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Effect of the Terminal Amino Group of a Linker Arm and Its Length at the C5 Position of a Pyrimidine Nucleoside on the Thermal Stability of DNA Duplexes

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2′-Deoxyuridine derivatives bearing a substituent at the C5-position, which has a different chain length and a different functional group (methyl or amino), were synthesized and incorporated into oligodeoxyribonucleotides. The effect of the substituent groups in the major groove on the stability of the duplexes was investigated by UV melting experiments. It was found that the stabilization of these duplexes by a terminal amino group depended on the length of a linker arm.

Key Words: oligodeoxyribonucleotide; C5-substituted 2′-deoxyuridine; thermal stability.

INTRODUCTION

There is great interest in modified oligodeoxyribonucleotides (ODN) as research tools for molecular biology and antisense molecules. An appropriate chemical modification of ODN leads to improved hybridization properties, nuclease resistance, and cell permeability. To date, various chemically modified ODNs have been synthesized and their ability for the antisense effect has been investigated. The modification at the 5′-end and 3′-end, internucleotide phosphodiester of ODNs, the 1′-position and 2′-position of the sugar, C5 of a pyrimidine, or N^4 of cytosine has been reported (*1*). The C5-position of a pyrimidine is a suitable site for the modification of oligonucleotides. The substituent at C5 of a pyrimidine does not inhibit the base pairing with adenine on a complementary strand, but the thermal stability of the duplex depends on the kind of tethers and functional groups. For example, C5-propagyl substitution of 2′-deoxyuridine conferred significant stability to duplexes and triplexes (*2*). This indicates that their modification should bring about perturbation to the stability of a duplex DNA. In a previous paper (*3*), we reported the effect of acridine-modification at the C5-position on duplex stability. The acridine modification causes stabilization

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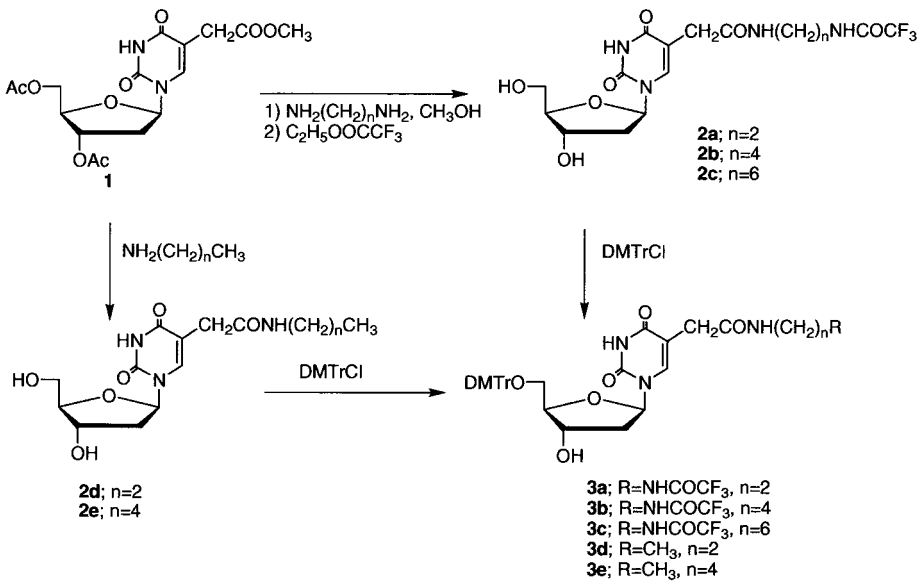
of a duplex-form by interaction between the acridine moiety and DNA, and the length of the spacer arm has a slight effect on the interaction. However, the relationship between the duplex stability and the length of spacer remains to be clarified. The structure of a tether to introduce a functional group on DNA is important for the preparation of hybridization probes or antisense molecules. Here we investigated the effect of the length of a tether between an oligodeoxyribonucleotide and a functional group on the duplex stability. An amino group was attached on the tether as the functional group because the amino group can interact with the DNA backbone and is useful for further functionalization.

