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SOLID PHASE SYNTHESIS OF OLIGORIBONUCLEOTIDES BY THE PHOSPHORAMIDITE APPROACH USING 2'-O-1-(2-CHLOROETHOXY)ETHYL PROTECTION

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ABSTRACT The new type protecting group, 1-(2-chloroethoxy)ethyl (Cee) group has been employed for the protection of the 2'-OH groups of ribonucleoside residues in the synthesis of oligoribonucleotides by the phosphoramidite approach on a solid support, using the acid-labile 5'-O-dimethoxytrityl (DMTr) group. This group is completely stable under the acidic conditions required to remove the 5'-terminal protecting groups in oligonucleotide synthesis on a solid support, and yet is easily removable under mild condition of acidic hydrolysis (pH 2.0) for the final unblocking step. The Cee-protected ribonucleoside 3'-phosphoramidite units were evaluated in the synthesis of a series of oligoribonucleotides consisting of the homopolymers of cytidine, the box 9R and 9R' sequences of Tetrahymena rRNA, and a leader sequence of phage Qβ-A protein mRNA. A full data for the deprotection and purification of synthetic oligoribonucleotides are also described.

INTRODUCTION

The chemical synthesis of oligo- and poly-ribonucleotides on a solid support is more elaborate and time-consuming than the oligo- and poly-deoxyribonucleotides primarily because of the need to protect the 2'-OH group of ribonucleosides. The choice of the protecting group for the 2'-OH group is a crucial point in polyribonucleotide synthesis and it should be completely stable under the conditions required for removal of the 5'-protecting groups in the solid-phase synthesis of oligoribonucleotides. Finally, it must be removed at the end of synthesis by a procedure that does not cause internucleotidic cleavage or phosphoryl migration under the deprotection conditions.

The tert-butyldimethylsilyl (TBDMS) and o-nitrobenzyl groups as the 2'-OH group have recently been described by Ogilvie et al.[1] and Tanaka et al.[2] for use with the phosphoramidite approach of long chain synthesis. The TBDMS group has been combined with 5'-O-DMTr group and used for the synthesis of a 43 and 77mers using the phosphoramidite approach. However, a few works demonstrated the lability of the 2'-O-silylated oligoribonucleotides towards concentrated aqueous ammonia due to the undesired removal of the TBDMS group, and subsequent loss of the silyl groups and cleavage of the phosphodiester bonds, during the alkaline removal of the base and phosphate protecting groups.[3-5] These side reactions were suppressed in ethanol containing ammonia solution or methanolic anhydrous ammonia.[4,5] The o-nitrobenzyl group has also been combined with 5'-O-DMTr group and used for the synthesis of oligoribonucleotides of up to 34mer using the

phosphotriester[6], phosphoramidite[7], and H-phosphonate[8] approaches. We have also investigated the use of substituted aryl ethers such as 4-methoxy-[9,10] and 3,4-dimethoxybenzyl[11,12] groups as the 2'-OH groups.

As some recent studies have shown[13,14] that an acid-labile 2'-acetal protecting group was insufficiently stable under acidic conditions required for the removal of either 5'-O-DMTr[15] or 9-phenylxanthen-9-y (Px)[16] groups to be useful for chain elongation on a solid support. However, the use of 2'-O-Thp-ribonucleosides for the synthesis of several 8mers on a solid support has been recently demonstrated by Keirzek et al.[17] Further, Tanimura et al.[18] have synthesized the oligoribonucleotides (10-13mers) using two different 5'-protecting groups [Px or (4-methoxy)phenylxanthen-9-yl] in combination with the 2'-O-Thp group.

In order to overcome this problem, a few workers have explored a new acetal protecting group for 2'-OH groups or have searched for a procedure without acid treatment during chain elongation. Reese et al.[19-21] have investigated the use of a new type of acetal group such as 1-aryl-4-methoxypyperidin-4-yl (1-[(2-chloro-4-methyl)-phenyl]-4-methoxypiperidin-4-yl (Ctmp) and 1-(2-fluoropheny)1-4-methoxypiperidin-4-yl group designed to be stable under the conditions required for the removal of a Px group but cleavable under mild conditions at pH 2.0. These protecting groups have been successfully applied in solid phase synthesis by the phosphoramidite[22,23] and H-phosphonate[24] approaches. The 3-methoxyl-1,5-carbomethoxypentanyl group described by Chattopadhyaya[25] has an advantage in that it is converted to the labile diamide group during ammonolysis step of deprotecting of the fully protected oligoribonucleotides. These groups can easily be removed under acidic conditions.

Recently, Iwai et al.[26] have reported the synthesis of a 21mer by the phosphoramidite approach using the base-labile levulinoyl[27] and tetrahydrofuranyl groups for the protection of 2'- and 5'-OH groups ,respectively. More recently, Lehmann et al.[28] have reported that a combination of the 9-fluorenylmethoxycarbonyl group (Fmoc)[29] for 5'-protection and 4-methoxytetrahydropyran-4yl (Mthp)[30] for 2'-protection was effective for the synthesis of oligoribonucleotides. However, these approaches introduce some problems, such as the low selectivity of introduction to the 5'-position of the levulinoyl and Fmoc groups. An evident advantage of 2'-O-acetal protecting groups is that acetal groups can be introduced to the 2'-OH group of ribonucleosides via the use of 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)ribonucleosides in high yields and removed easily by acid treatment (pH 2.0).

