Synthesis and Properties of Oligodeoxynucleotides Possessing *N*-Phosphoryl Groups

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This paper deals with the synthesis and properties of oligodeoxynucleotides of a Dickerson–Drew sequence, d(CGCGAATTCGCG), in which one of the four cytosine bases has been replaced with an *N*-phosphorylated or *N*-diethylphosphorylated cytosine base in order to study the effect of the *N*-phosphoryl or *N*-diethoxyphosphoryl group on their structural property and hybridization affinity. The CD spectra of these self-complementary duplexes showed the typical B DNA-type shape with very minor changes. In contrast, it was found from T_m experiments that *N*-phosphoryl groups destabilized the DNA duplexes significantly through steric hindrance and electronic repulsion.

Introduction

A number of nucleoside phosphate derivatives exist in living cells, playing essential roles in biological reactions as structural elements of DNA and RNA, phosphate donors in transphosphorylation, and second messengers. In contrast, *N*-phosphorylated nucleosides have not been discovered, except for the 6-*N*-phosphorylated adenine derivative agrocin 84.[1−5] In agrocin 84, *N*-phosphorylation is required for permeation of agrocin 84 into the target cells.[3] Therefore, if in nature the nucleobase can be phosphorylated by some biological process, there is a possibility that the presence of a phosphoryl residue attached to the amino group has a certain biological meaning. We have recently reported the synthesis of *N*-phosphorylated ribonucleosides, phosphorylation being at the *exo*-amino groups of adenosine, cytidine, and guanosine.[6]

It is known that 4-*N*-acetylcytidine (ac^4C), found as a minor modified nucleoside in tRNA, has an electron-withdrawing acetyl group on its amino moiety.[7] We have also reported that 4-*N*-acetyldeoxycytidine can form a stable base pair with deoxyguanosine upon incorporation into DNA in place of deoxycytidine.[8] It might therefore be expected that *N*-phosphorylated deoxycytidine derivatives should have hybridization affinities similar to that of *N*-acyldeoxycytidine when incorporated into DNA. We had previously reported that *N*-phosphorylated cytidine derivatives are fairly stable in comparison with *N*-phosphorylated adenosine and guanosine derivatives. To synthesize oligodeoxynucleotides incorporating *N*-phosphorylated deoxycytidine derivatives, an appropriately protected *N*-phosphorylated deoxycytidine 3′-phosphoramidite unit was required as a building block.

Here we report the synthesis and hybridization properties of oligodeoxynucleotides incorporating *N*-phosphorylated deoxynucleoside derivatives.

Results and Discussion

Thermal Stability of *N*-Phosphorylated Ribonucleoside Derivatives

It is well known that phosphate monoesters are hydrolyzed under thermal conditions, via metaphosphate intermediates.[9−20] This dephosphorylation is accelerated by neighboring group participation by proximal phosphate esters, or by addition of divalent metal cations.[21] It seems that the *N*-phosphorylated ribonucleosides are also hydrolyzed by simple heating, to give the parent corresponding nucleosides. We therefore studied the thermal stabilities of *N*-phosphorylated ribonucleosides **1**−**5** (Figure 1). Compounds **1**−**5** were dissolved in 0.1 M ammonium acetate buffer (pH = 7.0), and these solutions were heated at 90 °C. The products were analyzed by reversed-phase HPLC; the results are summarized in Table 1. The hydrolysis of the *N*-phosphorylated nucleoside derivatives **1**−**3** occurred extremely rapidly and was complete within 10 min in the cases of all three nucleoside derivatives. The rates of hydrolysis of these *N*-phosphorylated nucleoside derivatives **1**−**3** could not be determined because of the extremely fast hydrolysis, so the rate of the hydrolysis at 70 °C was measured in the same solutions. No obvious difference in reaction rate between the nucleosides was observed, the hydrolysis reaction appearing to proceed through metaphosphate intermediates and the rates of formation of the metaphosphate intermediates therefore being very similar in these *N*-phosphorylated

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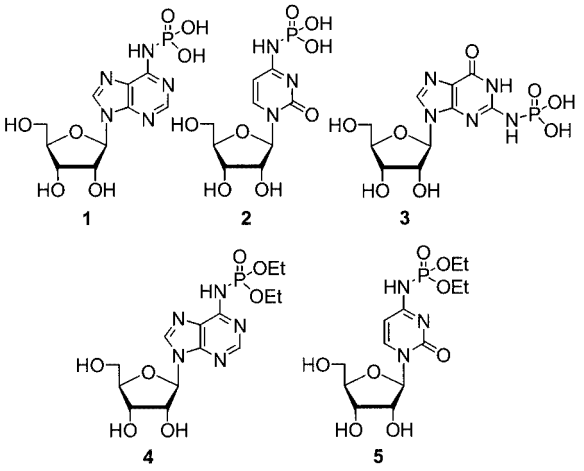


Figure 1. *N*-Phosphorylated ribonucleoside derivatives

Table 1. The hydrolysis of *N*-phosphorylated ribonucleosides

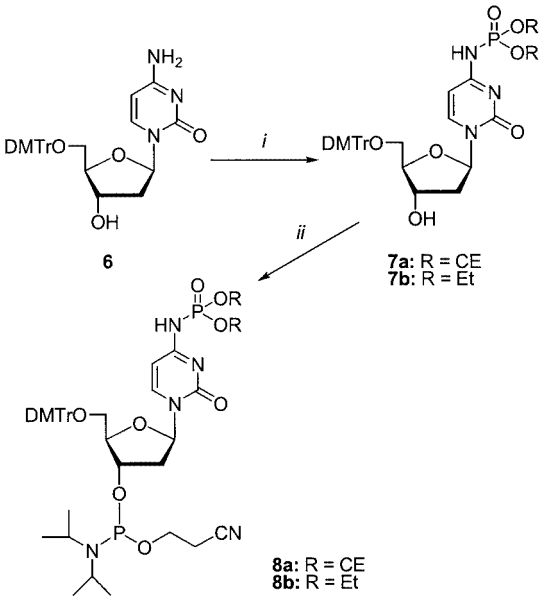
	90 °C		70 °C		
	$t_{1/2}$	t_{comp}	$t_{1/2}$	t_{comp}	k [10^{-4} s^{-1}]
1	–	< 10 min	–	60 min	9.8[a]
2	–	< 10 min	–	60 min	9.8[a]
3	–	< 10 min	–	60 min	11[a]
4	18 h	70 h	–	–	0.12[b]
5	15 h	60 h	–	–	0.18[b]

[a] 70 °C. [b] 90 °C.

nucleosides **1**–**3**. In contrast, the diethyl ester derivatives **4** and **5** were relatively stable and so were hydrolyzed much more slowly than the *N*-phosphoryl derivatives **1**–**3**, the half-life times of the hydrolysis being 15 and 18 h for the adenosine and cytidine derivatives **4** and **5**, respectively. It is obvious that there is a difference in the mechanisms of hydrolysis of non-esterified and esterified *N*-phosphoramidate derivatives. In the case of the diethyl ester derivatives, the hydrolysis proceeded without the formation of the intermediate metaphosphate.

