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Synthesis and properties of 2′-*O*,4′-*C*-methyleneoxymethylene bridged nucleic acid

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Abstract—A novel bridged nucleic acid (BNA) analogue, 2′-*O*,4′-*C*-methyleneoxymethylene bridged nucleic acid (2′,4′-BNA^{COC}), was synthesized and incorporated into oligonucleotides. The 2′,4′-BNA^{COC} modified oligonucleotides showed high binding affinity with an RNA complement and significant enzymatic stability against snake venom phosphodiesterase.

1. Introduction

For a practical application of antisense and/or antigene methodologies, it is essential to develop modified oligonucleotides, which strongly interact with single-stranded RNA (ssRNA) and/or double-stranded DNA (dsDNA) in a sequence-specific manner.[1–5] In addition, high resistance against enzymatic degradation is also required for in vivo use. We have so far developed various kinds of bridged nucleic acids (BNAs),[6–8] the sugar conformation of which is restricted or locked by introduction of an additional bridged structure to the furanose skeleton, and it was observed that one of the BNAs with a locked N-type sugar conformation, 2′,4′-BNA[6–11]/LNA,[5,12–15] was prominent in hybridization with ssRNA and dsDNA targets (Fig. 1). Recently, other nucleic acid analogues with a different type of bridged structure between the 2′- and 4′-positions, which have a six- or seven-membered ring, were reported (Fig. 1).[16–21] These studies revealed a significant effect of the size and composition of the ring structure on hybridization ability and/or enzymatic stability of the nucleic acid analogues. Thus, modification of the bridged structure in 2′,4′-BNA/LNA would be a workable strategy for development of practical antisense and/or antigene molecules. Here, we designed a novel bridged nucleic acid analogue, 2′,4′-BNA^{COC}, which has a methyleneoxymethylene (–C–O–C–) linkage between the O2′ and C4′ atoms (Fig. 1). The facile synthesis of 2′,4′-BNA^{COC} monomers and the properties of the corresponding oligonucleotide derivatives are described.

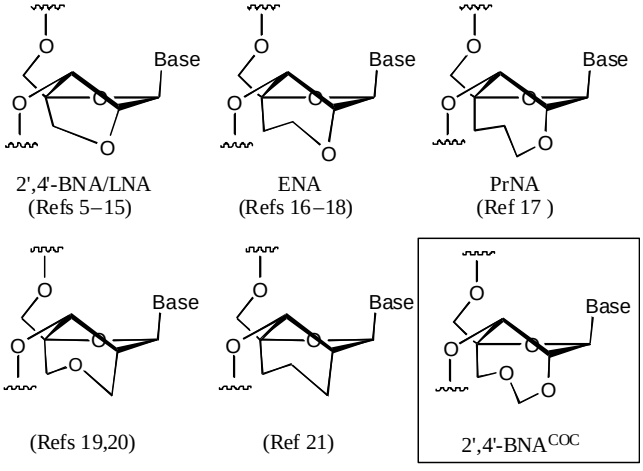

Figure 1. Structures of 2′,4′-BNA^{COC} and other bridged nucleic acids.

Keywords: Bridged nucleic acids; Conformation; Nucleosides; Oligonucleotides.
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2. Results and discussion

As shown in Scheme 1, an amidite **9** was synthesized by using **1** as the starting material, which was prepared according to our previous report.[22] Treatment of **1** with Ac_2O in AcOH in the presence of concd H_2SO_4 afforded triacetate **2**. According to Vorbrüggen's procedure,[23,24] the triacetate **2** was coupled with thymine to give a β-anomer $\mathbf{3}^{\mathbf{T}}$. Exposure of $\mathbf{3}^{\mathbf{T}}$ to K_2CO_3/MeOH provided a diol $\mathbf{4}^{\mathbf{T}}$, which was then treated with paraformaldehyde to yield the desired cyclic acetal product $\mathbf{5}^{\mathbf{T}}$. The *tert*-butyldiphenylsilyl (TBDPS) group was removed by treatment with TBAF and subsequent hydrogenolysis furnished the 2′,4′-BNA^{COC}-thymine monomer $\mathbf{7}^{\mathbf{T}}$ via an intermediate $\mathbf{6}^{\mathbf{T}}$. The structural features of $\mathbf{7}^{\mathbf{T}}$ were well confirmed by ^{1}H NMR measurement and X-ray crystallography.[25] It was observed that the sugar conformation existed in a typical N-form (C3′-*endo*, pseudorotation phase angle $P = 17°$) and the methyleneoxymethylene (C–O–C) linkage surrounded the O3′ atom in $\mathbf{7}^{\mathbf{T}}$. Furthermore, the maximum out-of-plane pucker (ν_{max}), γ, and δ values of $\mathbf{7}^{\mathbf{T}}$ were 38°, 58°, and 78°, respectively. These values are in good agreement with those observed in a typical A-type RNA duplex ($P = 14°$, $\nu_{max} = 38°$, $\gamma = 45°$ and $\delta = 83°$).[26,27] Reaction of a primary hydroxyl group in $\mathbf{7}^{\mathbf{T}}$ with DMTrCl in pyridine gave $\mathbf{8}^{\mathbf{T}}$, and subsequent phosphitylation led to the desired amidite $\mathbf{9}^{\mathbf{T}}$. The phosphoramidite $\mathbf{9}^{\mathbf{C}}$ bearing an N^4-benzoylcytosine nucleobase was synthesized in a similar manner (Fig. 2).

As shown in Scheme 2, the nucleobase of the 2′,4′-BNA^{COC}-thymine monomer was converted to 5-methylcytosine. The thymidine analogue $\mathbf{5}^{\mathbf{T}}$ was reacted with 1*H*-triazole, $POCl_3$, and Et_3N in MeCN to afford the corresponding 4-triazolylpyrimidine intermediate, which was then treated with aqueous NH_3 to give the 5-methylcytosine derivative **10**. Protection of the nucleobase moiety with a benzoyl group and removal of the 5′-*O*-TBDPS and 3′-*O*-benzyl groups gave the desired 2′,4′-BNA^{COC}-5-methylcytosine monomer **13**. Dimethoxytritylation and subsequent phosphitylation of the 5-methylcytosine monomer **13** afforded the phosphoramidite **15**. Thus, we achieved the synthesis of novel bridged nucleic acid monomers and their amidite derivatives via a convenient synthetic route. This facile route would be readily applicable to the synthesis of the 2′,4′-BNA^{COC} bearing other natural or unnatural nucleobases.

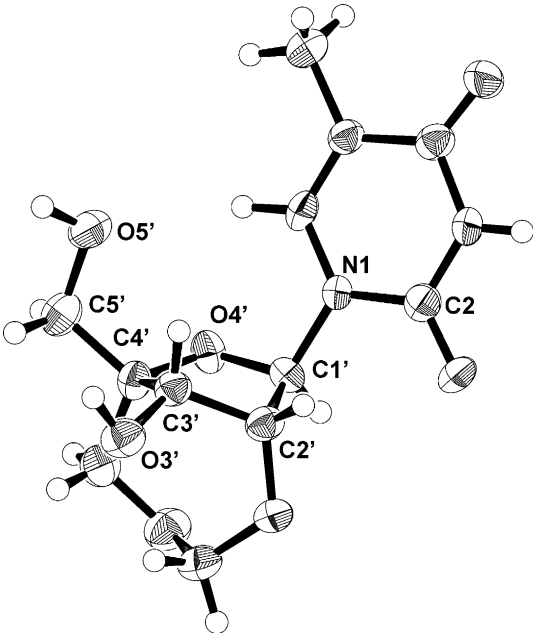
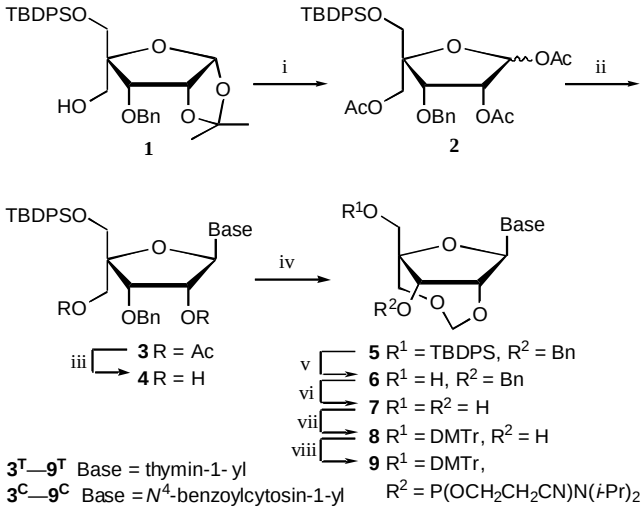


