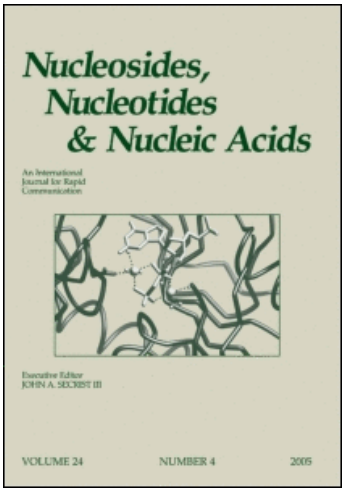

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Kittaka, Atushi , Kuze, Tesuya , Amano, Miori , Tanaka, Hiomichi , Miyasaka, Taashi , Hirose, Kuihiko , Yoshida, Taao , Sarai, Aknori , Yasukawa, Taashi and Ishii, Shnsuke(1999) 'Introduction of 6-Formylcytidine into a Myb Binding Sequence', Nucleosides, Nucleotides and Nucleic Acids, 18: 11, 2769 — 2783
To link to this Article: DOI: 10.1080/07328319908044640
URL: http://dx.doi.org/10.1080/07328319908044640

NUCLEOSIDES & NUCLEOTIDES, 18(11&12), 2769–2783 (1999)

INTRODUCTION OF 6-FORMYLCYTIDINE INTO A MYB BINDING SEQUENCE

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ABSTRACT: Single 6-formylcytidine was introduced into a oligonucleotide duplex (23 mers) as a substitute for thymidine in the Myb binding sequence of 3'-TTGAC-5'. The modified duplex showed Tm of 67 °C, which was six degrees lower than the Tm of the native duplex. Binding affinity of the 23-mers to the Myb protein was estimated by electrophoretic mobility shift assays, and the binding was almost completely abolished.

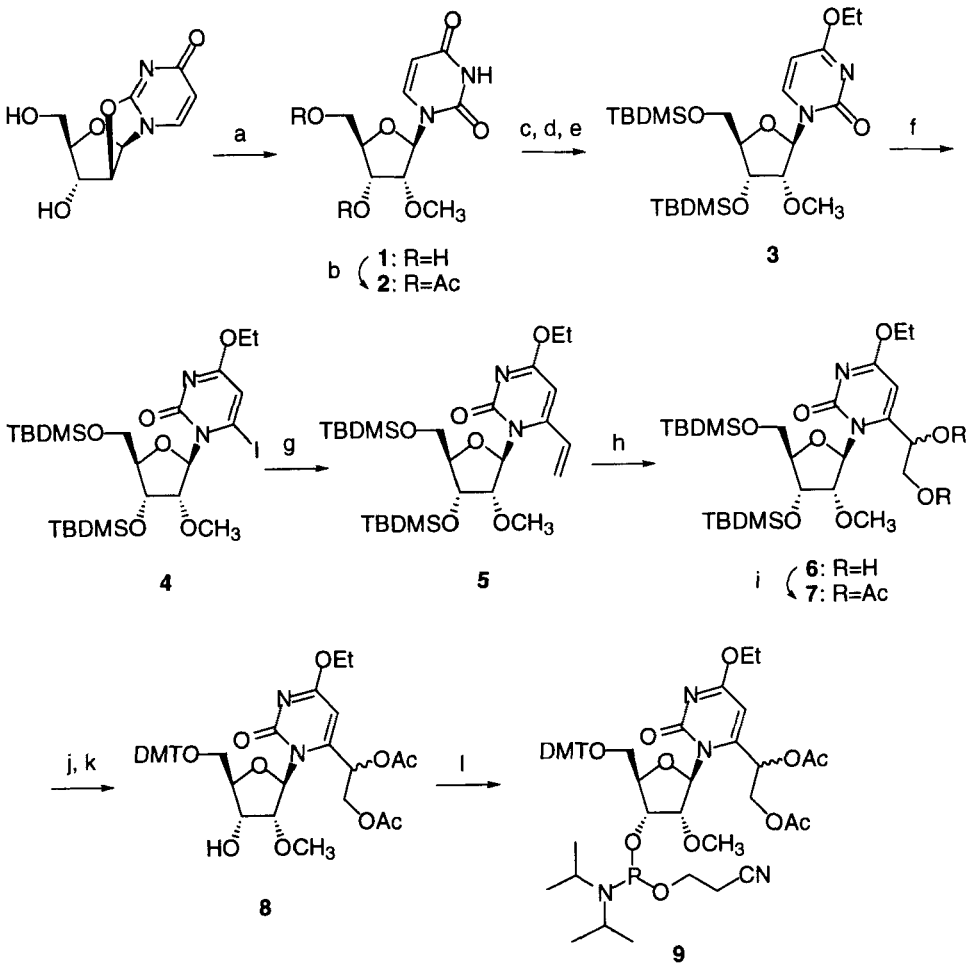
The c-*myb* proto-oncogene product (c-Myb) is a transcriptional activator that binds to the specific DNA sequence AACNG.[1] The DNA-binding domain located in the N-terminal region of c-Myb consists of three imperfect tandem repeats of 51-52 amino acids (R1, R2, and R3 from the N-terminus).[2] The three-dimensional structures of the DNA-binding domain of c-Myb in the free and DNA-bound forms show that each repeat has a very similar folding architecture, containing three well-defined helices.[3-6] Portions of the second and third helix in each repeat form a variant of the helix-turn-helix motif. The third helices of R2 and R3 are involved in specific base recognition in the major groove of the DNA, while R1 is not essential for the specific DNA recognition. To understand the biological role and target sequence specificity of the Myb protein, how they recognize the DNA-binding structure should be investigated.