Synthesis of Deoxycytidine Phosphoramidite Units Containing *N*-Phosphoryl Groups

To synthesize oligodeoxynucleotides containing *N*-phosphorylated deoxycytidine, the phosphoramidite derivative of *N*-phosphorylated deoxycytidine was prepared. The phosphorylation of the amino group of deoxycytidine was carried out by a method similar to that described previously in the synthesis of *N*-phosphorylated ribonucleosides. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-deoxycytidine (**6**) was heated at reflux with hexamethyldisilazane in MeCN/pyridine for 30 min, and the resulting *O*-silylated species was allowed to react with bis(2-cyanoethyl) *N*,*N*-diisopropylphosphoramidite in the presence of 1*H*-tetrazole as a promote



Scheme 1. (*i*) (**7a**) (a) HMDS, CH_3CN, pyridine reflux, 1 h; (b) $(iPr)_2NP(OCH_2CH_2CN)_2$, 1*H*-tetrazole, CH_3CN, room temp., 1 h; (c) *t*BuOOH, room temp., 15 min; (**7b**) (a) TMSCl, Et_3N, CH_2Cl_2, room temp., 30 min; (b) $(EtO)_2PCl$, CH_2Cl_2, room temp., 30 min; (c) *t*BuOOH, CH_2Cl_2, room temp., 15 min; (*ii*) $ClP(OCH_2CH_2CN)(NiPr_2)$, Et_3N, CH_2Cl_2, room temp., 30 min (**8a**), 1 h (**8b**); CE = 2-cyanoethyl

(Scheme 1). The subsequent oxidation of the trivalent phosphite intermediate with *tert*-butyl hydroperoxide gave the *N*-phosphorylated deoxycytidine derivative **7a** in 37% yield. The phosphoramidite unit **8a** was obtained in 82% yield by the standard method.

Next, the diethyl ester derivative **8b** was synthesized by an alternative strategy. Firstly, the 3′-OH and amino groups were trimethylsilylated by treatment with chlorotrimethylsilane in pyridine. Phosphitylation of the amino group was performed in pyridine through the use of a highly reactive reagent, diethyl phosphorochloridite. When *tert*-butyl hydroperoxide was used as the oxidizing agent in the following oxidation step, however, the *N*-phosphoryl group was found to be hydrolyzed completely. Since it had been found that pyridine promoted the decomposition of the phosphite intermediate, CH_2Cl_2 was used as a solvent and triethylamine was additionally used as a base at the *N*-phosphitylation. As the 3′-*O*-trimethylsilyl group was relatively stable during the usual workup, the resulting mixture was further treated with MeOH/H_2O/pyridine (8:1:1, v/v/v) to remove the trimethylsilyl group. The *N*-phosphorylated derivative **7b** was thus obtained in 74% yield. The 3′-*O*-phosphitylation of **7b** by the standard method gave **8b** in 52% yield.

The synthesis of deoxyadenosine derivatives containing an *N*-diethoxyphosphoryl group by the same procedure as described in the case of deoxycytidine derivatives was attempted. Phosphorylation by the phosphoramidite reagent indeed gave the corresponding *N*-diethoxyphosphoryl derivative, but this *N*-(phosphoryl)deoxyadenosine derivative was found to be unstable and decomposed during purification by silica gel column chromatography. It was also found that the *N*-[bis(2-cyanoethoxy)phosphoryl]deoxy-

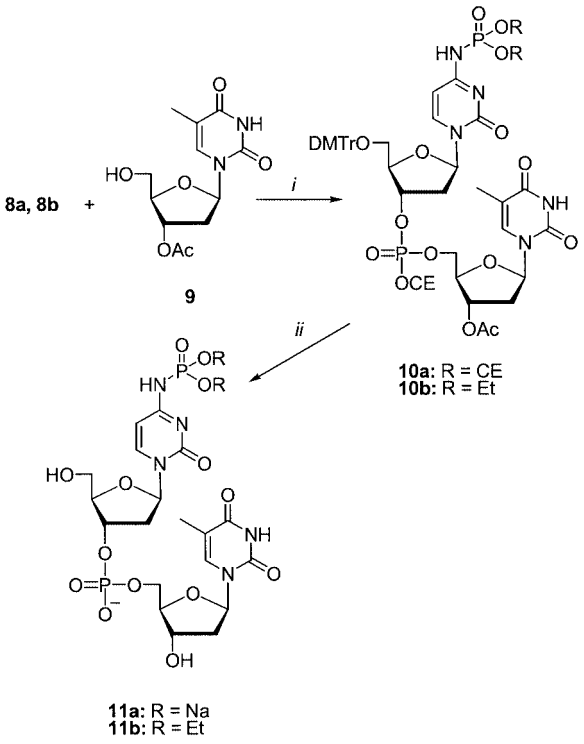
adenosine derivative decomposed during chromatographic separation. In addition, an non-esterified species of *N*-(phosphoryl)deoxyadenosine exhibited instability in aqueous solution. These modified phosphorylated deoxyadenosine building blocks were therefore not introduced into oligodeoxynucleotides.

Synthesis and Properties of dC*pT Dimers Containing *N*-Phosphorylated Deoxycytidine Derivatives

To study the chemical properties of the *N*-phosphoryl group in oligodeoxynucleotides, the deoxycytidyl(5′-3′)thymidine dimers d[C*pT] (**11a**) and d[C**pT] (**11b**), containing *N*-phosphoryl and *N*-diethoxyphosphoryl groups, were synthesized by the liquid-phase method. Deoxycytidinyl phosphoramidite units **8a** and **8b** were condensed with 3′-*O*-acetylthymidine (**9**) in the presence of 1*H*-tetrazole to give the fully protected dimers **10a** and **10b** in satisfactory yields Scheme 2). Deprotection of the fully protected dimers **10a** and **10b** was performed by the standard procedure to give d[C*pT] **11a** and d[C**pT] **11b**, where * refers to the phosphoryl or diethoxyphosphoryl group.

Some chemical properties of **11a** and **11b** were studied by use of ^{1}H NMR and CD spectroscopy. We have previously reported that the *N*-phosphoryl moieties of *N*-phosphorylated ribonucleosides acted as electron-withdrawing groups. It turned out that the 5-H and 6-H protons of the *N*-phosphorylated deoxycytidine derivatives are shifted to lower magnetic field than those of cytidine. It has also been reported that low magnetic field shifts of these base protons were observed when electron-withdrawing groups such as acetyl and benzoyl groups were introduced onto the amino function of the cytosine moiety. In the case of d(C*pT) dimer **11a**, it was also observed that the protons of the base moiety were shifted to low magnetic field at the same level.