In our continuing studies[24,31,32] to develop acetals as 2'-protecting groups for the solid phase synthesis of oligoribonucleotides using the phosphoramidite approach, we have also found that 1-(2-chloroethoxy)ethyl (Cee) group is stable under the acidic conditions required for the complete removal of a DMTr group at every cycle of oligoribonucleotide addition; however, under hydrolytic conditions (pH 2.0), the Cee group is removed. 2-Chloroethyl vinyl ether is an easily accessible compound, and the required 2'-O-Cee-ribonucleoside derivatives can be prepared in high yields. The results of this study have

been used successfully to synthesize a series of oligoribonucleotides of up to 20 residues.

RESULTS AND DISCUSSION

Synthesis and properties of 2'-O-acetaluridine derivatives.

First we examined the synthesis of uridine derivatives (**2a-c**) bearing three different 2'-acetal protecting groups. Further, we carried out investigation without separation of the diastereoisomers. The reaction between 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-uridine (**1a**)[33] and alkyl vinyl ethers in the presence of p-toluenesulfonic acid and the conversion of the products obtained into **2a-c** (80-83%) are indicated in outline in Figure 1. 2'-O-Acetaluridine derivatives (**2d-f**) were prepared according to the published procedure.[19,34,35] The location of the substitution was determined by ^{1}H-NMR.

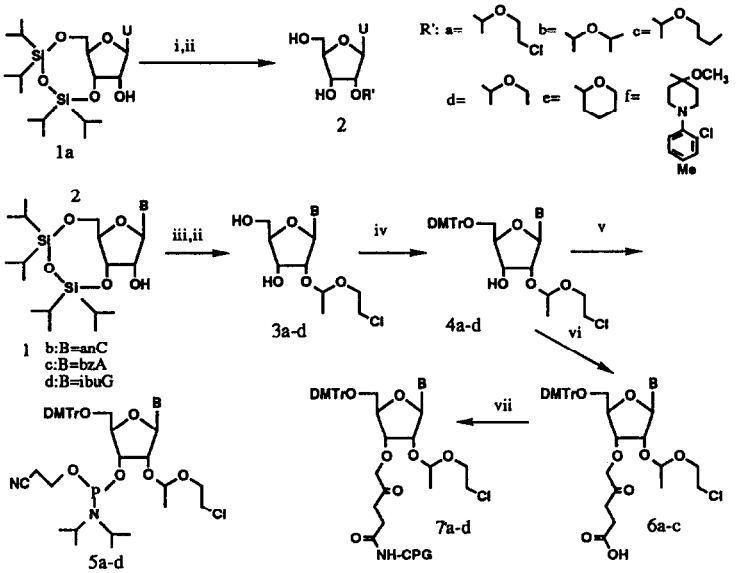

Figure 1. Reaction scheme for the preparation of the 2'-O-acetaluridine derivatives (**2**) and the 2'-O-Cee protected building blocks. Reagents: i, alkyl (2-chloroethyl, isopropyl, n-butyl, ethyl) vinyl ethers, 2,3-dihydropyran, 1-(2-chloro-4-methylphenyl)-4-methoxy-1,2,5,6-tetrahydropyridine, and PPTs in CH_2Cl_2: ii, tetraethylammonium hydrogen fluoride in THF: iii, 2-chloroethyl vinyl ether and TsOH (or PPTs) in CH_2Cl_2: iv, dimethoxytrityl chloride in pyridine: v, 2-cyanoethyl N,N-diisopropylphosphoramidochloridite and N,N-diisopropylethylamine in THF: vi, succinic anhydrine and 4-dimethylaminopyridine in CH_2Cl_2: vii, long-chain alkylamine CPG, 4-dimethylaminopyridine (DMAP), and DCC in DMF.

It was then interesting to explore the relative stabilities of these 2'-O-acetal groups in 2'-O-acetaluridine derivatives (**2a-f**) under the acidic conditions to evaluate their possible use in the chemical synthesis of oligoribonucleotides in conjunction with other acid-labile (5'-O-DMTr and 5'-O-Px) protecting groups on the pentose sugar. The relative rates of removal of the acetal groups from the corresponding 2'-O-acetal derivatives (**2a-f**) are shown in Table 1. It can be seen from Table 1 that both Cee and

Ctmp may be used as 2'-protecting groups in view of its relatively high stability under acidic conditions. However, the Cee group is ca. 1.2 times more stable to acidic hydrolysis than the Ctmp groups at pH 2.0. Further, there is a significant difference in

Table 1. The relative rates of hydrolysis of acetal groups from uridine derivatives **(2a-f)**[a].

comp.	0.01 N HCl (pH 2.0) $t_{1/2}$ (min)	t_{∞} (min)
2a	96	360
2b	1	4
2c	12	34
2d	5	18
2e	32	150
2f	55	295

a) These reactions were carried out with diastereomeric mixture of 2'-O-acetaluridine derivatives **(2)** at room temperature. The reactions were monitored by TLC and the reversed phase HPLC.

the stability of the Cee and Ctmp groups under acidic condition (1.5% dichloroacetic acid (DCA) in CH_2Cl_2) required for complete removal of a 5'-O-DMTr group. For example, in 1.5% DCA at 22°C ca 20% of the Ctmp group from **2f** was lost after 30 min, whereas the loss of the Cee groups from **2a** amounts to only ca 5% after 50 min.[36] From these results, during the 1 min acid treatment required for the cleave of a 5'-O-DMTr group the concomitant loss of 2'-O-Cee group will be considerably less than 0.1%. The Cee group is somewhat greater stable to acid (1.5% DCA) than the Ctmp group. This is advantageous in that the use of the Cee group would be expected to lead to even greater selectivity in the 5'-deblocking step than the Ctmp group.

