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Chemical synthesis of RNA via 2′-*O*-cyanoethylated intermediates

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Abstract—It was found that 2′-*O*-cyanoethyl group could be removed from 2′-*O*-cyanoethylated ribonucleoside derivatives by treatment with Bu_4NF. This finding was successfully applied to the synthesis of oligoribonucleotides via their 2′-*O*-cyanoethylated derivatives as key intermediates where a cyanoethyl group was used as the 2′-hydroxyl protecting group. The rate of condensation using this protecting group in the presence of various activators was generally faster than that observed when a TBDMS group was used as the protecting group.

1. Introduction

Naturally occurring and chemically modified oligoribonucleotides have been synthesized for a wide variety of studies in molecular biology, gene diagnosis, and gene therapy.[1] Recently, new strategies for gene regulation using small interfering RNA (siRNA)[2] have attracted growing interest among molecular biologists and medical researchers.[3] To respond to the current strong demand for synthetic RNAs in the biological and medicinal fields, much attention has been paid to the development of new strategies for the chemical synthesis of RNA.[4–10]

Because the chemical synthesis of RNA requires an additional protecting group for the 2′-hydroxyl group compared with that of DNA, several problems have been pointed out. For example, the necessity of prolonged coupling times due to the steric hindrance of the 2′-protecting group,[5] difficulty in deprotection of the 2′-protecting group,[4c,11] 3′→2′ migration of internucleotidic phosphate groups,[12] difficulty in purification of crude 2′-*O*-protected intermediates,[13] and instability of RNA.[14] Various kinds of 2′-protecting groups using silyl,[15] acetal,[16–19] orthoester,[20,21] and *o*-nitrobenzyl ether,[22] triisopropylsilyloxymethyl,[5] *tert*-butyldithiomethyl, phenylsulfonylethoxymethyl,[9] and *p*-toluenesulfonyloxyethoxymethyl[10] functions have been reported over the past 50 years.

Independently, we have recently established a facile method for the synthesis of 2′-*O*-cyanoethylated RNA derivatives as a new type of 2′-*O*-modified RNA. 2′-*O*-Cyanoethylated RNA derivatives could be synthesized using appropriately protected 2′-*O*-cyanoethylribonucleoside 3′-phosphoramidite derivatives.[23] These monomer building blocks were conveniently obtained by a series of reactions involving the Michael reaction of the 2′-hydroxyl group of ribonucleoside derivatives with acrylonitrile in *t*-BuOH in the presence of Cs_2CO_3. Interestingly, in the study of the synthesis of 2′-*O*-cyanoethylated RNAs, we observed that the 2-cyanoethyl group remained intact upon treatment with $Et_3N\cdot 3HF$ but was immediately cleaved upon treatment with Bu_4NF, the reagent, which has been widely used in the deprotection of the protecting groups for the 2′-*O*-hydroxyl groups.[23] These unexpected but intriguing results led us to the idea that the 2′-*O*-cyanoethylated RNAs, which are useful as 2′-*O*-modified functional RNAs, can be, at the same time, useful as intermediates for natural-type RNA synthesis.

In this paper, we examined the usefulness of 2′-*O*-cyanoethylated RNA as a synthetic intermediate for unmodified RNAs. There are several papers on the 2′-*O*-protecting groups containing a 2-cyanoethyl group such as the 1-(2-cyanoethoxy)ethyl group[6] and more recently, the (2-cyanoethoxy)methyl group,[7,8] which can be removed by Bu_4NF.[19] In comparison to these protecting groups, our 2′-*O*-cyanoethyl group has two advantages; first, the group can be introduced directly using very cheap acrylonitrile, and second, cyanoethylated RNAs can be used as either functional RNAs or synthetic intermediates.

Keywords: RNA synthesis; Hydroxyl protecting group; β-Elimination; siRNA.
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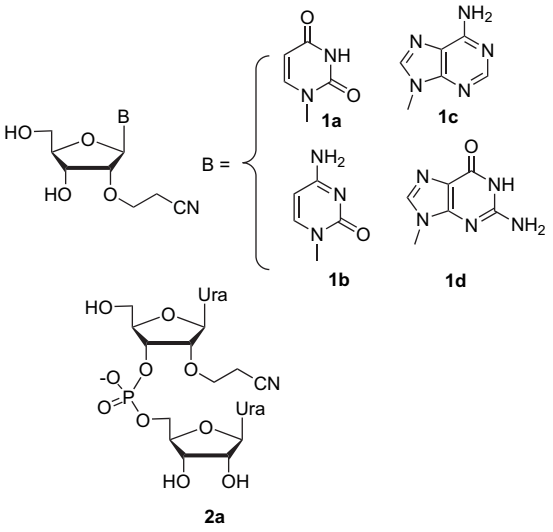
2. Results and discussion

2.1. Stability of 2′-*O*-cyanoethyl group toward various basic reagents

As reported in our previous paper,[23] we have synthesized various 2′-*O*-cyanoethylated ribonucleosides **1a**–**1d** and a partially 2′-*O*-cyanoethylated UpU **2a** (Scheme 1) via the Michael reaction of 2′-*O*-free ribonucleoside derivatives with acrylonitrile in the presence of Cs_2CO_3 in *t*-BuOH.



Scheme 1. Examples of the 2′-*O*-cyanoethylnucleoside derivatives.[23]

Initially, we checked the stability of the uridine derivative **1a** toward various basic conditions using TLC analysis. First of all, the stability toward K_2CO_3–MeOH solution used for oligonucleotide synthesis and 0.5 M DBU as an example of highly basic amines was examined. As a result, the cyanoethyl group of **1a** was cleaved very slowly to give uridine in 50% yield after 6 days with K_2CO_3 and 70% yield in 24 h with DBU. In contrast, the cyanoethyl group of **1a** was completely cleaved within 10 min by treatment with 1 M Bu_4NF in THF. The milder fluoride reagents such as 1 M Bu_4NF–AcOH and 1 M $Et_3N\cdot 3HF$ were not effective in cleaving the cyanoethyl group.

