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Oligonucleotide Analogues Containing the Internucleotide Linker C3'-NH-CO-CH_2-N(CH_3)-C5'

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Abstract—Oligonucleotide analogues were synthesized whose internucleoside linker contains an amide bond and a methylamino group (C3'-NH-CO-CH_2-N(CH_3)-C5'). Melting curves for duplexes formed by modified oligonucleotides and natural oligonucleotides complementary to them were measured, and the melting temperatures and thermodynamic parameters of duplex formation were calculated. The introduction of one modified dinucleoside linker into the oligonucleotide only slightly decreases the melting temperatures of these duplexes compared with unmodified ones. The CD spectra of modified duplexes were studied, and their spatial structures are discussed.

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

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INTRODUCTION

The therapy by synthetic analogues of oligonucleotides ("antisense" therapy) is a promising method in the treatment of viral and oncological diseases [1–4].[2] The idea of the method consists in the suppression of gene translation by the specific binding of a synthetic oligonucleotide to the corresponding region of mRNA. Owing to high hybridization specificity, it is possible to selectively suppress the expression of genes involved in the pathogenesis of disease. However, the "antisense" oligonucleotide should possess particular properties, namely: it should be stable to intracellular nucleases and, at the same time, specifically and firmly bind to the target sequence on mRNA; it should readily penetrate the cell membrane, have a low toxicity, and produce no side effects. Over the past 15 years, a great number of oligonucleotide analogues modified at the nucleic acid base, the carbohydrate residue, and the internucleotide bond have been synthesized (see reviews [5–7]). Oligonucleotide analogues wherein the phosphate internucleotide bond is substituted for by an amide linkage have received particular attention of investigators in the last few years. In a natural oligonucleotide, the atoms C3' of the preceding nucleoside and C5' of the next nucleoside are linked through three atoms (C3'-O-P-O-C5'). Therefore, the major efforts of investigators have been directed toward the synthesis of oligonucleotide analogues with amide internucleoside linkages of three atoms [8–16].

There are very few works devoted to the synthesis of amide analogues of oligonucleotides containing internucleoside linkages of four atoms. To date oligonucleotides have been synthesized wherein one or several dinucleotides are replaced by a dinucleoside residue (**A**) [17], (**B**) [18], (**C**) [19–21], (**D**) or (**E**) [20, 21] (Fig. 1). A detailed thermodynamic analysis of the melting of duplexes formed by deoxyoligonucleotides containing one modified dimer with complementary natural ribooligonucleotides has been performed [21]. It was found that the modification (**B**) only slightly decreases the stability of the duplex compared with the natural, and modifications (**C**), (**D**), and (**E**), depending on the sequence, either do not affect the duplex stability or somewhat increase it. The X-ray analysis of a duplex containing one modification (**E**) showed that it does not disturb the stacking interaction of bases and helical parameters of the duplex.

This work is concerned with the synthesis of oligonucleotide analogues containing an amide bond and an aminomethyl group in the internucleoside unit of four atoms [structure (**F**) in Fig. 1] and the study of their physicochemical and hybridization properties.

We assumed that the introduction into the internucleoside bond of an aminomethyl group carrying a positive charge at neutral pH values would increase the stability of a duplex formed by a modified and a natural oligonucleotide due to electrostatic attraction.

RESULTS AND DISCUSSION

The carboxy component (**III**) (scheme) was obtained by the alkylation of 5'-deoxy-5'-methylaminothymidine (**I**) [22] by the ethyl ester of bromoacetic acid with subsequent