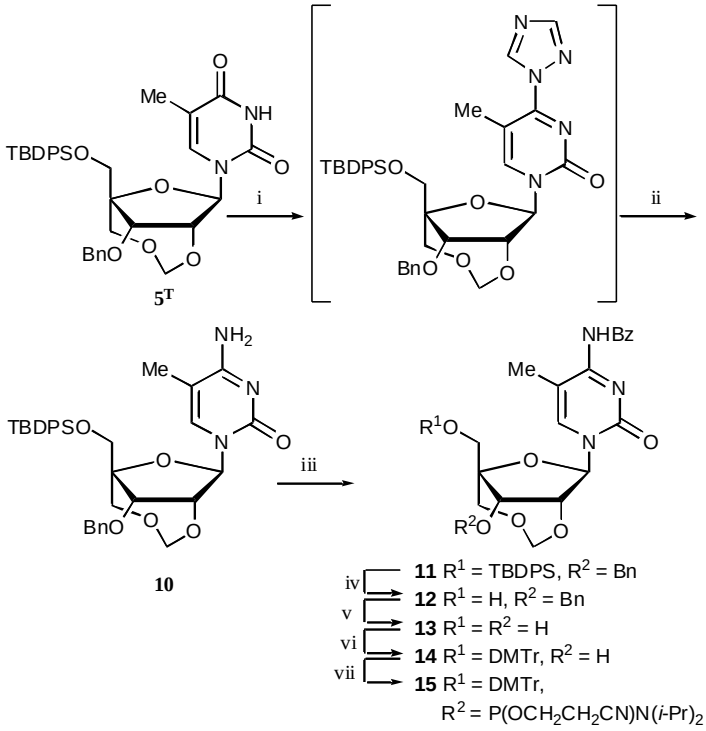
Figure 2. X-ray structure of $\mathbf{7}^{\mathbf{T}}$.



Scheme 1. Regents and conditions: i, Ac_2O, AcOH, concd H_2SO_4, 99%; ii, thymine, *N*,*O*-bis(trimethylsilyl)acetamide, TMSOTf, MeCN, 93% for $\mathbf{3}^{\mathbf{T}}$; N^4-benzoylcytosine, *N*,*O*-bis(trimethylsilyl)acetamide, TMSOTf, MeCN, 76% for $\mathbf{3}^{\mathbf{C}}$; iii, K_2CO_3, MeOH, 90% for $\mathbf{4}^{\mathbf{T}}$; LiOH·H_2O, THF/H_2O, 84% for $\mathbf{4}^{\mathbf{C}}$; iv, paraformaldehyde, *p*-TsOH·H_2O, 1,2-dichloroethane, 81% for $\mathbf{5}^{\mathbf{T}}$ and 70% for $\mathbf{5}^{\mathbf{C}}$; v, TBAF, THF, 85% for $\mathbf{6}^{\mathbf{T}}$ and 92% for $\mathbf{6}^{\mathbf{C}}$; vi, 20% $Pd(OH)_2$-C, cyclohexene, EtOH, 89% for $\mathbf{7}^{\mathbf{T}}$; 20% $Pd(OH)_2$-C, cyclohexene, MeOH, 71% for $\mathbf{7}^{\mathbf{C}}$; vii, DMTrCl, pyridine, quant. for $\mathbf{8}^{\mathbf{T}}$ and 88% for $\mathbf{8}^{\mathbf{C}}$; viii, (*i*-$Pr_2N)_2POCH_2CH_2CN$, 4,5-dicyanoimidazole, MeCN, 94% for $\mathbf{9}^{\mathbf{T}}$; (*i*-$Pr_2N)_2POCH_2CH_2CN$, 5-ethylthio-1*H*-tetrazole, MeCN, 81% for $\mathbf{9}^{\mathbf{C}}$.

Next, the phosphoramidite $\mathbf{9}^{\mathbf{T}}$ was incorporated into oligonucleotides by using conventional phosphoramidite chemistry on an automated DNA synthesizer. Although a decrease in the coupling efficiency of $\mathbf{9}^{\mathbf{T}}$ was supposed to be due to a steric hindrance of a seven-membered ring structure with a methyleneoxymethylene linkage, the corresponding oligonucleotides **16**–**20** containing one or more 2′,4′-BNA^{COC}-thymine monomers ($T^{B(COC)}$) were obtained by a standard phosphoramidite chemistry using 1*H*-tetrazole as an activator. The coupling time was prolonged to 45 min for $T^{B(COC)}$, and the coupling efficiency for $T^{B(COC)}$ was ca. 80%.[28] The coupling efficiency was remarkably improved (over 95% yield) when 4,5-dicyanoimidazole or 5-ethylthio-1*H*-tetrazole was used as an activator even with shorter coupling time (20 min).

Triplex-forming ability of the oligonucleotides **16**–**20** with dsDNA was evaluated under near physiological conditions by UV melting experiments (Table 1). In a triplex formation with a 21 mer dsDNA target, **16**–**18** containing one or three $T^{B(COC)}$ increased in the T_m



Scheme 2. Regents and conditions: i, 1,2,4-1*H*-triazole, $POCl_3$, Et_3N, MeCN; ii, 28% NH_3 aq, 1,4-dioxane, 82% for 2 steps; iii, BzCl, pyridine then 28% NH_3 aq, 87%; iv, TBAF, THF, 97 %; v, 20% $Pd(OH)_2$-C, cyclohexene, THF, 68%; vi, DMTrCl, pyridine, 93%; vii, $[(i\text{-}Pr)_2N]_2POCH_2CH_2CN$, 5-ethylthio-1*H*-tetrazole, MeCN, 95%.

Table 1. Thermal stability of the triplexes and duplexes comprising the oligonucleotides and dsDNA, ssDNA and ssRNA targets[a]

Oligonucleotides	T_m(ΔT_m/modification) (°C)		
	dsDNA target	ssDNA target	ssRNA target
5′-T<u>C</u>TT<u>C</u>TTTTT<u>C</u>T<u>C</u>T-3′ (natural oligonucleotide)	38	47	50
5′-T<u>C</u>TT<u>C</u>TTT$^{B(COC)}$TT<u>C</u>T<u>C</u>T-3′ (**16**)	39 (+1.0)	45 (−2.0)	51 (+1.0)
5′-T<u>C</u>TT<u>C</u>TT$^{B(COC)}$T$^{B(COC)}$T$^{B(COC)}$T<u>C</u>T<u>C</u>T-3′ (**17**)	41 (+1.0)	41 (−2.0)	55 (+1.7)
5′-T<u>C</u>TT<u>C</u>T$^{B(COC)}$TT$^{B(COC)}$TT$^{B(COC)}$<u>C</u>T<u>C</u>T-3′ (**18**)	42 (+1.3)	40 (−2.3)	56 (+2.0)
5′-T<u>C</u>TT<u>C</u>T$^{B(COC)}$T$^{B(COC)}$T$^{B(COC)}$T$^{B(COC)}$T$^{B(COC)}$<u>C</u>T<u>C</u>T-3′ (**19**)	36 (−0.4)	39 (−1.6)	60 (+2.0)

[a] Conditions: for a triplex formation with a dsDNA target, 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl and 10 mM $MgCl_2$; for a duplex formation with a ssDNA or ssRNA, 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl. The sequences of targets: dsDNA, 5′-d(GCTAGAAGAAAAAGAGATCG)-3′/3′- d(CGATCTTCTTTTTCTCTAGC)-5′; ssDNA, 5′-d(AGAAGAAAAAGAGA)-3′; ssRNA, 5′-r(AGAAGAAAAAGAGA)-3′. $T^{B(COC)}$ and <u>C</u> mean 2′,4′-BNA^{COC} unit bearing thymine nucleobase and 2′-deoxy-5-methylcytidine, respectively.