Recently, we succeeded in introducing a 5-formyl-2'-*O*-methyluridine unit into the various positions of oligonucleotides containing transcription factor NFκB binding sequence (κB site) to study the binding affinity of NFκB homo- and heterodimers to the modified oligonucleotides.[7] Some of modified oligonucleotides showed preference in binding affinity to the homo- and heterodimers. The 5-formyl group of 5-formyl-2'-deoxyuridine, which is known as one of the oxidatively damaged forms of thymidine,[8] possesses the possibility of forming DNA-protein crosslinks through Schiff bases with certain amino groups in the protein such as the ε-amino group of lysine.[9] However, oligonucleotides bearing 6-functionalized pyrimidine nucleosides and their interaction with DNA binding proteins have not been thoroughly studied.[10] We were interested in investigating whether the 6-formyl-pyrimidine base containing oligonucleotide could form a stable duplex with its complementary strand, and if possible, whether a DNA binding protein, for example the proto-oncogene product c-Myb, could contact the modified protein-binding sequence, in which the 6-formyl group would be able to interact with proximal basic amino acid residues in the modified oligonucleotide-protein complex.

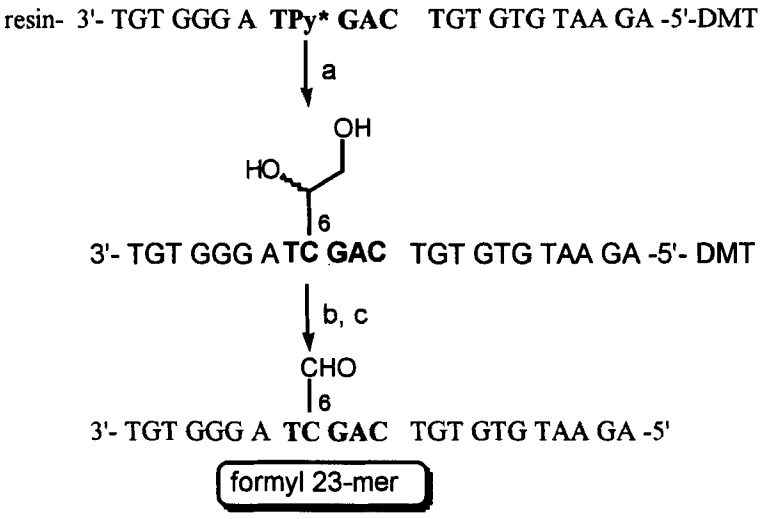
Computer calculation, based on the three-dimensional structure of a specific DNA complex of the Myb DNA-binding domain,[4] showed that only one particular position could be possible for replacement of thymine base by 6-formylcytidine to set a short distance (3.49 Å) between the 6-formyl group and the basic amino acid residue, which was the guanidino group of Arg-190 (data not shown).

In an initial attempt for synthesis of a precursor of 6-formyl-2'-deoxyuridine, it was found that 2'-deoxy-6-(1,2-diacetoxyethyl)uridine was unstable even in storage at ambient temperature.[11] Therefore, we decided to use stable 6-(1,2-diacetoxy)ethyl-4-ethoxy-1-(2-*O*-methylribofuranosyl)-2-pyrimidinone[12] (Py*) as the precursor of the 6-formyl-2'-*O*-methylcytidine[13] unit for oligonucleotide synthesis. Synthesis of the oligonucleotide building block **9** is shown in Scheme 1.

O^2,2'-Cyclouridine was treated under ISIS conditions for selective 2'-*O*-methylation to give 2'-*O*-methyluridine **1** in 92% yield.[14] The two hydroxyl groups were



Scheme 1. *Reagents and conditions*: (a) $B(OCH_3)_3$ (2 eq), $CH(OCH_3)_3$ (1 eq), Na_2CO_3 (0.003 eq), MeOH, 145-150 °C, in a sealed tube. (b) Ac_2O (5 eq), py. (c) $SOCl_2$ (10 eq), *cat.* DMF, $CHCl_3$, reflux. (d) NaOEt (5 eq), EtOH. (e) TBDMSCl (3 eq), imidazole (4.5 eq), DMF. (f) LDA (3 eq), THF, –78 °C; and then I_2 (2.5 eq). (g) $Bu_3SnCH{=}CH_2$ (1.2 eq), 5 mol% $Pd(CH_3CN)_2Cl_2$, THF, rt. (h) *cat.* OsO_4, NMO (5 eq), acetone-^{t}BuOH-H_2O (4:1:1). (i) Ac_2O (3 eq), DMAP (5 eq), CH_2Cl_2. (j) TBAF (3 eq), AcOH (2 eq), THF. (k) DMTCl (2 eq), py. (l) $[(i\text{Pr})_2N]_2POCH_2CH_2CN$ (2 eq), 4,5-dicyanoimidazole (0.7 eq), CH_2Cl_2.



Scheme 2. *Reagents and conditions*: (a) conc. NH_4OH, 55 °C, 12 h; and then, HPLC purification. (b) 3% CCl_3COOH in THF, rt, 10 min; and then, dialysis. (c) $NaIO_4$ (150 eq), H_2O, 0 °C, 15 min; and then dialysis followed by HPLC purification.

acetylated (93%), and the base moiety of **2** was converted to 4-ethoxy-2-pyrimidinone under Matsuda's procedure.[15] The resulting free nucleoside was protected by TBDMS groups to give **3** in 75% yield for 3 steps from **2**. To introduce the carbon functionality at the C6-position, lithiated **3** reacted with I_2 to afford **4** in 87% yield. Stille coupling with tributyl(vinyl)tin gave **5** in 74% yield,[16] and subsequent dihydroxylation of the resulting C6-vinyl group using OsO_4 afforded **6** in 84% yield. Acetylation of the vicinal diol followed by selective deprotection from 3',5'-dihydroxyl groups by TBAF, and dimethoxytritylation of the 5'-hydroxyl group provided **8** in 80% yield from **6**. The building block synthesis of the modified oligonucleotide was completed by 3'-*O*-phosphitylation in 65% yield.[17]

