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Oligonucleotide Analogues Containing Internucleotide C3'-CH_2-C(O)-NH-C5' Bonds

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Abstract—A dinucleoside bearing an amide internucleotide C3'-CH_2-C(O)-NH-C5' bond was synthesized by the interaction of 3'-deoxy-3'-carboxylmethylribothymidine-2',3'-lactone obtained by hydrolysis of 2'-*O*-acetyl-5'-*O*-benzoyl-3'-deoxy-3'-ethoxycarboxylmethylribothymidine with 5'-deoxy-5'-amino-3'-*O*-(*tert*-butyldimethylsilyl)thymidine. After standard manipulations with protective groups, the dinucleoside was converted into 3'-*O*-(2-cyanoethyl-*N,N'*-diisopropylphosphoroamidite), which was used for the synthesis of modified oligonucleotides on an automatic synthesizer. Duplex melting curves formed by modified and complementary natural oligonucleotides were measured and the melting temperatures and thermodynamic parameters of duplex formation were calculated. The introduction of one modified bond into oligonucleotides caused only an insignificant decrease in the duplex melting temperatures compared with the nonmodified ones.

Key words: amide internucleotide bond, analogues, hybridization, oligonucleotides, thermal stability

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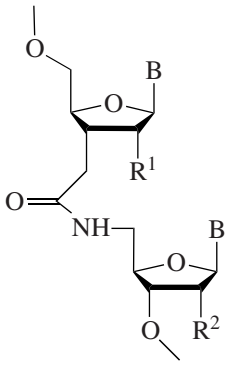
INTRODUCTION

Therapy with synthetic oligonucleotide analogues (antisense therapy) is one of the promising methods in the treatment of viral and oncologic diseases [1, 2].[2] The method is based on the inhibition of gene translation due to specific binding of a synthetic oligonucleotide to the mRNA corresponding site. The basic mechanism of action of synthetic oligonucleotides is an attack of the resulting DNA–RNA hybrid with intracellular endonuclease RNase H, which breaks a ribo-chain of the hybrid complex and inhibits translation [3, 4]. High hybridization specificity provides selective inhibition of the expression of genes involved in pathogenesis.

During last 15 years, a large number of base-, carbohydrate- or internucleotide bond-modified oligonucleotide analogues were synthesized (see reviews [5–7]). In recent years, researchers paid significant attention to oligonucleotide analogues, in which a phosphate internucleotide bond was replaced by an amide bond. Oligonucleotides in which one or several dinucleotides are replaced by a dinucleoside residue with a C3'–CH_2–C(O)–NH–C5' bond [8–12] (structures (**A**) and (**B**) in Scheme 1) are of special interest, because these oligonucleotides form duplexes with complementary natural oligonucleotides whose stability is practically the same as that of natural duplexes.

We describe in this work the synthesis and study of the hybridization properties of oligonucleotide analogues, in which a dinucleotide residue is replaced by a dinucleoside residue containing an amide internucleoside bond (structure (**D**) in Scheme 1).



(**A**): R^1 = H, R^2 = H
(**B**): R^1 = OH, R^2 = OH
(**D**): R^1 = OH, R^2 = H, B = Thy

Scheme 1. Dinucleosides with internucleotide amide bonds.

RESULTS AND DISCUSSION

Earlier, we published an improved method of synthesis of 2'-*O*-acetyl-5'-*O*-benzoyl-3'-deoxy-3'-ethoxycarboxylmethylnucleosides [13]. The use of these compounds in oligonucleotide synthesis requires the preparation of properly protected dinucleosides (**D**) (Scheme 1). At first