values by +1.0 to +1.3 °C per modification compared with natural oligonucleotide. However, five successive modifications in the middle of the oligonucleotide showed no increment in the binding affinity with the dsDNA target. On the other hand, duplex-forming ability of the oligonucleotides **16**–**20** with ssDNA and ssRNA targets was also examined (Table 1). The T_m values of the duplexes comprising the oligonucleotides **16**–**19** and the DNA complement were slightly lower than that of the natural DNA/DNA duplex, while **16**–**19** formed stable duplexes with the RNA complement. The increase in the T_m values per modification (ΔT_m) ranged from +1.0 to +2.0 °C. Thus, the 2′,4′-BNA^{COC} modification stabilized the duplexes formed with the RNA complement but destabilized the duplexes with the DNA complement. This RNA selective hybridization property may be due to the appropriate restriction of the sugar conformation (*P*, ν_{max}, γ and δ values) by the C–O–C linkage. However, the duplex and triplex stabilization effects by the 2′,4′-BNA^{COC} modification were less than that by the 2′,4′-BNA/LNA[5–15] or ENA.[16–18] One major structural difference between 2′,4′-BNA/LNA, ENA, and 2′,4′-BNA^{COC} is that the former has a relatively larger ν_{max} value (ν_{max} of 2′,4′-BNA[9] and ENA[17] are 57° and 48°, respectively), while the latter has the ν_{max} value similar to that of the typical A-type duplex (ν_{max} of 2′,4′-BNA^{COC} is 37°). Although the detail is still unclear, the combination between C3′-*endo* sugar puckering and larger ν_{max} value, in other words, the magnified N-type sugar conformation may be suitable for acquiring high binding affinity with the DNA and RNA complements and also with the dsDNA target. Alternatively, an increase in the steric hindrance of the additional linkage between the 2′-oxygen and 4′-carbon atoms might negatively affect the hybridization property. It is also noteworthy that the related nucleic

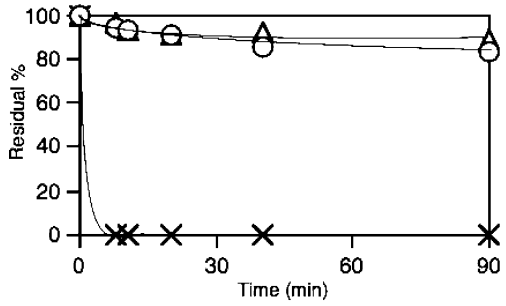


Figure 3. Degradation of the oligonucleotides by snake venom phosphodiesterase (SVPDE). Conditions: 50 mM Tris·HCl buffer (pH 8.0, 350 μl) containing 10 mM $MgCl_2$, 5 nmol oligonucleotides and 0.2 μg SVPDE. $T_8T^{B(COC)}T$ (**20**), circle; T_{10}, cross; T_9-ps-T, triangle, where $T^{B(COC)}$ and ps mean 2′,4′-BNA^{COC} unit bearing thymine nucleobase and phosphorthioate linkage, respectively.

acid analogue, PrNA, which possesses a propylene (C–C–C) linkage instead of a C–O–C linkage (Fig. 1), showed no significant increase in binding affinity with the RNA complement.[17] The hydration of dsDNA is well known to be a key factor in the stabilization of secondary and tertiary structure.[27,29,30] The results obtained here clearly indicate an importance of the hydrophilicity of the additional bridged structure.

Enzymatic stability of the oligonucleotide **20** containing one $T^{B(COC)}$ unit against snake venom phosphodiesterase (SVPDE) was examined. After reaction with SVPDE for certain time periods, the amount of the intact oligonucleotides was evaluated by RP-HPLC analysis (Fig. 3). An immediate degradation of the natural oligothymidylate T_{10} was observed, whereas the oligonucleotide **20** showed almost no degradation under the same conditions even after 90 min reaction. The excellent enzymatic stability of **20** against SVPDE, which is comparable to that of the corresponding phosphorothioate analogue, is probably due to the steric hindrance of the C–O–C linkage surrounding the O3′ atom. This result clearly indicates that the 2′,4′-BNA^{COC} modified oligonucleotide is promising for in vitro and/or in vivo use.

3. Conclusion

In conclusion, 2′,4′-BNA^{COC} monomers bearing a thymine, cytosine or 5-methylcytosine nucleobase were easily synthesized, and the 2′,4′-BNA^{COC}-thymine monomer was successfully incorporated into oligonucleotides. The corresponding 2′,4′-BNA^{COC} modified oligonucleotides showed interesting features, such as high binding affinity towards an RNA complement and excellent nuclease resistance. These results show that the 2′,4′-BNA^{COC} is a potential material for antisense and/or antigene strategies. Further application of the 2′,4′-BNA^{COC} is now in progress.

4. Experimental

All melting points were measured with a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were recorded on a JASCO DIP-370 instrument. IR spectra were recorded on a JASCO FT/IR-200 spectrometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-EX270 (^1H, 270 MHz; ^{13}C, 67.8 MHz) or JEOL JNM-EX300 (^1H, 300 MHz; ^{13}C, 75.5 MHz), and ^{31}P NMR spectrum was recorded on a Varian VXR-200 (^{31}P, 86.4 MHz). MS spectra of nucleoside analogues were recorded on a JEOL JMS-600 or JMS-700 MS spectrometer. For flash column, Fuji Silysia FL60D or BW-300 was used. MALDI-TOF-MS spectra were recorded on an Applied Biosystems Voyeger®-DE.

4.1. 4-*C*-Acetoxymethyl-1,2-di-*O*-acetyl-3-*O*-benzyl-5-*O*-*tert*-butyldiphenylsilyl-β-D-*erythro*-pentofuranose (2)

Ac_2O (1 ml) and concd H_2SO_4 (0.1 ml) were added to a solution of compound **1** (900 mg, 1.64 mmol) in AcOH (4 ml) at room temperature and the mixture was stirred at room temperature for 1 h. After addition of the reaction mixture to saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [hexane/AcOEt (3:1, v/v)] to give compound **2** (1.03 g, 99%). Colorless oil. IR ν_{max} (KBr): 3059, 2943, 2865, 1749, 1461, 1371, 1230, 1107 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.07 (9H, s), 1.84 (3H, s), 1.95 (3H, s), 2.10 (3H, s), 3.66, 3.88 (2H, AB, J = 11 Hz), 4.38, 4.44 (2H, AB, J = 12 Hz), 4.50 (1H, d, J = 5 Hz), 4.55, 4.61 (2H, AB, J = 11 Hz), 5.40 (1H, d, J = 5 Hz), 6.19 (1H, s), 7.23–7.45 (11H, m), 7.63–7.66 (4H, m). ^{13}C NMR ($CDCl_3$) δ 19.3, 20.8, 20.9, 20.9, 26.8, 63.9, 65.1, 73.6, 74.2, 79.2, 85.6, 97.6, 127.3, 127.6, 127.6, 127.7, 128.2, 129.6, 129.7, 132.5, 132.8, 135.3, 135.4, 137.0, 168.6, 169.4, 170.4. MS (FAB): *m*/*z* 657 (MNa^+). High-resolution MS (FAB): calcd for $C_{35}H_{42}N_2O_9SiNa$ (MNa^+): 657.2496. Found: 657.2504.