The nucleoside phosphoramidite unit **9** obtained as above was incorporated with an automated DNA synthesizer into the 23-mer including the Myb binding sequence as depicted in Scheme 2, in which the modified Myb binding sequence is written in bold letters. After ammonolysis, the 23-mer was purified by reverse phase HPLC, and the 5'-end of DMT group was removed under acidic conditions. This oligonucleotide including

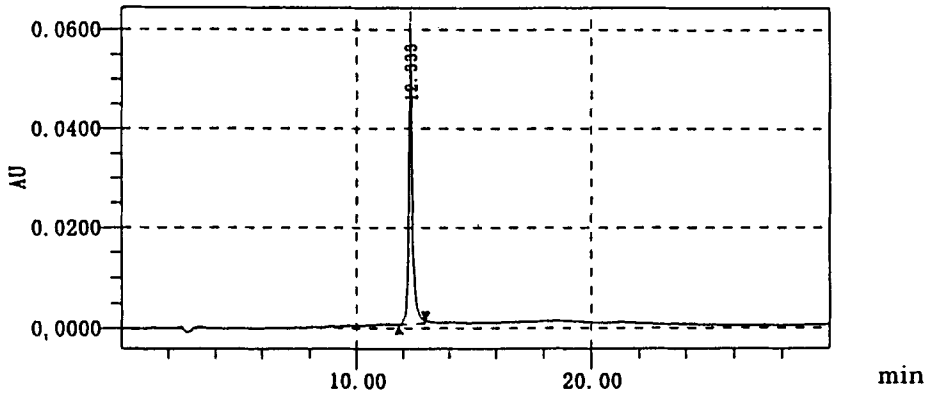


Figure 1. HPLC chart of formyl 23-mer on Wakopak WS-DNA column (4.0x150 mm) at 40 °C, eluted with 5-30% CH_3CN in 0.1 M triethylammonium acetate buffer (pH 7.2), linear gradient 25 min, at a flow rate of 1.0 mL/min and detected at 260 nm.

6-(1,2-dihydroxy)ethyl-2'-*O*-methylcytidine was treated with 150 equivalent of $NaIO_4$ in H_2O at 0 °C for 15 min to synthesize formyl 23-mer.[11]

Excess amount of oxidant and inorganic salts were removed by dialysis, and the resulting oligonucleotide, formyl 23-mer, was purified by HPLC (Figure 1).

Enzymatic hydrolysis and HPLC analysis proved the existence of 6-formyl-2'-*O*-methylcytidine in the modified strand.[18] Stability of the duplex between *a* and *b* in Figure 2 was evaluated by Tm measurement.

The Tm value of the duplex *ab* was 67 °C in the Tm buffer [6 mM sodium phosphate buffer (pH 6.7), 120 mM NaCl, 12 mM $MgCl_2$], which was 6 °C lower than that of the natural duplex between *a* and its complementary strand. The computer modeling displayed the structure of the modified duplex *ab* was somewhat between A- and B-forms of DNA. Fine structural features of the DNA double helix are considered to be the primary determinants of sequence specific recognition by DNA binding proteins, rather than the recognition of the bases.[19] To examine whether c-Myb binds to the modified sequence with an affinity similar to unmodified sequence, gel mobility shift assays were performed using the recombinant c-Myb protein.[20] The c-Myb protein bound to the ^{32}P-labeled unmodified duplex and generated protein-DNA

a. 5'-ACA CCC TAA CTG ACA CAC ATT CT-3'
b. 3'-TGT GGG ATC*GAC TGT GTG TAA GA-5'

Figure 2. Nucleoside sequence of the duplex of formyl 23-mer is shown. C* indicates 6-formyl-2'-*O*-methylcytidine, and the modified Myb binding sequence is written in bold letters.

complex (Figure 3, the most left lane). By using the unmodified or modified oligonucleotide as cold DNA competitors, competition analysis was done. Addition of a 20-fold molar excess of the unmodified competitor duplex almost abolished the complex formed with the DNA probe. In contrast, a significant amount of the complex was remained in the presence of 100-fold molar excess of the modified competitor oligonucleotide. These results indicate that the affinity between c-Myb and the modified oligonucleotide is much lower than that with the unmodified duplex. Consistent with this, the c-Myb-DNA complex was not detected using up to 5 ng of the ^{32}P-labeled modified duplex *ab*, when the ^{32}P-labeled modified duplex was used as a probe in gel mobility shift assays (data not shown).

In summary, we have demonstrated oligonucleotide synthesis containing 6-formyl-2'-*O*-methylcytidine in the Myb binding sequence. The binding affinity of the modified and unmodified natural duplexes toward the DNA binding domain of c-Myb was evaluated by electrophoretic mobility shift assays. The modified duplex abolished significantly binding to the protein. The possibility of introducing 5-formyl pyrimidine nucleosides into the Myb binding sequence is currently under investigation to target an ε-amino group of a certain lysine residue in the c-Myb protein.

Experimental

General Information. Melting points were determined with a Yanagi-moto micro melting point apparatus and are uncorrected. ^{1}H NMR spectra were measured at 23 °C with a JEOL JNM-GX 400 or a JEOL Lamda 500 spectrometer. Chemical shifts (δ) are in ppm relative to TMS and coupling constants are in Hz. Mass spectra (MS) were taken on a JEOL SX-102A spectrometer in FAB mode (*m*-nitrobenzylalcohol as a matrix).