Next, in order to study the effect of the *N*-phosphoryl groups on the structure, the CD spectra of these dimers were measured at pH = 7.0 at 0, 20 and 60 °C. The CD spectra of these dimers are shown in Figure 2. All three showed large positive Cotton effects around 280 nm and large negative Cotton effects around 220 nm. The intensity of the positive Cotton effect at 0 °C in the case of **11a** and **11b** was apparently lower than in that of d(CpT). However, with an increase in temperature to 60 °C, the CD spectra of all three derivatives became more similar to one another. These results suggest that the base-base stacking interaction



Scheme 2. (*i*) (a) 1*H*-tetrazole, CH_3CN, room temp., 30 min; (b) *t*BuOOH, CH_3CN, room temp., 15 min. (*ii*) (a) concd. NH_3/pyridine (3:1), room temp., 12 h, (b) 80% AcOH, room temp., 30 min; CE = 2-cyanoethyl

is weakened at 0 °C, due to steric hindrance around the *N*-phosphorylated site in the case of **11a** and **11b**, and that the contribution of the base-base stacking interaction to CD disappears at 60 °C.

Synthesis and Properties of Oligodeoxynucleotides Containing *N*-Phosphorylated Deoxycytidine Derivatives

In order to study the thermal properties of the *N*-phosphoryl function, oligodeoxynucleotides containing *N*-(phosphoryl)deoxycytidine were prepared. For this study, the Dickerson−Drew sequence of the dodecadeoxynucleotide[22] d(CGCGAATTCGCG)$_2$ was chosen. The structures of many variants of this sequence, containing either matched or severely mismatched base pairs, have been studied extensively by X-ray crystallography, NMR spectroscopy, and T_m experiments. We therefore decided to use

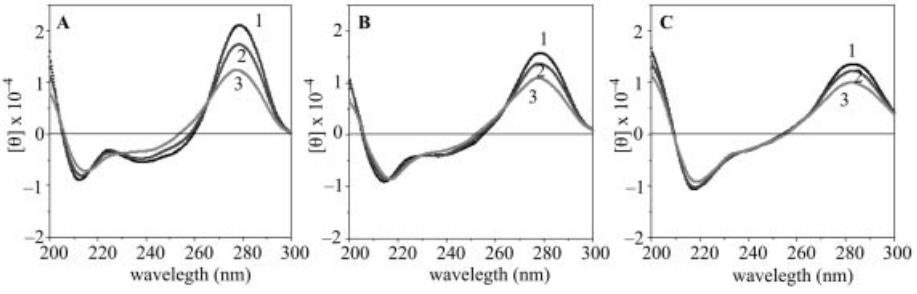


Figure 2. CD spectra of d(C*pT) dimers observed at various temperatures: 1: 0 °C; 2: 20 °C; 3: 60 °C; **A**: d(CpT); **B**: d(C*pT) (**11a**); **C**: d(C**pT) (**11b**)

this well-known dodecamer in this study, and so the *N*-(phosphoryl)deoxycytidine was introduced into the four sites of deoxycytidine. Since the *N*-phosphoryl group is unstable under thermal conditions, the deprotection procedure had to be carried out at room temperature, so the easily removable dimethylformamidine group was selected as the protecting group for the guanine moiety. The synthesis of the oligodeoxynucleotides containing the *N*-phosphorylated deoxycytidine was performed by the solid-phase method on an automated synthesizer. The coupling efficiencies at the *N*-(phosphoryl)deoxycytidine were similar to those of other canonical nucleoside derivatives, and the average coupling yields and isolated yields of all oligodeoxynucleotides are shown in Table 2. These modified oligodeoxynucleotides were purified by use of Sep−Pak cartridges with the DMTr-on mode and isolated by HPLC after detritylation by treatment with 2% TFA. The isolated yields of oligodeoxynucleotides **12**−**20** were 15−37%. The structures of these modified oligodeoxynucleotides were confirmed by MALDI-TOF mass spectrometry.

Table 2. The average yields and isolated yields of DNA 12mers containing *N*-phosphorylated deoxycytidine derivatives

	Sequences (5′-3′)	Average yield (%)	Isolated yield (%)
12	d(CGCGAATTCGCG)	99.5	28
13	d(**C***GCGAATTCGCG)	99.2	15
14	d(CG**C***GAATTCGCG)	98.5	25
15	d(CGCGAATT**C***GCG)	98.5	37
16	d(CGCGAATTCG**C***G)	99.4	22
17	d(**C****GCGAATTCGCG)	99.2	15
18	d(CG**C****GAATTCGCG)	99.3	30
19	d(CGCGAATT**C****GCG)	98.7	19
20	d(CGCGAATTCG**C****G)	99.9	30

In order to elucidate the global structures of the oligodeoxynucleotides **12**−**20**, their CD spectra were observed at 20 °C (Figure 3).

All the oligodeoxynucleotides showed typical CD spectra, with positive Cotton effects around 280 nm and negative Cotton effects around 250 nm, indicating the formation of B-type duplexes. A slight difference in the intensity of the Cotton effect of the unmodified oligodeoxynucleotide **12** was observed. This suggests that the presence of *N*-phosphoryl groups attached to the amino group of 2′-deoxycytidine residues might slightly affect the conformation of the duplexes.

Next, the thermal stabilities of the modified duplexes were investigated by the melting temperature. The UV melting curves of all the duplexes exhibited two-state profiles with shapes similar to those of the unmodified duplexes (Figure 4). The T_m values are listed in Table 3, and those of the DNA duplexes were dramatically lower than that of the unmodified DNA duplex. After addition of the *N*-diethoxyphosphoryl group, the T_m value dropped by approximately 5 °C. In general, introduction of a steric hindered group on an amino group of a cytosine moiety causes destabilization

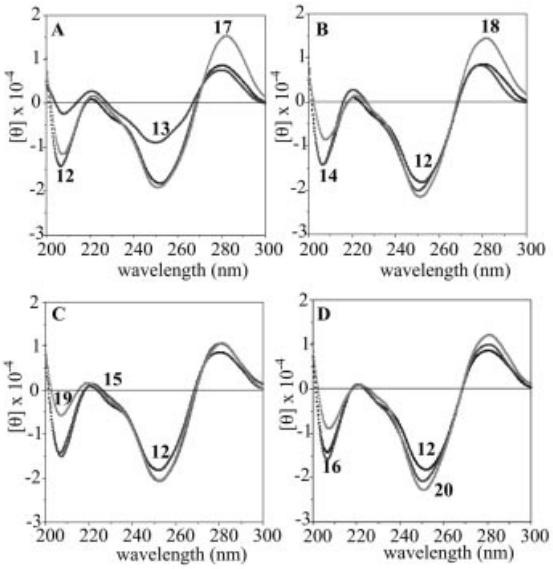


Figure 3. The CD spectra of oligodeoxynucleotides containing *N*-phosphorylated deoxycytidine derivatives: **A**: d(**C**GCGAATTCGCG); **B**: d(CG**C**GAATTCGCG); **C**: d(CGCGAATT**C**GCG) **D**: d(CGCGAATTCG**C**G)

of DNA duplexes,[23−26] and similar results were observed in our experiments. This large destabilization affect was obviously attributable to the introduction of the *N*-diethoxyphosphoryl group. A change of the *N*-diethoxyphosphoryl group to the *N*-phosphoryl group caused a more drastic destabilization effect, as shown in Table 3. Since the *N*-phosphoryl group retains negative charges, a significant electrostatic effect destabilizing the duplex was also to be expected. The decrease in the T_m value per modification by the introduction of this negatively charged phosphoryl group is approximately 3 °C.