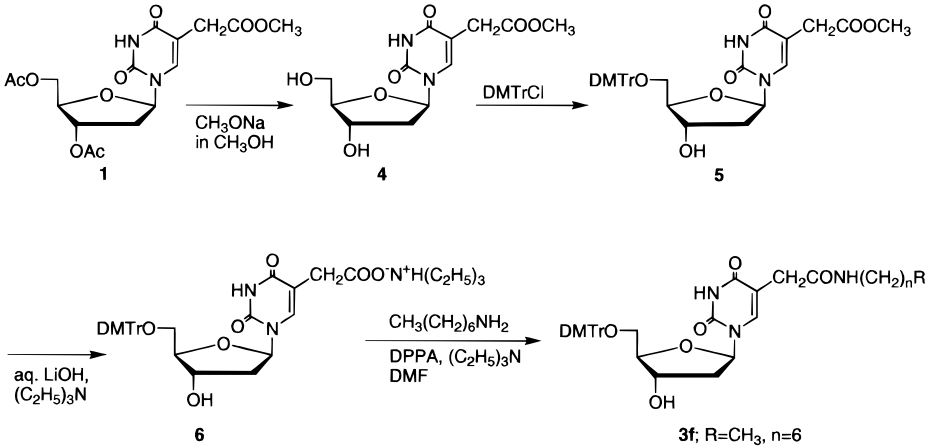
RESULTS AND DISCUSSION

Synthesis of Modified Nucleotides

The C5-Substituted nucleosides bearing an amine linker were prepared from 5-methoxycarbonylmethyl-2′-deoxyuridine (**1**) as described previously (*4*). Most of the amines reacted with **1** in satisfactory yields by a direct aminolysis of a methyl ester group as shown in Scheme 1. In the reactions with diamines, the terminal amino group at the C5-substituent was protected with a trifluoroacetyl group without purification. Heptylamine could not react with **1** by aminolysis because of its low nucleophilicity. Therefore, the corresponding nucleoside was synthesized by another route as shown in Scheme 2. The methoxycarbonyl group in **4** was hydrolyzed after protection of the 5′-hydroxyl group, followed by condensation of the resulting carboxyl group with heptylamine using diphenylphosphoryl azide (DPPA) as a condensing reagent. The 5′-hydroxyl group of the modified nucleosides was protected with a dimethoxytrityl



SCHEME 1.

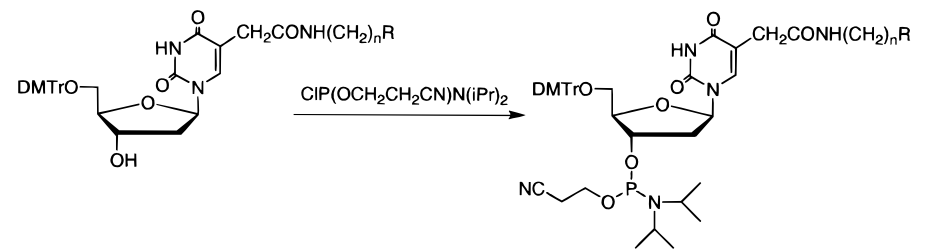


SCHEME 2.

group and all 5′-protected modified nucleosides were converted to nucleoside 3′-phosphoramidites as shown in Scheme 3.

Synthesis of the Modified Oligodeoxyribonucleotides

The modified oligodeoxyribonucleotides (ODNs) were synthesized on a DNA synthesizer. The coupling yields of the modified nucleoside 3′-phosophoramidites on a CPG support are shown in Table 1, with abbreviations and retention times of ODNs on a reversed-phase HPLC. The coupling yields decreased with an increase in the chain length of the C5-substituent, particularly for the nucleosides bearing a terminal methyl group (**7d–f**). The synthesized ODNs were deprotected and purified in the usual manner. The longer retention time on a reversed-phase HPLC column correlates



3a; R=NHCOCF$_3$, n=2:
3b; R=NHCOCF$_3$, n=4:
3c; R=NHCOCF$_3$, n=6:
3d; R=CH$_3$, n=2
3e; R=CH$_3$, n=4
3 f; R=CH$_3$, n=6

7a; R=NHCOCF$_3$, n=2:
7b; R=NHCOCF$_3$, n=4:
7c; R=NHCOCF$_3$, n=6:
7d; R=CH$_3$, n=2
7e; R=CH$_3$, n=4
7 f; R=CH$_3$, n=6

SCHEME 3.

TABLE 1

Abbreviations, Coupling Yields, and Retention Times of Modified ODNs

Abbreviations	X^a	Coupling yield (%)[b]	T_R/min[c]
N-ODN	T	99	16.5[d]
ODN-2N	**2a′**	100	15.8
ODN-4N	**2c′**	97	15.9
ODN-6N	**2e′**	96	16.7
ODN-2C	**2f**	100	18.0
ODN-4C	**2g**	97	21.1
ODN-6C	**2h**	87	26.0

Note. ODN sequence: 5′d(CATAGGAGAXGCCTA)3′.

[a] **2a′**, **2c′**, and **2e′** were the deprotected nucleosides of **2a**, **2c**, and **2d** by an alkaline treatment, respectively.

[b] Coupling yields are for incorporation of the modified nucleosides.

[c] HPLC condition is in the experimental section except the gradient. Gradient: 10% B to 35% B in 35 min.

[d] HPLC condition is in the experimental section except the gradient. Gradient: 3% B to 53% B in 35 min.

with the stronger hydrophobicity of the substituent. The structure of the ODNs was confirmed by a nuclease digestion.

Thermal Stability of the Duplexes Consisted of the Modified ODNs and Its Complementary DNA

Thermal stabilities of the ODN/DNA duplexes were investigated by UV melting experiments. The melting temperatures (T_m) of the duplexes are summarized in Table 2. For the ODN/DNA duplexes, most of the ODNs, except **ODN-2N**, showed lower T_m values than a normal ODN (**N-ODN**). The T_m values decreased with increasing chain length of the side arm, especially for **ODN-2N**, **-4N**, and **-6N**, which has a terminal amino group on the C5-substituent. In comparison of the different terminal groups with the same linker length, the duplexes formed from the ODN bearing a terminal amino group always had higher T_m values than those from the ODN bearing a terminal methyl group. This result suggests that the alkyl linker arm interfered with