Table 1. Stability of the cyanoethyl group of **1a**–**1d** and **2a**

Compound	Conditions	Results
1a	0.05 M K_2CO_3–MeOH	50% deprotected (6 days)
1a	0.5 M DBU–CH_3CN	70% deprotected (24 h)
1a	1 M Bu_4NF–THF	100% deprotected (10 min)
1a	1 M Bu_4NF–AcOH–THF	24 h stable
1a	1 M $Et_3N\cdot 3HF$–THF	24 h stable
1b–1d	1 M Bu_4NF–THF	100% deprotected (5 min)
2a	1 M Bu_4NF–THF	100% deprotected (30 min)

Next, we examined the influences of nucleobase structures and the presence of internucleotidic phosphate on this deprotection using **1b–d** and **2a**. As shown in Table 1, the cytidine (**1b**), adenosine (**1c**), and guanosine (**1d**) derivatives[23] were slightly more labile than the uridine derivatives and cyanoethyl group was removed completely within 5 min.

We also checked the stability of dimer **2a** against 1 M Bu_4NF and revealed that the removal of cyanoethyl group took 30 min to give UpU (**3a**), as shown in Panels A and B of Figure 1, and in Table 1. Even when the reaction time was prolonged to 24 h, no decomposition or migration of the phosphodiester linkage was observed (data not shown). This result indicated that the lability of 2′-*O*-cyanoethyl depends on the base moieties and the presence of 3′-phosphate, but it can be removed within 30 min. These results clearly showed the possibility of 2′-*O*-cyanoethyl group as the 2′-*O*-protecting group.

2.2. Effects of 2′-hydroxyl protecting group on condensation

Next, we examined the effect of 2′-*O*-protecting group on the kinetics of internucleotidic bond formation as shown in Scheme 2. Generally, 2′-*O*-protecting groups significantly affect the coupling efficiency because of their own steric effect. The effect of 2′-*O*-protecting group on the coupling reaction was studied using the 2′-*O*-cyanoethyluridine 3′-phosphoramidite derivatives **4a** and the commercially available 2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-phosphoramidite derivatives **5**.

In this study, we have chosen 1*H*-tetrazole,[24,25] 5-benzylthio-1*H*-tetrazole (BnS-tet),[24] benzimidazolium triflate (BIT),[26,27] *N*-phenylimidazolium triflate (PhIMT),[26] 2-sulfobenzimide-*N*-methylimidazolium salt (SBIM),[28] which have been widely used as activators of the P–N bond of phosphoramidite building blocks. The reaction of 2′,3′-*O*,2-*N*-triacetylguanosine (**6**) with **4a** or **5** in the presence of these activators was monitored by ^{31}P NMR. The coupling efficiency was calculated by the amount of the phosphite

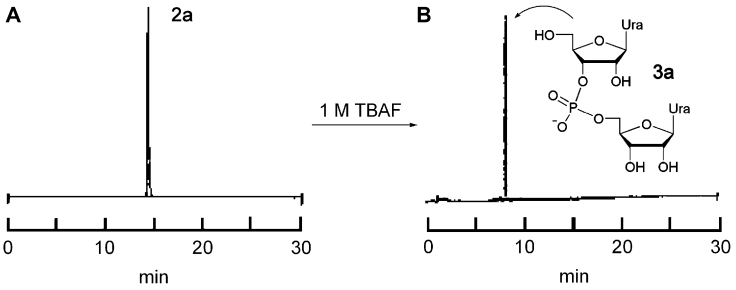
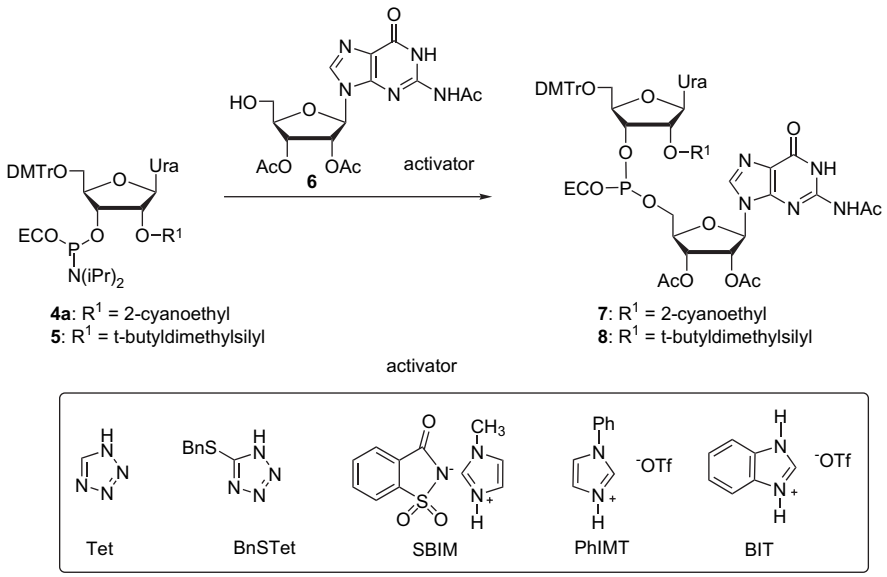


Figure 1. Reversed-phase HPLC profiles of UcepU (**2a**) and the mixture obtained after removal of 2-cyanoethyl group from **2a** by treatment with Bu_4NF. Panel A: purified **2a**; Panel B: crude UpU (**3a**) after decyanoethylation.