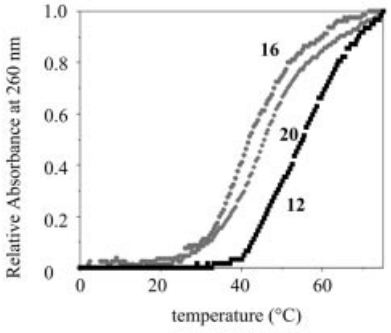


Figure 4. Melting temperatures of oligodeoxynucleotides containing *N*-phosphorylated deoxycytidine derivatives

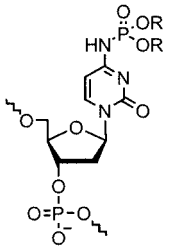
Conclusion

In order to elucidate the chemical properties of the *N*-phosphoryl and *N*-diethoxyphosphoryl group in DNA, oligodeoxynucleotides containing *N*-phosphorylated deoxycytidine derivatives were synthesized by the phosphoramidite approach. Some chemical properties of *N*-phosphoryl groups included in oligodeoxynucleotides were revealed by

Table 3. The T_m values of oligodeoxynucleotides containing *N*-phosphorylated deoxycytidine derivatives

		T_m [°C]	ΔT_m [°C]
12	d(CGCGAATTCGCG)	56.5	
16	d(CGCGAATTCGC*G)	39.5	–17.0
20	d(CGCGAATTCGC**G)	45.7	–10.8

C* : R = H, C**: R = Et.



the spectroscopic experiments. The CD spectra showed slight changes in the global structure of modified forms of DNA from that of the canonical B-type DNA structure. Tm experiments, however, showed that drastic destabilization occurred on the introduction of the phosphoryl groups. This destabilization seems to be caused by the two factors: steric effects and the electrostatic repulsion of the phosphoryl groups.

Experimental Section

General Remarks: CH_2Cl_2 and MeCN were distilled from CaH_2 after being heated at reflux for several hours, and were stored over molecular sieves (4 Å). Pyridine was distilled after being heated at reflux in the presence of *p*-toluenesulfonyl chloride for several hours, redistilled from CaH_2, and stored over molecular sieves (4 Å). *tert*-Butyl hydroperoxide (containing 20% di-*tert*-butyl peroxide) was purchased from Merck & Co. Inc. Bis(2-cyanoethoxy)(-diisopropylamino)phosphane was synthesized by a previously reported method.[27] ^{1}H NMR spectra were obtained at 270 MHz with a JEOL-EX-270 spectrometer with tetramethylsilane as an internal standard in $CDCl_3$ and with sodium 3-(trimethylsilyl)propanesulfonate (DSS) as an external standard in D_2O. ^{13}C NMR spectra were obtained at 67.8 MHz with a JEOL-EX-270 spectrometer with tetramethylsilane as an internal standard and with DSS as an external standard in D_2O. ^{31}P NMR spectra were obtained at 109.25 MHz with a JEOL-EX-270 spectrometer, with 85% H_3PO_4 as an external standard. UV spectra were recorded with a Hitachi 220A spectrophotometer. Thin layer chromatography was performed on precoated glass plates of Kieselgel 60 F_{254} (Merck, no. 5715). Silica gel column chromatography was carried out on Wakogel C-200. Reversed-phase column chromatography was performed with Waters μBondapak C18. MALDI-TOF mass spectra were obtained with a Voyager™ spectrometer (PerSeptive Biosystems). CE = 2-cyanoethyl.

***N*^{4}-Bis(2-cyanoethoxy)phosphoryl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)cytidine (7a):** 2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)cytidine (**6**) (1.06 g, 2.0 mmol) was dissolved in dry pyridine (10 mL)/dry MeCN (10 mL). Hexamethyldisilazane (1.27 mL, 6.0 mmol) was added to this solution, and the mixture was heated at reflux for 1 h. The reaction mixture was allowed to cool to room temp. and concentrated to give a colorless foam. The resulting foam was dissolved in dry MeCN (20 mL), and bis(2-cyanoethoxy)(diisopropylamino)phosphane (814 mg, 3.0 mmol) and 1*H*-tetrazole (280 mg, 4.0 mmol) were added. After the mixture had been stirred at room temp. for 1 h, *tert*-butyl hydroperoxide (containing 20% di-*tert*-butyl peroxide, 1.25 μL, 10.0 mmol) was added, and the mixture was stirred at room temp. for 15 min. It was concentrated to a small volume, diluted with $CHCl_3$, and washed three times with 5% $NaHCO_3$ (aq.), and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and washings were combined, dried with Na_2SO_4, and filtered, and the solvents were evaporated to dryness. The residue was placed on a silica gel column. Column chromatography with CH_2Cl_2 containing 0.5% pyridine, with a gradient of methanol (3−3.5%), gave **7a** (452 mg, 32%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 2.21−2.31 (m, 1 H, 2"-H), 2.44−2.53 (m, 1 H, 2′-H), 2.75 (t, J = 6.3 Hz, 4 H, CH_2CN), 3.42 (dd, $J_{5'',4'}$ = 3.3, $J_{5'',5'}$ = 10.6 Hz, 1 H, 5"-H), 3.49 (dd, $J_{5',4'}$ = 3.0, $J_{5',5''}$ = 10.6 Hz, 1 H, 5′-H), 3.80 (s, 6 H, OCH_3 of DMTr), 4.03−4.06 (m, 1 H, 4′-H), 4.19−4.28 (m, 4 H, 2 × $POCH_2$), 4.52−4.57 (m, 1 H, 3′-H), 5.68 (d, $J_{5,6}$ = 7.9 Hz, 1 H, 5-H), 6.24 (dd, $J_{1',2'}$ = 6.3, $J_{1',2''}$ = 5.9 Hz, 1 H, 1′-H), 6.80−6.87 (d, J = 8.9 Hz, 4 H, *o*-H of DMTr), 7.13−7.40 (m, 9 H, ArH), 7.84 (dd, 1 H, $J_{6,5}$ = 7.9, J = 2.0 Hz, 6-H) ppm. ^{13}C NMR ($CDCl_3$): δ = 19.6 (d, $^3J_{POCC}$ = 6.4 Hz, CH_2CN), 41.3 (2′-C), 55.2 (OMe of DMTr), 61.2 (d, $^2J_{POC}$ = 3.7 Hz, $POCH_2$), 62.8 (5′-C), 71.0 (3′-C), 85.6, 86.2, 86.9 (1′-C, 4′-C and *tert*-C of DMTr), 101.4, 101.7 (5-C), 113.2, 116.8 (CN), 125.2−130.0, 135.2, 135.3 (1,1′-C of DMTr), 140.7 (1"-C of DMTr), 144.2 (6-C), 148.5, 158.6 (2-C), 160.2, 160.3 (4,4′-C of DMTr) ppm. ^{31}P NMR ($CDCl_3$): δ = 5.56 ppm. $C_{36}H_{38}N_5O_9P{\cdot}1.5H_2O$ (742.7): calcd. C 58.22, H 5.56, N 9.43; found calcd. C 57.80, H 5.75, N 9.79.