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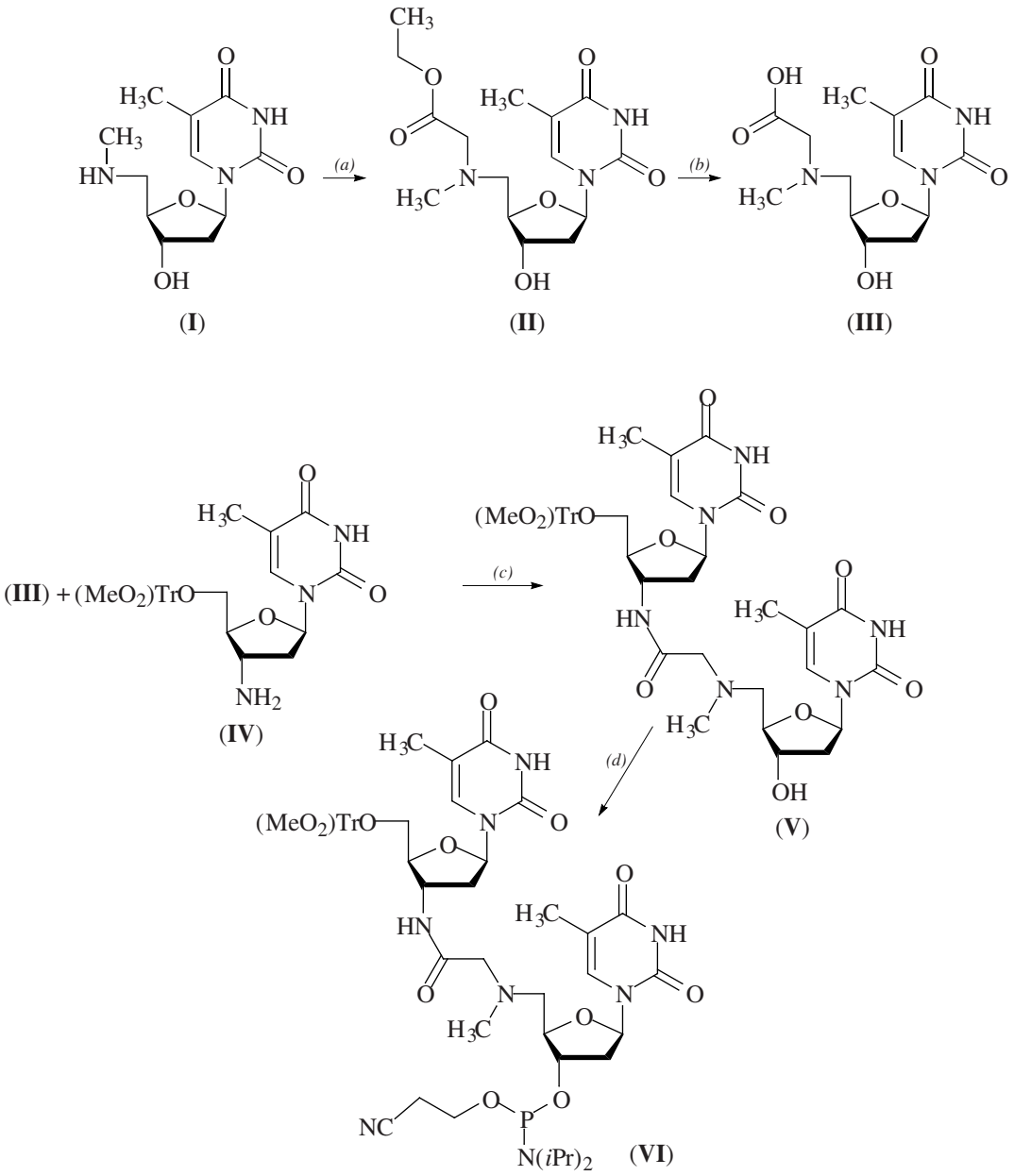
[2] Abbreviations: Im_2CO, carbonyldiimidazole; TEA, triethylamine.

saponification of ester. Protected 3'-deoxy-3'-aminothymidine (**IV**) was synthesized by the method described [23].



(*a*) $BrCH_2COOEt$, TEA, DMF; (*b*) NaOH, Py/EtOH; (*c*) Im_2CO, DMF; (*d*) $NCCH_2CH_2OP(NiPr_2)_2$, tetrazole.

The condensation of the carboxy component (**III**) with the amino component (**IV**) in the presence of Im_2CO gave dinucleoside (**V**). Finally, dinucleoside (**V**) was converted to 2-cyanethyl-*N*,*N*-diisopropyl-phosphoamidite (**VI**) by the method described [22].

The structures of all compounds synthesized were confirmed by ^1H NMR spectra. Thus, the presence of the ethoxycarbonylmethyl group in compound (**II**) was confirmed by the presence in the spectrum of a broadened singlet at 3.355 ppm from the protons of the $N\underline{CH_2}CO$-group (broadening due to diastereotopism of protons) as well as a triplet (1.172 ppm, ^3J = 7.13 Hz) and a quartet (4.063 ppm, ^3J = 7.13 Hz) of the ethyl group. In the spectrum of acid (**III**), the signals from the protons of the ethyl group are absent, with all other signals being retained.