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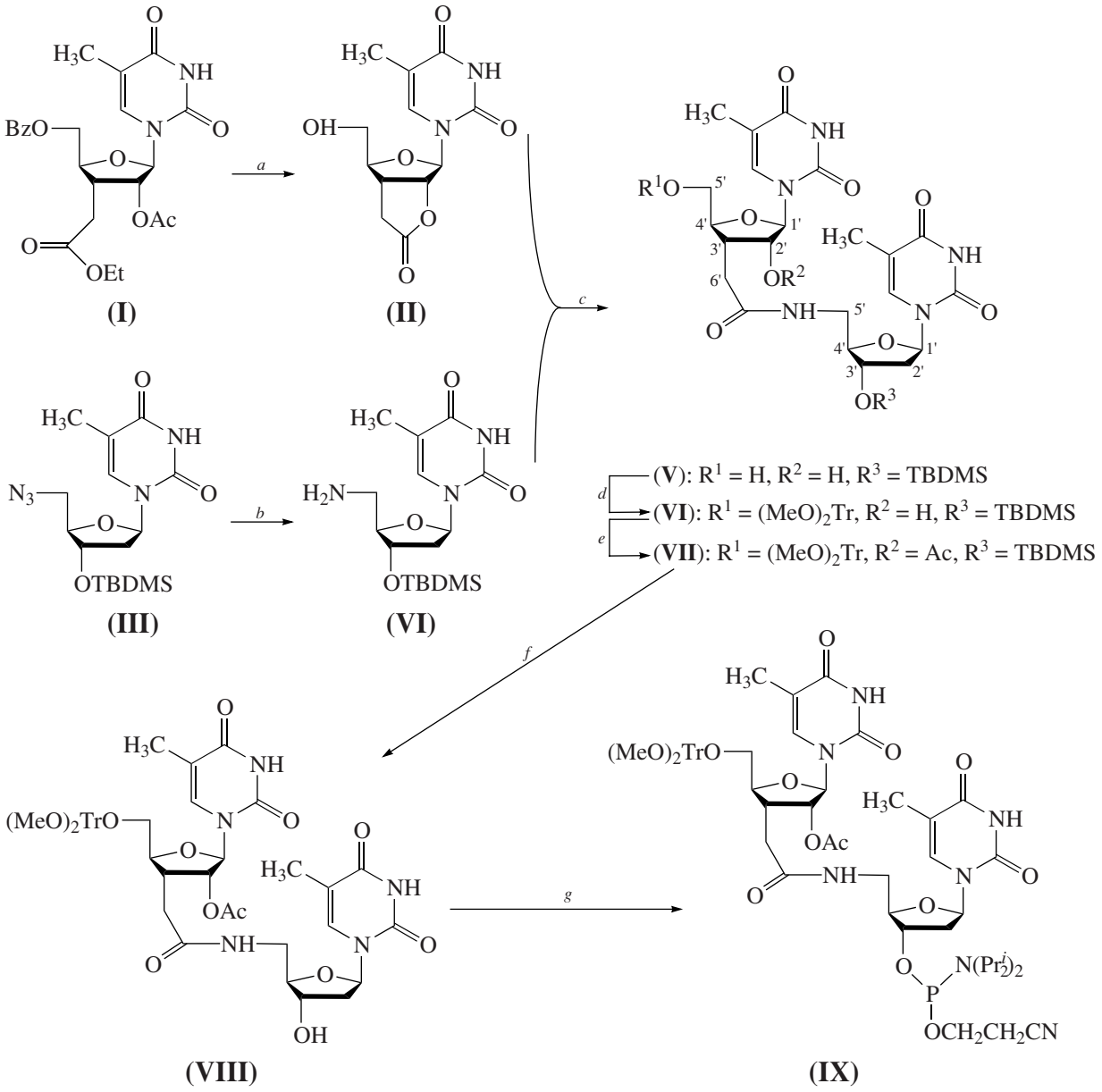
[2] Abbreviations: TBAF, tetra-*n*-butylammonium fluoride; TEAA, triethylammonium acetate; TBDMS, *tert*-butyldimethylsilyl; aT, 5'-amino-5'-deoxythymidine; cT, 3'-deoxy-3'-carboxymethylribothymidine.



Scheme 2. *a*) 1 – KOH, H_2O/EtOH, 2 – AcOH. 70°C; *b*) $HCOONH_4$, Pd/C, MeOH; *c*) 2-hydroxypyridine, DMF, 70°C 30 h; *d*) $(MeO)_2TrCl$, Py; *e*) Ac_2O, Py; *f*) TBAF, THF; *g*) $NCCH_2CH_2OP(NPr^i_2)_2$, tetrazole.

glance, the simplest method of dinucleoside synthesis is the following sequence: alkaline hydrolysis, successive protection of a 5'-hydroxyl with a dimethoxytrityl and 2'-hydroxyl with a *tert*-butyldimethylsilyl group, and final coupling with 5'-amino-5'-deoxynucleoside. However, this route proved to be ineffective in the case of 3'-deoxy-3'-ethoxycarboxylmethylnucleosides. The reason being that ester hydrolysis was accompanied by 2',3'-lactone closure. It was shown in [14] that hydrolysis of 2',5'-di-*O*-acetyl-3'-deoxy-3'-ethoxycarboxylmethylribothymidine followed by tritylation only resulted in protected 2',3'-lactone. Saponification of the lactone and interaction with a large excess of *tert*-butyldimethylsilyl chloride for 3 days resulted in two major products: target protected 3'-deoxy-3'-carboxylmethylribothymidine and its bissilyl derivative. This mixture was hydrolyzed with an acetone–water–triethylamine mixture (3 days) to give 5'-*O*-$(MeO)_2$Tr–2'-*O*-TBDMS-3'-deoxy-3'-carboxylmethylribothymidine. The above procedure is time consuming and is of little use for the large-scale synthesis of dinucleosides.

We used 2',3'-lactone as a key compound for the synthesis of dinucleoside (Scheme 2). Lactone (**II**) was