Preparation of ribonucleoside 3'-O-phosphoramidite units.

The N-acyl-2'-O-Cee-ribonucleosides **3b-d** were key intermediates for the preparation of ribonucleoside 3'-O-phosphoramidite units. The cytidine derivative **3b** was obtained from 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-N-anisoylcytidine **(1b)** in 87% yield by reaction with 2-chloroethyl vinyl ether in CH_2Cl_2 in the presence of p-toluenesulfonic acid, followed by treatment with 1 M tetraethylammonium hydrogen fluoride (TEAHF).[34] However, the 2'-O-1-(2-chloroethoxy)ethylation of **1c** and **d** did not proceed smoothly under the conditions described above. In the presence of pyridinium p-toluenesulfonate (PPTs) in CH_2Cl_2, **1c** and **d** reacted almost quantitatively with 2-chloroethyl vinyl ether and the conversion of products obtained into **3c** and **d** (83% and 81%) are indicated in Figure 1. The location of the Cee groups were determined by ^{1}H-NMR. Further, evidence of the site of the Cee group was obtained by determining the structure of the oligoribonucleotide with hydrolysis of ribonuclease T_2 as described later in this paper.

The general procedure of the preparation of N-acyl-5'-O-DMTr-2'-O-Cee-nucleoside 3'-O-phosphoramidite units **(5a-d)** is shown in Figure 1. The protected ribonucleosides **4a-d** were dissolved in dry CH_2Cl_2 to which was added N,N-diisopropylethylamine followed by 2-cyanoethyl N,N-diisopropylphosphoramidochloridite[37] at 0°C. After 2h at room temperature,

the workup was an extraction between ethyl acetate and saturated sodium chloride. The crude products were purified by short column chromatography on silica gel. However, the phosphitylating reaction did not procced smoothly and the yeild became lower. When THF was used in place of CH_2Cl_2, the reaction proceed smoothly and gave the phosphoramidite units (**5a-d**) in good (82-87%) yields. The spectroscopic and physical properties of **5a-d** are shown in the experimental section. The ^{31}P-NMR data clearly show that there is, as expected, two pairs of diastereomeric signals for all of the phosphoramidite units, thereby establishing the isomeric purity of these compounds. These diastereomers should have no influence on the coupling reaction.

Solid phase synthesis of oligoribonucleotides.

The preparation of nucleoside-controlled pore glass (CPG) resins was performed by the method reported previously.[24] The amount of loaded nucleosides was 44.0 μmol/g for **7a**, 39.5 μmol/g for **7b** and 37.5 μmol/g for **7c**, as estimated from the DMTr cation release after treatment with 1.5% DCA in CH_2Cl_2. The proceduces for synthesis was a modification of the procedure described previously.[38,39] The reaction was carried out on a small column of nucleoside-functionalized glass (0.2 μmoles) with an Applied Biosystems Model 381A DNA synthesizer using the cycle as described in the experimental section.

The utility of ribonucleoside 3'-phosphoramidites (**5**) in which the 2'-hydroxyl functions are protected with the Cee group is now demonstrated by the synthesis of 2-20 mers consisting of the homopolymers of cytidylic acids, the octamers of mixed base composition corresponding to the box9R and 9R' of Tetrahymena rRNA[40], and a leader sequence (rAGUAUAAGAGGACAUAUGCA) of phage Qβ-A protein as well as the AUG initiation codon.[41,42] In all cases, the average coupling yields were excellent, ranging from 95 to 98%. On the other hand, the 2'-O-Thp-cytidine 3'-O-phosphoramidite[17] was used in the synthesis of rC_{18}. The average coupling yield from the 2'-O-Thp amidite was similar, 94%, when compared with 95% yield obtained with the 2'-Cee amidite **5b**. As will be discussed bellow, the yield following deprotection was slightly higher in the case of the 2'-O-Cee protected oligomer.

Deprotection of chemically synthesized oligoribonucleotides.

In order to study both internucleotidic cleavage and phosphoryl migration under the deprotection conditions, the dimer, rUpU was treated first with ammonia and then with 0.01 N HCl (pH 2.0). The 2',5'-protected dimer was dissolved in 0.01 N HCl and the pH adjusted to 2.0 by addition of 0.1 N HCl. The mixture was stirred at room temperature for 6 h and neutralized with aqueous ammonia, followed by the reversed-phase HPLC analysis (Figure 2). Integration of the main peak in Figure 2a reveals that the dimer, rUpU account for 98% of the total absorbance at 254 nm. Further, no isomerization to rU2'p5' can be detected under the deprotection conditions (Figure 2b). The ratio of rUp and rU were estimated by the reversed-phase HPLC after digestion of rUpU with ribonuclease T_2 and was found to be rUp:rU=1.00:1.04 (theoretical, 1.00:1.00). Under the conditions of digestion, ribonuclease T_2 did not cleave rU2'p5'U. No peak corresponding to rUpU was observed in the chromatogram of the digests, indicating the absence of any 2'-5' internucleotidic bonds.