Scheme 2. Effects of the protecting groups on the coupling reaction at the 3′-reaction site.

intermediate **7** or **8**. The results obtained when 1*H*-tetrazole and 5-benzylthio-1*H*-tetrazole were used are shown in Figure 2. Panel A shows the results of the comparison of the condensation of the TBDMS building unit **5** in acetonitrile in the presence of 1*H*-tetrazole with that of the cyanoethyl building unit **4a**. Apparently, the cyanoethyl derivative was activated more quickly than the TBDMS unit. The same is true for the use of 5-benzylthio-1*H*-tetrazole, as shown in Panel B. Similar experiments were performed using other activators described above and the

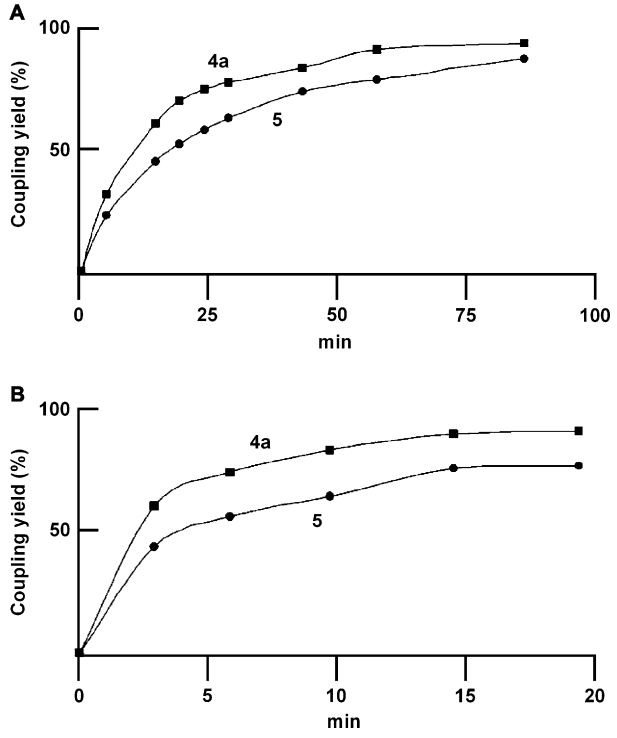


Figure 2. Effects of TBDMS and cyanoethyl groups as 2′-protecting groups on the coupling efficiency in the synthesis of **7** and **8**. Panel A: 3 equiv of 1*H*-tetrazole was used as the activator; Panel B: 3 equiv of 5-benzylthio-1*H*-tetrazole was used as the activator.

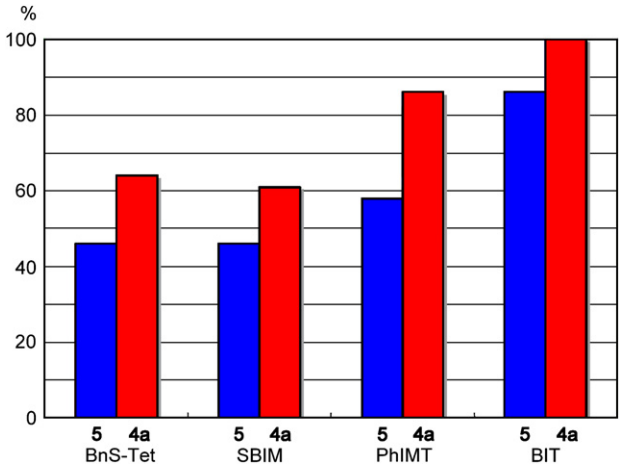


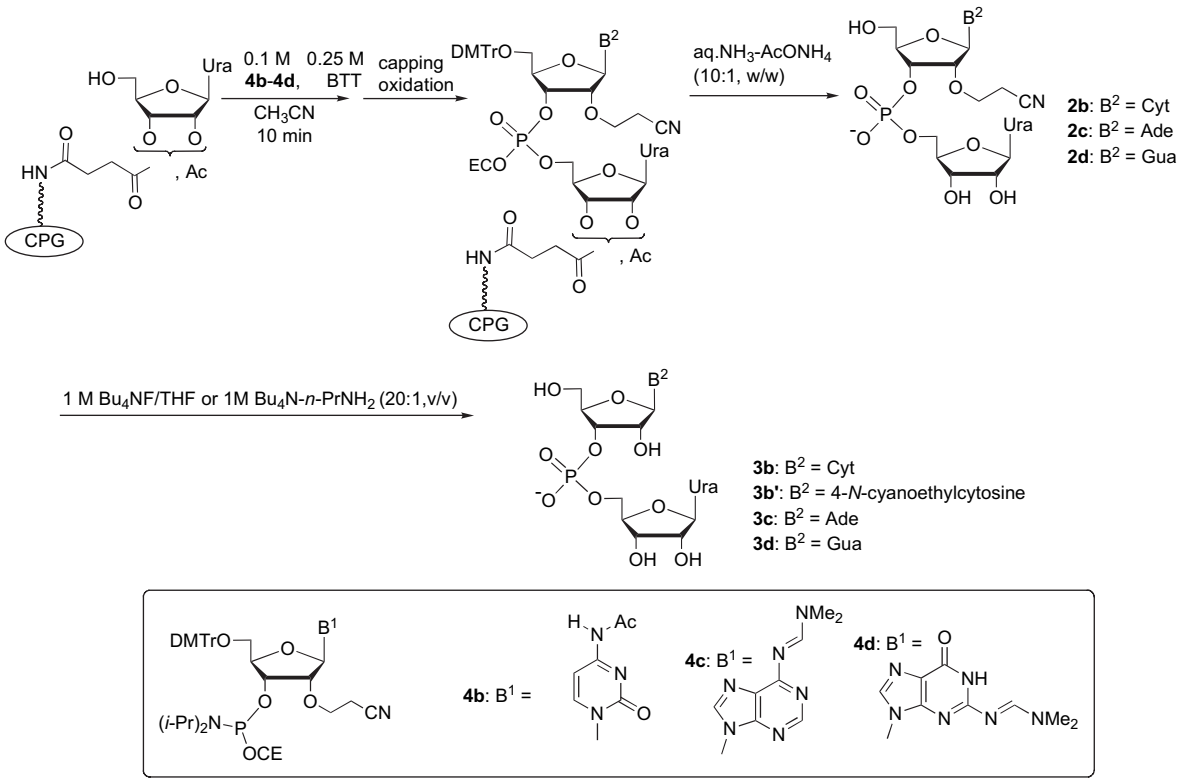
Figure 3. Coupling efficiency in the synthesis of **7** and **8** using various activators. The coupling yields after 3 min were estimated by ^{31}P NMR.

results are summarized in Figure 3 as the percentage formation of dimers **7** and **8** within 3 min. In all cases, the cyanoethyl building block proved superior to the one having a TBDMS group in terms of the kinetics of phosphite triester bond formation.