2′-Deoxy-*N*^{4}-diethoxyphosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-cytidine (7b): Compound **6** (530 mg, 1.0 mmol) was dried by repeated coevaporation with dry pyridine and dissolved in dry CH_2Cl_2 (10 mL). Trimethylsilyl chloride (381 μL, 3.0 mmol) and triethylamine (694 μL, 5.0 mmol) were added, and the resulting mixture was stirred at room temp. for 30 min. Diethyl phosphorochloridite (289 μL, 2.0 mmol) was added. After the mixture had then been stirred at room temp. for 30 min, *tert*-butyl hydroperoxide (containing 20% di-*tert*-butyl peroxide, 626 μL, 5.0 mmol) was added. The solution was stirred at room temp. for 15 min. The mixture was diluted with $CHCl_3$ and washed three times with 5% $NaHCO_3$ (aq.), and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and washings were combined, dried with Na_2SO_4, filtered, and concentrated to dryness. The residue was placed on a silica gel column. Column chromatography with CH_2Cl_2 containing 1% pyridine, with a gradient of methanol (2−3%), gave **7b** (494 mg, 74%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.32 (t, J = 7.3 Hz, 3 H, CH_3 of Et), 2.20−2.30 (m, 1 H, 2"-H), 2.43−2.53 (m, 1 H, 2"-H), 3.41 (dd, $J_{5'',4'}$ = 3.6, $J_{5'',5'}$ = 10.9 Hz, 1 H, 5"-H), 3.47 (dd, $J_{5',4'}$ = 3.3, $J_{5',5''}$ = 10.9 Hz, 1 H, 5′-H), 3.80 (s, 6 H, OCH_3 of DMTr), 4.02−4.13 (m, 5 H, 4′-H and 2 × $POCH_2$), 4.51−4.56 (m, 1 H, 3′-H), 5.67 (d, $J_{5,6}$ = 7.9 Hz, 1 H, 5-H), 6.26 (dd, $J_{1',2'}$ = 6.3, $J_{1',2''}$ = 5.9 Hz, 1 H, 1′-H), 6.84 (d, J = 8.9 Hz, 4 H, *o*-H of DMTr), 7.19−7.43 (m, 9 H, ArH), 7.77 (dd, 1 H, $J_{6,5}$ = 7.9, J = 1.7 Hz, 6-H) ppm. ^{13}C NMR ($CDCl_3$): δ = 16.1 (d, $^3J_{POCC}$ = 7.3 Hz, CH_3 of Et), 41.4 (2′-C), 55.2 (OCH_3 of DMTr), 62.8 (d, $^2J_{POC}$ = 7.3 Hz, CH_2 of Et and 5′-C), 71.0 (3′-C), 85.5, 86.1, 86.8 (1′-C, 4′-C and *tert*-C of DMTr), 101.7 (5-C), 113.2 (3,3′,5,5′-C of DMTr), 127.0, 128.0, 128.1, 130.0 (2,6-C of DMTr), 135.2, 135.4 (1,1′-C of DMTr), 140.2 (6-C), 144.3 (1"-C of DMTr), 149.2 (2-C), 158.6, 159.5 (4,4′-C of DMTr) ppm. ^{31}P NMR ($CDCl_3$): δ = 6.34 ppm. $C_{34}H_{40}N_3O_9P$ (665.7): calcd. C 61.35, H 6.06, N 6.31; found calcd. C 61.01, H 6.30, N 6.11.

***N*^{4}-Bis(2-cyanoethoxy)phosphoryl-2′-deoxy−5′-*O*-(4,4′-dimethoxytrityl)cytidin-3′-yl 2-Cyanoethyl-*N*,*N*-diisopropylphosphoramidite (8a):** Compound **7a** (451 mg, 0.63 mmol) was dried by repeated coevaporation with dry pyridine and then dry toluene, and was then dissolved in dry CH_2Cl_2 (6 mL). Chloro(2-cyanoethoxy)(diisoprop-

ylamino)phosphane (211 μL, 0.95 mmol) and *N,N*-diisopropylethylamine (220 μL, 1.26 mmol) were added, and the mixture was stirred at room temp. for 30 min. The mixture was diluted with $CHCl_3$ and washed three times with 5% $NaHCO_3$ (aq.), and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and washings were combined, dried with Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was placed on a silica gel column. Column chromatography with CH_2Cl_2 containing 1% pyridine, with a gradient of methanol (2−3%), gave **8a** (287 mg, 50%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.15−1.19 (m, 12 H, CH_3 of *i*Pr), 2.28−2.37 (m, 1 H, 2"-H), 2.46, 2.62 (2 t, *J* = 6.3 Hz, 2 H, CH_2CN), 2.52−2.65 (m, 1 H, 2′-H), 2.72−2.77 (2 t, *J* = 6.3 Hz, 4 H, 2 × CH_2CN), 3.40−3.83 (m, 6 H, $POCH_2$, 5′-H, 5"-H and 2 × CH of *i*Pr), 3.80, 3.80 (2 s, 6 H, OCH_3 of DMTr), 4.10−4.27 [m, 5 H, 2 × $P(O)OCH_2$, 4′-H], 4.67 (m, 1 H, 3′-H), 5.66−5.70 (m, 1 H, 5-H), 6.23−6.27 (m, 1 H, 1′-H), 8.83−8.87 (2 d, *J* = 8.9 Hz, 4 H, *o*-H of DMTr), 7.15−7.41 (m, 10 H, ArH), 7.84−7.92 (2 dd, $J_{6,5}$ = 7.9, *J* = 1.7 Hz,1 H, 6-H) ppm. ^{13}C NMR ($CDCl_3$): δ = 19.5 [d, $^3J_{POCC}$ = 7.3 Hz, β-C of P(O)OCE], 20.1, 20.3 (d, $^3J_{POCC}$ = 7.3 Hz, β-C of POCE), 24.3−24.5 (CH_3 of *i*Pr), 40.2−40.4 (2′-C), 43.0, 43.2 (d, $^2J_{PNC}$ = 2.4 Hz, CH of *i*Pr), 55.1 (OCH_3 of DMTr), 57.9, 58.1 (d, $^2J_{POC}$ = 4.9 Hz, $POCH_2$), 61.1 [d, $^2J_{POC}$ = 4.9 Hz, $P(O)OCH_2$], 62.1 (d, $^2J_{POC}$ = 7.3 Hz, 5′-C), 71.8−72.5 (3′-C), 85.1−86.8 (1′-C, 4′-C and *tert*-C of DMTr), 101.7, 102.0 (5-C), 113.1 (3,3′5,5′-C of DMTr), 116.7 [CN of P(O)OCE], 117.4, 117.6 (CN of POCE), 125.1−130.0 (2,6-C of DMTr), 134.9−135.2 (1,1′-C of DMTr), 140.4 (1"-C of DMTr), 144.0 (6-C), 148.1, 158.5 (2-C), 160.1 (4,4′-C of DMTr) ppm. ^{31}P NMR ($CDCl_3$): δ = 6.15, 6.17, 149.48, 149.69 ppm. $C_{45}H_{55}N_7O_{10}P_2{\cdot}H_2O$ (932.9): calcd. C 57.88, H 6.15, N 10.49; found calcd. C 57.15, H 5.98, N 10.25.