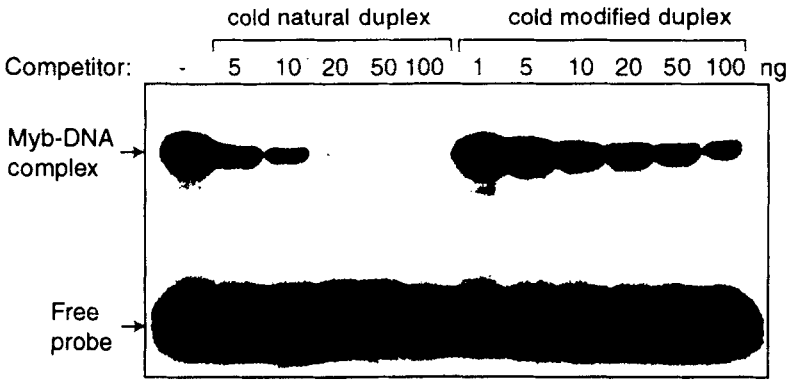


Figure 3. Competition analysis in gel mobility shift assay. The recombinant c-Myb protein containing the DNA binding domain (R1R2R3) was expressed in *E. coli* and purified as described in reference 20. The ^{32}P-labeled natural duplex (probe, 1 ng) between *a* and its complementary strand was incubated with the recombinant c-Myb protein (50 ng) in 10 μL of the binding buffer [100 mM $KHPO_4$-K_2PO_4 (pH 6.8), 5 mg/mL BSA] at 37 °C for 15 min, in the presence of 0-100 ng of the cold natural duplex or the cold modified duplex *ab*. DNA-protein complexes were analyzed by electrophoresis with non-denaturing gel followed by autoradiography.

Ultraviolet spectra (UV) were recorded on a JASCO Ubest-55 spectro-photometer. A commercially available hexane solution of BuLi was titrated before use with diphenyl acetic acid in THF. THF was distilled from benzophenone ketyl. Column chromatography was carried out on silica gel (Silica Gel 60, Merck). Thin layer chromatography (TLC) was performed on precoated silica gel plate (F_{254}, Merck). Preparative HPLC was carried out on a Shimadzu LC-6AD with a Shim-pack PREP-SIL(H)•KIT column (2x25 cm).

2'-*O*-Methyluridine (1). This compound was prepared by the ISIS method.[14] O^2,2'-cyclouridine (5.90 g, 26.1 mmol) was converted to **1** (4.39 g) with recrystallization from isopropanol, and additional 1.78 g of **1** was obtained from the mother liquor by silica gel column purification (2-8% MeOH in $CHCl_3$) as a foam. Total yield of **1** was 6.17 g (92%). ^{1}H NMR (DMSO-d_6) δ 11.33 (1H, br, NH), 7.93 (1H, d, $J_{5,6}$ = 7.9, H6), 5.86 (1H, d, $J_{1',2'}$ = 4.9, H1'), 5.65 (1H, d, H5), 5.15 (1H, d, $J_{3',OH}$ = 5.2, 3'-OH), 4.12

(1H, dt, $J_{2',3'} = J_{3',4'} = 4.3$, H3'), 3.85 (1H, dt, $J_{4',5'} = 3.1$, H4'), 3.78 (1H, dd, H2'), 3.64 (1H, dd, $J_{gem} = 12.2$, H5'), 3.56 (1H, dd, H5'), 3.35 (3H, s, OCH_3), 3.33 (1H, br, 5'-OH).

3',5'-Di-*O*-acetyl-2'-*O*-methyluridine (2). To the solution of **1** (3.37 g, 13.0 mmol) in pyridine (26 mL), acetic anhydride (6.15 mL, 65.0 mmol) was added dropwise. The mixture was stirred at rt for 6 h and concentrated *in vacuo* with toluene (30 mL). The residue was partitioned between EtOAc (200 mL) and H_2O (80 mL). The organic layer was washed with 0.5 M HCl, saturated aqueous $NaHCO_3$, water, and brine (80 mL each). Purification with column chromatography (30-50% EtOAc in hexane) gave **2** as a white foam (4.14 g, 93%). UV (MeOH) λ_{max} 260 nm (ε 9600), λ_{min} 229 nm (ε 2400). ^{1}H NMR ($CDCl_3$) δ 8.77 (1H, br, NH), 7.53 (1H, d, $J_{5,6} = 7.9$, H6), 5.91 (1H, d, $J_{1',2'} = 3.5$, H1'), 5.76 (1H, dd, $J_{5,NH} = 2.0$, H5), 4.99 (1H, dd, $J_{2',3'} = 5.2$, $J_{3',4'} = 6.1$, H3'), 4.3–4.35 (3H, m, H4' and 2xH5'), 4.04 (1H, dd, H2'), 3.48 (3H, s, OCH_3), 2.15 and 2.13 (6H, each as s, 2xOAc). FAB MS *m/z* 343 $(M+H)^+$. Anal. Calcd for $C_{14}H_{18}N_2O_8$: C, 49.12; H, 5.30; N, 8.18. Found: C, 49.50; H, 4.90; N, 7.18.