2.3. Deprotection of cyanoethyl group from XcepU (2b–2d: X=C, A, G) using Bu_4NF

To examine if cyanoethyl group could be removed at the level of oligoribonucleotides, the dimers XcepU (**2b–2d**: X=C, A, and G) were synthesized using the previously reported phosphoramidites **4b–4d** by solid-phase synthesis using CPG, as shown in Scheme 3, and treated with 1 M Bu_4NF solutions to remove the 2′-*O*-cyanoethyl group.

The deprotection of AcepU (**2c**) and GcepU (**2d**) was tested (Fig. 4). In this case, the fully protected dimers, which were synthesized on CPG supports were first treated with NH_3–$AcONH_4$[23] to cleave the protecting groups on the



Scheme 3. Solid-phase synthesis of UcepU, CcepU, AcepU, and GcepU.

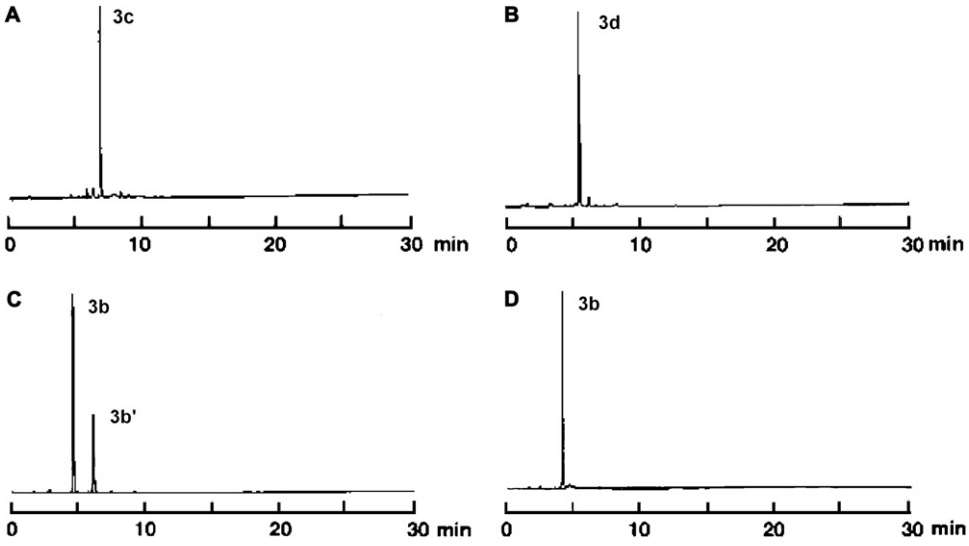


Figure 4. Reversed-phase HPLC profiles of ApU **3c** (Panel A), GpU **3d** (Panel B), and CpU **3b** (Panel C) obtained by treatment of AcepU, GcepU, and CcepU, respectively, with Bu_4NF. Panel D: **3b** obtained in the presence of $PrNH_2$.

nucleobase, phosphates, and the linker between 3′ terminal nucleotide residues and CPG supports. As described in the previous paper, 2′-*O*-cyanoethyl group remained intact in this buffered aqueous ammonia solution. After this ammonia treatment, the dimers were subjected to 1 M Bu_4NF solution. Similarly, it was confirmed that AcepU (**2c**) and GcepU (**2d**) were converted successfully to ApU (**3c**) and GpU (**3d**), respectively.

However, it was observed that treatment of CcepU with Bu_4NF was accompanied by a byproduct, a 4-*N*-cyanoethylated species **3b**′ of the cytosine moiety, in 32% yield. This result is explained in terms of the alkylation of once-formed acrylonitrile on the cytosine residue under basic conditions (Fig. 4C).

The mechanism was supported by the fact that the addition of *n*-$PrNH_2$, which trapped the acrylonitrile effectively reduced the side reaction. Thus, when 1 M Bu_4NF–*n*-$PrNH_2$ (20:1, v/v) was used instead of 1 M Bu_4NF, the *N*-cyanoethylated product **3b**′ could not be observed as shown in Figure 4D. Side reactions on the cytosine moiety concerning

the *N*-cyanoethylation have already been reported by some research groups.[29]

2.4. Synthesis of longer RNA fragments using 2′-*O*-cyanoethyl intermediates

We first studied the synthesis of a RNA fragment of U_{30}. A 2′-*O*-cyanoethylated intermediate of oligouridylate was synthesized using the phosphoramidite **4a** and BnS-Tet as an activator. Unexpectedly, a significant amount of a 29mer was observed with HPLC (Fig. 5A) probably due to incomplete detritylation process repeated at each coupling cycle. As expected, this problem was resolved by changing detritylation time from 15 s (5 s×3) to 30 s (5 s×6), as shown in Figure 5B. Subsequently, the cyanoethyl group was removed by treatment with 1 M Bu_4NF–THF to give the desired 30mer whose structure was confirmed by MALDI TOF mass analyses and enzymatic digestion as shown in Figure 6C.

When we tried to synthesize longer oligoribonucleotides having mixed sequences, we noticed that the dimethylaminomethylene (dmf) group of the adenine base did not have sufficient stability during the oligonucleotide synthesis. Therefore, the protecting group for the *exo*-amino group of adenosine was changed to a phenoxyacetyl group. A new adenosine building block **4c′** was synthesized, as shown in Scheme 4. The *N*-dmf protected derivative **9**[24] was converted to the *N*-unprotected adenosine derivative **10** by the action of hydrazine monohydrate without loss of the cyanoethyl group. Compound **10** was allowed to react successively with phenoxyacetic anhydride, $Et_3N\cdot 3HF$, and DMTrCl to give the 3′-free derivative **11**. The building block **5c′** was synthesized by phosphitylation of **11** in the usual manner.

