.9, 66.0, 68.8, 82.2, 84.7, 88.6, 102.8, 120.3, 142.2, 152.1, 166.8; HRMS calcd for $C_{12}H_{15}N_3O_6$ (M + H$^+$) 298.1039, found 298.1033. Anal. Calcd for $C_{12}H_{15}N_3O_6$: C, 47.91; H, 5.16; N, 13.97. Found: C, 47.66; H, 5.27; N, 13.48.

2′-*O*-(2-Cyanoethyl)-5′-*O*-(4,4′-dimethoxytrityl)-uridine (5a). Compound **4a** (1.97 g, 6.63 mmol) was coevaporated five times with dry toluene and finally dissolved in dry pyridine (70 mL). To the solution was added 4,4′-dimethoxytrityl chloride (2.47 g, 7.29 mmol). After being stirred at room temperature for 4 h, the mixture was quenched by addition of water and evaporated in vacuo. The residue was dissolved with $CHCl_3$. The solution was washed with brine and aqueous $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on a column of silica gel with $CHCl_3$−MeOH (95:5, v/v) containing 0.5% triethylamine to give compound **5a** as white foam (3.91 g, 98%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.68−2.71 (2 H, m), 3.53−3.58 (2 H, m), 3.90−3.98 (2 H, m), 4.03−4.06 (1 H, m), 4.17−4.22 (1 H, m), 4.49 (1 H, dd, J = 5.1, 8.8), 5.31 (1 H, d, J = 8.1), 5.89 (1 H, s), 6.84−6.86 (4H, m), 7.21−7.40 (9H, m), 8.06 (1 H, d, J = 8.1); ^{13}C NMR ($CDCl_3$) δ 19.0, 55.3, 60.8, 65.4, 68.2, 83.0, 87.1, 87.8, 102.3, 113.4, 117.7, 127.2, 128.1, 128.2, 130.1, 130.2, 135.1, 135.3, 139.8, 144.4, 150.7, 158.7, 158.8, 163.9; HRMS calcd for $C_{33}H_{33}N_3O_8$ (M + Na^+) 622.2165, found 622.2162.

Loading of 2′-*O*-(2-Cyanoethyl)uridine on CPG Resin. Compound **5a** (180 mg, 0.30 mmol) was dissolved in dry CH_2Cl_2 (30 mL). To the solution were added 4-(dimethylamino)-pyridine (48 mg, 0.39 mmol) and succinic anhydride (60 mg, 0.60 mmol). After being stirred at room temperature for 20 h, the mixture was washed with aqueous 10% citric acid and H_2O. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on a column of silica gel with $CHCl_3$−MeOH (90:10, v/v) to give the 3′-*O*-succinate as white foam (151 mg, 73%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.38−2.42 (1 H, m), 2.51−2.55 (1 H, m), 2.54−2.93 (4 H, m), 3.38 (1 H, dd, J = 2.0, 11.7), 3.75−3.80 (7 H, m), 3.90−3.94 (1 H, m), 4.02−4.06 (1 H, m), 4.38−4.41 (1 H, m), 5.13 (1H, dd, J = 4.9, 9.8), 5.32 (1 H, dd, J = 1.7, 8.1), 5.79 (1 H, s), 6.81−6.85 (4H, m), 7.22−7.38 (9H, m), 8.21 (1 H, d, J = 8.1), 11.17 (1H, br); ^{13}C NMR ($CDCl_3$) δ 19.4, 28.0, 28.2, 55.4, 59.9, 67.2, 68.5, 80.1, 81.7, 87.4, 89.7, 101.0, 113.4, 113.5, 118.7, 127.3, 128.2, 130.3, 135.1, 140.8, 144.3, 149.4, 158.9, 166.7, 172.0, 177.4.

An LCAA CPG resin (500 mg, 101.9 μmol/g) was suspended in CH_2Cl_2 (5 mL). To the solution were added 3′-*O*-succinate (18 mg, 26 μmol) and DCC (16 mg, 78 μmol). After being stirred at room temperature for 15 h, the resin was filtered and washed with dry CH_2Cl_2 and dry CH_3CN. To the resin was added a capping solution (pyridine−Ac_2O, 9:1, v/v) in advance and stirred at room temperature for 12 h. The resin was washed with dry CH_2Cl_2 and dry CH_3CN and dried in vacuo. The amount of Uce loaded to the solid support was calculated to be 16 μmol/g from calculation of the released dimethoxytrityl cation by use of a solution of 60% $HClO_4$−EtOH (3:2, v/v).