1-[3,5-Bis-*O*-(*tert*-butyldimethylsilyl)-2-*O*-methyl-β-D-ribo-furanosyl]-4-ethoxy-2-pyrimidinone (3). The mixture of **2** (4.24 g, 12.4 mmol), DMF (0.75 mL), and $SOCl_2$ (9.9 mL, 136 mmol) in $CHCl_3$ (62 mL) was refluxed for 6 h, and the solution was concentrated *in vacuo* to dryness. The residual solid was dissolved in EtOH (20 mL) and the mixture was cooled in an ice bath. The EtOH solution of NaOEt, which was prepared from Na (1.43 g, 62.0 mmol) and EtOH (30 mL), was added to the ice cooled solution prepared above, and then the mixture was stirred at rt for 30 min. Precipitates were filtered off by celite-pad, and the eluate was passed through a silica gel (75 g) short column. The neutral eluate was concentrated *in vacuo*. Column chromatography (1-10% EtOH in $CHCl_3$) gave **4-ethoxy-1-(2-*O*-methyl-β-D-ribofuranosyl)-2-pyrimidinone** (2.95 g, 83%) as a solid: UV (MeOH) λ_{max} 276 nm (ε 6600), λ_{min} 240 nm (ε 1300). ^{1}H NMR (DMSO-d_6) δ 8.32 (1H, d, $J_{5,6} = 7.5$, H6), 6.03 (1H, d, H5), 5.86 (1H, d, $J_{1',2'} = 3.1$, H1'), 5.19 (1H, t, $J_{5',OH} = 5.0$, 5'-OH), 5.09 (1H, d, $J_{3',OH} = 6.7$, 3'-OH), 4.30 (2H, q, $J = 7.0$, CH_2), 4.06 (1H, dt, $J_{2',3'} = 4.9$, $J_{3',4'} = 6.6$, H3'), 3.88 (1H, dt, $J_{4',5'} = 2.8$, H4'),

3.72 (1H, ddd, J_{gem} = 12.5, H5'), 3.69 (1H, dd, H2'), 3.58 (1H, ddd, H5'), 3.43 (3H, s, OCH_3), 1.29 (3H, t, CH_3). FAB MS *m/z* 287 $(M+H)^+$. Anal. Calcd for $C_{12}H_{18}N_2O_6$: C, 50.35; H, 6.34; N, 9.79. Found: C, 50.33; H, 6.47; N, 9.71.

The mixture of 4-ethoxy-1-(2-*O*-methyl-β-D-ribofuranosyl)-2-pyrimidinone (2.78 g, 9.71 mmol) obtained above, imidazole (2.97 g, 43.7 mmol), and TBDMSCl (5.12 g, 34.0 mmol) in DMF (30 mL) was stirred at rt for 8 h. The solution was partitioned between EtOAc (400 mL) and H_2O (100 mL), and the organic layer was washed with H_2O and brine (100 mL each), and dried over Na_2SO_4. Column chromatography (10-33% EtOAc in hexane) gave **3** (3.73 g, 75%) as an oil. UV (MeOH) λ_{max} 276 nm, λ_{min} 240 nm. ^{1}H NMR ($CDCl_3$) δ 8.37 (1H, d, $J_{5,6}$ = 7.3, H6), 5.95 (1H, s, H1'), 5.81 (1H, d, H5), 4.42 (2H, q, *J* = 7.0, CH_2), 4.18 (1H, dd, $J_{2',3'}$ = 4.9, $J_{3',4'}$ = 9.0, H3'), 4.11–4.05 (2H, m, H4' and H5'), 3.79 (1H, dd, $J_{4',5'}$ = 1.4, J_{gem} = 11.8, H5'), 3.66 (3H, s, OCH_3), 3.61 (1H, d, H2'), 1.36 (3H, t, CH_3), 0.94 and 0.88 (18H, each as s, 2xSi^{t}Bu), 0.13, 0.11, 0.07, and 0.04 (12H, each as s, 2xSiMe$_2$). FAB MS *m/z* 515 $(M+H)^+$, 499 $(M-Me)^+$, and 457 $(M-{}^tBu)^+$. HRMS (FAB+) calcd for $C_{24}H_{47}N_2O_6Si_2$ $[M+H]^+$ 515.2972. found 515.2888.

1-[3,5-Bis-*O*-(*tert*-butyldimethylsilyl)-2-*O*-methyl-β-D-ribo-furanosyl]-4-ethoxy-6-iodo-2-pyrimidinone (4). To a THF (32.0 mL) solution of LDA (19.1 mmol), compound **3** (3.29 g, 6.38 mmol) in THF (32.0 mL) was added *via* a syringe while maintaining the temperature below –70 °C. After stirring for 15 min at –78°C, I_2 (4.05 g, 16.0 mmol) in THF (20 mL) was added and the mixture was stirred for 30 min at the same temperature. The reaction was quenched with AcOH (1.1 mL, 19.1 mmol), diluted with EtOAc (400 mL), and the whole solution was washed with 0.1 M $Na_2S_2O_3$, saturated aqueous $NaHCO_3$, H_2O, and brine (100 mL each), successively, and dried over Na_2SO_4. Column chromatography (10-17% EtOAc in hexane) gave **4** (3.56 g, 87%) as a pale yellow solid. UV (MeOH) λ_{max} 290 nm (ε 8100), λ_{min} 249 nm (ε3200). ^{1}H NMR ($CDCl_3$) δ 6.48 (1H, s, H5), 5.95 (1H, d, $J_{1',2'}$ = 3.4, H1'), 4.59 (1H, t, $J_{2',3'} = J_{3',4'}$ = 5.8, H3'), 4.48 (1H, dd, H2'), 4.41–4.34 (2H, m, CH_2), 3.95–3.75 (3H, m, H4' and 2xH5'),

3.41 (3H, s, OCH_3), 1.32 (3H, t, J = 7.0, CH_3), 0.93 and 0.88 (18H, each as s, 2xSi^{t}Bu), 0.11 and 0.05 (12H, each as s, 2x$SiMe_2$). FAB MS *m/z* 583 $(M-{}^tBu)^+$. Anal. Calcd for $C_{24}H_{45}IN_2O_6Si_2 \cdot 4/3H_2O$: C, 43.37; H, 6.23; N, 4.21. Found: C, 42.98; H, 6.51; N, 4.08.