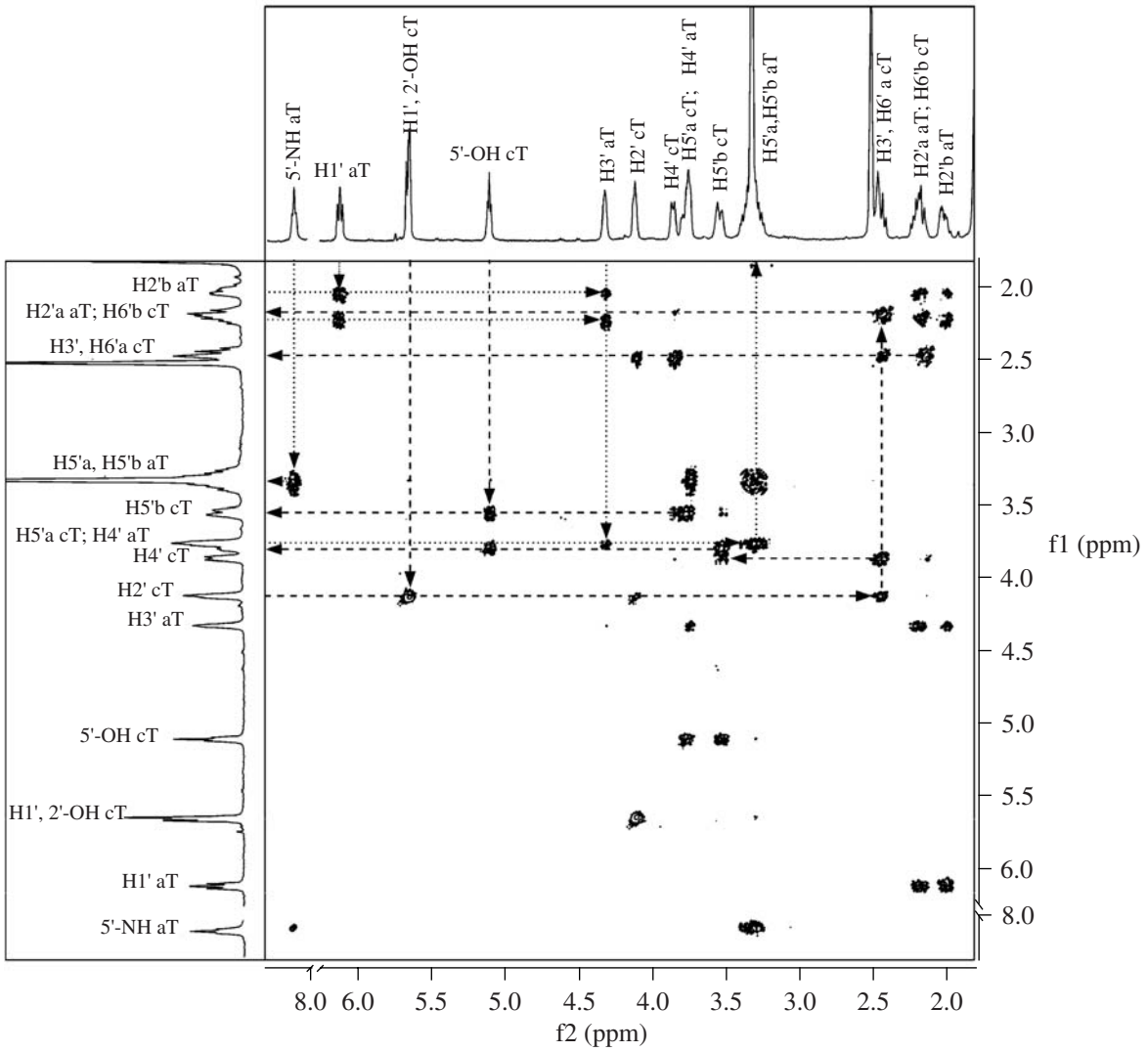


Fig. 1. A COSY DQF spectral fragment demonstrating correlations among protons of carbohydrate cycles of dinucleoside (**V**) (DMSO-d_6 solution). cT, a 3'-deoxy-3'-carboxymethylribothymidine residue; aT, 5'-amino-5'-deoxythymidine residue. The assignment of resonances to the cT residue is shown with a dotted line, and to the aT residue, with dots.

obtained in a yield of 84% by alkaline hydrolysis of nucleoside (**I**) followed by short heating of the intermediate acid in a weak acidic medium. Peterson et al., showed that the lactone interaction with amines occurred with great difficulty and good results could only be achieved in the presence of a bifunctional catalyst, 2-hydroxypyridine [15]. Indeed, the heating of lactone (**II**) with a fivefold excess of 5'-amino-5'-deoxy-3'-*O*-butyldimethylsilylthymidine (**IV**) and a twofold excess of 2-hydroxypyridine in dimethylformamide led to 86% dinucleoside (**V**). Further routine procedures of tritylation, acetylation, and the removal of silyl protective groups resulted in dinucleoside (**VIII**) with a total yield of 78% relative to starting dinucleoside (**V**). Phosphoroamidite (**IX**) was obtained as was earlier described in [16].

The structures of all synthesized compounds were confirmed by ^{1}H NMR spectra. In the case of dinucleosides (**V**)–(**VIII**), the resonances were assigned using COSY DQF spectra (as an example, a two-dimensional spectrum of dinucleoside (**V**) is given in Fig. 1).

The two-dimensional spectrum (Fig. 1) provided unambiguous identification of the resonances of terminal 5' and 3'dinucleoside residues. Since the spectrum was registered in DMSOd_6, it contained resonances of the changing protons, namely, those of 2' and 5'-hydroxy groups, with the T residue (identified by disappearance of proton resonances of hydroxyl groups when ^2H_2O was added to the sample). In the 1D spectrum the 2'-OH resonance was partially overlapped with the H1' resonance; therefore they gave a united peak with H2', which interacted in turn with H3', whose resonance was overlapped with that of the solvent. The

Table 1. Mass spectra of the synthesized oligonucleotides

Code	Sequence, 5'⟶3'	Mass spectra	
		found, *m/z*	calculated $[M - H]^-$
Modified oligonucleotides			
M1	G-**D**-TT-TT-TT-TT-G	3614.9	3614.45
M2	G-TT-TT-TT-TT-**D**-G	3614.6	3614.45
M3	G-TT-TT-**D**-TT-TT-G	3615.8	3614.45
M4	G-AC-AC-**D**-CT-AA-G	3606.7	3607.47
M5	G-AC-AA-**D**-AT-CA-G	3629.3	3629.50
M6	G-AC-AG-**D**-GT-CA-G	3662.5	3661.49
M7	G-AA-GT-**D**-GA-CA-G	3683.5	3685.52
Natural oligonucleotides			
S1	G-TT-TT-TT-TT-TT-G	3637.8	6337.38
S2	C-AA-AA-AA-AA-AA-C	3647.3	3647.46
S3	G-AC-AC-TT-CT-AA-G	3628.9	3628.4
S4	G-AC-AA-TT-AT-CA-G	3653.2	3652.42
S5	G-AC-AG-TT-GT-CA-G	3683.1	3684.42
S6	G-AA-GT-TT-GA-CA-G	3709.2	3708.54
S7	C-TT-AG-AA-GT-GT-C	3660.7	3659.41
S8	C-TG-AT-AA-TT-GT-C	3622.5	3633.4
S9	C-TG-AC-AA-CT-GT-C	3606.4	3604.37
S10	C-TG-TC-AA-AC-TT-C	3679.7	3579.36