2-Cyanoethyl 2′-*O*-(2-Cyanoethyl)-5′-*O*-(4,4′-dimethoxytrityl)uridine 3′-(*N*,*N*-Diisopropyl)phosphoramidite (6a). Compound **5a** (1.20 g, 2.00 mmol) was coevaporated 5 times with dry toluene and dissolved in dry CH_2Cl_2 (10 mL) under argon atmosphere. To the solution were added ethyldiisopropylamine (0.5 mL, 2.87 mmol) and a solution of chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine (521 mg, 2.20 mmol) in dry CH_2Cl_2 (2 mL). After being stirred at room temperature for 2 h, the mixture was diluted with $CHCl_3$. The solution was washed with brine and aqueous $NaHCO_3$. The organic layer was dried over Na_2SO_4 and filtered. The solution was evaporated in vacuo. The residue was chromatographed on a column of silica gel with $CHCl_3$−MeOH (98:2, v/v) containing 0.5% triethylamine to give compound **6a** as white foam (1.33 g, 83%): ^{1}H NMR $CDCl_3$, 500 MHz) δ 1.11−1.29 (12 H, m), 2.42−2.73 (4 H, m), 3.42−4.66 (16 H, m), 5.19−5.26 (1 H, m), 5.87−5.88 (1 H, m), 6.82−6.87 (4 H, m), 7.23−7.43 (9 H, m), 8.05−8.11 (1 H, m); ^{13}C NMR ($CDCl_3$) δ 19.0, 19.1, 20.6, 20.7, 22.0, 22.8, 23.1, 24.6, 24.7, 24.8, 43.3, 43.4, 45.4, 55.4, 58.0, 59.4, 60.5, 65.6, 65.9, 69.0, 69.7, 69.8, 80.5, 80.6, 81.7, 82.3, 82.8, 87.2, 88.9, 89.2, 102.2, 102.3, 113.4, 113.5, 117.8, 118.1, 127.3, 127.5, 128.1, 128.2, 128.4, 130.4, 134.7, 135.1, 135.2, 139.4, 139.9, 143.81, 144.3, 150.3, 150.4, 158.8, 158.9, 159.0, 163.3, 163.4; ^{31}P NMR ($CDCl_3$) δ 151.1, 150.0; HRMS calcd for $C_{42}H_{50}N_5O_9P$ (M + H^+) 800.3424, found 800.3419

Oligonucleotide Synthesis. Oligoribonucleotides were synthesized on an Applied Biosystems 392 oligonucleotide synthesizer on a 1 μmol, using 2′-O-cyanoethylated phosphoramidite building blocks **6a**−**6d** and/or 2′-*O*-TBDMS PAC phosphoramidites (PacA, isopropyl-PAC-G, and Acetyl-C) from Glen Research. A 0.1 M solution of each 2′-*O*-Ce or 2′-*O*-TBDMS nucleoside phosphoramidite was used, and the time for coupling time was set to be 10 min. 1*H*-Tetrazole (0.45 M; for fully 2′-O-cyanoethylated RNA) and 5-ethylthio-1*H*-tetrazole (0.25 M; for one point modified RNAs) were used as the activator. Deprotection was carried out by use of ammonium hydroxide at room temperature for 20 min (for fully 2′-O-cyanoethylated oligouridylate), NH_4OH−NH_4OAc (10:1, w/w) at room temperature for 90 min (for fully cyanoethylated RNAs containing U, C, A, and G), propylamine−THF (1:1, v/v) at 40 °C for 24 h, and Et_3N·3HF at room temperature for 24 h (one-point modified RNAs).

The products were analyzed after C18 cartridge purification by use of anion-exchange HPLC as shown in Figure 2 (chromatographic conditions: a linear gradient of 25 mM sodium phosphate buffer containing 1 M NaCl (pH 6.0), 0−50% for 50 min, in 25 mM sodium phosphate buffer, pH 6.0, at a flow rate of 1 mL/min at 50 °C). The pure materials of the following oligoribonucleotides were obtained after additional reversed-phase HPLC (chromatographic conditions: a linear gradient of acetonitrile, 0−30% for 30 min, in 100 mM ammonium acetate, pH 7.0, at a flow rate of 1 mL/min at 50 °C) for UceU or anion-exchange HPLC for the other oligonucleotides in the following yields. UceU (80%), (Uce)12 (21%), GceAceCceUce (58%), GceAceCceUceGceAceCceUce-GceAceCceUce (6%), GCUAGceACUAUCUA (10%), GCUA-GAceCUAUCUA (12%), GCUAGACceUAUCUA (13%), GC-UAGACUceAUCUA (14%), GAGCCAceAGCIUCGGCUC (33%).

Nuclease Resistance Assay. The nuclease stability of the 2′-O-cyanoethylated oligonucleotides was evaluated by treatment with snake venom phosphodiesterase (Purchased from Sigma) or bovine spleen phosphodiesterase (Purchased from Sigma). The enzyme assay using snake venom phosphodiesterase (5×10^{-4} u/mL) was performed in a buffer of 50 mM Tris-HCl at pH 8.5, 72 mM NaCl and 14 mM $MgCl_2$ at 37 °C by use of 50 μM oligonucleotide. The enzyme assay using bovine spleen phosphodiesterase (0.2 U/mL) was performed in a buffer 30 mM NaOAc at pH 6.0 at 37 °C by use of 50 μM oligonucleotide. After the enzyme was deactivated by heating at 100 °C for 2 min, the solution was diluted and filtered by a 0.45-μm filter (Millex-HV, Millipore). The mixture was analyzed by reverse-phase HPLC or anion exchange HPLC.

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Supporting Information Available: General procedure and the procedures for the synthesis of 2′-O-cyanoethylated adenosine, guanosine, cytidine, and their phosphoroamidites. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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