1-[3,5-Bis-*O*-(*tert*-butyldimethylsilyl)-2-*O*-methyl-β-D-ribo-furanosyl]-4-ethoxy-6-vinyl-2-pyrimidinone (5). The mixture of compound **4** (3.66 g, 5.72 mmol), tributyl(vinyl)tin (3.1 mL, 10.6 mmol), and $Pd(CH_3CN)_2Cl_2$ (164.9 mg, 0.572 mmol) in THF (57 mL) was stirred at rt overnight. After paper-filtration, the solution was concentrated *in vacuo*, and the residue was purified by column chromatography (10-20% EtOAc in hexane) to yield **5** (2.28 g, 74%) as a pale yellow oil. UV (MeOH) λ_{max} 291 nm, λ_{min} 252 nm. ^{1}H NMR ($CDCl_3$) δ 6.82 (1H, dd, $J_{7,8}$ = 11.0 and 17.1, H7), 5.84 (1H, dd, $J_{7,8}$ = 17.1, J_{gem} = 0.9, H8), 5.83 (1H, s, H5), 5.72 (1H, d, $J_{1',2'}$ = 3.7, H1'), 5.59 (1H, dd, $J_{7,8}$ = 11.0, H8), 4.60 (1H, t, $J_{2',3'} = J_{3',4'}$ = 6.3, H3'), 4.46–4.36 (3H, m, H2' and CH_2), 3.93 (1H, dd, $J_{4',5'}$ = 4.0, J_{gem} = 11.0, H5'), 3.88 (1H, ddd, $J_{4',5'}$ = 5.2, H4'), 3.76 (1H, dd, H5'), 3.33 (3H, s, OCH_3), 1.34 (3H, t, J = 7.0, CH_3), 0.92 and 0.89 (18H, each as s, 2xSi^{t}Bu), 0.11 and 0.05 (12H, each as s, 2x$SiMe_2$). FAB MS *m/z* 541 $(M+H)^+$, 525 $(M-Me)^+$, and 483 $(M-{}^tBu)^+$. HRMS (FAB+) calcd for $C_{26}H_{48}N_2O_6Si_2$ $[M+H]^+$ 541.3129. found 541.3148.

1-[3,5-Bis-*O*-(*tert*-butyldimethylsilyl)-2-*O*-methyl-β-D-ribo-furanosyl]-6-(1,2-dihydroxyethyl)-4-ethoxy-2-pyrimidinone (6). Compound **5** (924.0 mg, 1.71 mmol), 4-methylmorpholine *N*-oxide (NMO, 500.8 mg, 4.28 mmol), and a solution of 2% OsO_4 in H_2O (0.5 mL) were stirred in a solution of acetone-H_2O-^{t}BuOH (4:1:1, 48 mL) at rt for 18 h. Additional NMO (500.8 mg, 4.28 mmol) was added and the solution was stirred for 24 h. The reaction mixture was partitioned between EtOAc (250 mL) and H_2O (100 mL). The organic layer was washed with H_2O and brine (80 mL each). Column chromatography (10-33% EtOAc in hexane) afforded **6** (828.7 mg, 84%) as a solid. UV (MeOH) λ_{max} 280 nm (ε 6700), λ_{min} 243 nm (ε 1600). ^{1}H NMR ($CDCl_3$) δ 6.12 and 6.11 (1H, each as s, H5), 5.61 (1H, d, $J_{1',2'}$ = 4.3, H1'), 4.92–4.84 (1H, m, H7), 4.70–4.42 (2H, m, H2' and H3'), 4.41-4.37 (2H, m, CH_2), 4.36–3.89 (3H, m, H8, H4'

and H5'), 3.81–3.70 (2H, m, H8 and H5'), 3.62 and 3.43 (1H, each as d, $J_{7,OH}$ = 4.0, 7-OH), 3.39 (3H, s, OCH_3), 2.64 and 2.58 (1H, each as t, $J_{8,OH}$ = 5.8, 8-OH), 1.34 (3H, t, J = 7.2, CH_3), 0.93 and 0.88 (18H, each as s, 2xSi^{t}Bu), 0.13, 0.12, 0.05, and 0.04 (12H, each as s, 2x$SiMe_2$). FAB MS *m/z* 575 $(M+H)^+$, 559 $(M-Me)^+$, and 517 $(M-{}^t Bu)^+$. Anal. Calcd for $C_{26}H_{50}N_2O_8Si_2$: C, 54.41; H, 8.61; N, 4.88. Found: C, 54.30; H, 8.99; N, 4.75.