H3' proton formed cross-peaks with H4' and H6'a and H6'b. Proton H4' interacted with H5'a and H5'b. Additional information was obtained from cross peaks between 5'-OH, H5'a and H5'b. In the case of an aT proton, H1' formed cross peaks with H2'a and H2'b, which interacted with H3'. With the above data, it was easy to assign the H4' resonance and resonances of H5'a and H5'b, whose signals in the 1D spectrum were completely overlapped with the signal of residual water. In addition, the cross peak between H5'a and H5'b and 5'-NH was observed.

All the dinucleosides were characterized by MALDI mass spectra.

Phosphoramidite (**IX**) was used for the synthesis of oligonucleotide analogues (**D**-modified dinucleoside (Scheme 1)) (Table 1). In addition, complementary natural oligonucleotides were also prepared (Table 1).

The synthesized oligonucleotides were characterized by mass spectra (Table 1).

Profiles of the thermal dissociation of natural and modified duplexes were measured (Fig. 2a). Duplex experimental melting temperatures were determined by the derivative maximum after the differentiation of experimental melting curves (Fig. 2b).

Based on the curves, thermodynamic parameters of duplex formation from individual chains were calculated using a model of two states (Table 2). When calculating, temperature dependences of the duplex and individual chains were taken into account. Since melting temperatures of short duplexes depended on the concentration, experimental melting temperatures were recalculated to a standard chain concentration of 10^{-5} M (the next to last row in Table 2).

As is seen in Table 2, one modification caused a decrease in the duplex melting temperatures by 0.4–1.3°C, depending on the sequence if compared with that of natural duplexes. The data on the thermal stability of duplexes formed by antisense and complementary natural oligonucleotides were summarized in [17]. Of the analogues with amide internucleotide bonds, oligonucleotides containing modified dinucleoside (**A**) (Scheme 1) or internucleotide C3'–CH_2–NH–C(O)–C5' bonds formed the most stable duplexes. In the first case, δT_m was –0.1 – +0.9, and in the second, –0.8 – +0.4°C. Other modifications of amide, carbamide, and carbamate internucleotide bonds decreased duplex T_m by more than 2°C. To summarize, stability of the duplexes formed by the synthesized with complementary natural oligonucleotides was only insignificantly lower than that of the corresponding natural duplexes and comparable to the stability of the above-described most stable modified duplexes.

EXPERIMENTAL

Pyridine, chloroform, methylene chloride, dimethylformamide, diethyl ether, ethyl acetate, acetonitrile, tetrahydrofurane, and acetic acid of a chemically pure grade were from Khimmed, Russia; ammonium formate, triethylamine, and acetic anhydride were from Fluka, Switzerland; dimethoxytrityl chloride, *tert*-butyldimethylsilyl chloride, imidazole, tetrazole, and 2-hydroxypyridine, from Aldrich, United States; anhydrous methanol, from Pancreac, Spain. Dimethylformamide, methylene chloride, and acetonitrile were dried by distillation over phosphorus pentachloride; pyridine, over calcium hydride; and tetrahydrofurane, over lithium aluminium hydride. 2-Cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropylphosphoroamidite was prepared as described in [18].

NMR spectra (δ, ppm, *J*, Hz) of DMSO-d_6 solutions (if not stated otherwise) were registered on a Bruker AMXIII-400 spectrometer with a working frequency of 400 MHz. The spectra were treated using the MestReNova program, version 5.2.3 (Mestrelab Research SL). The multiplet centers are shown.

Duplex melting curves were registered on a UV 160-A spectrophotometer (Shimadzu, Japan) supplied with a thermostatic device. Duplex absorption A_{260} was registered at intervals of 0.5°C.

MALDI analysis of modified and natural oligonucleotides was performed on a MS-30 mass-spectrometer (Kratos, Japan) in a linear mode and registration of negative peaks.

Oligonucleotides were synthesized on an automatic ASM-102U DNA synthesizer (Biosun, Russia) using the standard protocol. Oligonucleotides were purified by reverse-phase HPLC on columns (25×4 mm^2) with Hypersil C18 in 0.05 M TEAA buffer (pH 7.8). DMTr-protected oligomers were isolated in a gradient of acetonitrile (10–50%) for 30 min. Exhaustively deblocked oligonucleotides were additionally purified in a gradi-

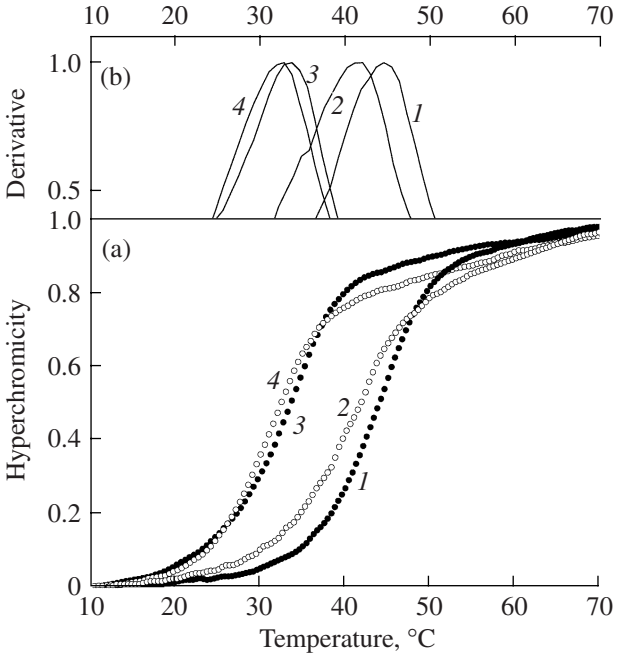


Fig. 2. a, Experimental melting curves of natural duplexes: *1*, **S5·S9**, *3*, **S1·S2**; and modified duplexes *2*, **M6·S9**, *4*, **M2·S2**. b, a derivative obtained by differentiation of experimental curves. For the convenience of comparison, hyperchromicity and the derivative were normalized to 1.