2′-Deoxy-*N*^{4}-diethoxyphosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-cytidin-3′-yl 2-Cyanoethyl-*N,N*-diisopropylphosphoramidite (8b): Compound **7b** (239 mg, 0.36 mmol) was dried by repeated coevaporation with dry pyridine and then dry toluene, and was then dissolved in dry CH_2Cl_2 (4 mL). Chloro(2-cyanoethoxy)(diisopropylamino)phosphane (120 μL, 0.54 mmol) and *N,N*-diisopropylethylamine (125 μL, 0.72 mmol) were added, and the mixture was stirred at room temp. for 1 h. The reaction mixture was diluted with $CHCl_3$ and washed three times with 5% $NaHCO_3$ (aq.), and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and washings were combined and dried with Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was placed on a silica gel column. Column chromatography with CH_2Cl_2 containing 1% pyridine, with a gradient of methanol (1−2%), gave **8b** (158 mg, 51%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.15−1.19 (m, 12 H, CH_3 of *i*Pr), 1.33 (t, *J* = 7.3 Hz, 3 H, CH_3 of Et), 2.28−2.38 (m, 1 H, 2"-H), 2.55 (m, 2 H, CH_2CN), 2.50−2.64 (m, 1 H, 2′-H), 3.30−3.88 (m, 6 H, $POCH_2$, 5′-H, 5"-H and 2 × CH of *i*Pr), 3.80, 3.81 (2 s, 6 H, OCH_3 of DMTr), 4.05−4.22 [m, 5 H, 2 × $P(O)OCH_2$, 4′-H], 4.69 (m, 1 H, 3′-H), 5.67 (m, 1 H, 5-H), 6.25 (m, 1 H, 1′-H), 8.83−8.88 (2 d, *J* = 8.9 Hz,4 H, *o*-H of DMTr), 7.17−7.45 (m, 10 H, ArH), 7.85−7.92 (2 dd, $J_{6,5}$ = 7.9, *J* = 1.7 Hz, 1 H, 6-H) ppm. ^{13}C NMR ($CDCl_3$): δ = 16.0 (d, $^3J_{POCC}$ = 7.3 Hz, CH_3 of Et), 20.1−20.3 (β-C of CE), 24.35, 24.39 (d, $^3J_{PNCC}$ = 7.3 Hz, CH_3 of *i*Pr), 40.3 (2′-C), 43.1, 43.2 (d, $^2J_{PNC}$ = 12.2 Hz, CH of *i*Pr), 55.1 (OCH_3 of DMTr), 62.3−62.9 (CH_2 of Et, 5′-C and α-C of CE), 72.8, 73.1 (3′-C), 85.0−87.0 (1′-C, 4′-C, *tert*-C of DMTr), 113.1, 113.2 (3,3′,5,5′-C of DMTr), 117.4 (CN), 127.0−130.1 (2,6-C of DMTr), 134.8, 135.0, 135.2 (1′-C of DMTr), 39.4, 139.6 (6-C), 143.9, 144.1 (1"-C of DMTr), 148.9 (2-C), 158.5, 158.6 (4,4′-C of DMTr), 159.2 (4-C) ppm. ^{31}P NMR ($CDCl_3$): δ = 6.33, 149.37, 149.76 ppm. $C_{45}H_{55}N_7O_{10}P_2{\cdot}H_2O$ (882.9): calcd. C 57.88, H 6.15, N 10.49; found calcd. C 57.15, H 5.98, N 10.25.