TABLE 2

Melting Points and Thermodynamic Parameters of ODN/DNA Duplexes

ODN	T_m(°C)	ΔH^0(kJ mol^{-1})	ΔS^0(J mol^{-1} K^{-1})	ΔG_{37}(kJ mol^{-1})
N-ODN	50.6	−496	−1420	−55.5
ODN-2N	53.7	−516	−1460	−63.1
ODN-4N	49.8	−521	−1500	−55.7
ODN-6N	47.9	−559	−1630	−53.4
ODN-2C	47.6	−526	−1530	−51.4
ODN-4C	46.1	−481	−1390	−49.8
ODN-6C	47.1	−414	−1180	−48.0

Note. Measurement condition under Experimental.

the duplex formation and the terminal amino group stabilized the duplexes. This effect of the amino linker arm on the duplex stability is consistent with our previous results (*5*), which indicated that the stability of the duplexes depends on the number of amino groups in the linker arm and the length of the arm.

To gain detailed information on the stability of duplexes formed from the ODNs, thermodynamic parameters for the duplex formation were estimated from the curve-fitting for each melting curve. The calculation was performed by a nonlinear, least squares calculation program, "TMSPEC," which was developed by Kodama *et al.* (*6*). The results are shown in Table 2. In the ODNs bearing a terminal amino group on the linker arm, ODNs bearing the longer linker arm showed a more negative ΔH, which suggests the stabilization of the duplex, and a more negative ΔS, which suggests the destabilization of the duplexes. Hence, this destabilization is governed by a decrease in ΔS for the ODN bearing the longer linker arm. The ODNs bearing a terminal methyl group and the longer linker arm showed a less negative ΔH and ΔS. Since their duplexes had almost the same T_m values, an unfavorable ΔH of the duplex formation bearing a longer alkyl group was compensated by a favorable ΔS. This suggests that the long alkyl linker destabilizes the duplex in terms of enthalpy, but the long linker with a terminal amino group stabilizes the duplex in terms of enthalpy. In comparison of the different terminal groups bearing the same linker length, the differences between the enthalpy change of a terminal amino group and a methyl group ($\Delta\Delta H^0$), are $+10$ kJ mol^{-1}, for **ODN-2N** and **-2C**, -40 kJ mol^{-1} for **ODN-4N** and **-4C**, and -145 kJ mol^{-1} for **ODN-6N** and **-6C** from Table 2. It is suggested that the duplexes consisting of **ODN-4N** or **-6N**, which have a long linker, were stabilized by the contribution of enthalpy change compared with those consisting of **ODN-4C** or **-6C**, although the alkyl group is disadvantageous for the duplex formation in terms of entropy change. On the other hand, the **ODN-2N**/DNA duplex was stabilized by an increase in ΔS value compared with the **ODN-2C**/DNA. That is to say, the large negative $\Delta\Delta H^0$ in the long linker arms suggests that the terminal amino groups at the side chain interact with moieties of the complementary strand on the DNA duplex. Dande *et al.* reported that the side chain of 5-(aminohexyl)-2′-deoxyuridine was placed at the 3′-side in the major groove could have an electrostatic interaction with an electronegative atom in the major groove (*7*). In their model, the terminal amino group at the linker arm interacted with *O*6 of 5′-side G on the complementary strand. Therefore, in our duplexes it is also estimated that the terminal amino group at the side chain of ODN could interact with electronegative atoms in the major groove such as *O*6 and *N*7 of G-12 and G-13 and *N*7 of A14 in the complementary stand. One of the possible structures of each duplex, which were constructed by modeling of the modified ODN/DNA duplex based on Dande's report, is shown in Fig. 1.

In conclusion, the side chain at the C5-position of a pyrimidine nucleoside could interact with the DNA backbone and affect the duplex stability according to the length of the linker arm. The terminal amino group on the linker arm has a stabilization effect by the entropy for the ODN bearing a short linker and by the enthalpy for the ODN bearing a long linker. This knowledge is useful in the development of DNA probes or antisense molecules.

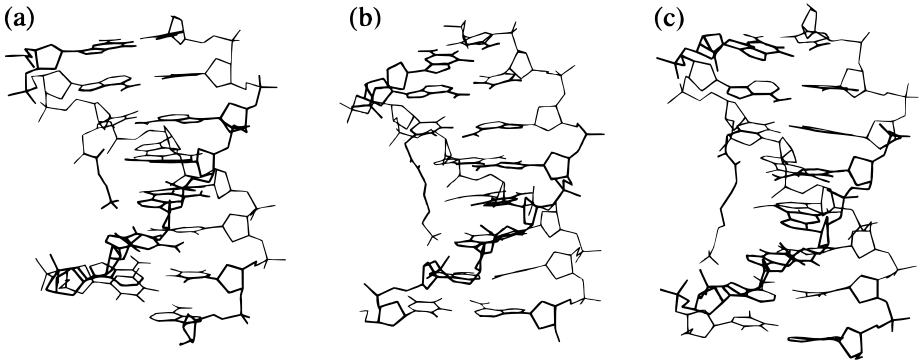


FIG. 1. Parts of possible structures of **ODN-2N**/DNA (a), **ODN-4N**/DNA (b), and **ODN-6N**/DNA (c) duplexes. Eight base-pairs from 3′-side of the modified ODN strand were illustrated.