Table 2. Thermodynamic parameters of duplex formation from single chains

Duplex	$c \times 10^6$, M	$\Delta H°$, kJ/mol	$\Delta S°$, J/mol^{-1} K^{-1}	T_m, °C ±0.5	T_m, °C scaled*	ΔT_m, °C**
S1·S2	7.46	–368.6 ± 5.4	–962.1 ± 17.2	33.6	34.3	
M1·S2	9.96	–294.3 ± 5.4	–851.6 ± 29.7	33.9	33.9	–0.4
M2·S2	9.42	–275.9 ± 12.7	–791.7 ± 42.2	33.7	33.9	–0.4
M3·S2	1.12	–289.7 ± 7.9	–838.2 ± 32.2	33.7	33.4	–0.9
S3·S7	8.82	–344.5 ± 3.7	–987.7 ± 12.9	41.3	41.6	
M4·S7	4.84	–275.9 ± 5.0	–737.3 ± 16.3	38.2	40.3	–1.3
S4·S8	8.76	–364.7 ± 3.4	–1053.0 ± 10.9	41.0	41.3	
M5·S8	9.04	–265.9 ± 12.6	–740.6 ± 40.6	40.2	40.5	–0.8
S5·S9	7.53	–338.7 ± 5.0	–965.1 ± 15.5	42.2	42.9	
M6·S9	8.62	–292.7 ± 5.9	–822.3 ± 18.1	41.4	41.8	–1.1
S6·S10	7.70	–357.4 ± 5.4	–1016.6 ± 18.5	44.4	45.0	
M7·S10	5.53	–249.9 ± 7.1	–681.2 ± 22.2	42.0	44.0	–1.0

Notes: * Recalculated to a concentration of 10^{-5} M.

** Differences between T_m of modified and natural duplexes.

ent of acetonitrile (10–25%) for 30 min in the above buffer.

For column chromatography, a Kieselgel 60 (Merck, Germany) was used. TLC was performed on Kieselgel 60 F_{254} plates (Merck, Germany) in 1 : 49 ethanol–chloroform (system A); 1 : 19 ethanol–chloroform (system B); 1 : 9 ethanol–chloroform (system C); and 1 : 19 ethanol–chloroform + 0.5% triethylamine (system D).

3'-Deoxy-3'-carboxymethylribothymidine 2',3'-lactone (II). A solution of potassium hydroxide (1.51g, 27 mmol) in water (5 ml) was added under stirring to a solution of 2'-*O*-acetyl-5'-*O*-benzoyl-3'-deoxy-3'-ethoxycarbonylmethylribothymidine (1.28 g, 2.7 mmol) (**I**) in ethanol (20 ml) [13]. The mixture was kept under stirring at room temperature overnight. Concentrated HCl (2.23 ml) and then water (20 ml) were added, the mixture was evaporated to half a volume, and the solution was extracted with ether (3 × 20 ml). Acetic acid (5 ml) was added to the aqueous layer and heated at 70–80°C for 1 h. The mixture was evaporated to dryness; the residue was evaporated with ethanol (4 × 10 ml) and dissolved in absolute ethanol (10 ml). Silica gel (6 g) was added to the solution and the mixture was evaporated to dryness. The residue was loaded onto the column (3.5 × 20 cm) with silica gel and chromatographed with elution with system C. The fractions containing lactone were evaporated to dryness to give 0.64 g (84%) of lactone (**II**) as thick oil. R_f 0.25 (C). ^{1}H NMR: 11.366 (1 H, br. s, H3), 7.623 (1 H, br d, *J* 1.16, H6), 5.913 (1 H, d, $J_{1',2'}$ 1.63, H1'), 5.104 (1 H, dd, $J_{1',2'}$ 1.63, $J_{2',3'}$ 7.12, H2'), 5.043 (1 H, t, *J* 5.04, 5'-OH), 3.854 (1 H, m, H4'), 3.678 (1 H, dd, $J_{4',5'a}$ 3.54, $^2J_{5'a,5'b}$ 12.08, H5'a), 3.597 (1 H, dd, $J_{4',5'b}$ 4.39, $^2J_{5'a,5'b}$ 12.08, H5'b), 3.144 (1 H, m, H3'), 2.847 (1 H, dd, $J_{3',6'a}$ 8.61, $^2J_{6'a,6'b}$ 18.07, H6'a), 2.464 (1 H, dd, $J_{3',6'b}$ 1.51, $^2J_{6'a,6'b}$ 18.07, H6'b), 1.769 (3 H, d, *J* 1.26, 5-CH_3).