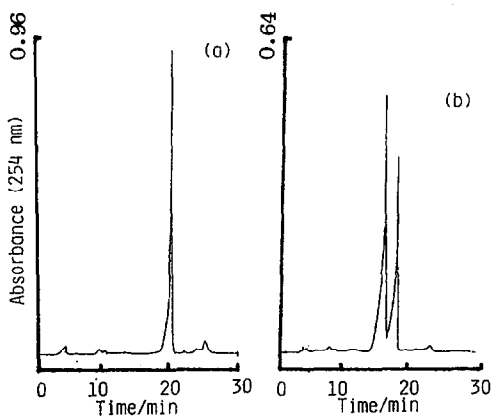

Figure 2. HPLC analysis of the crude mixture containing $rU^{3'}p^{5'}U$ after deprotection (a), using TSKgel oligo-DNA RP column with a linear gradient of 50% aqueous methanol (from 0 to 50% during 30 min) in 0.05 M ammonium phosphate (pH 7.0), and after addition of $rU^{2'}p^{5'}U$ (b),using the same gradient.

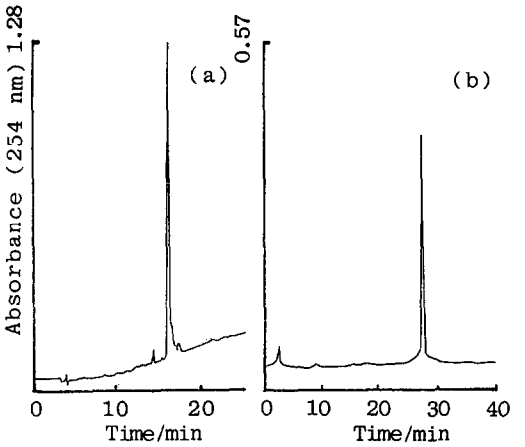

Figure 3. Purification and analysis of the 20 mer. (a) The deprotected 20 mer was purified by the reverse phase HPLC using a Inertsil ODS column with a linear gradient of CH_3CN (from 5 to 20% during 25 min) in 0.1 M triethylammonium acetate (pH 7.0). (c) The purified oligomer, rC_{18} was analyzed by anion exchange HPLC using a TSKgel DEAE-2SW column with a linear gradient of ammonium formate (from 0.5 to 1.5 M during 40 min) in 20% aqueous CH_3CN.

The oligomers synthesized above were treated in a similar manner with ammonia at 55°C for 5-8 h affect the concomitant cleave of the oligomer from support and to complete the removal of the exocyclic aminoacyl protecting groups, and then 0.01 N-HCl (pH 2.0) at room temperature for 6-24 h. The reaction mixture was then neutralized with aqueous ammonia and analyzed by the reversed-phase HPLC. The HPLC profiles of the purified oligomers are shown in Figure 3. Further, the crude oligomer was checked by analytical polyacrylamide gel electrophoresis (PAGE). Figure 4 shows the 20% polyacrylamide gels of the 18 and 20mers, lane 1 and 2, respectively, (a) crude and (b) after purification. The PAEG showed

Table 2. Isolated Yields in the Synthesis of Oligoribonucleotides.

sequence	length	yields[a]
rCCCCCCCCCCCC	12	3.50 A_{260} unit (25%)
rCCCCCCCCCCCCCCCCCC	18	3.66 A_{260} unit (24%)
rCCCCCCCCCCCCCCCCCC	18	1.40 A_{260} unit (10%)[b]
rUGUCGGUC	8	6.34 A_{260} unit (36%)
rGACCGUCA	8	5.34 A_{260} unit (27%)
rGACCGACA	8	4.89 A_{260} unit (30%)
rAGUAUAAGAGGACAUAUGCA	20	2.01 A_{260} unit (9%)

a) The overall yield from the 3'-terminal ribonucleoside on CPG.
b) The coupling reaction was carried out by use of DMTranCThp-3'-O-phosphoramidite unit.

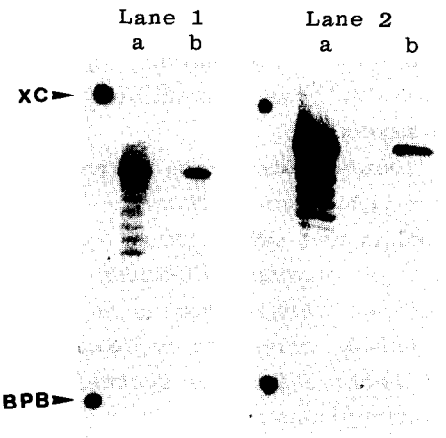

Figure 4. 20% polyacrylamide/7 M urea gel electrophoresis of fully deptotected oligoribonuccleotides. Lane 1=rC_{18} [(a) crude; (b) pure]. Lane 2=AGUAUAAGAGGACAU-AUGCA [(a) crude; (b) pure].