1-[3,5-Bis-*O*-(*tert*-butyldimethylsilyl)-2-*O*-methyl-β-D-ribo-furanosyl]-6-(1,2-diacetoxyethyl)-4-ethoxy-2-pyrimidinone (7). To the mixture of compound **6** (1.87 g, 3.25 mmol) and DMAP (1.82 g, 16.3 mmol) in CH_2Cl_2 (45 mL), Ac_2O (0.92 mL, 9.75 mmol) was added, and the solution was stirred at rt for 4 h. The mixture was partitioned between EtOAc (350 mL) and H_2O (100 mL). The organic layer was washed successively with saturated aqu. $NaHCO_3$, H_2O, and brine (100 mL each), and dried over Na_2SO_4. Column chromatography (10-33% EtOAc in hexane) afforded **7** (2.15 g, quant) as a foam. Diastereomer-mixture of **7** (*ca.* 50 mg) was separated to each component **7a** (HPLC retention time: t_R 8.5 min, 26.7 mg) and **7b** (t_R 9.1 min, 22.7 mg) by HPLC (40% EtOAc in hexane) for assignment of the ^{1}H NMR peaks. Compound **7**: UV (MeOH) λ_{max} 282 nm(ε 6700), λ_{min} 245 nm (ε 2000). ^{1}H NMR ($CDCl_3$) for **7a** δ 5.98 (1H, dd, $J_{7,8}$ = 3.7 and 7.6, H7), 5.90 (1H, s, H5), 5.64 (1H, d, $J_{1',2'}$ = 2.9, H1'), 4.64 (1H, t, $J_{2',3'} = J_{3',4'}$ = 5.8, H3'), 4.59 (1H, dd, H2'), 4.52 (1H, dd, $J_{7,8}$ = 3.7, J_{gem} = 12.2, H8), 4.41 and 4.37 (2H, each as dq, J = 7.0, J_{gem} = 10.8, CH_2), 4.28 (1H, dd, H8), 3.93–3.87 (2H, m, H4' and H5'), 3.72 (1H, dd, $J_{4',5'}$ = 5.6, J_{gem} = 11.0, H5'), 3.43 (3H, s, OCH_3), 2.16 and 2.07 (6H, each as s, 2xOAc), 1.34 (3H, t, CH_3), 0.92 and 0.85 (18H, each as s, 2xSi^{t}Bu), 0.12 and 0.01 (12H, each as s, 2x$SiMe_2$). FAB MS *m/z* 659 $(M+H)^+$, 643 $(M-Me)^+$, and 601 $(M-{}^t Bu)^+$. For **7b** δ 5.97 (1H, dd, $J_{7,8}$ = 3.4 and 6.4, H7), 5.93 (1H, s, H5), 5.84 (1H, d, $J_{1',2'}$ = 5.5, H1'), 4.69 (1H, t, $J_{2',3'}$ = 5.5, H2'), 4.49 (1H, dd, $J_{3',4'}$ = 4.1, H3'), 4.45 (1H, dd, $J_{7,8}$ = 3.4, J_{gem} = 12.2, H8), 4.43–4.35 (3H, m, H8 and CH_2), 3.94–3.87 (2H, m, H4' and H5'), 3.72 (1H, dd, $J_{4',5'}$ = 4.1, J_{gem} = 10.2, H5'), 3.37 (3H, s, OCH_3), 2.15 and 2.06 (6H, each as s, 2xOAc), 1.34 (3H, t, J = 7.0,

CH_3), 0.92 and 0.88 (18H, each as s, 2xSi^{t}Bu), 0.12, 0.11, 0.05, and 0.04 (12H, each as s, 2xSiMe$_2$). FAB MS *m/z* 659 $(M+H)^+$, 643 $(M-Me)^+$, and 601 $(M-{}^tBu)^+$. Compound 7: Anal. Calcd for $C_{30}H_{54}N_2O_{10}Si_2$: C, 54.68; H, 8.26; N, 4.25. Found: C, 54.69; H, 8.54; N, 4.28.

6-(1,2-Diacetoxyethyl)-1-[5-*O*-(4,4'-dimethoxytrityl)-2-*O*-methyl-β-D-ribofuranosyl]-4-ethoxy-2-pyrimidinone (8). Compound 7 (2.18 g, 3.30 mmol) was dissolved in THF (33 mL), and AcOH (0.38 mL, 6.60 mmol) and TBAF•nH_2O (2.59 g, 9.90 mmol) were added. The solution was stirred at rt overnight. Silica gel (*ca.* 15 g) was added and the mixture was concentrated *in vacuo*. The resulting powder was applied on a column chromatography (1-4% EtOH in $CHCl_3$) to afford 1-(2-*O*-methyl-β-D-ribofuranosyl)-6-(1,2-diacetoxyethyl)-4-ethoxy-2-pyrimidinone (1.44 g, quant) as a foam. This compound was used without further purification. FAB MS *m/z* 431 $(M+H)^+$.

A mixture of 1-(2-*O*-methyl-β-D-ribofuranosyl)-6-(1,2-diacetoxyethyl)-4-ethoxy-2-pyrimidinone (145.0 mg, 0.337 mmol) obtained above and DMTCl (228.3 mg, 0.674 mmol) in dry pyridine (5 mL) was stirred at rt for 2 h. EtOH (3 mL) was added and the solution was concentrated *in vacuo*, and the residue was partitioned between EtOAc (20 mL) and saturated aqueous $NaHCO_3$ (5 mL). The organic layer was washed with H_2O and brine (5 mL each), and dried over Na_2SO_4. Column chromatography (20-60% EtOAc in hexane) gave **8** (197.4 mg, 80%) as a foam. Diastereomer-mixture of **8** (*ca.* 80 mg) was separated to each component **8a** (t_R 11.0 min, 38.6 mg) and **8b** (t_R 11.9 min, 31.7 mg) by HPLC (20% hexane in EtOAc). *Compound* **8a** : UV (MeOH) λ_{max} 281 nm (ε 7900), 275 nm (ε 8100), and 232 nm (ε 22600). λ_{min} 278 nm (ε 7800) and 256 nm (ε 4900). ^{1}H NMR ($CDCl_3$) δ 7.44–7.42 (2H, m), 7.33–7.30 (4H, m), 7.24–7.21 (2H, m), 7.17–7.14 (1H, m), 6.79–6.75 (4H, m), 6.08 (1H, dd, $J_{7,8}$ = 3.5 and 7.9, H7), 5.95 (1H, s, H5), 5.71 (1H, s, H1'), 4.63 (1H, dd, J_{gem} = 12.2, H8), 4.53 (2H, m, H2' and H3'), 4.41 and 4.38 (2H, each as dq, J = 7.0, J_{gem} = 10.4, CH_2), 4.24 (1H, dd, H8), 3.97 (1H, dt, $J_{3',4'} = J_{4',5'}$ = 6.7, $J_{4',5'}$ = 4.3, H4'), 3.77 (6H, s, 2xOCH_3), 3.50 (3H, s, 2'-OCH_3), 3.41 (1H, dd, $J_{4',5'}$ = 4.3, J_{gem} = 10.1, H5'), 3.37 (1H, dd, H5'), 2.65 (1H, br, 3'-OH), 2.19 and 2.08 (6H, each as s, 2xOAc), 1.35 (3H, t, CH_3). FAB MS *m/z* 733 $(M+H)^+$ and