We synthesized both the analogues of oligonucleotides [X is the residue of the modified dinucleoside (**F**)

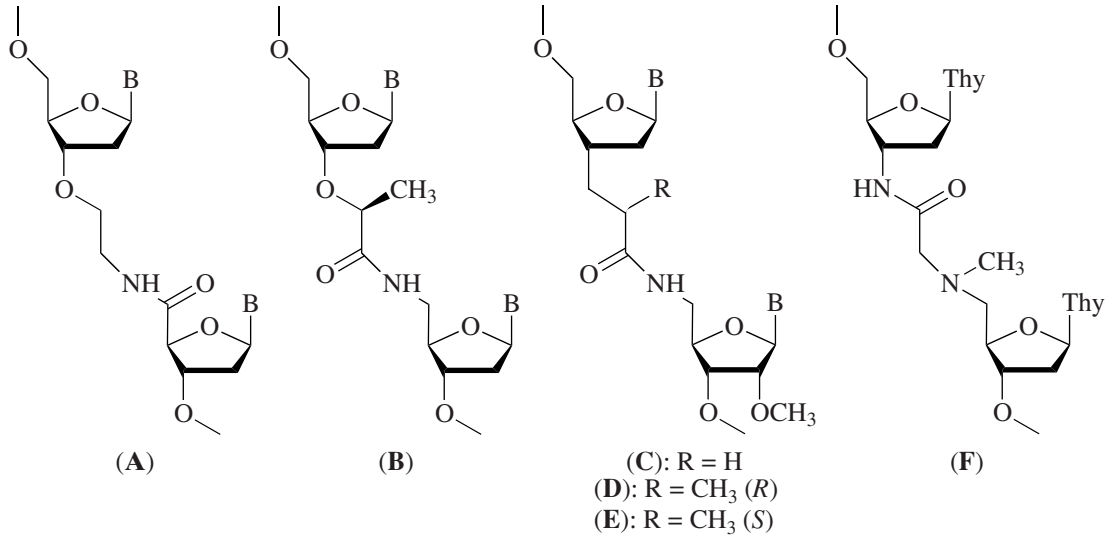


Fig. 1. Dinucleosides with the amide (3 ⟶ 5)-linkage of four atoms.

in Fig. 1] and natural oligonucleotides complementary to them (Table 1).

The structures of all oligonucleotides synthesized were confirmed by mass spectra (Table 1) and by PAGE under denaturing conditions (Fig. 2).

As follows from Fig. 2, the modified oligonucleotides have a lower mobility in the electric field, which is consistent with a decrease in the total negative charge as the phosphate bond is substituted for by the amide.

A comparison of the CD spectra of modified duplexes with those of the corresponding natural duplexes (Fig. 3 and Table 2) enables some conclusions about the structure of the duplexes to be drawn. All duplexes formed by modified oligonucleotides with the complementary natural duplexes, irrespective of the position of the modified bond in the strand and the number of these bonds, have the spectrum shape very similar to that of the corresponding natural oligonucleotide. It is seen from Fig. 3 that the spectrum of a duplex containing even five nonphosphate internucleoside bonds in the modified strand insignificantly differs from the spectrum of the natural duplex. It has been

Table 1. Synthesized oligonucleotides and their mass spectra

Code	Sequence 5' ⟶ 3'	Mass spectra, *m/z* $[M + H]^+$	
		calculated	found
Modified oligonucleotides			
MS1	G-X-TT-TT-TT-TT-G	3629.53	3630
MS2	G-TT-X-TT-TT-TT-G	3629.53	3629
MS3	G-TT-TT-X-TT-TT-G	3629.53	3630
MS4	G-TT-TT-TT-X-TT-G	3629.53	3631
MS5	G-TT-TT-TT-TT-X-G	3629.53	3630
MS6	G-X-TT-TT-TT-X-G	3619.65	3618
MS7	G-X-X-X-X-X-G	3589.99	3591
MS8	G-AA-GT-X-GA-CA-G	3700.60	3701
Natural oligonucleotides			
S1	G-TT-TT-TT-TT-TT-G	3639.43	3639
S2	C-AA-AA-AA-AA-AA-C	3649.51	3549
S3	G-AA-GT-TT-GA-CA-G	3700.48	3703
S4	C-TG-TC-AA-AC-TT-C	3581.40	3581

* X, residue of thymidine dinucleoside with a modified internucleoside linkage [structure (**F**) in Fig. 1].