***N*^{4}-Bis(2-cyanoethoxy)phosphoryl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-*P*-(2-cyanoethyl)cytidylyl-(3′→5′)-3′-*O*-acetylthymidine (10a):** 3′-*O*-Acetylthymidine (**9**) (28 mg, 0.10 mmol) and compound **8a** (110 mg, 0.12 mmol) were dried by repeated coevaporation with dry pyridine and then dry toluene, and were then dissolved in dry MeCN (1 mL). 1*H*-Tetrazole (14 mg, 0.20 mmol) was added to the mixture of **9** and **8a** in dry MeCN. After the mixture had been stirred at room temp. for 30 min, *tert*-butyl hydroperoxide (containing 20% di-*tert*-butyl peroxide, 63 μL, 0.50 mmol) was added and the mixture was stirred at room temp. for 15 min. It was then diluted with $CHCl_3$ and washed three times with 5% $NaHCO_3$ (aq.), and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and washings were combined and dried with Na_2SO_4, filtered, and concentrated to dryness. The residue was placed on a silica gel column. Column chromatography with CH_2Cl_2 containing 1% pyridine, with a gradient of methanol (1−2%), gave **10a** (104 mg, 93%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.90 (s, 3 H, 5-Me of Th), 2.08, 2.09 (2 s, 3 H, Ac), 2.26−2.44 (m, 4 H, 2"-H of C and T, CH_2CN of CE), 2.65−2.78 (m, 6 H, 2′-H of C and T, 2 × CH_2CN of CE), 3.47 (m, 2 H, 5"-H of C and T), 3.80 (s, 6 H, OCH_3 of DMTr), 4.13−4.35 (m, 10 H, 4′-H, 5′-H of C and T, 3 × $POCH_2$), 5.16−5.25 (m, 2 H, 3′-H of C and T), 5.78 (d, $J_{5,6}$ = 7.9 Hz, 1 H, 5-H of C), 6.24−6.31 (m, 2 H, 1′-H of C and T), 6.85 (d, *J* = 8.6 Hz, 4 H, ArH of DMTr), 7.22−7.36 (m, 10 H, ArH of DMTr and 6-H of T), 7.69 (m, 1 H, 6-H of C), 9.40, 9.47 (2 s, NH of T) ppm. ^{13}C NMR ($CDCl_3$): δ = 12.4 (5-Me of T), 19.6 (d, $^3J_{POCC}$ = 4.7 Hz, β-C of CE), 36.6 (2′-C of T), 39.3 (d, $^3J_{POCC}$ = 2.4 Hz, 2′-C of C), 55.2 (MeO of DMTr), 61.3−62.6 (5′-C of C and T, α-C of CE), 67.6 (3′-C of T), 73.5 (3′-C of C). 82.3−85.2 (1′-C, 4′-C of C and T), 87.3 (*tert*-C of DMTr), 102.2 (5-C of C), 111.5, 111.7 (4-C of T), 113.1, 113.3 (3,3′-C of DMTr), 116.3, 116.4, 116.8 (CN), 127.2−130.0 (2,6-C of DMTr), 134.8, 134.9, 135.2 (1,1′-C of DMTr), 140.0 (6-C of C, 1"-C of DMTr, 2-C of T), 150.4 (2-C of C), 158.7 (4,4′-C of DMTr), 160.1 (4-C of C), 163.65, 163.68 (6-C of T), 170.46, 170.53 (C=O) ppm. ^{31}P NMR ($CDCl_3$): δ = −2.12, −2.07, 5.77 ppm. $C_{51}H_{56}N_8O_{17}P_2$ (1115.0): calcd. C 54.94, H 5.06, N 9.77; found calcd. C 56.34, H 5.20, N 9.77.

2′-Deoxy-*N*^{4}-diethoxyphosphoryl-5′-*O*-(4,4′-dimethoxytrityl)-*P*-(2-cyanoethyl)cytidylyl-(3′→5′)-3′-*O*-acetylthymidine (10b): 3′-*O*-Acetylthymidine (**9**) (28 mg, 0.10 mmol) and compound **8b** (110 mg, 0.12 mmol) were dried by repeated coevaporation with dry pyridine and then dry toluene, and were then dissolved in dry MeCN (1 mL). 1*H*-Tetrazole (14 mg, 0.20 mmol) was added to the mixture of **9** and **3b** in dry MeCN. After the mixture had been stirred at room temp. for 30 min, *tert*-butyl hydroperoxide (containing 20% di-*tert*-butyl peroxide, 63 μL, 0.50 mmol) was added and stirring was continued at room temp. for an additional 15 min. The reaction mixture was diluted with $CHCl_3$ and washed three times with 5% $NaHCO_3$ (aq.), and the aqueous layer was back-extracted with $CHCl_3$. The organic layer and washings were combined, dried with Na_2SO_4, filtered, and concentrated to dryness. The residue was placed on a silica gel column. Column chromatography with CH_2Cl_2 containing 1% pyridine, with a gradient of methanol (1−2%), gave **10b** (94 mg, 84%) as a colorless foam. ^{1}H NMR ($CDCl_3$): δ = 1.32 (t, *J* = 6.9 Hz, 6 H, CH_3 of Et), 1.91 (s, 3 H, 5-Me of T), 2.08, 2.09 (2 s, 3 H, Ac), 2.27−2.41 (m, 4 H, 2"-H of C and T, 2 × CH_2CN), 2.65−2.78 (m, 2 H, 2′-H of C and T), 3.43−3.47 (m, 2 H, 5"-H of C and T), 3.79, 3.80 (2 s, 6 H, MeO of DMTr), 4.03−4.35 [m, 10 H, 4′-H, 5′-H of C and T, 3 ×

P(O)OCH$_2$], 5.18−5.29 (m, 2 H, 3′-H of C and T), 5.69 (d, $J_{5,6}$ = 7.9 Hz, 1 H, 5-H of C), 6.26−6.33 (m, 2 H, 1′-H of C and T), 6.86 (2 d, J = 7.6 Hz, 4 H, ArH of DMTr), 7.15−7.35 (m, 10 H, ArH of DMTr and 6-H of T), 7.62−7.66 (m, 1 H, 6-H of C), 9.42 (s, 1 H, NH of T) ppm. ^{13}C NMR (CDCl$_3$): δ = 2.4 (5-Me of T), 16.1 (d, $^3J_{POCC}$ = 6.1 Hz, CH$_3$ of Et), 19.5−19.6 (2 d, $^3J_{POCC}$ = 6.1 Hz, β-C of CE), 36.6 (2′-C of T), 39.3 (d, $^3J_{POCC}$ = 5.0 Hz, 2′-C of C), 55.2 (MeO of DMTr), 62.4−62.9 (CH$_2$ of Et, 5′-C of C and T, α-C of CE), 73.5 (3′-C of T), 79.0 (d, $^2J_{POC}$ = 6.1 Hz, 3′-C of C), 82.3−87.2 (1′-C, 4′-C of C and T, *tert*-C of DMTr), 101.8, 101.9, 102.2 (5-C of C), 111.6, 111.7 (4-C of T), 113.3 (3,3′,5,5′-C of DMTr), 116.1, 116.2, 116.3 (CN), 127.2−130.0 (2,6-C of DMTr), 134.8, 135.1 (1,1′-C of DMTr), 139.3 (6-C of C), 143.88, 143.92 (1"-C of DMTr), 149.2 (2-C of T), 150.3 (2-C of C), 158.6, 158.7 (4,4′-C of DMTr), 159.3 (4-C of C), 163.5 (6-C of T), 170.4, 170.5 (C=O) ppm. ^{31}P NMR (CDCl$_3$): δ = 5.79, −2.02, −2.04 ppm.