5'-Deoxy-5'-azido-3'-*O*-(*tert*-butyldimethylsilyl)thymidine (III). A mixture of 5'-deoxy-5'-azidothymidine (3.2 g, 12 mmol) [19] and imidazole (1.63 g, 24 mmol) was evaporated with anhydrous acetonitrile (3 × 10 ml). The residue was dissolved in anhydrous acetonitrile (25 ml) and *tert*-butyldimethylsilyl chloride (2.7 g, 24 mmol) was added under stirring. The mixture was kept at room temperature overnight, water (10 ml) was added, evaporated, and water and ethyl acetate (50 ml of each) were added to the residue. The aqueous layer was extracted with ethyl acetate (20 ml), the united organic fractions were washed with 10% $KHSO_4$ (2 × 10 ml), saturated $NaHCO_3$ (2 × 10 ml), and water, dried with anhydrous Na_2SO_4, and evaporated to give 4.5 g (98%) of azide (**III**) as crystalline mass, R_f 0.51 (A), mp 89–90°C (2 : 1 ethyl acetate–hexane). ^{1}H NMR ($CDCl_3$): 9.433 (1 H, br. s, H3), 7.281 (1 H, br. d, *J* 1.24, H6), 6.214 (1 H, t, *J* 6.56, H1'), 4.325 (1 H, m, H3'), 3.905 (1 H, m, H4'), 3.661 (1 H, dd, $J_{4',5'a}$ 3.29, $^2J_{5'a,5'b}$ 13.27, H5'a), 3,468 (1 H, dd, $J_{4',5'b}$ 3.62, $^2J_{5'a,5'b}$ 13.27, H5'b), 2.256 (1 H, m, H2'a), 2.149 (1 H, m, H2'b), 1.911 (3 H, d, *J* 1.24, 5-CH_3), 0.861 (9 H, s, $(CH_3)_3$CSi), 0.058 (6 H, s, $(CH_3)_2$Si).

5'-Deoxy-5'-amino-3'-O-(tert-butyldimethylsilyl)thymidine (IV). A mixture of 10% Pd/C (0.42 g) and a solution of azide (**III**) (4.2 g, 11 mmol) in anhydrous methanol (50 ml) was stirred at room temperature. In approximately 10 min, a gas was liberated and the mixture was heated. After 2 h stirring, the mixture was filtered through Cellit, the Cellit was washed with anhydrous methanol (2 × 15 ml) on the filter, and the filtrate was evaporated. The residue was mixed with saturated $NaHCO_3$ (50 ml) and extracted with chloroform (3 × 30 ml). Chloroform extracts were washed with water, dried with anhydrous Na_2SO_4, and evaporated to give amine (**IV**) (3.82 g, 98%) as a thick oil; R_f 0.28 (B). ^{1}H NMR ($CDCl_3$): 7.325 (1 H, br. d, *J* 1.26, H6), 6.176 (1 H, t, *J* 6.6, H1'), 4.316 (1 H, m, H3'), 3.811 (1 H, m, H4'), 3.051 (1 H, dd, $J_{4',5'a}$ 3.75, $^2J_{5'a,5'b}$ 13.45, H5'a), 2.881 (1 H, dd, $J_{4',5'b}$ 5.76, $^2J_{5'a,5'b}$ 13.45, H5'b), 2.258 (1 H, m, H2'a), 2.175 (1 H, m, H2'b), 1.916 (3 H, d, *J* 1.26, 5-CH_3), 0.881 (9 H, s, $(CH_3)_3$CSi), 0.073 (6 H, s, $(CH_3)_2$Si).

3'-Deoxy-3'-{[*N*-(3'-*O*-*tert*-butyldimethylsilyl)-5'-deoxythymidine-5'-yl)carboxamido]methyl}-ribothymidine(V). A solution of lactone (**II**) (0.56 g, 2 mmol), amine (**IV**) (3.56 g, 10 mmol), and 2-hydroxypyridine (0.38 g, 4 mmol) in anhydrous DMF (20 ml) was heated at 70°C for 30 h. The solvent was removed in vacuum, and the residue was dissolved in chloroform (100 ml). The solution was washed with 10% $KHSO_4$ (3 × 30 ml), saturated with $NaHCO_3$ (2 × 30 ml) and water, and dried with anhydrous Na_2SO_4. The solvent was evaporated, and the residue was dissolved in chloroform and loaded on a silica gel column (20 × 4 cm). The product was eluted with a mixture of chloroform–EtOH (gradient from 5 to 15% EtOH) to give two substances with R_f 0.38 and 0.66 (C). The compound with a higher mobility (1.4 g) was a side product, 5'-deoxy-5'-formamido-3'-*O*-(*tert*-butyldimethylsilyl)thymidine, whereas the fraction with lower mobility contained the target dinucleoside (**V**) as solid foam (1.1 g, 86%). ^{1}H NMR: 11.261 (1 H, br. s, H3 cT), 11.233 (1 H, br. s, H3 aT), 8.102 (1 H, br. t, *J* 5.57, NH aT), 7.998 (1 H, br. d, *J* 1.36, H6 cT), 7.437 (1 H, br. d, *J* 1.14, H6 aT), 6.112 (1 H, dd, $J_{1',2'a}$ 8.25, $J_{1',2'b}$ 5.79, H1' aT), 5.654 (1 H, d, *J* 4.91, 2'-OH cT), 5.637 (1 H, d, $J_{1',2'}$ 1.41, H1' cT), 5.103 (1 H, d, *J* 4.94, 5'-OH cT), 4.312 (1 H, m, H3' aT), 4.137 (1 H, m, H2' cT), 3.848 (1 H, m, H4' cT), 3.756 (1 H, m, H5'a cT), 3.748 (1 H, m, H4' aT), 3.530 (1 H, m, H5'b cT), 3.39–3.23 (2 H, m, H5'a,b aT), 2.49–2.38 (2 H, m, H3', H6'a cT), 2.25–2.11 (2 H, m, H6'b cT, H2'a aT), 2.007 (1 H, m, H2'b aT), 1.799 (3 H, d, *J* 1.36, 5-CH_3 cT), 1.758 (3 H, d, *J* 1.14, 5-CH_3 aT), 0.862 (9 H, s, $(CH_3)_3$CSi), 0.067 (6 H, s, $(CH_3)_2$Si). Mass: m/z 637.3. Calculated 636.8 $[M–H]^-$ ($C_{26}H_{42}N_5O_{10}Si$).