EXPERIMENTAL

General. Thin-layer chromatography (TLC) was performed on Kieselgel $60F_{254}$ (Art. 5554, E. Merck). Silica-gel column chromatography was performed on Wako gel C-200 (Wako Pure Chemical Industries Ltd., Wako), silica gel 60 (63–200 or 40–63 μm, Merck). High-performance liquid chromatography (HPLC) was carried out on Wakosil 5C18 columns (4 mm i.d. × 250 mm length or 10 mm i.d. × 250 mm length, Wako) by use of a system consisting of JASCO 880-PU pump, 875-UV UV/vis detector, 801-SC system controller, and Shimadzu C-R5A chromatopac. The eluent was acetonitrile gradient in 50 mM triethylammonium acetate (TEAA, pH 7.0). ^{1}H NMR spectra and ^{31}P NMR spectra were obtained with a Varian Gemini 200 or a JEOL α-500 spectrometer. ^{1}H NMR spectra were recorded relative to internal tetramethylsilane and ^{31}P NMR spectra were recorded relative to external 85% H_3PO_4. Mass spectra were measured by a Perkin Elmer Sciex API-100 instrument in ESI mode. Molecular modeling studies were carried out using MacroModel Ver. 6.0 (Schrödinger, Inc.). The AMBER* algorithm (*8*) was used during minimization of oligonucleotide conformations. Oligodeoxyribonucleotides were synthesized by a phosphoramidite chemistry on an Applied Biosystems 381A DNA synthesizer. Normal nucleoside phosphoramidites were purchased from Glen Research. Dichloro(2-cyanoethoxy)phosphine and chloro(diisopropylamino)-2-cyanoethoxyphosphine were prepared by the described method (*9*). 3′,5′-Di-*O*-acetyl-5-(methoxycarbonylmethyl)-2′-deoxyuridine (**1**) was prepared by the method described previously (*4*). The following compounds were also prepared by the previously reported methods (*3,5*): 5-[*N*-(2-trifluoroacetylaminoethyl)]carbamoylmethyl-2′-deoxyuridine (**2a**), 5-[*N*-(4-trifluoroacetylaminobutyl)]carbamoylmethyl-2′-deoxyuridine (**2b**), 5-[*N*-(6-trifluoroacetylaminohexyl)]carbamoylmethyl-2′-deoxyuridine (**2c**), 5′-*O*-(4,4′-dimethoxytrityl)-5[*N*-(2-trifluoroacetylaminoethyl)]carbamoylmethyl-2′-deoxyuridine (**3a**), 5′-*O*-(4,4′-dimethoxytrityl)-5-[*N*-(6-trifluoroacetylaminohexyl)]carbamoylmethyl-2′-deoxyuridine (**3c**), 3′-*O*-[(2-cyanoethyl)(diisopropylamino)]phosphino-5′-*O*-(4,4′-dimethoxytrityl)-5-[*N*-(2-trifluoroacetylaminoethyl)]carbamoylmethyl-2′-deoxyuridine

(**7a**), 3′-*O*-[(2-cyanoethyl)(diisopropylamino)]phosphino-5′-*O*-(4,4′-dimethoxytrityl)-5-[*N*-(6-trifluoroacetylaminohexyl)]carbamoylmethyl-2′-deoxyuridine (**7c**). Snake venom phosphodiesterase (SVPD) was purchased from Worthinton. Nuclease P1 was purchased from Yamasa Co. Alkaline phosphatase (AP) was purchased from Boehringer Mannheim BmbH. All other reagents were purchased from Wako or Kanto Chemical Co., Inc. All organic solvents for reactions were dried and distilled in the usual manner.

*5-(N-Propyl)carbamoylmethyl-2′-deoxyuridine (**2d**).* A mixture containing **1** (0.500 g, 1.30 mmol), propylamine (1.29 mL, 15.6 mmol), and 4-dimethylaminopyridine (0.020 g, 0.16 mmol) in methanol (5 mL) was stirred at 50°C for 23 h. After the reaction solution was evaporated and coevaporated with methanol, the residue was dissolved in a small amount of methanol and added dropwise to benzene to precipitate 5-(*N*-propyl)carbamoylmethyl-2′-deoxyuridine (**2d**) as an oily residue. Further, the purified product from the supernatant was obtained by silica-gel column chromatography using 15% methanol in dichloromethane as an eluent. Yield of **2d** was 86.2% (0.367 g). ^{1}H NMR (CD_3OD) δ 7.89 (s, 1H, H6), 6.27 (t, 1H, H1′, J = 6.6 Hz), 4.38 (m, 1H, H3′), 3.90 (m, 1H, H4′), 3.75 (m, 2H, H5′), 3.19 (m, 2H, C5–CH_2–), 3.12 (t, 2H, –NH$\underline{CH_2}$–, J = 7.2 Hz), 2.26 (m, 2H, H2′), 1.51 (q, 2H, –$CH_2\underline{CH_2}CH_3$, J = 7.2 Hz), 0.91 (t, 3H, –CH_3, J = 7.5 Hz); Mass *m*/*z* 328.3 ($[M + H]^+$, 328.2 Calcd for $C_{14}H_{22}N_3O_6$).