For further demonstration of the chemical synthesis of RNAs using our strategy, an RNA 21mer (GCUUUCAAAAUGAU CUCACUG) known as an antisense strand of miR-bantam,[30] one of the microRNAs, was synthesized using our improved adenosine building block and BnS-Tet. It should be noted that a 2′-*O*-cyanoethylated oligoribonucleotide intermediate with a 5′-terminal DMTr group could be separated from the failure sequences without the DMTr

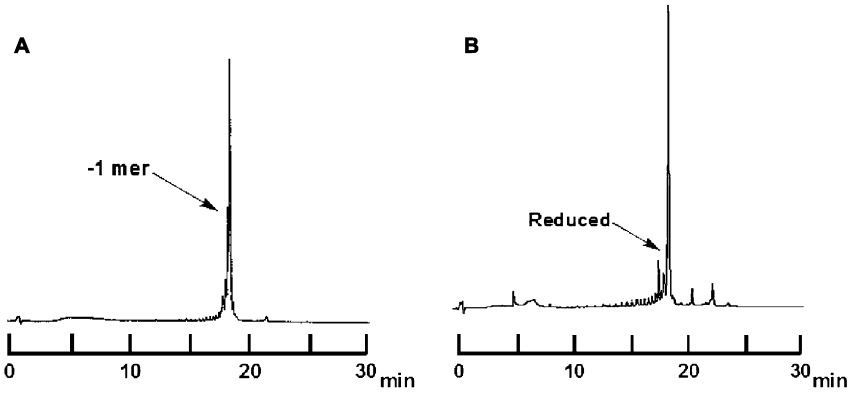


Figure 5. Anion-exchange HPLC profiles of crude $(Uce)_{30}$ obtained using a detritylation time of 15 s (Panel A) and prolonged time of 30 s (Panel B).

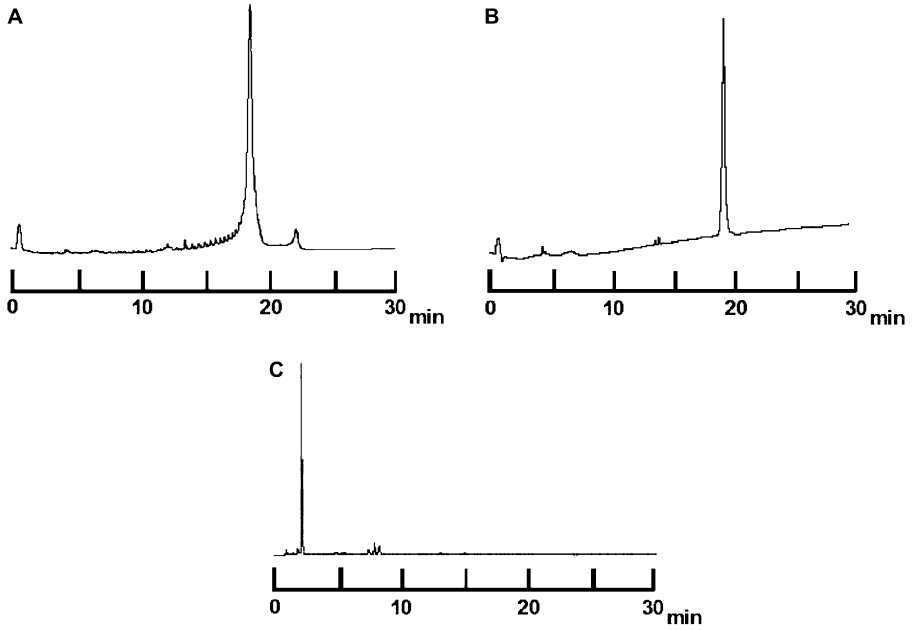
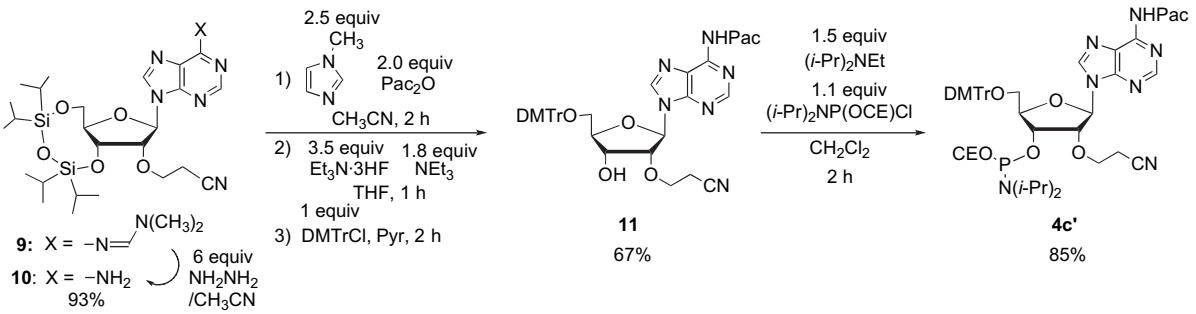


Figure 6. Anion-exchange HPLC profiles of crude U_{30} (Panel A), purified U_{30} (Panel B) and reversed-phase HPLC profile of enzymatic digestion of U_{30} with snake venom phosphodiesterase and phosphatase (Panel C).



Scheme 4. Synthesis of *N*-phenoxyacetyladenosine phosphoramidite derivative.