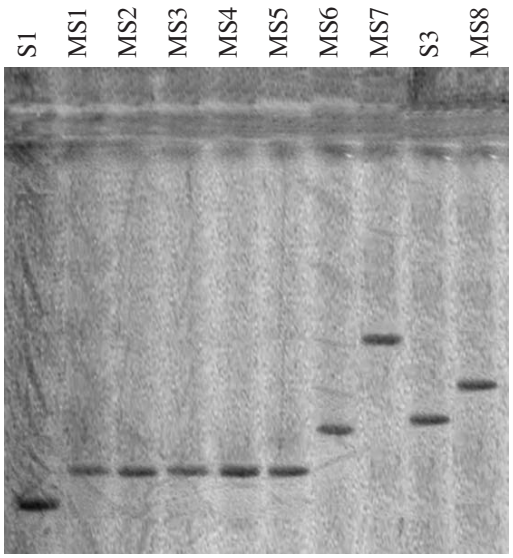


Fig. 2. Gel electrophoresis of oligonucleotides under denaturing conditions. The position of bands was determined from the absorption in UV light (~254 nm) after applying the gel to a fluorescent support (TLC Kieselgel 60 F_{254} plates).

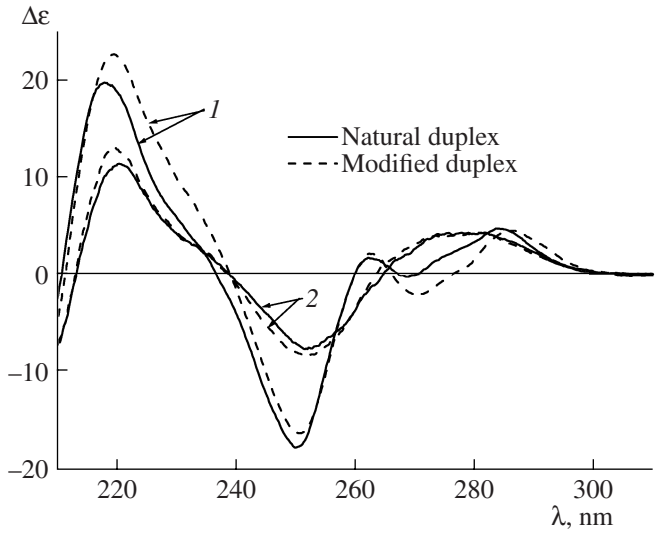


Fig. 3. CD spectra of a natural (**S1·S2**) and a modified duplex (**MS7·S2**) at (*1*) 0°C and (*2*) 70°C (phosphate buffer, pH 7, and 0.1 M NaCl).

shown earlier that such double-humped CD spectra with maxima at ~263 and ~285 nm are characteristic of the B-form of oligo(dT) · oligo(dA)-sequences [24]. Thus, it can be assumed that the modification proposed in this work does not practically distort the structure of the DNA duplex.

The profiles of the thermal dissociation of natural and modified duplexes were measured (Fig. 4*a*). The experimental melting temperatures for duplexes were determined from the derivative maxima after numerical differentiation of experimental melting curves (Fig. 4*b*).

From the curves obtained, the thermodynamic parameters of duplex formation from single strands were calculated (Table 3). In the calculation, the temperature dependence of absorption of a duplex (A_d) and a single strand (A_{ss}) was taken into account. Because the melting temperature for short duplexes depends on concentration, the experimental melting temperatures were recalculated with respect to the standard concentration of strands of 10^{-5} M (column next to the last in Table 3).