*5-(N-Pentyl)carbamoylmethyl-2′-deoxyuridine (**2e**).* **2e** was similarly prepared from **1** and 1-aminopentane. Yield 83.5%. ^{1}H NMR (D_2O) δ 7.75 (s, 1H, H6), 6.26 (t, 1H, H1′, J = 6.6 Hz), 4.42 (q, 1H, H3′, J = 5.2 Hz), 3.99 (m, 1H, H4′), 3.73 (m, 2H, H5′), 3.66 (m, 2H, C5–CH_2–), 3.13 (t, 2H, –NH$\underline{CH_2}$–, J = 6.7 Hz), 2.35 (m, 2H, H2′), 1.48–1.18 (m, 6H, –$(CH_2)_3$–), 0.82(t, 3H, –CH_3, J = 6.7 Hz); Mass *m*/*z* 356.2 ($[M + H]^+$, 356.2 Calcd for $C_{16}H_{26}N_3O_6$).

*5′-O-(4,4′-Dimethoxytrityl)-5-[N-(4-trifluoroacetylaminobutyl)]carbamoylmethyl-2′-deoxyuridine (**3b**).* **2b** (0.354 g, 0.780 mmol) was reacted with 4,4′-dimethoxytrityl chloride (DMTr-Cl, 0.317 g, 0.94 mmol) in pyridine (4.0 mL) containing 4-dimethylaminopyridine (10 mg, 0.082 mmol) at room temperature for 10 h. The reaction mixture was poured into cold water and extracted 5 times with dichloromethane (5 × 50 mL). After the organic layer was dried with anhydrous sodium sulfate, the solvent was removed by evaporation and coevaporation with toluene. The product was purified by silica-gel column chromatography using 5–8% methanol in dichloromethane containing 0.2% triethylamine as an eluent. The appropriate fractions were collected, evaporated, and precipitated with a small amount of dichloromethane into hexane to give white precipitates, **3b**. Yield of **3b** was 45.0% (0.266 g). ^{1}H NMR ($CDCl_3$) δ 9.09 (br.s, 1H, imido-NH), 7.75 (s, 1H, H6), 7.38–6.81 (m, 13H, Ar), 6.25 (t, 1H, H1′, J = 5.1 Hz), 4.56 (m, 1H, H3′), 4.00 (m, 1H, H4′), 3.78 (s, 6H, CH_3O–), 3.38–3.09 (m, 8H, H5′, –$NHCH_2$– × 2, and C5–CH_2–), 2.36 (m, 2H, H2′), 1.57–1.26 (m, 8H, –$(CH_2)_2$–); ^{13}C NMR ($CDCl_3$) δ 169.93 (C = O), 164.03 (C4), 158.67 (DMTr), 157.44 (J = 37 Hz, *C*OCF3), 150.27 (C2), 144.29 (DMTr), 138.86 (C6), 135.54 (DMTr), 135.35 (DMTr), 130.08 (DMTr), 128.11 (DMTr), 127.14 (DMTr), 115.99 (J = 288 Hz, CO*C*F3), 113.37 (DMTr), 109.02 (C5), 86.93 (DMTr), 86.15 (C4′), 84.95 (C1′), 72.24 (C3′), 63.48 (C5′), 55.27 (OCH_3 in DMTr), 41.12 (C2′),

39.47 (CH_2), 38.85 (CH_2), 34.53 (C5–CH_2), 26.57 (CH_2), 25.53 (CH_2); Mass *m/z* 753.5 ($[M - H]^-$, 753.3 Calcd for $C_{35}H_{40}F_3N_4O_9$).

*5′-O-(4,4′-Dimethoxytrityl)-5-(N-propyl)carbamoylmethyl-2′-deoxyuridine (**3d**).* Protection of the 5′-OH of **2d** with 4,4′-dimethoxytrityl group was carried out in a similar manner. Yield of **3d** was 53.9%; ^{1}H NMR ($CDCl_3$) δ 8.14 (br.s, 1H, imido-NH), 7.65 (s, 1H, H6), 7.43–6.85 (m, 13H, Ar), 6.31 (t, 1H, H1′, J = 6.7 Hz), 4.54 (m, 1H, H3′), 3.99 (m, 1H, H4′), 3.79 (s, 6H, CH_3O–), 3.40 (d, 2H, H5′, J = 3.7 Hz), 3.11 (dd, 2H, C5–CH_2–, J = 6.8 and 13.1 Hz), 2.72 (m, 2H, –NHC$\underline{H}_2$–), 2.37 (m. 2H, H2′), 1.98 (d, 1H, –CON$\underline{H}$CH2–, J = 3.7 Hz), 1.45 (m, 2H, –NHC$\underline{H}_2$C$\underline{H}_2$–), 0.87 (t, 3H, CH_3–, J = 7.4 Hz); ^{13}C NMR ($CDCl_3$) δ 169.59 (C = O), 163.95 (C4), 158.56 (DMTr), 150.29 (C2), 144.45 (DMTr), 138.59 (C6), 135.42 (DMTr), 130.06 (DMTr), 128.00 (DMTr), 126.97 (DMTr), 113.27 (DMTr), 109.17 (C5), 86.75 (DMTr), 85.99 (C4′), 84.92 (C1′), 71.99 (C3′), 63.59 (C5′), 55.19 (OCH3 in DMTr), 41.42 (CH_2), 40.75 (C2′), 34.51 (C5–CH_2), 22.53 (CH_2), 11.32 (CH_3); Mass *m/z* 630.4 ($[M + H]^+$, 630.28 Calcd for $C_{35}H_{40}N_3O_8$).