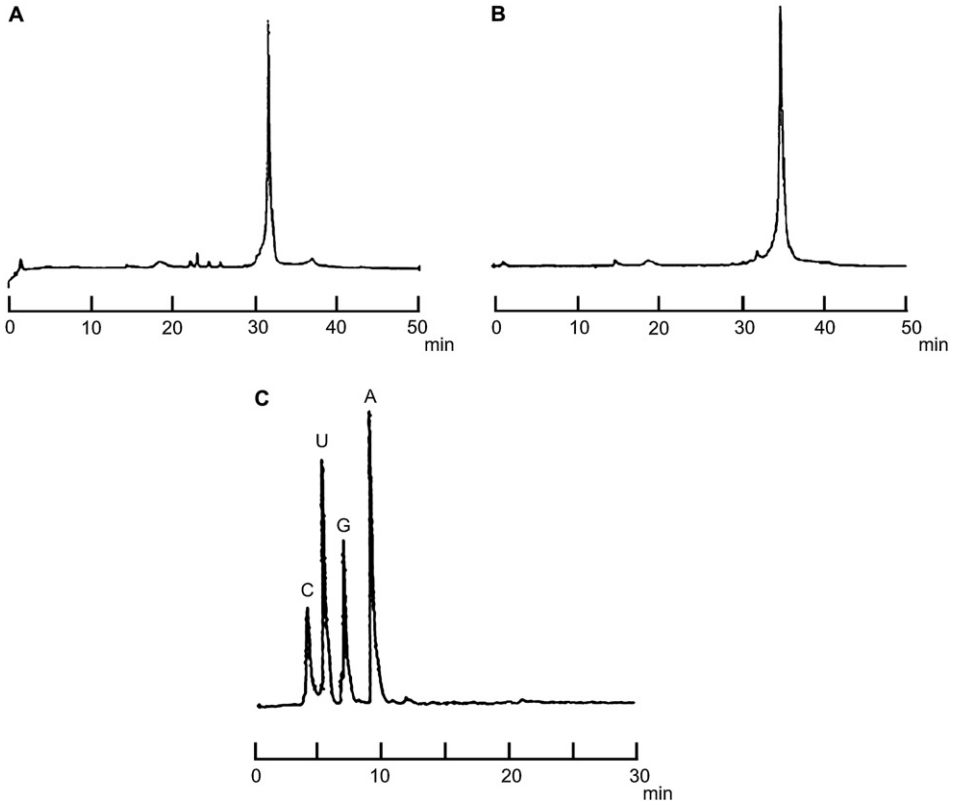


Figure 7. Anion-exchange HPLC profiles of the 2′-*O*-cyanoetylated RNA 21mer (Panel A) and the decyanoethylated RNA 21mer (Panel B) obtained using BnS-Tet and reversed-phase HPLC profile of enzymatic digestion of the RNA 21mer with snake venom phosphodiesterase and phosphatase.

group using a simple reversed-phase cartridge column because the very polar cyanoethyl group did not interact with C18 column surfaces.

At this stage, the desired 2′-*O*-cyanoethylated oligoribonucleotide could be obtained as the main peak in HPLC (Fig. 7A). Treatment of this intermediate with 1 M Bu_4NF in the presence of *n*-propylamine resulted in fully deprotected RNA, isolated in 34% yield (Fig. 7B). The structure of this product was characterized by MALDI TOF mass analyses and enzymatic digestion analysis as shown in Figure 7C.

3. Conclusion

In this study, we developed a new method for the synthesis of oligoribonucleotides using the cyanoethyl group as a protecting group for the 2′-hydroxyl group. When Bu_4NF was used as a reagent for removal of the cyanoethyl group, it was immediately deprotected. The present finding that the cyanoethyl group can be removed by treatment with Bu_4NF would be useful for the synthesis of naturally occurring products since this protecting group can be easily introduced by reaction of alcoholic functions with acrylonitrile in *t*-BuOH in the presence of Cs_2CO_3 as reported previously.[23] These results clearly demonstrated that the 2′-*O*-cyanoethylated RNA previously reported as a new 2′-*O*-modified functional RNA could also be used as a synthetic intermediate for natural-type RNA. Further development of the cyanoethyl protection strategy for RNA synthesis will be reported in the near future to achieve the synthesis of much longer and purer natural-type RNAs. We are now studying the mechanism of elimination of the cyanoethyl group by Bu_4NF using theoretical calculations. These results will be also reported in the near future.

4. Experimental section

4.1. General

^{1}H NMR spectra were recorded at 500 MHz and the chemical shifts were measured from the solvent peak as an internal standard (in $CDCl_3$) or HDO (in D_2O) as an external standard. ^{13}C NMR spectra were recorded at 500 MHz and the chemical shifts were measured from the solvent peak and dioxane (D_2O) as an internal standard. ^{31}P NMR spectra were recorded at 109 MHz and the chemical shifts were measured from 85% H_3PO_4 as an external standard. Pyridine was distilled twice from *p*-toluenesulfonylchloride and CaH_2 after being reluxed for several hours and stored over molecular sieves 4 Å. Triethylamine was distilled from CaH_2 and stored over molecular sieves 4 Å. TLC was performed on Merck Kieseigel 60 F_{254} precoated glass plates. Column chromatography was performed with silica gel C-200, C-300 (Wako Co. Ltd), 60N (Kanto Chemical, Co., Inc.), and NH (Fuji Silysia Chemical Ltd), and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. The solid-phase synthesis of oligonucleotides was carried out on a DNA/RNA synthesizer (ABI 392) using normal phosphoramidite protocol. HPLC was performed using the following systems. System A: reversed-phase HPLC was performed on a Waters Aliance system with a Waters 3D UV detector and a μBondasphere 5μ C_{18} 100 Å column (Waters, 3.9×150 mm). A linear gradient (0–30% or 0–10%) starting from 0.1 M NH_4OAc and applying CH_3CN was used at a flow rate of 1 mL/min for 30 min at 50 °C. System B: anion-exchange HPLC was performed on a Waters Aliance system with a Waters 3D UV detector and a Gen-Pak FAX column (Waters, 4.6×100 mm). A linear gradient (0–60%, A; 10–80%, C, C′) starting from 25 mM sodium phosphate buffer (pH 6.0) and applying 25 mM sodium phosphate buffer (pH 6.0) containing 1 M NaCl (pH 6.0) was used at a flow rate of 1 mL/min for 50 min (A) or 45 min at 50 °C (B, C) or 80 °C (C′). High resolution ESI mass spectrometry was performed by use of a Mariner (PerSeptive Biosystems, Inc.). The MALDI TOF mass spectrometry was carried out by use of a Voyger RP (PerSeptive Biosystems, Inc.).