4.2. 4′-*C*-Acetoxymethyl-2′-*O*-acetyl-3′-*O*-benzyl-5′-*O*-*tert*-butyldiphenylsilyl-5-methyluridine (3^T)

Under N_2 atmosphere, thymine (296 mg, 2.35 mmol) and *N*,*O*-bis(trimethylsilyl)acetamide (0.78 ml, 5.48 mmol) were added to a solution of compound **2** (1.00 g, 1.57 mmol) in anhydrous MeCN (10 ml) at room temperature and the mixture was refluxed for 1 h. TMSOTf (0.14 ml, 0.78 mmol) was added to the reaction mixture at 0 °C and the mixture was refluxed for 1 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [hexane/AcOEt (3:2, v/v)] to give compound $\mathbf{3^T}$ (1.07 g, 93%). White powder; mp 68–70 °C. $[\alpha]_D^{25}$ +24.3 (*c* 0.50, $CHCl_3$). IR ν_{max} (KBr): 3175, 3066, 2957, 2860, 1711, 1465, 1375, 1239, 1114 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.11 (9H, s), 1.63 (3H, s), 1.93 (3H, s), 2.09 (3H, s), 3.71, 3.89 (2H, AB, J = 11 Hz), 4.10, 4.40 (2H, AB, J = 12 Hz), 4.47, 4.59 (2H, AB, J = 11 Hz), 4.52 (1H, d, J = 6 Hz), 5.42 (1H,

dd, J = 6, 6 Hz), 6.22 (1H, d, J = 6 Hz), 7.27–7.48 (12H, m), 7.63–7.67 (4H, m), 9.37 (1H, s). ^{13}C NMR ($CDCl_3$) δ 12.1, 19.4, 20.8, 20.8, 27.1, 63.1, 65.3, 74.3, 74.8, 77.4, 86.3, 86.4, 111.6, 127.7, 127.9, 128.0, 128.4, 130.0, 130.1, 131.9, 132.4, 135.2, 135.4, 135.5, 136.9, 150.0, 163.2, 170.1, 170.2. MS (FAB): m/z 701 (MH^+). Anal. Calcd for $C_{38}H_{44}N_2O_9$ Si·H_2O: C, 63.49; H, 6.45; N, 3.89. Found: C, 63.43; H, 6.16; N, 3.86.

4.3. 4′-*C*-Acetoxymethyl-2′-*O*-acetyl-4-*N*-benzoyl-3′-*O*-benzyl-5′-*O*-*tert*-butyldiphenylsilylcytidine (3^C)

Under N_2 atmosphere, N^4-benzoylcytosine (8.13 g, 37.8 mmol) and N,O-bis(trimethylsilyl)acetamide (16.3 ml, 0.11 mol) were added to a solution of compound **2** (16.0 g, 25.2 mmol) in anhydrous MeCN (75 ml) at room temperature and the mixture was refluxed for 3 h. TMSOTf (11.4 ml, 53.0 mmol) was added to the reaction mixture at 0 °C and the mixture was refluxed for 9 h. After addition of saturated aqueous $NaHCO_3$ solution at 0 °C, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [hexane/AcOEt (3:2, v/v)] to give compound $\mathbf{3^C}$ (15.1 g, 76%). White powder; mp 113–115 °C. $[\alpha]_D^{24}$ +57.5 (c 1.03, $CHCl_3$). IR ν_{max} (KBr): 3069, 1744, 1671, 1555, 1484, 1370, 1311, 1242, 1109 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.12 (9H, s), 1.94 (3H, s), 2.12 (3H, s), 3.78, 4.04 (2H, AB, J = 11 Hz), 4.12, 4.53 (2H, AB, J = 13 Hz), 4.40, 4.61 (2H, AB, J = 11 Hz), 4.54 (1H, d, J = 6 Hz), 5.53 (1H, dd, J = 5, 6 Hz), 6.26 (1H, d, J = 5 Hz), 7.21–7.53 (14H, m), 7.58–7.67 (5H, m), 7.89 (2H, d, J = 7 Hz), 8.13 (1H, d, J = 7 Hz), 8.67 (1H, br s). ^{13}C NMR ($CDCl_3$) δ 19.2, 20.7, 20.7, 27.0, 63.1, 64.1, 74.2, 74.9, 76.7, 86.8, 89.0, 96.9, 127.5, 127.8, 127.8, 128.2, 128.7, 130.0, 130.0, 131.8, 132.1, 132.9, 135.2, 135.4, 136.8, 144.3, 154.2, 162.2, 166.6, 169.5, 170.1. MS (FAB): m/z 790 (MH^+). Anal. Calcd for $C_{44}H_{47}N_3O_9Si$: C, 66.90; H, 6.00; N, 5.32. Found: C, 66.52; H, 6.06; N, 5.33.

4.4. 3′-*O*-Benzyl-5′-*O*-*tert*-butyldiphenylsilyl-4′-*C*-hydroxymethyl-5-methyluridine (4^T)

K_2CO_3 (355 mg, 2.56 mmol) was added to a solution of compound $\mathbf{3^T}$ (360 mg, 0.52 mmol) in MeOH (10 ml) at room temperature and the mixture was stirred at room temperature for 4 h. After being neutralized by diluted aqueous HCl solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [hexane/AcOEt (2:1, v/v)] to give compound $\mathbf{4^T}$ (288 mg, 90%). White powder; mp 63–65 °C. $[\alpha]_D^{22}$ +43.2 (c 1.01, MeOH). IR ν_{max} (KBr): 3174, 3069, 2937, 2860, 1701, 1468, 1260, 1119 cm^{-1}.^{1}H NMR ($CDCl_3$) δ 1.06 (9H, s), 1.50 (3H, s), 3,42 (1H, br s), 3.55, 3.77 (2H, AB, J = 12 Hz), 3.64, 3.80 (2H, AB, J = 11 Hz), 4.31 (1H, d, J = 6 Hz), 4.38 (1H, dd, J = 5,6 Hz), 4.52, 4.87 (2H, AB, J = 12 Hz), 4.96 (1H, br d), 6.14 (1H, d, J = 5 Hz), 7.30–7.44 (12H, m), 7.57–7.60 (4H, m), 10.1 (1H, br s). ^{13}C NMR ($CDCl_3$) δ 12.0, 19.4, 27.0, 62.7, 73.5, 74.5, 77.0, 88.3, 89.7, 111.2, 127.8, 127.8, 128.4, 129.8, 129.9, 132.0, 132.6, 135.1, 135.3, 135.4, 137.1, 151.0, 163.9. MS (FAB): m/z 617 (MH^+). Anal. Calcd for $C_{34}H_{40}N_2O_7Si \cdot \frac{3}{4}H_2O$: C, 64.79; H, 6.64; N, 4.44. Found: C, 64.66; H, 6.48; N, 4.41.

4.5. 4-*N*-Benzoyl-3′-*O*-benzyl-5′-*O*-*tert*-butyldiphenylsilyl-4′-*C*-(hydroxymethyl)cytidine (4^C)

$LiOH·H_2O$ (1.60 g, 8.23 mmol) was added to a solution of compound $\mathbf{3^C}$ (6.50 g, 8.23 mmol) in THF/H_2O (1:1, v/v, 90 ml) at room temperature and the mixture was stirred at room temperature for 2 h. The mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by recrystallization from AcOEt to give compound $\mathbf{4^C}$ (5.24 g, 84%). Colorless crystals; mp 144–145 °C (AcOEt). $[\alpha]_D^{25}$ +61.5 (c 1.02, AcOEt). IR ν_{max} (KBr): 3265, 3069, 1700, 1651, 1561, 1485, 1388, 1314, 1256, 1116 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.06 (9H, s), 3.57, 3.84 (2H, AB, J = 12 Hz), 3.68, 3.87 (2H, AB, J = 12 Hz), 4.36 (1H, d, J = 6 Hz), 4.38 (1H, d, J = 6 Hz), 4.42, 4.78 (2H, AB, J = 11 Hz), 4.49 (1H, br s), 5.75 (1H, br s), 6.23 (1H, s), 7.24–7.62 (19H, m), 7.96 (2H, d, J = 7 Hz), 8.25 (1H, d, J = 7 Hz), 9.46 (1H, br s). ^{13}C NMR ($CDCl_3$) δ 19.2, 26.9, 60.3, 61.4, 63.8, 71.9, 74.8, 75.1, 90.3, 93.8, 97.0, 127.1, 127.5, 127.7, 127.8, 128.1, 128.6, 129.9, 131.8, 132.2, 132.8, 135.1, 135.3, 137.2, 143.9, 155.9, 162.4, 166.5. MS (FAB): m/z 706 (MH^+). Anal. Calcd for $C_{40}H_{43}N_3O_7Si \cdot \frac{1}{5}H_2O$: C, 67.72; H, 6.17; N, 5.92. Found: C, 67.55; H, 6.20; N, 5.88.