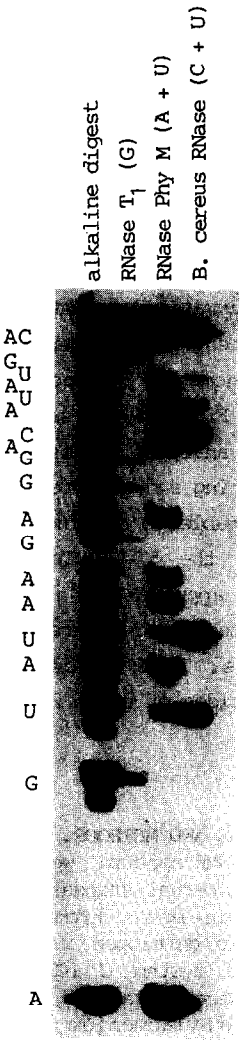

Figure 5. Autoradiogram of electrophoresis on a 20% polyacrylamide gel containing 7 M urea for RNA sequence. The 5'-end-^{32}P-labeled-20mer was partially digested with ribo-nucleases according to the method of Donis-Keller.

the shorter fragment spots, presumaly due to the direct acid treatment without separation of trityl-containing oligomers after ammonia treatment. The purity of the oligoribonucleotides of up to 20 residues was assessed by electrophoresis on a 20% polyacrylamide gel containing 7M urea, and when a single band could not be detected, the purification was conducted again by the polyacrylamide gel electrophoresis. Isolated yields of deprotected

oligoribonucleotides after the reversed-phase HPLC or PAGE are as shown in Table 2. Comparison of the overall yields showed syntheses using 2'-O-Cee protection to be 14% higher than employing 2'-O-Thp protection.

It is important to ensure in any new procedure for oligoribonucleotide synthesis that all internucleotidic bonds are 3'-5' and that no base modification has taken place. For example, the deprotected octamer, rGACCGUCA was completely digested with RNase T_2 and then treated with alkaline phosphatase. The reversed-phase HPLC analysis showed complete conversion into rA, rC, rU , and rG in the expected proportions. The homopolymer rC_{18} was subjected to enzymatic degradation by RNase T_2 to give the correct ratio of rCp/rC (17:1). The base sequences of the 20mer was confirmed by partial enzymatic degradation of the 5'-end-labeled oligomer on polyacrylamide gel electro-phoresis.[43] The autoradiogram of the gel is shown in Figure 5 and substaniates the expected sequence.

From these analyses it is clear that the 3'-5' phosphodiester bonds is preserved during the coupling and deprotection procedures of the oligoribonucleotides.

It is reasonable to conclude from the work described in this paper the Cee group is compatible with the 5'-O-DMTr group in the synthesis of oligoribonucleotides by the phosphoramidite approach on a solid support. 2-Chloroethyl viniyl ether is an easily accessible compound, and the required 2'-O-Cee-ribonucleoside derivatives can be prepared in high yields. This procedure is facile and effective for the synthesis of oligoribonucleotides of up to 20 residues with an automatic synthesizer.

EXPERIMENTAL

GENERAL MATERIALS AND METHODS.

^1H and ^{31}P-NMR spectra were recorded on a Bruker AMX 400 spectrometer with TMS and 80%H_3PO_4 as an internal standard. Ultraviolet spectra were recorded on a Shimadzu UV-160 spectrometer. Thin layer chromatography (TLC) was carried out on Merck Kieselgel $60F_{254}$ plates which were developed in system A (CH_2Cl_2-MeOH, 9:1, v/v), system B (CH_2Cl_2-MeOH, 95:5, v/v), and system C (CH_2Cl_2-EtOAc-Et_3N (45:45:10, v/v). Reversed-phase TLC was carried out on Merck silanized silica gel; [RP-8F $60F_{254}$]plates with a mixture of acetone and 0.02 M triethylammonium acetate (TEAA) (6:4, v/v) as the eluting agent. Column chromatography was carried out on silica gel (BW-300; Fuji Davison Co.Ltd.) and alkylated silica gel (C-18, Waters Associates Inc.).

All anhyrous solvents were obtained using standard techniques.[44] Tetrazole and 2-chloroethyl vinyl ether were obtained from Tokyo Kasei Kogyo Co.Ltd. Dicylcohexylcarbodi-imide (DCC) was distilled before use. 1,3-Dichlorotetraisoprppyldisiloxane was obtained from Shin-Etsu Sillicon Chemical. Long-chain alkylamino controlled pore glass was purchased from Electro Nucleonics Inc. Enzymes were purchased from Beohringer Mannheim and Pharmacia.

Electrophoretic gels were either 20% polycarylamide/7 M urea or 10% polyacrylamide/8 M urea and run at 400V.

Reversed-phase HPLC was performed on a Yosoh CCPM system using a TSKgel oligo-DNA RP for analysis and Inertsil ODS for purification with a linear gradient of CH_3CN in 0.1 M triethylammonium acetate (pH 7.0). For anion exchange HPLC, a TSKgel DEAE-2SW column was used with a linear gradient of ammonium formate in 20% CH_3CN.

N-Acyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)nucleosides (**1**) were prepared as described previously.[45] 3'-O-[(2-Cyanoethyl)(diisopropylamino)phosphino]-5'-O-DMTr-2'-O-

Thp-N^4-anisolylcytidine and 3'-O-CPG-5'-O-DMTr-2'-O-Thp-N^4-anisolylcytidine (25 μmol/g were prepared according to the desribed procedure by Keirzek et al.[17]

Synthesis of N-acyl-[1-(alkoxy)ethyl]nucleosides (2a-c, 3).