4.2. 2′-*O*-(2-Cyanoethyl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (10)

Compound **9**[23] (618 mg, 1.00 mmol) was dissolved in anhydrous CH_3CN (5 mL). To the solution was added NH_2NH_2 monohydrate (291 μL, 6.00 mmol). After being vigorously stirred at room temperature for 2 h, the mixture was diluted with ethyl acetate. The mixture was washed six times with brine and dried over Na_2SO_4. The mixture was evaporated in vacuo. The residue was precipitated from diisopropyl ether and $CHCl_3$ to give compound **10** as a white solid (521 mg, 93%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.98–1.13 (28H, m), 2.72–2.75 (2H, m), 3.95–4.04 (2H, m), 4.15–4.18 (1H, m), 4.21–4.26 (2H, m), 4.30 (1H, d, *J*=4.40), 4.75 (1H, dd, *J*=4.40, 9.28), 5.67 (2H, br), 5.99 (1H, s), 8.10 (1H, s), 8.30 (1H, s); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 12.7, 12.9, 13.0, 13.5, 17.0, 17.1, 17.2, 17.3, 17.4, 17.6, 19.3, 59.7, 66.2, 69.7, 81.3, 82.6, 88.6, 117.7, 120.3, 139.0, 149.0, 153.2, 155.6; HRMS calcd for $C_{25}H_{42}N_6O_5Si_2$ ($M+H^+$) 563.2833, found 563.2787.

4.3. 6-*N*-Phenoxyacetyl-5′-*O*-dimethoxytrityl-2′-*O*-cyanoethyladenosine (11)

Compound **10** (563 mg, 1.0 mmol) was dissolved in anhydrous CH_3CN (5 mL). To the solution were added phenoxyacetic anhydride (573 mg, 2 mmol) and *N*-methylimidazole (0.2 mL, 2.5 mmol). The mixture was stirred at room temperature for 2 h and evaporated. The residue was dissolved in THF–MeOH–Et_3N–H_2O (5.5 mL, 2:2:1:0.5, v/v). The solution was stirred at room temperature for 30 min. The solution was evaporated in vacuo and diluted with $CHCl_3$. The solution was washed with brine, dried over Na_2SO_4 and filtered. The solution was evaporated in vacuo. The residue was dissolved in anhydrous THF (5 mL). To the solution were added $Et_3N\cdot 3HF$ (570 μL, 3.5 mmol) and Et_3N (246 μL, 1.8 mmol). The mixture was stirred at room temperature for 1 h. The solution was evaporated. The residue was chromatographed on a silica gel column ($CHCl_3$–MeOH, 100:0 to 95:5, v/v). The residue was coevaporated five times with anhydrous pyridine and dissolved in anhydrous pyridine (100 mL). To the solution was added DMTrCl (338 mg, 1 mmol). The mixture was stirred at room temperature for 2 h. The reaction was quenched with H_2O and evaporated. The residue was diluted with ethyl acetate and washed with brine and aq $NaHCO_3$. The solution was dried over Na_2SO_4 and evaporated. The residue was chromatographed on a silica gel column ($CHCl_3$–hexane, 1:1 to 1:0, v/v) to give **11** (505 mg, 67%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.64–2.75 (3H, m), 3.43 (1H, dd, *J*=4.15, 10.74), 3.55 (1H, dd, *J*=3.17, 10.74), 3.78 (6H, s), 3.91–3.95 (1H, m), 4.02–4.07 (1H, m), 4.23–4.25 (1H, m), 4.52–4.55 (1H, m), 4.63 (1H, dd, *J*=3.17, 4.59), 6.19 (1H, d, *J*=3.17), 6.80–7.44 (18H, m), 8.28 (1H, s), 8.72 (1H, s), 9.49 (1H, br); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 19.2, 55.4, 62.7, 65.9, 68.2, 69.9, 82.2, 83.8, 86.8, 87.5 113.4, 115.1, 117.4, 122.6, 123.4, 127.1, 128.1, 128.2, 128.3, 129.2, 130.0, 135.6, 135.7, 142.1, 144.6, 148.6, 151.3, 152.7, 157.1, 158.7, 166.8; HRMS calcd for $C_{42}H_{40}N_6O_8$ ($M+H^+$) 757.2986, found 757.2947.

4.4. 6-*N*-Phenoxyacetyl-5′-*O*-dimethoxytrityl-2′-*O*-cyanoethyl adenosine 3′-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) (4c′)

Compound **11** (412 mg, 0.54 mmol) was coevaporated five times with anhydrous toluene and replaced into argon atmosphere and dissolved in anhydrous CH_2Cl_2 (4.4 mL). To the solution were added ethyldiisopropylamine (142 μg, 0.82 mmol) and chloro(2-cyanoethyl)(*N,N*-diisopropylamino)phosphine (142 mg, 0.60 mmol) in CH_2Cl_2. The mixture was stirred at room temperature for 2 h and diluted with Et_2O (30 mL). The solution was washed with brine three times, satd aq $NaHCO_3$, and finally brine. The solution was dried over Na_2SO_4 and filtered. The solution was evaporated in vacuo. The residue was chromatographed on a silica gel column (hexane–EtOAc, 2:1 to 1:1 to 1:2, v/v) to give **4c′** (440 mg, 85%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.06–1.19 (12H, m), 2.59–2.65 (4H, m), 3.34–3.37 (1H, m), 3.55–3.76 (4H, m), 3.77–4.07 (10H, m), 4.37–4.38 (1H, m), 4.59–4.63 (1H, m), 4.82–4.87 (3H, m), 6.16–6.17 (1H, m), 6.78–6.82 (4H, m), 7.03–7.06 (3H, m), 7.19–7.43 (11H, m), 8.26–8.29 (1H, m), 8.70–8.71 (1H, m), 9.51 (1H, br); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 19.0, 20.2, 20.3, 20.5,