*5′-O-(4,4′-Dimethoxytrityl)-5-(N-pentyl)carbamoylmethyl-2′-deoxyuridine (**3e**).* Protection of the 5′-OH of **2e** with 4,4′-dimethoxytrityl group was carried out in a similar manner. Yield of **3e** was 16.4%; ^{1}H NMR ($CDCl_3$) δ 8.05 (br.s, 1H, imido-NH), 7.65 (s, 1H, H6), 7.43–6.75 (m, 13H, Ar), 6.32 (t, 1H, H1′, J = 6.9 Hz), 4.53 (m, 1H, H3′), 3.99 (m, 1H, H4′), 3.79 (s, 6H, CH_3O–), 3.39 (d, 2H, H5′, J = 3.7 Hz), 3.12 (dd, 2H, C5–CH_2–, J = 6.9 and 13.0 Hz), 2.71 (m, 2H, –NHC$\underline{H}_2$–), 2.35 (m. 2H, H2′), 1.92 (d, 1H, –CON$\underline{H}$CH_2–, J = 3.7 Hz), 1.42 (m, 2H, –NHCH_2C$\underline{H}_2$–), 1.26 (m, 4H, –$(CH_2)_2$–), 0.87 (t, 3H, CH_3–, J = 6.7 Hz); ^{13}C NMR ($CDCl_3$) δ 169.06 (C = O), 165.81 (C4), 158.70 (DMTr), 149.54 (C2), 144.43 (DMTr), 138.24 (C6), 135.47 (DMTr), 130.07 (DMTr), 128.06 (DMTr), 127.09 (DMTr), 113.36 (DMTr), 109.28 (C5), 86.55 (DMTr), 85.67 (C4′), 84.86 (C1′), 72.23 (C3′), 63.49 (C5′), 55.25 (OCH3 in DMTr), 40.71 (C2′), 39.70 (CH_2), 34.75 (C5–CH_2), 29.07 (CH_2), 29.00 (CH_2), 22.29 (CH_2), 13.97 (CH_3); Mass *m/z* 656.65 ($[M - H]^-$, 656.30 Calcd for $C_{37}H_{42}N_3O_8$).

*5-Methoxycarbonylmethyl-2′-deoxyuridine (**4**).* A mixture of 3′,5′-di-*O*-acetyl-5-(methoxycarbonylmethyl)-2′-deoxyuridine (**1**, 512 mg, 1.30 mmol) and sodium methoxide (84 mg, 1.56 mmol) was stirred at room temperature for 2 h. The reaction mixture was evaporated, redissolved in water, and passed through a cation-exchange resin (Diaion SKB1B, Mitsubishi Chemical Corp.). The effluent was evaporated to dryness and the product was recrystallized from chloroform–ethanol. Yield of **4** was 79.9% (0.312 g); ^{1}H NMR (D_2O) δ 7.74 (s, 1H, H6), 6.19 (t, 1H, H1′, J = 6.8 Hz), 4.36 (q, 1H, H3′, J = 5.1 Hz), 3.94 (q, 1H, H4′, J = 4.2 Hz), 3.69 (m, 2H, H5′), 3.62 (s, 3H, –OCH_3), 3.35 (s, 2H, C5–CH_2–), 2.30 (m, 2H, H2′); Mass *m/z* 301.3 ($[M + H]^+$, 301.1 Calcd for $C_{12}H_{17}N_2O_7$).

*5′-O-(4,4′-Dimethoxytrityl)-5-methoxycarbonylmethyl-2′-deoxyuridine (**5**).* **4** (0.5 g, 1.67 mmol) was reacted with 4,4′-dimethoxytrityl chloride (DMTr-Cl, 0.691 g, 2.404 mmol) in pyridine (3.0 mL) containing 4-dimethylaminopyridine (10 mg, 0.082 mmol) at room temperature for 13 h. The reaction mixture was poured into cold water and extracted five times with dichloromethane (5 × 50 mL). After the organic layer was dried with anhydrous sodium sulfate, the solvent was removed by evaporation

and coevaporation with toluene. The product was purified by silica-gel column chromatography using 8% methanol in dichloromethane containing 0.2% triethylamine as an eluent. The appropriate fractions were collected, evaporated, and precipitated with a small amount of dichloromethane into hexane to give white precipitates, **5**. Yield of **5** was 80.8% (0.813 g). ^{1}H NMR ($CDCl_3$) δ 7.76 (s, 1H, H6), 7.35–6.81 (m, 13H, Ar), 6.44 (t, 1H, H1′, J = 6.7 Hz), 4.60 (m, 1H, H3′), 4.05 (m, 1H, H4′), 3.79 (s, 6H, CH_3O–), 3.58 (s, 3H, CH_3O–), 3.43 (m, 2H, H5′), 2.71–2.39 (m, 4H, H2′ and C5–CH_2–); Mass *m*/*z* 603.2 ([M + H]$^+$, 603.2 Calcd for $C_{33}H_{35}N_2O_9$).