4.6. 3′-*O*-Benzyl-5′-*O*-*tert*-butyldiphenylsily-5-methyl-2′-*O*,4′-*C*-(methylenoxymethylene)uridine (5^T)

Under N_2 atmosphere, paraformaldehyde (1.20 g) and $TsOH·H_2O$ (180 mg, 0.94 mmol) were added to a solution of compound $\mathbf{4^T}$ (360 mg, 0.52 mmol) in anhydrous 1,2-dichloroethane (30 ml) at room temperature and the mixture was reflued for 3 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [hexane/AcOEt (3:1, v/v)] to give compound $\mathbf{5^T}$ (664 mg, 81%). White powder; mp 59–62 °C. $[\alpha]_D^{23}$ +42.7 (c 0.34, $CHCl_3$). IR ν_{max} (KBr): 3189, 3067, 2933, 2860, 1691, 1466, 1270, 1177, 1120 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ :1.09 (9H, s), 1.57 (3H, s), 3.67, 3.80 (2H, AB, J = 12 Hz), 3.74, 3.92 (2H, AB, J = 12 Hz), 4.57 (1H, d, J = 6Hz), 4.59, 4.83 (2H, AB, J = 11 Hz), 4.61 (1H, d, J = 6 Hz), 5.25, 5.33 (2H, AB, J = 6 Hz), 6.22 (1H, s), 7.28–7.43 (12H, m), 7.59–7.67 (4H, m), 9.78 (1H, br s). ^{13}C NMR ($CDCl_3$) δ 12.0, 19.3, 26.9, 62.2, 71.4, 72.5. 75.2, 76.3, 88.7, 93.1, 95.8, 110.2, 127.6, 127.7, 127.9, 128.3, 129.8, 129.8, 131.9, 132.5, 135.0, 135.1, 135.4, 136.6, 149.9, 164.0. MS (FAB): m/z 629 (MH^+). Anal. Calcd for $C_{35}H_{40}N_2O_7Si \cdot \frac{3}{4}H_2O$: C, 65.45; H, 6.51; N, 4.36. Found: C, 65.27; H, 6.35; N, 4.22.

4.7. 4-*N*-Benzoyl-3′-*O*-benzyl-5′-*O*-*tert*-butyldiphenylsily-2′-*O*,4′-*C*-(methylenoxymethylene)cytidine (5^C)

Under N_2 atmosphere, $TsOH{\cdot}H_2O$ (130 mg, 0.68 mmol) was added to a solution of compound $\mathbf{4^C}$ (324 mg, 0.46 mmol) in anhydrous 1,2-dichloroethane (30 ml) at room temperature and paraformaldehyde (41 mg) was added at 60 °C. The mixture was stirred at 60 °C for 1.5 h. After addition of saturated aqueous $NaHCO_3$ solution at 0 °C, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [hexane/AcOEt (2:3, v/v)] to give compound $\mathbf{5^C}$ (230 mg, 70%). White powder; mp 210–212 °C. $[\alpha]_D^{25}$ +85.2 (*c* 0.99, THF). IR ν_{max} (KBr): 3069, 1667, 1556, 1484, 1363, 1301, 1262, 1179, 1126 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.05 (9H, s), 3.56, 3.63 (2H, AB, J = 12 Hz), 3.64, 3.86 (2H, AB, J = 12 Hz), 4.38 (1H, d, J = 6 Hz), 4.45, 4.73 (2H, AB, J = 11 Hz), 4.48 (1H, d, J = 6 Hz), 5.19, 5.22 (2H, AB, J = 6 Hz), 6.21 (1H, s), 7.15–7.59 (19H, m), 7.81 (2H, d, J = 7 Hz), 8.33 (1H, d, J = 7 Hz), 8.63 (1H, br s). ^{13}C NMR ($CDCl_3$) δ 19.3, 26.9, 61.7, 71.0, 72.5, 74.3, 76.4, 89.5, 93.2, 95.8, 96.1, 127.5, 127.8, 128.0, 128.1, 128.2, 128.6, 129.0, 130.2, 130.2, 131.8, 132.2, 133.1, 135.3, 135.5, 136.7, 143.9, 154.8, 162.2, 166.3. MS (FAB): *m*/*z* 718 (MH^+). Anal. Calcd for $C_{41}H_{43}N_3O_7Si \cdot \frac{3}{4}H_2O$: C, 67.33; H, 6.13; N, 5.75. Found: C, 67.43; H, 5.95; N, 5.72.

4.8. 3′-*O*-Benzyl-5-methyl-2′-*O*,4′-*C*-(methyleneoxymethylene)uridine (6^T)

Under N_2 atmosphere, TBAF (1 M solution in THF, 4.0 ml, 4.00 mmol) was added to a solution of compound $\mathbf{5^T}$ (2.20 g, 3.50 mmol) in anhydrous THF (50 ml) at room temperature and the mixture was stirred at room temperature for 4 h. After removal of the solvent under reduced pressure, the residue was purified by flash silica gel column chromatography [$CHCl_3$/MeOH (20:1, v/v)] to give compound $\mathbf{6^T}$ (1.16 g, 85%). White powder; mp 103–105 °C. $[\alpha]_D^{22}$ +57.5 (*c* 1.05, MeOH). IR ν_{max} (KBr): 3167, 3073, 1715, 1470, 1274, 1179, 1127 cm^{-1}. ^1H NMR ($CDCl_3$) δ :1.86 (3H, s), 2.99 (1H, br s), 3.65–3.81 (4H, m), 4.55 (1H, d, J = 6 Hz), 4.63, 4.77 (2H, AB, J = 10 Hz), 4.68 (1H, d, J = 6 Hz), 5.19, 5.36 (2H, AB, J = 6 Hz), 6.02 (1H, s), 7.31–7.39 (6H, m), 9.54 (1H, br s). ^{13}C NMR ($CDCl_3$) δ 12.3, 60.9, 71.5, 72.8, 75.4, 76.4, 88.7, 95.2, 95.9, 110.5, 127.8, 128.5, 137.1, 137.9, 150.2, 164.2. MS (FAB): *m*/*z* 391 (MH^+). Anal. Calcd for $C_{19}H_{22}N_2O_7 \cdot \frac{1}{2}H_2O$: C, 57.14; H, 5.80; N, 7.01. Found: C, 57.25; H, 5.77; N, 7.01.

4.9. 4-*N*-Benzoyl-3′-*O*-benzyl-2′-*O*,4′-*C*-(methyleneoxymethylene)cytidine (6^C)

Under N_2 atmosphere, TBAF (1 M solution in THF, 5.0 ml, 5.00 mmol) was added to a solution of compound $\mathbf{5^C}$ (2.37 g, 3.30 mmol) in anhydrous THF (50 ml) at room temperature and the mixture was stirred at room temperature for 18 h. After removal of the solvent under reduced pressure, the residue was purified by flash silica gel column chromatography [hexane/AcOEt (1:3, v/v)] to give compound $\mathbf{6^C}$ (1.45 g, 92%). White powder; mp 242–243°C. $[\alpha]_D^{24}$ +116.7 (*c* 1.06, THF). IR ν_{max} (KBr): 3247, 1658, 1558, 1493, 1362, 1306, 1265, 1178, 1124 cm^{-1}. ^1H NMR ($CDCl_3$) δ 3.23 (1H, br s), 3.72, 3.80 (2H, AB, J = 12 Hz), 3.72, 3.90 (2H, AB, J = 12 Hz), 4.50 (1H, d, J = 6 Hz), 4.55 (1H, d, J = 6 Hz), 4.57, 4.77 (2H, AB, J = 11 Hz), 5.22, 5.36 (2H, AB, J = 6 Hz), 6.17 (1H, s), 7.31–7.59 (9H, m), 7.80 (2H, d, J = 8 Hz), 8.36 (1H, d, J = 7 Hz), 8.93 (1H, br s). ^{13}C NMR (DMSO-d_6) δ 59.1, 70.5, 71.8, 74.5, 76.0, 89.0, 91.9, 94.6, 95.6, 127.3, 127.6, 128.2, 128.3, 132.6, 133.0, 137.6, 144.2, 154.1, 162.9, 167.1. MS (FAB): *m*/*z* 480 (MH^+). Anal. Calcd for $C_{25}H_{25}N_3O_7 \cdot \frac{3}{2}H_2O$: C, 59.28; H, 5.57; N, 8.29. Found: C, 59.43; H, 5.34; N, 8.31.