N-Acyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)nucleosides (1) (5 mmol) were dissolved in CH_2Cl_2 (40 mL) and alkyl vinyl ether (50 mmol) in the presence of p-toluenesulfonic acid or pyridinium p-toluenesulfonate with cooling. The mixture was checked by TLC (system A) after it had been kept at room temperature for 2-24 h and quenched with triethylamine (3 mL) and saturated $NaHCO_3$. The mixture was shaken and the CH_2Cl_2 layer was washed with water and dried over Na_2SO_4. The solution was concentrated and the residue was dissolved in 1 M TEAHF in THF (20 mL). After 90 min, TLC analysis (system B) showed the complete removal of tetraisopropyldidiloxane-1,3-diyl (TIDSi) groups and the mixture was washed with saturated $NaHCO_3$. The solution was concentrated and the residue was coevaporated with toluene, precipitated as a syrup with hexane from its solution of CH_2Cl_2, and applied to a column of silica gel. Elution was performed in CH_2Cl_2 containing MeOH (0-5%) to give **2a-c,3** (80-87%) as foam.

2'-O-[1-(2-chloroethoxy)ethyl]uridine 2a: Rf 0.25 (system A), UV λmax (MeOH) 263 nm, λmin (MeOH) 232 nm; ^{1}H-NMR (DMSO-d_6) δ 7.90 (dd, 1H, $J_{5,6}$=9 Hz, C-6), 5,72 (d, 1H, $J_{1',2'}$=6 Hz, H-1_1', 5.59 (d, 1H, $J_{5,6}$=9 Hz, C-5), 5.35-5.00 (m, 1H, HO-3'), 4.82 (d, 1H, CH), 4.20-3.68 (m, 3H, HO-5', H-2', H-3'), 3.75 (m, 1H, H-4'), 3.55 (br s, 6H, H-5', CH_2CH_2), 1.23 (dd, 3H, CH_3). Anal. Calcd for $C_{13}H_{19}N_2O_7Cl.1/2CH_3OH$: C, 44.22; H, 5.77; N, 7.64. Found: C, 43.99; H, 5.82; N, 7.62.

2'-O-(isopropoxyethyl)uridine 2b: Rf 0.26 (system A), UV λmax (MeOH) 261 nm, λmin (MeOH) 230 nm, ^{1}H-NMR (DMSO-d_6) δ 8.10 (dd, 1H, $J_{5,6}$=9 Hz, C-6), 6.05 (d, 1H, $J_{1',2'}$=6 Hz, H-1'), 5.80 (d, 1H, $J_{5,6}$=9 Hz, C-5), 5.50-4.90 (m, 3H, OH-3', H-2', CH), 4.30 (m, 3H, HO-5', H-3', H-4'), 4.00 (m, 3H, $\underline{CH}(CH_3)_2$, H-5'), 1.75 (d, 3H, CH_3), 1.15 (d, 6H, $CH(\underline{CH_3})$. Anal. Calcd for $C_{14}H_{22}N_2O_7.1/4CH_3OH$: C, 50.60; H, 6.98; N, 7.61. Found: C, 50.68; H,7.20; N, 7.69.

2'-O-(butoxyethyl)uridine 2c: Rf 0.28 (system A), UV λmax (MeOH) 262 nm, λmin (MeOH) 239 nm, ^{1}H-NMR (DMSO-d_6) δ 7.95 (dd, 1H, $J_{5,6}$=9 Hz, C-6), 5.94 (d, 1H, $J_{1',2'}$=6 Hz, H-1'), 5.65 (d, 1H, $J_{5,6}$=9 Hz, C-5), 5.20-4.72 (m, 2H, HO-3', CH), 4.20-3.82 (m, 4H, HO-5', H-2', H-3', 4'-H), 3.65 (m, 2H, H-5'), 3.30 (m, 2H, CH_2), 1.30 (m, 7H, CH_2CH_2, $CHCH_3$), 0.81 (t, 3H, CH_3). Anal. Calcd for $C_{15}H_{24}N_2O_7.CH_3CH_2OH$: C, 52.97; H, 7.74; N, 7.17. Found: C, 52.70; H, 7.74; N, 7.41.

N^4-anisoyl-2'-O-[1-(2-chloroethoxy)ethyl]cytidine 3b: Rf 0.28 (system A), UV λmax (MeOH) 301, 258 nm, λmin (MeOH) 279 nm, ^{1}H-NMR (DMSO-d_6) δ 8.42 (d, 1H, $J_{5,6}$=8Hz, H-6), 7.95 (d, 2H, Ar), 7.25 (d, 1H, $J_{5,6}$=8Hz, H-5), 6.95 (d, 2H, Ar), 5.90 (d, 1H, $J_{1',2'}$=6.1Hz, H-1'), 5.12 (d, 2H, HO-3', CH), 4.10 (m, 3H, H-2', H-3', H-4'), 3.75 (s, 3H, OCH_3), 3.68 (brs, 4H, CH_2CH_2), 3.45 (m, 2H, H-5'), 1.35 (d, 3H, CH_3). Anal. Calcd for $C_{21}H_{26}N_3O_8Cl$: C, 52.15; H, 5.42; N, 8.68. Found: C, 52.30; H, 5.46; N, 8.58.