*5′-O-(4,4′-Dimethoxytrityl)-5-carboxymethyl-2′-deoxyuridine triethylammonium salt (**6**).* 1 M aq. Lithium hydroxide (9.0 mL) was added to a solution of **5** (0.540 g, 0.90 mmol) in 0.5 mL of methanol. After the solution was stirred at room temperature for 1 h, the reaction mixture was applied on a cation-exchange resin (Dowex 50W-X8, 100–200 mesh, triethylammonium form) and washed by water. The product was eluted by 1% aq. thriethylamine solution and the effluent was evaporated to dryness. Yield of **6** was 83% (0.510 g); ^{1}H NMR ($CDCl_3$) δ 7.44 (s, 1H, H6), 7.41–6.79 (m, 13H, Ar), 6.30 (t, 1H, H1′, J = 6.6 Hz), 4.42 (m, 1H, H3′), 3.96 (m, 1H, H4′), 3.75 (s, 6H, CH_3O–), 3.33 (m, 2H, H5′), 2.99–2.75 (m. 8H, ($\underline{CH_2}$ CH_3)$_3$ and C5–CH_2–), 2.30 (m, 1H, H2′) 1.16 (t, 9H, –($CH_2\underline{CH_3}$)$_3$, J = 7.2 Hz); Mass *m*/*z* 690.3 ([M − H]$^-$, 690.3 Calcd for $C_{38}H_{48}N_3O_9$).

*5′-O-(4,4′-Dimethoxytrityl)-5-(N-heptyl)carbamoylmethyl-2′-deoxyuridine (**3f**).* A solution of **6** (0.20 g, 0.29 mmol), heptylamine (0.126 mL, 0.86 mmol), diphenylphosphoryl azide (0.182 mL, 0.86 mmol), and triethylamine (0.118 ml) in DMF (3.0 mL) was stirred at 0°C for 3 h and then stirred at room temperature for an additional 3 days. The reaction mixture was evaporated and coevaporated with methanol. The residue was redissolved in ethyl acetate, washed with 5% aq. sodium bicarbonate and sat. aq. sodium chloride, and the organic layer was dried over anhydrous sodium sulfate. The product was purified by silica-gel column chromatography using 8% methanol in dichloromethane containing 0.2% triethylamine as an eluent. The appropriate fractions were collected, evaporated, and precipitated with a small amount of dichloromethane into hexane to give white precipitates, **3f**. Yield of **3f** was 60.3% (0.120 g). ^{1}H NMR ($CDCl_3$) δ 8.20 (br.s., 1H, imido-NH), 7.66 (s, 1H, H6), 7.43–6.85 (m, 13H, Ar), 6.32 (t, 1H, H1′, J = 6.6 Hz), 4.54 (m, 1H, H3′), 3.99 (m, 1H, H4′), 3.79 (s, 6H, CH_3O–), 3.39 (d, 2H, H5′, J = 3.6 Hz), 3.12 (dd, 2H, C5–CH_2–, J = 7.1 and 12.7 Hz), 2.72 (m, 2H, –NHC$\underline{H_2}$–), 2.35 (m, 2H, H2′), 2.04 (d, 1H, –CON$\underline{H}$CH2–, J = 3.7 Hz), 1.42 (m, 2H, –NHCH$_2$C$\underline{H_2}$–), 1.24 (m 8H, –(CH_2)$_4$–), 0.86 (t, 3H, CH_3–, J = 6.5 Hz); ^{13}C NMR ($CDCl_3$) δ 169.29 (C = 0), 163.61 (C4), 158.65 (DMTr), 149.96 (C2), 144.49 (DMTr), 138.39 (C6), 135.53 (DMTr), 130.08 (DMTr), 128.02 (DMTr), 127.04 (DMTr), 113.33 (DMTr), 109.26 (C5), 86.86 (DMTr), 85.84 (C4′), 84.88 (C1′), 72.13 (C3′), 63.56 (C5′), 55.24 (OCH3 in DMTr), 40.73 (C2′), 39.77 (CH_2), 34.66 (C5–CH_2), 31.71 (CH_2), 29.37 (CH_2), 28.90 (CH_2), 26.82 (CH_2), 22.55 (CH_2), 14.05 (CH_3); Mass *m*/*z* 684.65 ([M − H]$^-$, 684.3 Calcd for $C_{39}H_{46}N_3O_8$).