732 $(M)^+$. Anal. Calcd for $C_{39}H_{44}N_2O_{12}\cdot 1/2H_2O$: C, 63.15; H, 6.11; N, 3.78. Found: C, 62.99; H, 6.14; N, 3.65. *Compound* **8b**: UV (MeOH) λ_{max} 281 nm (ε 7700), 276 nm (ε 7700), and 232 nm (ε 20900). λ_{min} 278 nm (ε 7600) and 258 nm (ε 4600). ^{1}H NMR ($CDCl_3$) δ 7.44–7.42 (2H, m), 7.34–7.31 (4H, m), 7.25–7.23 (2H, m), 7.19–7.16 (1H, m), 6.81–6.76 (4H, m), 6.03 (1H, d, $J_{1',2'}$ = 4.0, H1'), 5.96 (1H, s, H5), 5.87 (1H, dd, $J_{7,8}$ = 3.4 and 6.1, H7), 4.55 (1H, dd, $J_{2',3'}$ = 6.9, H2'), 4.44–4.39 (3H, m, H3' and CH_2), 4.33 (1H, dd, $J_{7,8}$ = 6.1, J_{gem} = 12.2, H8), 4.29 (1H, dd, H8), 3.90 (1H, br dt, $J_{4',5'}$ = 4.3 and 6.1, H4'), 3.78 and 3.77 (6H, each as s, 2xOCH_3), 3.49 (3H, s, 2'-OCH_3), 3.44 (1H, dd, $J_{4',5'}$ = 6.1, J_{gem} = 10.7, H5'), 3.39 (1H, dd, H5'), 2.73 (1H, d, $J_{3',OH}$ = 7.6, 3'-OH), 2.16 and 1.96 (6H, each as s, 2xOAc), 1.36 (3H, t, J = 7.0, CH_3). FAB MS *m/z* 733 $(M+H)^+$ and 732 $(M)^+$. Anal. Calcd for $C_{39}H_{44}N_2O_{12}\cdot 1/2H_2O$: C, 63.15; H, 6.11; N, 3.78. Found: C, 63.06; H, 5.93; N, 3.66.

6-(1,2-Diacetoxyethyl)-1-[5-*O*-(4,4'-dimethoxytrityl)-2-*O*-methyl-β-D-ribofuranosyl]-4-ethoxy-2-pyrimidinone-3'-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)-phosphoramidite (9). To a mixture of compound **8** (139.6 mg, 0.191 mmol) and 2-cyanoethyl tetraisopropylphosphorodiamidite (0.12 mL, 0.382 mmol) in CH_2Cl_2 (3.5 mL), a solution of 4,5-dicyano-imidazole (15.7 mg, 0.134 mmol) in CH_3CN (0.35 mL) was added. The mixture was stirred at rt for 1.5 h and diluted with CH_2Cl_2 (40 mL) and washed with saturated aqu. $NaHCO_3$ (2–10 mL), and the organic layer dried (Na_2SO_4). Column chromatography (1% Et_3N in 33-60% EtOAc in hexane) gave **9** (189.0 mg) as an oil, which was dissolved in toluene (1.5 mL), and hexane (25 mL) was added to the solution with stirring. After decantation, 115.1 mg of **9** was obtained as a form (65%).

^{1}H NMR ($CDCl_3$) δ 7.44–7.41 (2H, m), 7.33–7.29 (4H, m), 7.27–7.13 (3H, m), 6.80–6.74 (4H, m), 6.04 and 6.01 (1H, each as dd, $J_{7,8}$ = 3.7 and 7.3, H7), 5.95 and 5.94 (1H, each as s, H5), 5.70 and 5.67 (1H, each as d, $J_{1',2'}$ = 2.4 and 2.8, H1'), 4.69–4.54 (3H, m, H2' and 2xH8), 4.43–4.32 (3H, m, H3' and CH_2), 4.22–4.18 (1H, m, H4'), 3.94–3.29 (6H, m, PO-CH_2, 2xH5', and 2x*N*-methyne), 3.77 and 3.76 (6H, each as s, 2xOCH_3), 3.46 and 3.44 (3H, each as s, 2'-OCH_3), 2.66, 2.64, 2.60, and 2.38 (2H, each

as t, J = 6.1 and 7.0, CH_2CN), 2.18, 2.08, and 2.07 (6H, each as s, 2xOAc), 1.35 (3H, t, J = 7.0, CH_3), 1.27–0.98 [12H, m, 2x(NCH($C\underline{H}_3$)$_2$)]. FAB MS *m/z* 955 $(M+Na)^+$ and 933 $(M+H)^+$.

Acknowledgment: The authors would like to thank Dr. Katsutoshi Ito (Showa University) for supporting us with HPLC analysis and Dr. Masahisa Nakada (Waseda University) for arrangements for high resolution mass spectra measurement of compounds **3** and **5**. This work was partially supported by the High-Technology Research Center Project from the Ministry of Education, Science, Sports and Culture of Japan.

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Received : 3 / 12 / 99
Accepted : 6 / 1 / 99