As follows from the data in Table 3, one modification decreases the melting temperature of the duplex on the average by 2°C; in this case, the additivity between the decrease in the melting temperature of the duplex and the number of modified bonds is retained within the

Table 2. CD spectra of duplexes at 0°C*

Duplex	λ_{max}, nm (Δε*)	λ_{min}, nm (Δε*)	λ_{max}, nm (Δε*)	λ_{min}, nm (Δε*)	λ_{max}, nm (Δε*)
S1·S2	218.0 (19.6)	250.3 (–17.6)	262.7 (1.5)	268.8 (–0.4)	284.5 (4.7)
MS1·S2	218.7 (21.8)	250.1 (–19.2)	263.1 (2.0)	269.2 (–0.1)	284.5 (6.1)
MS2·S2	218.5 (22.5)	250.6 (–18.2)	263.0 (1.9)	269.1 (–0.2)	284.6 (6.0)
MS3·S2	218.7 (23.4)	250.5 (–17.7)	262.9 (2.1)	269.2 (0.5)	284.9 (5.6)
MS4·S2	218.4 (22.2)	250.4 (–18.1)	262.9 (2.1)	269.1 (0.1)	284.7 (5.2)
MS5·S2	218.8 (22.4)	250.5 (–19.0)	263.0 (1.7)	269.3 (–0.5)	284.8 (5.9)
MS6·S2	218.5 (21.9)	250.2 (–17.9)	262.8 (1.9)	269.3 (–0.2)	284.6 (5.5)
MS7·S2	219.2 (22.6)	250.8 (–16.4)	262.9 (2.2)	270.2 (–2.1)	286.5 (4.4)

* $\Delta\varepsilon = (\varepsilon_L - \varepsilon_R)$ (ε, M^{-1} cm^{-1}).

limits of experimental error (–3.6°C for two and –10.0°C for five bonds).

Thus, the oligonucleotides obtained form a rather stable duplex with complementary natural oligonucleotides, and the structure of this duplex does not substantially differ from the structure of the natural duplex.

EXPERIMENTAL

The following preparations were used: pyridine, chloroform, dimethylformamide (all of chemical grade) (Khimmed, Russia); ethyl ester of bromoacetic acid, thymidine, Im_2CO (Fluka, Switzerland); and dimethoxytrityl chloride (Aldrich, United States). Dimethylformamide was dried by distillation over phosphorus pentoxide, and pyridine, over calcium hydride.

NMR spectra of substances in DMSO-d_6 were measured on a Bruker AMXIII-400 spectrometer with an operating frequency of 400 MHz. Duplex melting curves were recorded on a UV 160-A spectrophotometer (Shimadzu, Japan) equipped with a thermostating accessory. The absorption of a duplex A_{260} was recorded in steps of 0.5°C. CD spectra were measured on a J-715 spectropolarimeter (Jasco, Japan).

Modified and natural oligonucleotides were analyzed by MALDI spectrometry using an MS-30 mass spectrometer (Kratos, Japan).

Oligonucleotides were synthesized by an ASM-102U automatic DNA synthesizer (Biosun, Russia) according to a standard protocol. Oligonucleotides were purified by reverse-phase HPLC using columns (25×4 mm^2) of Hypersil C18 in 0.05 I TEAB buffer. DMTr-protected oligomers were isolated using a gradient of acetonitrile from 10 to 50% in 30 min. Completely unblocked oligonucleotides were additionally purified using a gradient of acetonitrile concentration from 0 to 25% (30 min) in a buffer indicated.

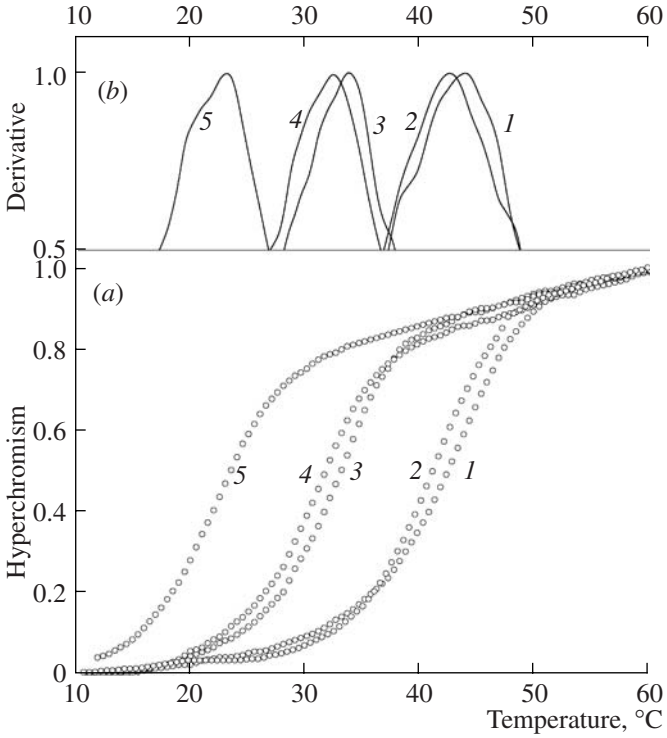


Fig. 4. (*a*) Experimental melting curves for natural duplexes: *1*, **S3·S4** S4; *3*, **S1·S2** S2 and modified duplexes: *2*, **MS8·S4**, *4*, **MS1·S2**; *5*, **MS7·S2**. (*b*) A derivative obtained by numerical differentiation of the experimental curves. For convenience of comparison, hyperchromism and the derivative are normalized to unity.