24.6, 24.7, 24.8, 24.9, 43.3, 43.4, 43.5, 55.3, 57.9, 58.1, 58.3, 58.4, 62.6, 65.5, 65.7, 68.2, 71.0, 71.1, 80.7, 81.1, 83.3, 83.5, 83.6, 86.7, 87.7, 87.8, 113.2, 114.9, 115.0, 117.2, 117.5, 117.6, 117.9, 122.1, 122.4, 123.4, 127.0, 127.1, 128.0, 128.3, 129.8, 129.9, 130.2, 135.6, 135.7, 142.5, 142.6, 144.5, 144.6, 148.5, 151.5, 152.6, 157.1, 158.7, 166.8; ^{31}P NMR ($CDCl_3$) δ 150.8, 150.7; HRMS calcd for $C_{51}H_{57}N_8O_9P$ (M+H$^+$) 957.4064, found 957.4066.

4.5. Steric effects of the 2′-*O*-protecting group in the coupling reaction

2′,3′-*O*,2-*N*-Triacetylguanosine (**6**)[31] (25 mg, 0.06 mmol) and a protected phosphoramidite **4a** or **5** (0.03 mmol) were co-evaporated three times each with anhydrous pyridine, anhydrous toluene, and anhydrous CH_3CN under reduced pressure. The dried mixture was dissolved in CD_3CN (1 mL or 840 μL, 800 μL), and to the solution was added a 0.25 M solution of 1*H*-tetrazole, 5-benzylthio-1*H*-tetrazole, 0.2 M benzimidazolium triflate, *N*-phenylimidazolium triflate, or *o*-sulphobenzimide *N*-methylimidazolium salt in CH_3CN. The reaction was monitored by ^{31}P NMR. These results are summarized in Figures 2 and 3.

4.6. Oligonucleotide synthesis

Oligoribonucleotides were synthesized on an Applied Biosystems 392 DNA synthesizer on a 1 μmol scale, using 2′-*O*-cyanoethylated ribonucleoside 3′-phosphoramidite building blocks **4a**, **4b**, **4c**′, and **4d**. A 0.1 M solution of each 2′-*O*-cyanoethylated ribonucleoside 3′-phosphoramidite was used and the time for condensation was set to be 10 min. A 0.25 M 5-benzylthio-1*H*-tetrazole in acetonitrile was used. Release of the oligonucleotide from the resin and deprotection of the protecting groups of the phosphate and base moieties were carried out by use of NH_4OH–NH_4OAc (10:1, w/w, 2 mL, System A) or *n*-$PrNH_2$–THF (1:1, v/v, 2 mL, System B) at room temperature for 60–120 min (CpU, ApU, GpU, and U_{30}, Method A) and for 15 h (RNA21mer: GCUUUCAAAAUGAUCUCACUG Method B). The mixture was diluted in 0.1 M NH_4OAc buffer (10 mL) and the solution was subjected to a C18-cartridge (DMTr-On). The cartridge was washed with 10–20% CH_3CN–0.1 M NH_4OAc buffer (10 mL), detritylation with 2% aq TFA (5 mL×3), washed with 0.1 M NH_4OAc buffer (10 mL), H_2O (10 mL) and elution with 20% CH_3CN–H_2O (10 mL) was done. The eluate was lyophilized. The powder of the 2′-*O*-cyanoethylated oligomer thus obtained was treated with a 1 M solution of TBAF in THF–*n*-$PrNH_2$ (20:1, v/v, 210 μL for 2mer, 630 μL for 12mer, 2 mL for 21mer) at room temperature for 0.5 h (2mer), 12 h (12mer, U30mer), 15 h (21mer). The mixture was diluted with 0.1 M ammonium acetate buffer (10–20 mL), and THF component of the solution was evaporated under reduced pressure. The solution was subjected to a cation exchange cartridge (OASISMCX cartridge) pre-washed with CH_3CN and 0.1 M NH_4OAc buffer, passed through it. Further purification was carried out with C18-cartridge, which was washed with 0.1 M NH_4OAc buffer and eluted with 20% CH_3CN–H_2O (10 mL). The eluate was lyophilized. The residue was dissolved in H_2O and purified with anion-exchange HPLC. The fractions containing the product were combined. For desalt, the obtained fraction was lyophilized. The residue was dissolved in 0.1 M NH_4OAc buffer (10 mL). The solution was subjected to a C18-cartridge. After the cartridge was washed with 0.1 M NH_4OAc buffer (10 mL), H_2O (10 mL), elution was performed with 20% CH_3CN–H_2O (10 mL). The fractions containing the product were combined and lyophilized: U_{30}, 15%, MALDI TOF mass calcd for $C_{270}H_{332}N_{60}O_{238}P_{29}$ 9119.81, found 9119.32; GCUUUCAAAAUGAUCUCACUG, 34% MALDI TOF mass calcd for $C_{198}H_{247}N_{74}O_{146}P_{20}$ 6615.89, found 6620.11.

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Supplementary data

The ^{1}H, ^{13}C, and ^{31}P NMR data of all new products. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2007.07.102.

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13. For example, when fully protected oligoribonucleotides synthesized by the TBDMS strategy were treated with ammonia–EtOH for removal of the phosphate and base protecting groups, the resulting oligoribonucleotides having the 5′-terminal DMTr group and the 2′-*O*-TBDMS group at each 2′-positon could not be purified by a C18 cartridge at this stage. This is because the lipophilicity of the TBDMS group is so high that shorter 2′-*O*-TBDMS-oligoribonucleotides formed owing to the insufficient coupling were contaminated. Contrary to this fact, 2′-*O*-cyanoethylated oligoribonucleotides exhibited less liphophilicity so that the DMTr-on 2′-*O*-cyanoethylated oligoribonucleotides could be easily separated from the undesired shorter sequences. If necessary, further purification could be done after the decyanoethylation by a C18-cartridge.
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