5'-*O*-(4,4'-Dimethoxytriryl)-3'-deoxy-3'-{[N-(3'-*O*-*tert*-butyldimethylsilyl-5'-deoxythymidine–5'-yl)carboxamido]methyl}-ribothymidine (VI). A solution of

dinucleoside (**V**) (0.6 g, 0.94 mmol) in anhydrous pyridine (10 ml) was evaporated to dryness. The residue was dissolved in anhydrous pyridine (10 ml) and $(CH_3O)_2TrCl$ (0.41 g, 1.2 mmol) was added. The mixture was allowed to stay overnight under stirring at room temperature; water (1 ml) and triethylamine (0.2 ml) were added, kept for 30 min, and evaporated. The residue was dissolved in ethyl acetate (50 ml), washed with saturated $NaHCO_3$ (2 × 20 ml), water, and dried with anhydrous Na_2SO_4. The residue was dissolved in chloroform containing 0.2% triethylamine and loaded on a silica gel column (20 × 4 cm). The product was eluted with a chloroform–EtOH mixture containing 0.2% triethylamine (gradient from 2 to 10% EtOH) to give compound (**VI**) as solid foam (0.74 g, 94%); R_f 0.52 (C). ^{1}H NMR: 11.304 (1 H, br. s, H3 cT), 11.272 (1 H, br. s, H3 aT), 8.066 (1 H, br. t, *J* 5.83, NH aT), 7.525 (1 H, br. d, *J* 1.27, H6 cT), 7.46 (1 H, br. d, *J* 1.18, H6 aT), 7.41–6.86 (13 H, m, Ar-H, $(CH_3O)_2Tr$), 6.09 (1 H, dd, $J_{1',2'a}$ 8.05, $J_{1',2'b}$ 6.09, H1' aT), 5.671 (1 H, d, *J* 4.96, 2'-OH cT), 5.671 (1 H, d, $J_{1',2'}$ 1.73, H1' cT), 4.289 (1 H, m, H3' aT), 4.221 (1 H, m, H2' cT), 4.003 (1 H, m, H4' cT), 3.73 (6 H, s, CH_3, $(CH_3O)_2Tr$), 3.702 (1 H, m, H4' aT), 3.14–3.32 (4 H, m, H5'a,b cT, H5'a,b aT), 2.652 (1 H, m, H3' cT), 2.429 (1 H, dd, $J_{3',6'a}$ 8.01, $^2J_{6'a,6'b}$ 15.16, H6'a cT), 2.166 (1 H, m, H2'a aT), 1.95–2.04 (2 H, m, H6'b cT, H2'b aT), 1.784 (3 H, d, *J* 1.27, 5-CH_3 cT), 1.366 (3 H, d, *J* 1.18, 5-CH_3 aT), 0.849 (9 H, s, $(CH_3)_3CSi$), 0.047 (3 H, s, CH_3Si), 0.043 (3 H, s, CH_3Si). Mass: *m/z* 939.1. Calculated 939.1 $[M - H]^-$ ($C_{49}H_{60}N_5O_{12}Si$).

2'-*O*-Acetyl–5'-*O*-(4,4'-dimethoxytrityl)-3'-deoxy-3'-{[*N*-(3'-*O*-*tert*-butyldimethylsilyl-5'-deoxythymidine-5'-yl)carboxamido]methyl}-ribothymidine (VII). Dinucleoside (**VI**) (0.7 g, 0.75 mmol) was evaporated with anhydrous pyridine (2 × 5 ml). The residue was dissolved in anhydrous pyridine (8 ml), acetic anhydride (0.54 g, 0.44 ml, 5.25 mmol) was added, and the mixture was allowed to stay overnight. Water (0.5 ml) and triethylamine (0.1 ml) were added and the mixture was stirred for 30 min. The solvent was removed, and the residue was dissolved in ethyl acetate (50 ml), washed with saturated $NaHCO_3$ (2 × 20 ml) and water, and dried with anhydrous Na_2SO_4. The mixture was chromatographed on a silica gel column (20 × 3 cm) in a chloroform–EtOH mixture containing 0.2% triethylamine (gradient from 2 to 7% EtOH) to give dinucleoside (**VII**) as solid foam (0.66 g, 90%); R_f 0.27 (B). ^{1}H NMR: 11.334 (1 H, br. s, H3 cT), 11.249 (1 H, br. s, H3 aT), 8.082 (1 H, br. t, *J* 5.95, NH aT), 7.488 (1 H, br. d, *J* 1.22, H6 cT), 7.47 (1 H, br. d, *J* 1.17, H6 aT), 7.41–6.86 (13 H, m, Ar-H, $(CH_3O)_2Tr$), 6.092 (1 H, dd, $J_{1',2'a}$ 7.98, $J_{1',2'b}$ 6.06, H1' aT), 5.737 (1 H, d, $J_{1',2'}$ 3.06, H1' cT), 5.358 (1 H, dd, $J_{1',2'}$ 3.06, $J_{2',3'}$ 6.94, H2' cT), 4.27 (1 H, dd, H3' aT), 3.997 (1 H, m, H4' cT), 3.732 (6 H, s, CH_3, $(CH_3O)_2Tr$), 3.703 (1 H, m, H4' aT), 3.14–3.32 (4 H, m, H5'a,b cT, H5'a,b aT), 3.011 (1 H, m, H3'), 2.319 (1 H, dd, $J_{3',6'a}$ 8.75, $^2J_{6'a,6'b}$ 15.14, H6'a cT), 2.182 (1 H, m, H2'a aT), 2.113 (1 H, dd, $J_{3',6'b}$ 6.12, $^2J_{6'a,6'b}$ 15.14, H6'b cT), 2.026 (3 H, s, CH_3CO), 2.001 (1 H, m, H2'b aT), 1.781 (3 H, d, *J* 1.22, 5-CH_3 cT), 1.514 (3 H, d, *J* 1.17, 5-CH_3 aT), 0.852 (9 H, s, $(CH_3)_3CSi$), 0.05 (6 H, s, $(CH_3)_2Si$). Mass: *m/z* 981.2. Calculated 981.2 981.2 $[M–H]^-$ ($C_{51}H_{62}N_5O_{13}Si$).