N^6-benzoyl-2'-O-[1-(2-chloroethoxy)ethyl]adenosine 3c: Rf 0.33 (system A), UV λmax (MeOH) 280 nm, λmin (MeOH) 263 nm, ^{1}H-NMR (DMSO-d_6) δ 9.41 (s, 2H, H-8 or H-2), 9.00-7.52 (m, 5H, Ar-H), 6.10 (d, 1H, $J_{1',2'}$=6 Hz, H-1'), 5.50 (s, 1H, -O-CH-O-), 5.00 (m, 2H, H-2', HO-3'), 4.65 (m, 1H, H-3'), 4.20 (m, 1H, H-4'), 3.85 (brs, 2H, H-5'), 3.45 (brs, 4H, CH_2CH_2), 1.82 (d, 3H, CH_3). Anal. Calcd for $BC_{21}H_{24}N_5O_6Cl$: C,52.78; H, 5.06; N, 14.65. Found: C, 52.91; H, 5.14; N, 14.48.

N^2-isobutyryl-2'-O-[1-(2-chloroethoxy)ethyl]guanosine 3d: Rf 0.30 (system A), UV λmax (MeOH) 280, 259 nm, λmin (MeOH) 270 nm. ^{1}H-NMR (DMSO-d_6) δ 8.20 (s, 1H, H-8), 5.91 (d, 1H, $J_{1',2'}$=6 Hz, H-1'), 4.90-4.60 (m, 2H, H-2', H-3'), 4.28 (brs, 1H, CH), 3.78 (s, 5H, H-4', CH_2CH_2), (m, 2H, H-5'), 2.75 (m, 1H, $\underline{CH}(CH_3)_2$), 1.15 (d, 9H, CH_3, $CH(\underline{CH_3})_2$). Anal. Calcd for $C_{18}H_{26}N_5O_7Cl$: C, 47.01; H, 5.70; N, 15.23. Found: C, 47.20; H, 5.78; N, 15.19.

Tritylation of protected ribonucleosides (2a,3b-d).

The protected ribonucleosides (**2a,3b-d**) (1.0 mmol) was treated with DMTrCl (1.2 mmol) in dry pyridine (5 mL) for 2-5 h. The reaction mixture was quenched with ethanol (5 mL) and poured into water (25 mL). The mixture was extracted with CH_2Cl_2 (3 X 25 mL) and washed with water (2 X 25 mL). The CH_2Cl_2 layer was dried over Na_2SO_4 and evaporated in

vacuo. The residue was applied to a column of silica gel. Elution with a gradient of methanol (0-2%) in CH_2Cl_2 gave the 5'-O-tritylated materials **4a-d** in 85-87%.

General procedure for preparation of 3'-O-[(2-cyanoethyl)(diisopropylamino)phosphino]-5'-O-dimethoxytrityl-2'-O-[1-(2-chloroethoxy)ethyl]-N-acylribonucleosides (5a-d).

The ribonucleoside derivative (**4a-d**) (2 mmol) was dissolved in THF (10 ml) and N,N-diisopropylethylamine (2.8 mL, 8 mmol) was added. To this solution, 2-cyanoethyl N,N-diisopropyphosphoramidochloridite (0.96 mL, 4 mmol) was added dropwise with cooling. The mixture was stirred at room temperature for 1 h when TLC (system C) showed the complete conversion of 5 into high Rf products. The mixture was diluted with ethyl acetate (50 mL) and washed with saturated NaCl solution (3 X 25 mL). The organic layer was dried over Na_2SO_4 and evaporated in vacuo. The product was purified by a column of silica gel eluting with 50% ethyl acetate in CH_2Cl_2 containing 1% triethylamine. Fractions containing the desired products were combined, evaporated to an oil, dissolved in CH_2Cl_2 and precipitated with petroleum ether.

3'-O-[(2-cyanoethyl)(diisopropylamino)phosphino]-5'-O-DMTrO-2'-O-Cee-uridine (5a): Yeild: 85%, Rf 0.64 (system C), UV λ max (MeOH) 263nm; λ min (MeOH) 232 nm. ^{31}P-NMR ($CDCl_3$) δ 151.22, 151.10, 150.52.

3'-O-(2-cyanoethyl)(diisopropylamino)phosphino]-5'-DMTr-O-2'-O-Cee-N^{4}anisoylcytidine (5b): Yield: 82%, Rf o.58 (system C), UV λ max (MeOH) 304, 288 nm; λmin (MeOH) 258 nm. ^{31}P-NMR ($CDCl_3$) δ 151.68, 151.25, 150.92, 150.04.

3'-O-(2-cyanoethyl)(diisopropylamino)phosphino]-5'-O-DMTr-2'-O-Cee-N^6-benzoyladenosine (5c): Yield: 87%, Rf 0.76 (system C), UV λ max (MeOH) 280 nm,λ min (MeOH) 263mn. ^{31}P-NMR ($CDCl_3$) δ 151.68, 151.29, 151.04, 150.67.

3'-(2-cyanoethyl)(diisopropylamino)phosphino]-5'-O-DMTr-O-2'-O-Cee-N^2-isobutyrylguanosine (5d): Tield: 83%, Rf 0.43 (system C), UV λ max (MeOH) 280, 255nm; λ min (MeOH) 269 nm. ^{31}P-NMR ($CDCl_3$) δ 151.62, 150.89, 150.52, 150.22.