2′-Deoxy-*N*^{4}-(phosphoryl)cytidylyl-(3′→5′)thymidine (11a): The fully protected d(CpT) dimer **10a** was treated with NH$_3$/pyridine (20 mL, 3:1, v/v) at room temperature for 12 h. The mixture was concentrated to a small volume, and the residue was dissolved in dist. H$_2$O. The solution was washed five times with diethyl ether. The aqueous layer was concentrated to dryness under reduced pressure, and the residue was treated with 80% aqueous AcOH at room temperature for 30 min. The reaction mixture was concentrated and co-evaporated several times with dist. H$_2$O. The residue was dissolved in dist. H$_2$O, and the solution was washed five times with diethyl ether. The aqueous layer was concentrated to dryness under reduced pressure. The residue was dissolved in a small amount of water and placed on a gel filtration column (Sephadex G-10, 300 × 15 mm). Elution was performed with water. The eluent was collected and lyophilized to give a crude product. This crude product was further purified by C-18 reversed-phase column chromatography (80 × 20 mm, H$_2$O/MeCN, 100:0 to 85:15). The fractions containing **11a** were combined and lyophilized. The residue was treated with cation-exchange resin (Dowex 50W × 8, Na$^+$ form) and lyophilized to give **11a** (20 mg, 32%) as a white powder. ^{1}H NMR (D$_2$O): δ = 1.88 (d, 3 H, $J_{6\text{-Me},5}$ = 1.0 Hz, 6-Me of T), 2.26−2.37 (m, 3 H, 2′-H of C, 2"-H of C and T), 3.72 (dd, $J_{5'',5'}$ = 12.5, $J_{5'',4'}$ = 4.6 Hz, 1 H, 5"-H of C), 3.82 (dd, 1 H, $J_{5',5''}$ = 12.5, $J_{5',4'}$ = 3.3 Hz, 5′-H of C), 4.01−4.24 [m, 8 H, 4′-H of C and T, 5′-H and 5"-H of T, 2 × P(O)OCH$_2$], 4.52−4.57 (m, 1 H, 3′-H of T), 4.78−4.87 (m, 1 H, 3′-H of C), 6.13 (t, 1 H, $J_{1',2'}$ = $J_{1',2''}$ = 6.3 Hz, 1′-H of T), 6.23 (m, 1 H, 1′-H of C), 6.29 (d, $J_{5,6}$ = 7.6 Hz, 1 H, 5-H of C), 7.67 (d, 1 H, $J_{6,5\text{-Me}}$ = 1.3 Hz, 6-H of T), 7.91 (d, $J_{6,5}$ = 7.6 Hz, 1 H, 6-H of C) ppm. ^{31}P NMR (D$_2$O): δ = −2.12, −2.07, 5.77 ppm.

2′-Deoxy-*N*^{4}-diethoxyphosphorylcytidylyl-(3′→5′)thymidine (11b): A procedure similar to that described in the case of **11a** gave **11b** (26 mg, 46%): ^{1}H NMR (D$_2$O): δ = 1.31 (2 t, J = 7.3 Hz, 6 H, CH$_3$ of Et), 1.86 (d, 3 H, $J_{6\text{-Me},5}$ = 1.0 Hz, 6-Me of T), 2.24−2.37 (m, 3 H, 2′-H of C, 2"-H of C and T), 2.58−2.67 (m, 1 H, 2"-H of T), 3.74 (dd, $J_{5'',5'}$ = 12.5, $J_{5'',4'}$ = 4.6 Hz, 1 H, 5"-H of C), 3.82 (dd, 1 H, $J_{5',5''}$ = 12.5, $J_{5',4'}$ = 3.3 Hz, 5′-H of C), 4.02−4.24 [m, 8 H, 4′-H of C and T, 5′-H and 5"-H of T, 2 × P(O)OCH$_2$], 4.55 (m, 1 H, 3′-H of T), 4.77 (m, 1 H, 3′-H of C), 6.17 (t, 1 H, $J_{1',2'}$ = $J_{1',2''}$ = 6.6 Hz, 1′-H of T), 6.24−6.32 (m, 2 H, 1′-H and 5-H of C), 7.67 (d, 1 H, $J_{6,5\text{-Me}}$ = 1.3 Hz, 6-H of T), 8.00 (d, $J_{6,5}$ = 7.6 Hz, 1 H, 6-H of C) ppm. ^{13}C NMR (D$_2$O): δ = 14.2 (5-Me of T), 17.9 (d, $^3J_{POCC}$ = 6.1 Hz, CH$_3$ of Et), 41.2 (d, $^3J_{POCC}$ = 8.5 Hz, 2′-C of C), 41.3 (2′-C of T), 67.4, 67.5, 67.6 [P(O)OCH$_2$, 5′-C of T], 73.1 (3′-C of T), 77.3 (d, $^2J_{POC}$ = 4.8 Hz, 3′-C of C), 87.6, 89.1 (1′-C of C and T), 87.7, 88.7 ($^3J_{POCC}$ = 8.5 Hz, 6.1 Hz, 4′-C of C and T), 114.1 (6-C of C), 134.0 (6-C of T), 154.2 (2-C of C), 168.9 (4-C of C) ppm. ^{31}P NMR (D$_2$O): δ = −2.04, −2.02, 5.79 ppm.

Synthesis and Purification of Modified Oligodeoxynucleotides: Automated DNA synthesis was performed with an Applied Biosystems Model 392 DNA/RNA Synthesizer. Deoxynucleoside phosphoramidite units and dG-LCAA-CPG were purchased from Perkin−Elmer Japan, Inc. Synthesis of oligodeoxynucleotides was performed on a 1-μmol scale by use of standard β-cyanoethyl phosphoramidite chemistry. After the automated synthesis, the solid supports containing the DMTr-on oligodeoxynucleotides were transferred to a glass snap vial and treated with conc. NH$_3$ at room temp. for 12 h. The DMTr-on oligodeoxynucleotides were purified by a Sep-Pak cartridge by washing with distilled water. The detritylation was performed by treatment with 2% TFA/H$_2$O on the Sep-Pak cartridge, and the subsequent purification of the detritylated oligodeoxynucleotides was performed with a Shimadzu 6A system on a μBondapak column (Waters, C18-100A, 7.8 × 300 mm) by use of a linear gradient of 0−30% acetonitrile in 0.1 M NH$_4$OAc (pH = 7.0) for 30 min at a flow rate of 3.0 mL/min at room temp. The oligodeoxynucleotides synthesized were analyzed by MALDI-TOF mass spectrometry. Compound **16**: calcd. 3725.36; found 3726.43. Compound **20**: calcd. 3781.47, found 3782.96.

Melting Experiments: Thermal denaturation studies of oligodeoxynucleotides were carried out with a Hitachi U-2000 spectrophotometer with 1-cm optical path length quartz cuvettes. The sample temperature was increased from 0 to 80 °C at 1 °C/min with absorption readings at 260 nm taken every 0.5 °C. All samples were prepared in a buffer containing 10 mM sodium phosphate, pH = 7.0, 150 mM NaCl. The melting temperature (T_m) values were determined by the reading of the maximum point of the first derivative of the melting profiles.

CD Spectra: The CD spectra of the d(CpT) dimers and oligodeoxynucleotides containing *N*-phosphorylated deoxycytidine derivatives were obtained with a JASCO J-500C spectrometer with 0.5-cm optical path length quartz cuvettes. The d(CpT) dimers were dissolved in 10 mM sodium phosphate buffer (pH = 7.0), and the final concentration was adjusted to about 10 μM. In the cases of the deoxydodecamers, the solvent also contained 150 mM NaCl, and the sample concentration was 2 μM.

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