2'-*O*-Acetyl-5'-*O*-(4,4'-dimethoxytrityl)-3'-deoxy-3'-{[*N*-(5'-deoxythymidine-5'-yl)carboxamido]methyl}-ribothymidine (VIII). Anhydrous TBAF [20] (0.25 g, 0.97 mmol) was added to a solution of dinucleoside (**VII**) (0.63 g, 0.64 mmol) in absolute THF (10 ml), and the mixture was stirred for 3 h. The solvent was evaporated, and the residue was dissolved in ethanol (20 ml). Silicagel (10 g) was added to the solution, ethanol was evaporated, and the dry residue was loaded on a silica gel column (20 × 4 cm). The mixture was chromatographed with a chloroform–EtOH mixture containing 0.2% triethylamine (gradient from 5 to 15% EtOH) to give dinucleoside (**VIII**) as solid foam (0.51 g, 92%); R_f 0.24 (C). ^{1}H NMR: 11.283 (1 H, br. s, H3 cT), 11.170 (1 H, br. s, H3 aT), 7.99 (1 H, br. t, *J* 5.53, NH aT), 7.466 (1 H, br. d, *J* 1.08, H6 cT), 7.444 (1 H, br.d, *J* 1.18, H6 aT), 7.41–6.86 (13 H, m, Ar-H, $(CH_3O)_2Tr$), 6.111 (1 H, dd, $J_{1',2'a}$ 7.49, $J_{1',2'b}$ 6.39, H1' aT), 5.734 (1 H, d, $J_{1',2'}$ 3.09, H1' cT), 5.36 (1 H, dd, $J_{1',2'}$ 3.09, $J_{2',3'}$ 6.97, H2' cT), 4.094 (1 H, dd, H3' aT), 3.995 (1 H, m, H4' cT), 3.739 (6 H, s, CH (6 H, c, CH_3, $(CH_3O)_2Tr$), 3.715 (1 H, m, H4' aT), 3.14–3.32 (4 H, m, H5'a,b cT, H5'a,b aT), 3.026 (1 H, m, H3'), 2.331 (1 H, dd, $J_{3',6'a}$ 8.42, $^2J_{6'a,6'b}$ 15.19, H6'a cT), 2.132 (1 H, dd, $J_{3',6'b}$ 6.93, $^2J_{6'a,6'b}$ 15.19, H6'b cT), 2.04–2.12 (2 H, m, H2'a,b aT), 2.029 (3 H, s, CH_3CO), 1.775 (3 H, d, *J* 1.08, 5-CH_3 cT), 1.552 (3 H, d, *J* 1.18, 5-CH_3 aT). Mass: *m/z* 867.3. Calculated 866.9 866.9 $[M - H]^-$ ($C_{45}H_{48}N_5O_{13}$).

2'-*O*-Acetyl-5'-*O*-(4,4'-dimethoxytrityl)-3'-deoxy-3'-{[*N*-(3'-*O*-(*N,N*-diisopropylamino-2-cyanoethoxyphosphinyl)-5'-deoxythymidine-5'-yl)carboxamido]methyl}-ribothymidine (IX). Absolute pyridine (56 mg, 57 μl, 0.7 mmol), tetrazole (45 mg, 0.65 mmol) and then 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphoroamidite were added under stirring to a solution of dinucleoside (**VIII**) (0.47 g, 0.54 mmol) in dry methylene chloride (2.5 ml). Cold saturated $NaHCO_3$ (20 ml) and methylene chloride (50 ml) were added to the mixture in 1 h. The organic phase was separated, and the aqueous phase was extracted with methylene chloride (2 × 10 ml). United organic fractions were washed with saturated NaCl (20 ml), dried with anhydrous Na_2SO_4, and evaporated. The residue was loaded onto a silica gel column (15 × 2 cm) in a chloroform–EtOH mixture (1 : 49) containing 0.2% triethylamine and eluted with a chloroform–EtOH mixture containing 0.2% triethylamine (a gradient from 2 to 5% EtOH) to give phosphoroamidite (**IX**) (0.46 g, 80%) as solid foam; R_f 0.59 (D). ^{31}P NMR (acetonitrile-dd_3): 151.731, 151.465. Mass: *m/z* 1067.2. Calculated 1067.1 $[M - H]^-$ ($C_{54}H_{65}N_7O_{14}P$).

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