Procedure for preparation of N-acyl-5'-O-DMTr-2'-O-Cee-ribonucleoside 3'-O-succinic acid half esters (6b,c).

Compound **4a-c** (1 mmol), succinic anhydride (244 mg, 2 mmol), and DMAP (200 mg, 2 mmol) were dissolved in CH_2Cl_2 (5 mL) and stirred at room temperature. After 2 h, TLC (system A) showed a major product at lower Rf. The mixture was quenched with 0.1 M TEAB solution, dried over Na_2SO_4 and evaporated to dryness. The product was purified by short silica gel column chromatography eluting with a linear gradient of methanol (0-2%) in CH_2Cl_2 to give the corresponding products **6a** (679 mg, 81%), **6b** (771mg, 84%), and **6c** (687 mg, 85%) as white solids.

Derivatization of ribonucleoside on supports.

A mixture of long-chain alkylamine CPG (500 mg), the appropriate protected ribonucleoside 3'-O-succinates (**6**) (200 μmol), triethylamine (28 μL), DCC (206 mg, 1 mmol), a catalytic amount of DMAP, and DMF (1 mL) was shaken for 24 h. The support was filtered off and successively washed with DMF (15 mL), pyridine (15 mL), methanol (15 mL), and ether (15 mL). The unreacted amino groups were capped by treatment with acetic anhydride-pyridine (1:9, v/v) in the presence of a catalytic amount of DMAP for 30 min followed by washing of the support with pyridine, methanol, and ether. The derivatized supports were dried over P_2O_5 overnight. The loading of each support was determined by detritylation of 5 mg samples with 1.5% DCA/DCE solution. The loading was 4.40 μmol/g for **7a**, 39.6 μmol/g for **7b** and 37.5 μmol/g for **7c**.

Synthesis of oligoribonucleotides.

The LCAA-CPG support loaded with first nucleoside (0.2 μmol) was packed in a small ABI column which is part of an Applied Biosystems 381A DNA Synthesizer. The reaction cycle of chain elengation was were carried out by a programmed series of reagent and solvent washes based on a grogram of the DNA synthesis with the following modifications:

1) coupling: 0.07 M phosphoramidite unit (5) and 0.21 M tetrazole in dry CH_3CN in delivered in 4 sec burst (tetrazole) followed by 10 sec (tetrazole + phosphoramidite unit) with wait time 15-20 min.

2) capping: Solution A:THF/lutidine/acetic anhydride (8:1:1, v/v), solution B:0.27 M DMAP

in THF delivered in two 15 sec bursts with wait time 60 sec.

3) oxidation: 0.1 M iodine in THF/lutidine/water (40:10:1, v/v) delivered in 40 sec burst with wait time 30 sec.

4) unblocking: 1.5% dichloroacetic acid in CH_2Cl_2 delivered in 10 X 10 sec bursts with intermediate 1 sec reverse flushes.

Deprotection and isolation of oligoribonucleotides.

To the column, concentrated aqueous ammonia was added by syringe. After 1h at room temperature, the solution was eluated from column and heated in a sealed vial at 55°C for 5-8 h. The solution was concentrated and the residue was dissolved in 0.01 N HCl (0.5 mL) and 0.1 N HCl added to adjust to pH 2.0. The solution was left at room temperature for 6-24 h (depending on length), neutralized with aqueous ammonia to pH 7.0, and passed through a membrane filter (EKICRODISC 13, Gelman Sciences Japan). The deprotected oligoribonucleotide was analyzed and purified by the reversed-phase HPLC as shown in Figure 3. The appropriate fractions were collected and lyophilized from sterile water. Slightly crude oligomers (rC_{18} and 20mer) were further purified by electrophoresis on 20% polyacrylamide containing 7 M urea (Figure 4). After electrophoresis the desired band was sliced out and suspended in 0.5 M ammonium acetate at 37°C for 12 h. The solution was passed through a column of Sephadex G-25 eluted with 0.1 M TEAB and lyophilized from sterile water. Isolated yields of deprotected oligoribonucleotides after the reverse phase HPLC or PAGE are as shown in Table 2.

Enzymatic digestions.

The purified oligomer (0.2 A_{260} units) was dissolved in 50 mM ammonium acetate (30 µL) and treated with RNase T_2(25 units/mL, 2.5 µL)at 37°C for 4 h. The solution was concentrated by centrifugal concentrator VC-96, dissolved in 0.1 M Tris.HCl (pH 8.5, 25 µL) and treated with alkaline phosphatase (2 µL, 15 units/µL) at 37°C for 2 h. The ratio of nucleosides were estimated by the reverse-phase chromatography on a TSKgel oligo-DNA RP column using 0.1 M triethylammonium acetate containing 1% CH_3CN, flow rate 1 mL/min. In case of the homopolymers, after RNase T_2 hydrolysis, the digest was centrifuged and injected onto a TSKgel oligo-DNA RP. The HPLC analysis showed only two peaks corresponding to rCp and rC. The labeled oligomer were partially digested with RNases or alkali and sequenced by the 20% polyacrylamido-7M urea gel electrophoresis developed by Donis-Keller for distinguishing G,A+U, and C+U from each other (Figure 5).

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