Denaturing gel electrophoresis of the oligonucleotides was carried out in 20% (acrylamide–bis-acryla-

.

Table 3. Thermodynamic parameters of formation of duplexes from single strands

Duplex	$c \times 10^6$, M	$\Delta H°$, kcal/mol	$\Delta S°$, cal/mol^{-1} K^{-1}	T_{melt}, °C ± 0.5	T_{melt}, °C, reduce*	ΔT_{melt}, °C
S1·S2	4.64	–96.1 ± 2.0	–287.6 ± 6.3	33.8	35.6	
MS1·S2	5.94	–98.1 ± 1.8	–296.3 ± 5.9	32.4	33.6	–2.0
MS2·S2	8.60	–100.7 ± 1.0	–305.0 ± 3.2	32.8	33.1	–2.5
MS3·S2	7.62	–97.5 ± 1.0	–294.4 ± 3.4	32.5	33.1	–2.5
MS4·S2	9.03	–99.3 ± 0.9	–300.3 ± 3.0	32.6	32.8	–2.8
MS5·S2	5.06	–96.9 ± 2.2	–292.5 ± 7.1	32.2	33.8	–1.8
MS6·S2	7.14	–98.8 ± 1.2	–300.0 ± 4.0	31.5	32.2	–3.4
MS7·S2	4.82	–74.7 ± 1.5	–226.3 ± 5.1	23.2	25.4	–10.0
S3·S4	8.82	–86.8 ± 0.6	–249.2 ± 2.0	44.0	44.4	
MS8·S4	10.1	–97.7 ± 1.3	–284.8 ± 4.3	42.6	42.6	–1.8

Notes: * Recalculated with respect to the concentration value of 10^{-5} M.

** Difference between T_{melt} of the modified and natural duplexes.

mide 19 : 1) PAG in 100 mM Tris-borate buffer (pH 7) and 7 M urea.

Column chromatography was carried out using Kieselgel 60 (Merck, Germany). TLC was performed on Kieselgel 60 F_{254} plates (Merck, Germany) in systems (A) ethanol–chloroform 1 : 19, (B) ethanol–chloroform 1 : 4, and (C) *i*PrOH–NH_4OH–H_2O, 7 : 2 : 1.

Phosphoramidite (**VI**) was obtained by the method described in [22] and used without isolation for the synthesis of modified oligonucleotides.

5'-Deoxy-5'-*N*-methyl-*N*-ethoxycarbonylmethylaminothymidine (II). TEA (0.46 ml, 3.3 mmol) and ethyl ester of bromoacetic acid (0.55 g, 0.36 ml, 3.3 mmol) were added with stirring to a solution of 5'-deoxy-5'-methylaminothymidine (**I**) (0.77 g, 3 mmol) [22] in absolute DMF (5 ml), and the mixture was kept overnight at room temperature under stirring. The mixture was diluted with a saturated aqueous solution of $NaHCO_3$ (10 ml) and extracted with chloroform (5 × 10 ml). Chloroform extracts were combined, dried over anhydrous Na_2SO_4, and the solvent was thoroughly evaporated. Compound (**II**) was obtained as a dense oil. Yield: 0.97 g (94.7%); R_f 0.55 (B). ^{1}H NMR: 11.301 (1 H, s, H3), 7.517 (1 H, d, br, *J* 1.05, H6), 6.121 (1 H, t, *J* 6.83, H1'), 4.120 (1 H, m, H3'), 4.063 (2 H, q, *J* 7.13, $\underline{CH_2}CH_3$), 3.795 (1 H, m, H4'), 3.355 (2 H, s, br, $N\underline{CH_2}CO$), 2.746 (1 H, dd, *J* 4.99, *J* 13.45, H5'a), 2.682 (1 H, dd, *J* 7.17, *J* 13.45, H5'b), 2.358 (3 H, s, NCH_3), 2.136 (1 H, m, H2'a), 2.046 (1 H, m, H2'b), 1.787 (3 H, d, *J* 1.06, 5-CH_3), 1.172 (3 H, t, *J* 7.13, $\underline{CH_3}CH_2$).