4.10. 5-Methyl-2′-*O*,4′-*C*-(methylenoxymethylene) uridine (7^T)

Under N_2 atmosphere 20% $Pd(OH)_2$-C (25 mg) and cyclohexene (0.38 ml) were added to a solution of compound $\mathbf{6^T}$ (36 mg,75 μmol) in EtOH (2 ml) at room temperature and the mixture was refluxed for 3 h. The mixture was filtered, and after addition of SiO_2 (0.2 g) to the filtrate, the mixture was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [$CHCl_3$/MeOH (12:1, v/v)] to give compound $\mathbf{7^T}$ (25 mg, 89%). Colorless crystals; mp 294–295 °C (MeOH). $[\alpha]_D^{24}$ +48.4 (*c* 0.56, MeOH). IR ν_{max} (KBr): 3525, 3366, 3187, 1691, 1275, 1186, 1097 cm^{-1}. ^1H NMR (CD_3OD) δ 1.86 (3H, d, J = 1 Hz), 3.65, 3.71 (2H, AB, J = 12 Hz), 3.70 (2H, s), 4.18 (1H, d, J = 6 Hz), 4.49 (1H, d, J = 6 Hz), 5.07, 5.32 (2H, AB, J = 6 Hz), 6.05 (1H, s), 7.99 (1H, q, J = 1 Hz). ^{13}C NMR (DMSO-d_6) δ 12.4, 59.3, 67.6, 70.4, 78.6, 88.7, 90.4, 94.6, 108.4, 135.5, 150.0, 163.7. MS (FAB): *m*/*z* 301 (MH^+). Anal. Calcd for $C_{12}H_{16}O_7N_2$: C, 48.00; H, 5.37; N, 9.33. Found: C, 47.61; H, 5.39; N, 9.09.

4.11. 4-*N*-Benzoyl-2′-*O*,4′*C*-(methylenoxymethylene)cytidine (7^C)

Under N_2 atmosphere 20% $Pd(OH)_2$-C (50 mg) and cyclohexene (2 ml) were added to a solution of compound $\mathbf{6^C}$ (50 mg, 0.10 mmol) in MeOH (20 ml) at room temperature and the mixture was refluxed for 1 h. After filtration of the reaction mixture, 20% $Pd(OH)_2$-C (50 mg) was added to the filtrate and the mixture was further refluxed for 1 h. The mixture was filtered and after addition of SiO_2 (0.2 g) to the filtrate, the mixture was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [$CHCl_3$/MeOH (15:1, v/v)] to give compound $\mathbf{7^C}$ (29 mg, 71%). White powder; mp 244–246 °C. $[\alpha]_D^{23}$ +90.3 (*c* 1.00, DMSO). IR ν_{max} (KBr): 3376, 3157, 1712, 1653, 1485, 1246, 1123, 1094 cm^{-1}. ^1H NMR (C_5D_5N) δ 4.03, 4.10 (2H, AB, J = 12 Hz), 4.13, 4.21 (2H, AB, J = 12 Hz), 4.90 (1H, d, J = 6 Hz), 5.29 (1H, d, J = 6 Hz), 5.47, 5.85 (2H, AB, J = 6 Hz), 6.87 (1H, s), 7.42–7.67 (4H, m), 8.18 (2H, d, J = 7 Hz), 8.35 (1H,

br s), 9.11 (1H, d, J = 8 Hz), 12.19 (1H, br s). ^{13}C NMR (C_5D_5N) δ 60.2, 68.4, 71.6, 80.6, 90.8, 93.3, 95.8, 96.4, 128.8, 128.9, 132.6, 135.3, 144.6, 155.3, 163.7, 169.0. MS (FAB): m/z 390 (MH^+). Anal. Calcd for $C_{18}H_{19}N_3O_7 \cdot \frac{1}{5}H_2O$: C, 55.02; H, 4.98; N, 10.69. Found: C, 55.01; H, 4.97; N, 10.40.

4.12. 5′-*O*-(4,4′-Dimethoxytrityl)-5-methyl-2′-*O*,4′-*C*-(methylenoxymethylene)uridine (8^T)

Under N_2 atmosphere, DMTrCl (266 mg, 0.79 mmol) was added to a solution of compound 7^T (157 mg, 0.52 mmol) in anhydrous pyridine (3 ml) at room temperature and the mixture was stirred at room temperature for 3 h. After addition of saturated aqueous solution of sodium hydrogen carbonate, the mixture was extracted with ethyl acetate. The organic extracts were washed with water and brine, dried over sodium sulfate, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [$CHCl_3$/MeOH (50:1, v/v)] to give compound 8^T (315 mg, quant). White powder; mp 189–194 °C. $[\alpha]_D^{24}$ −16.4 (c 0.72, $CHCl_3$). IR ν_{max} (KBr): 3388, 1692, 1508, 1253, 1178, 1073 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 1.41 (3H, d, J = 1 Hz), 3.33, 3.40 (2H, AB, J =11 Hz), 3.68, 3.85 (2H, AB, J = 12 Hz), 3.79 (6H, s), 4.34 (1H, d, J = 6 Hz), 4.91 (1H, dd, J = 6, 6 Hz), 5.07, 5.30 (2H, AB, J = 6 Hz), 5.32 (1H, d, J = 6 Hz), 6.15 (1H, s), 6.89 (4H, d, J = 8 Hz), 7.22–7.39 (7H, m), 7.50 (2H, d, J = 10 Hz), 7.61 (1H, s), 10.04 (1H, s). ^{13}C NMR (acetone-d_6) δ 12.2, 55.5, 63.4, 70.8, 72.2, 80.0, 87.3, 89.3, 92.7, 96.0, 110.2, 114.0, 127.7, 128.7, 129.0, 131.0, 136.2, 136.2, 136.4, 145.8, 151.0, 159.7, 164.3. MS (FAB): m/z 625 [MNa^+]. Anal. Calcd for $C_{33}H_{34}N_2O_9 \cdot \frac{1}{2}H_2O$: C, 64.80; H, 5.77; N, 4.58. Found: C, 64.75; H, 5.77; N, 4.63.

4.13. 4-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*,4′-*C*-(methylenoxymethylene)cytidine (8^C)

Under N_2 atmosphere, DMTrCl (79 mg, 0.23 mmol) was added to a solution of compound 7^C (70 mg, 0.18 mmol) in anhydrous pyridine (3 ml) at room temperature and the mixture was stirred at room temperature for 5 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [$CHCl_3$/MeOH (20:1, v/v)] to give compound 8^C (110 mg, 88%). White powder; mp 155–161 °C. $[\alpha]_D^{24}$ +37.8 (c 1.03, MeOH). IR ν_{max} (KBr): 3269, 1653, 1611, 1556, 1483, 1302, 1254, 1179, 1119 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 3.38, 3.49 (2H, AB, J = 11 Hz), 3.71, 3.80 (2H, AB, J = 12 Hz), 3.82 (6H, s), 4.35 (1H, d, J = 6 Hz), 4.87 (1H, br d, J = 6 Hz), 5.08, 5.31 (2H, AB, J = 6 Hz), 5.25 (1H, br s). 6.12 (1H, s), 6.92 (4H, d, J = 9 Hz), 7.15 (1H, br s), 7.25–7.41 (7H, m), 7.49–7.68 (5H, m), 8.15 (2H, d, J = 7 Hz), 8.44 (1H, d, J = 8 Hz), 9.72 (1H, br s). ^{13}C NMR (acetone-d_6) δ 55.4, 55.5, 63.0, 70.1, 72.0, 79.9, 87.7, 89.7, 93.2, 95.6, 96.7, 114.0, 127.8, 128.8, 129.0, 129.1, 129.4, 130.9, 131.0, 133.5, 134.5, 136.2, 136.4, 145.1, 145.3, 155.0, 159.7, 159.7, 163.6, 168.1. MS (FAB): m/z 692 (MH^+). Anal. Calcd for $C_{39}H_{37}N_3O_9 \cdot \frac{1}{2}H_2O$: C, 66.85; H, 5.47; N, 6.00. Found: C, 66.45; H, 5.53; N, 5.96.