5'-Deoxy-5'-*N*-methyl-*N*-carboxymethylaminothymidine (III). Six milliliters of 2 M NaOH in ethanol was added under stirring to a solution of nucleoside (**II**) (0.95 g, 2.8 mmol) in pyridine (6 ml) and ethanol (6 ml) cooled to 0°C, the reaction mixture was kept for 30 min at room temperature, and an excess of Dowex 50 (Py-form) (80–100 ml) was added to bind Na ions. The resin was filtered off, washed with 50 ml of a 5% solution of pyridine in H_2O. The solvent was evaporated, and the residue was reevaporated with ethanol (3 × 20 ml). Yield of pyridinium salt (**III**) 0.88 g (80.5%) as a solid foam; R_f 0.32 (C). ^{1}H NMR: 11.234 (1 H, s, H3), 7.546 (1 H, d, br, *J* 0.89, H6), 6.134 (1 H, t, *J* 6.81, H1'), 4.147 (1 H, m, H3'), 3.840 (1 H, m, H4'), 3.263 (2 H, s, br, $N\underline{CH_2}CO$), 2.827 (1 H, dd, *J* 4.79, *J* 13.42, H5'a), 2.765 (1 H, dd, *J* 7.20, *J* 13.42, H5'b), 2.409 (3 H, s, NCH_3), 2.142 (1 H, m, H2'a), 2.063 (1 H, m, H2'b), 1.797 (3 H, d, *J* 0.91, 5-CH_3).

Dinucleoside (V). Acid (**III**) (0.156 g, 0.4 mmol) was evaporated first with absolute pyridine (3 × 3 ml) and then with dry toluene (3 × 3 ml). The residue was dissolved in absolute DMF (2 ml), and Im_2CO (0.081 g, 5 mmol) was added to the mixture under stirring. After 10 min, amine (**IV**) (0.217 g, 0.4 mmol) [23] was added, and the solution was kept under stirring at room temperature overnight. DMF was evaporated, and a saturated $NaHCO_3$ solution (10 ml) was added to the residue and extracted with chloroform. The extracts were washed with a saturated $NaHCO_3$ solution and water, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was dissolved in chloroform-0.1% Et_3N and applied to a column of silica gel (11 × 3.5 cm). The product was eluted with chloroform-EtOH (gradient from 2 to 15% EtOH) with the addition of 0.1% Et_3N. The yield: 0.176 mg (52.4%) as a solid foam; R_f 0.25 (B). ^{1}H NMR: 5'-terminal nucleoside: 11.276 (1 H, s, H3), 7.510 (1 H, d, br, *J* 0.96, H6), 7.41–6.34 (13 H, m, Ar), 6.176 (1 H, t, *J* 6.26, H1'), 4.549 (1 H, m, H3'), 4.103 (1 H, m, H4'), 3.731 (6 H, s, CH_3, Thy), 3.931 (2 H, m, H5'), 2.312 (1 H, m, H2'a), 2.212 (1 H, m, H2'b), 1.471 (3 H, d, *J* 1.01, 5-CH_3); 3'-terminal nucleoside: 11.235 (1 H, s, H3), 7.535 (1 H, d, br, *J* 0.91, H6), 6.114 (1 H, t, *J* 6.77, H1'), 3.930 (1 H, m, H3'), 3.930 (1 H, m, H4'), 3.249 (2 H, s, br, $N\underline{CH_2}CO$), 2.706 (1 H, dd, *J* 4.65, *J* 13.43, H5'a), 2.647 (1 H, dd, *J* 7.39, *J* 13.43, H5'b), 2.249 (3 H, s, NCH_3), 2.146 (1 H, m, H2'a), 2.056 (1 H, m, H2'b), 1.768 (3 H, d, *J* 0.98, 5-CH_3).

EIMS: *m/z* 861.9 $[M + Na]^+$. Calculated: 861.9 ($C_{44}H_{50}N_6O_{11}Na$).

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