4.14. 3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-5-methyl-2′-*O*,4′-*C*-(methylenoxymethylene)uridine (9^T)

Under N_2 atmosphere, $(i\text{-}Pr_2N)_2POCH_2CH_2CN$ (0.33 ml, 1.04 mmol) was added to a solution of compound 8^T (250 mg, 0.42 mmol), 4,5-dicyanoimidazole (98 mg, 0.83 mmol) in anhydrous MeCN (20 ml) at room temperature and the mixture was stirred at room temperature for 2 h. Then, $(i\text{-}Pr_2N)_2POCH_2CH_2CN$ (0.33 ml, 1.04 mmol) was added and the mixture was further stirred for 10 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [hexane/AcOEt (1:1, v/v)] and by the reprecipitation from hexane/AcOEt to give compound 9^T (328 mg, 94%). White powder; mp 81–87 °C. ^{31}P NMR (acetone-d_6) δ 150.7, 151.1. MS (FAB): m/z 803 (MH^+). High-resolution MS (FAB): calcd for $C_{42}H_{52}N_4O_{10}P$ (MH^+): 803.3421. Found: 803.3394.

4.15. 4-*N*-Benzoyl-3′-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*,4′-*C*-(methylenoxymethylene)cytidine (9^C)

Under N_2 atmosphere, $(i\text{-}Pr_2N)_2POCH_2CH_2CN$ (0.28 ml, 0.87 mmol) was added to a solution of compound 8^C (304 mg, 0.43 mmol), 5-ethylthio-1H-tetrazole (86 mg, 0.66 mmol) in anhydrous MeCN (20 ml) at room temperature and the mixture was stirred at room temperature for 20 h. Then, $(i\text{-}Pr_2N)_2POCH_2CH_2CN$ (0.14 ml, 0.43 mmol) was added and the mixture was further stirred for 6 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [hexane/AcOEt (1:2, v/v)] and by the reprecipitation from hexane/AcOEt to give compound 9^C (319 mg, 81%). White powder; mp 97–101 °C. ^{31}P NMR (acetone-d_6) δ 151.58, 151.65. MS (FAB): m/z 892 (MH^+). High-resolution MS (FAB): calcd for $C_{48}H_{55}N_5O_{10}P$ (MH^+): 892.3687. Found: 892.3688.

4.16. 3′-*O*-Benzyl-5′-*O*-*tert*-butyldiphenylsily-5-methyl-2′-*O*,4′-*C*-(methylenoxymethylene)cytidine (10)

Under N_2 atmosphere, $POCl_3$ (1.09 g, 7.15 mmol) was added dropwise to a suspension of 1,2,4-1H-triazole (1.98 g, 28.6 mmol) in anhydrous MeCN (20 ml) at 0 °C and the mixture was vigorously stirred at 0 °C for 10 min. Et_3N (2.89 g, 26.6 mmol) was added dropwise to the mixture and the mixture was further stirred at 0 °C for 35 min. Then, a solution of compound 5^T (300 mg, 0.48 mmol) in anhydrous MeCN (10 ml) was added dropwise and the mixture was stirred at room

temperature for 5.5 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. To the residue 28% aq NH_3/1,4-dioxane (1:6, v/v, 11 ml) was added and the mixture was stirred at room temperature for 2 h. After removal of the solvent under reduced pressure, the residue was purified by flash silica gel column chromatography [AcOEt/MeOH (10:1, v/v)] and by the reprecipitation from hexane/AcOEt to give compound **10** (246 mg, 82%). White powder; mp 63–66 °C. $[\alpha]_D^{23}$ +58.1 (*c* 1.00, $CHCl_3$). IR ν_{max} (KBr): 3072, 1658, 1602, 1483, 1178, 1121, 1080 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.09 (9H, s), 1.50 (3H,s), 3.67, 3.76 (2H, AB, J = 12 Hz), 3.76, 3.97 (2H, AB, J = 11 Hz), 4.46 (1H, d, J = 6 Hz), 4.52, 4.80 (2H, AB, J = 11 Hz), 4.56 (1H, d, J = 6 Hz), 5.26, 5.32 (2H, AB, J = 6 Hz), 6.22 (1H, s), 7.27–7.47 (10H, m), 7.59–7.68 (5H, m), 8.17 (1H, s). ^{13}C NMR ($CDCl_3$) δ 12.7, 19.5, 27.1, 62.2, 71.3, 72.4, 74.8, 76.7, 89.0, 93.2, 95.8, 101.9, 127.7, 127.9, 127.9, 128.1, 128.4, 129.9, 130.0, 132.0, 132.8, 135.0, 135.2, 136.7, 137.1, 155.9, 165.8. MS (FAB): *m*/*z* 628 (MH^+). High-resolution MS (FAB): calcd for $C_{35}H_{42}N_3O_6Si$ (MH^+): 628.2843. Found: 628.2861.

4.17. 4-*N*-Benzoyl-3′-*O*-benzyl-5′-*O*-*tert*-butyldiphenylsily-5-methyl-2′-*O*,4′-*C*-(methylenoxymethylene)cytidine (11)

Under N_2 atmosphere, BzCl (1.59 g, 11.3 mmol) was added to a solution of compound **10** (2.37 g, 3.77 mmol) in anhydrous pyridine (20 ml) at room temperature and the mixture was stirred at room temperature for 3.5 h. After addition of aq NH_3, the mixture was further stirred at room temperature for 1 h. After removal of the solvent under reduced pressure, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [hexane/AcOEt (3:1, v/v)] to give compound **11** (2.40 g, 87%). White powder; mp 56–58 °C. $[\alpha]_D^{21}$ +101.4 (*c* 1.09, $CHCl_3$). IR ν_{max} (KBr): 3069, 1700, 1647, 1571, 1329, 1270, 1174, 1129 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.11 (9H, s), 1.69 (3H, s), 3.67, 3.78 (2H, AB, J = 12 Hz), 3.76, 3.96 (2H, AB, J = 12 Hz), 4.53 (1H, d, J = 6 Hz), 4.57 (1H, d, J = 6 Hz), 4.58, 4.83 (2H, AB, J = 11 Hz), 5.25, 5.33 (2H, AB, J = 6 Hz), 6.23 (1H, s), 7.30–7.48 (14H, m), 7.60–7.68 (5H, m), 8.27–8.30 (2H, m). ^{13}C NMR ($CDCl_3$) δ 13.1, 19.5, 27.0, 62.0, 71.3, 72.5, 74.9, 76.4, 89.2, 93.3, 95.9, 111.2, 127.7, 127.8, 127.8, 127.9, 128.1, 128.4, 129.6, 129.9, 130.0, 131.9, 132.2, 132.6, 135.0, 135.2, 136.3, 136.5, 136.9, 147.4, 159.6, 179.0. MS (FAB): *m*/*z* 732 (MH^+). Anal. Calcd for $C_{42}H_{45}N_3O_7Si$: C, 68.92; H, 6.20; N, 5.74. Found: C, 68.72; H, 6.46; N, 5.54.

4.18. 4-*N*-Benzoyl-3′-*O*-benzyl-5-methyl-2′-*O*,4′-*C*-(methyleneoxymethylene)cytidine (12)

Under N_2 atmosphere, TBAF (1 M solution in THF, 1.9 ml, 1.90 mmol) was added to a solution of compound **11** (1.00 g, 1.37 mmol) in anhydrous THF (20 ml) at room temperature and the mixture was stirred at room temperature for 10 h. After removal of the solvent under reduced pressure, the residue was purified by flash silica gel column chromatography [$CHCl_3$/MeOH (60:1, v/v)] to give compound **12** (631 mg, 97%). White powder; mp 255–257 °C. $[\alpha]_D^{23}$ +159.0 (*c* 1.00, DMSO). IR ν_{max} (KBr): 3399, 3083, 1677, 1641, 1566, 1320, 1278, 1174, 1122 cm^{-1}. ^1H NMR (DMSO-d_6) δ 2.02 (3H,s), 3.58, 3.70 (2H, AB, J = 12 Hz), 3.59, 3.70 (2H, AB, J = 12 Hz), 4.42 (1H, d, J = 5 Hz), 4.61 (1H, d, J = 5 Hz), 4.64, 4.71 (2H, AB, J = 12 Hz), 5.11, 5.20 (2H, AB, J = 6 Hz), 5.67 (1H, br s), 5.97 (1H, s), 7.28–7.36 (5H, m), 7.47–7.62 (3H, m), 8.18 (2H, d, J = 8 Hz), 8.27 (1H, s), 12.90 (1H, br s). ^{13}C NMR (DMSO-d_6) δ 13.2, 58.7, 70.3, 71.6, 74.3, 75.6, 88.8, 91.3, 94.4, 108.6, 127.1, 127.4, 128.0, 128.1, 128.9, 132.2, 136.1, 137.4, 138.8, 147.9, 159.9, 176.2. MS (FAB): *m*/*z* 494 (MH^+). Anal. Calcd for $C_{26}H_{27}O_7N_3 \cdot \frac{1}{5}H_2O$: C, 62.82; H, 5.56; N, 8.45. Found: C, 62.86; H, 5.56; N, 8.44.

4.19. 4-*N*-Benzoyl-5-methyl-2′-*O*,4′-*C*-(methylenoxymethylene)cytidine (13)

Under N_2 atmosphere 20% $Pd(OH)_2$-C (800 mg) and cyclohexene (4 ml) were added to a solution of compound **12** (216 mg, 0.44 mmol) in anhydrous THF (20 ml) at room temperature and the mixture was refluxed for 4 h. After filtration of the mixture, the filtrate was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [$CHCl_3$/THF (8:1, v/v)] to give compound **13** (121 mg, 68%). Colorless crystals; mp 204–206 °C. $[\alpha]_D^{23}$ +87.9 (*c* 0.64, DMSO). IR ν_{max} (KBr): 3426, 3072, 1678, 1572, 1273, 1119 cm^{-1}. ^1H NMR (C_5D_5N) δ 1.93 (3H, s), 3.98, 4.07 (2H, AB, J = 12 Hz), 4.09, 4.18 (2H, ABX, J = 4, 12 Hz), 4.86 (1H, dd, J = 5, 6 Hz), 5.29 (1H, br s), 5.44, 5.82 (2H, AB, J = 6 Hz), 6.69 (1H, d, J = 3 Hz), 7.43–7.61 (4H, m), 8.46 (1H, br s), 8.55 (2H, dd, J = 1, 8 Hz), 8.66 (1H, s), 13.6 (1H, br s). ^{13}C NMR (C_5D_5N) δ 13.5, 59.9, 68.2, 71.5, 80.3, 90.8, 92.8, 95.8, 110.1, 128.5, 130.3, 132.6, 138.1, 138.9, 148.2, 160.4, 179.1. MS (FAB): *m*/*z* 404 (MH^+). Anal. Calcd for $C_{19}H_{21}N_3O_7 \cdot \frac{1}{5}H_2O$: C, 56.07; H, 5.30; N, 10.32. Found: C, 56.01; H, 5.27; N, 10.11.

4.20. 4-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-5-methyl-2′-*O*,4′-*C*-(methylenoxymethylene)cytidine (14)

Under N_2 atmosphere, DMTrCl (55 mg, 0.16 mmol) was added to a solution of compound **13** (50 mg, 0.12 mmol) in anhydrous pyridine (3 ml) at room temperature and the mixture was stirred at room temperature for 12 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [$CHCl_3$/MeOH (50:1, v/v)] to give compound **14** (81 mg, 93%). White powder; mp 123–126 °C. $[\alpha]_D^{20}$ +19.0 (*c* 1.04, $CHCl_3$). IR ν_{max} (KBr): 3267, 3067, 1700, 1642, 1566, 1254, 1177, 1120 cm^{-1}. ^1H NMR (acetone-d_6) δ 1.62 (3H, d, J = 1 Hz), 3.38, 3.46 (2H, AB,

J = 11 Hz), 3.71, 3.85 (2H, AB, J = 12 Hz), 3.79 (6H, s), 4.45 (1H, d, J = 6 Hz), 4.96 (1H, dd, J = 5, 6 Hz), 5.09, 5.31 (2H, AB, J = 6 Hz), 5.41 (1H, d, J = 5 Hz), 6.16 (1H, s), 6.89–6.93 (4H, m), 7.23–7.55 (12H, m), 7.95 (1H, d, J = 1 Hz), 8.26–8.30 (2H, m), 8.56 (1H, br s). ^{13}C NMR (C_5D_5N) δ 13.2, 55.5, 63.1, 70.4, 72.0, 79.7, 87.5, 89.8, 93.2, 96.0, 111.0, 114.0, 127.8, 127.8, 128.9, 129.0, 130.5, 131.1, 133.1, 136.1, 136.3, 138.3, 138.7, 145.7, 148.3, 150.6, 159.7, 159.7, 161.5, 179.9. MS (FAB): m/z 706 (MH^+). Anal. Calcd for $C_{40}H_{39}N_3O_9\cdot\frac{1}{2}H_2O$: C, 67.22; H, 5.64; N, 5.89. Found: C, 67.20; H, 5.58; N, 5.79.

4.21. 4-*N*-Benzoyl-3′-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-5-methyl-2′-*O*,4′-*C*-(methylenoxymethylene)cytidine (15)

Under N_2 atmosphere, (i-Pr_2N)$_2$POCH$_2$CH$_2$CN (45 μl, 0.14 mmol) was added to a solution of compound **14** (50 mg, 71 μl), 5-ethylthio-1*H*-tetrazole (14 mg, 0.11 mmol) in anhydrous MeCN (10 ml) at room temperature and the mixture was stirred at room temperature for 6 h. Then, (i-Pr_2N)$_2$POCH$_2$CH$_2$CN (23 μl, 71 μmol) was added and the mixture was further stirred for 6 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [hexane/AcOEt (3:1, v/v)] and by the reprecipitation from hexane/AcOEt to give compound **15** (61 mg, 95%). White powder; mp 65–68 °C. ^{31}P NMR (acetone-d_6) δ 149.5, 150.9. MS (FAB): m/z 906 (MH^+). High-resolution MS (FAB): calcd for $C_{49}H_{57}N_5O_{10}P$ (MH^+): 906.3843. Found: 906.3844.

4.22. Synthesis of oligonucleotides 16–20

Synthesis of oligonucleotides **16–20** was performed in 0.2 μmol scale on an automated DNA synthesizer (Applied Biosystems, Expedite® 8909) using the standard phosphoramidite protocol. The oligonucleotide synthesis was performed on DMTr-ON mode. Cleavage from the CPG support and removal of the protecting groups were accomplished by using 28% ammonia solution (55 °C for 20 h). The crude oligonucleotides bearing a DMTr group were detritylated and purified with Dupon Nensorb™ Prep according to the manufacturer's protocol. The obtained oligonucleotides were again purified by reversed-phase HPLC (Wako Wakopak® WS-DNA, 10 mm × 250 mm). The composition of the oligonucleotides was confirmed by MALDI-TOF-MS analysis. MALDI-TOF-MS data ([M−H]$^-$) for oligonucleotides **16–20**: **16**, found 4250.17 (calcd 4249.89); **17**, found 4366.38 (calcd 4365.97); **18**, found 4366.89 (calcd 4365.97); **19**, found 4481.12 (calcd 4482.04); **20**, found 3036.39 (calcd 3037.04).

4.23. T_m measurements

The UV melting experiments were carried out on a Beckman DU-650 spectrophotometer equipped with T_m analysis accessory using quartz cuvettes of 1 cm optical path length. The UV melting profiles were recorded in 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl and 10 mM $MgCl_2$ for triplexes and in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl for duplexes at a scan rate of 0.5 °C/min with detection at 260 nm. The final concentration of each oligonucleotide was 1.5 μM for triplexes and 4 μM for duplexes. The melting temperatures were obtained as the maxima of the first derivative of the melting curves.

4.24. Degradation of oligonucleotides by SVPDE

SVPDE (0.2 μg) was added to a solution of an oligonucleotide (5 nmol) in 50 mM Tris·HCl buffer (pH 8.0, 350 μl) containing 10 mM $MgCl_2$. The reaction was carried out at 37 °C. After certain periods of time (7, 10, 20, 40, and 90 min) passed, part of the mixture was heated to 90 °C for 2 min because of inactivation of SVPDE. The amount of the intact oligonucleotides was evaluated by reversed-phase HPLC (Wako Wakopak® WS-DNA, 4.6 mm × 250 mm) analysis.

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