*3′-O-[(2-Cyanoethyl)(diisopropylamino)]phosphino-5′-O-(4,4′-dimethoxytrityl)-5-[N-(3-tri-fluoroacetylaminobutyl)]carbamoylmethyl-2′-deoxyuridine (**7b**).* Chloro-(2-cyanoethoxy)diisopropylaminophosphine (0.13 mL, 0.57 mmol) was added dropwise to a solution of **3b** (0.200 g, 0.260 mmol) in dry dichloromethane (5 mL)

containing *N*-ethyl-*N*,*N*-diisopropylamine (0.11 mL, 0.65 mmol) under Ar atmosphere at room temperature. After this mixture was stirred for 0.5 h, ethyl acetate (prewashed with 5% aq. sodium hydrogencarbonate solution) was added to the reaction mixture, the solution was washed with 5% aq. sodium hydrogencarbonate solution and sat. aq. sodium chloride, dried with anhydrous sodium sulfate, and evaporated to dryness. The crude product was purified by silica-gel column chromatography using ethyl acetate/methanol/triethylamine (85/5/10, v/v/v) as an eluent. The appropriate fractions were collected, evaporated, and precipitated with a small amount of dichloromethane into hexane to give precipitates. Yield of **7b** was 73.1% (0.185 g); ^{31}P NMR ($CDCl_3$) δ 149.44 and 149.55 ppm.

*3′-O-[(2-Cyanoethyl)(diisopropylamino)]phosphino-5′-O-(4,4′-dimethoxytrityl)-5-(N-propyl)carbamoylmethyl-2′-deoxyuridine (**7d**).* The phosphitylation of **3d** was carried out in the same method as described above. Yield 30%; ^{31}P NMR ($CDCl_3$) δ 149.31 and 149.63 ppm.

*3′-O-[(2-Cyanoethyl)(diisopropylamino)]phosphino-5′-O-(4,4′-dimethoxytrityl)-5-(N-pentyl)carbamoylmethyl-2′-deoxyuridine (**7e**).* The phosphitylation of **3e** was carried out in the same method as described above. Yield 36%; ^{31}P NMR ($CDCl_3$) δ 149.32 and 149.66 ppm.

*3′-O-[(2-Cyanoethyl)(diisopropylamino)]phosphino-5′-O-(4,4′-dimethoxytrityl)-5-(N-heptyl)carbamoylmethyl-2′-deoxyuridine (**7f**).* The phosphitylation of **3f** was carried out in the same method as described above. Yield 64.9% ^{31}P NMR ($CDCl_3$) δ 149.31 and 149.66 ppm.

Oligodeoxyribonucleotide synthesis. Oligodeoxyribonucleotides and their analogs were prepared using normal phosphoramidite coupling procedure on a DNA synthesizer. The oligodeoxyribonucleotides bearing the C5-substituted 2′-deoxyuridine in place of thymidine were synthesized along with the normal and complementary oligodeoxyribonucleotides. The modified nucleoside phosphoramidite was incorporated into the oligodeoxyribonucleotides at the appropriate position by using the normal synthetic cycle except that the reaction time for the coupling step was 6 min. For the synthesis of an oligodeoxyribonucleotide containing the modified base (**ODN-2N, ODN-4N, ODN-6N, ODN-2C, ODN-4C,** or **ODN-6C**), normal deoxyribonucleoside phosphoramidites and modified deoxyribonucleoside phosphoramidite (**7a**, **7c**, **7e**, **7f**, **7g**, or **7h**) were used. The deprotection and cleavage from CPG support was carried out by treatment with conc. aq. ammonia solution at 50°C for 18 h. All modified oligodeoxyribonucleotides with 5′-(4,4′-dimethoxytrityl) group were isolated by reversed-phase HPLC on a Wakosil 5C18 column (10 mm i.d. × 250 mm length) using 50 mM TEAA (pH 7.0) with a gradient of 15.0–40.0% acetonitrile in 25 min. The isolated compound was treated with 10% acetic acid by the usual procedure to remove a 4,4′-dimethoxytrityl group followed by desalting on a Sephadex G-25 column. The modified oligodeoxyribonucleotides were further purified by reversed-phase HPLC after deprotection of 5′-(4,4′-dimethoxytrityl) group. Isolated yields: **ODN-2N**, 3.6% **ODN-4N**, 2.6%; **ODN-6N**, 4.8% **ODN-2C**, 2.4%; **ODN-4C**, 11.5%; **ODN-6C**, 4.8%.

Nuclease digestion of oligodeoxyribonucleotides containing the modified base. The modified oligomers (ca. 0.5 $OD_{260\ nm}$) were treated overnight with snake venom phosphodiesterase (0.5 units) and alkaline phosphatase (1 units) in 20 mM Tris–HCl

(pH 8.0) containing 10 mM $MgCl_2$ at 37°C. To the reaction mixtures, nuclease P1 (ca. 4 units) and 0.1 M sodium acetate (pH 4.75) were added, then the reaction mixtures were incubated at 37°C. The reaction mixtures were analyzed by reversed-phase HPLC. The nucleoside composition ratios were calculated from areas of the peaks in the HPLC chart.

T_m measurements. UV absorbance was measured with a Hitachi UV-3000 spectrophotometer equipped with a Hitachi SPR-10 temperature controller. The solution temperature in a cuvette was measured directly with a temperature data collector AM-7002 (Anritsu Meter Co., Ltd.). Absorbance and temperature data were recorded on an NEC PC-9821 personal computer. The rate of heating or cooling was 0.5°C/min. T_m values were obtained in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride at a duplex concentration of 2×10^{-6} M. T_m values were determined by the described method (*10*) from the absorbance vs temperature plot using an Igor graphing and data analysis program (WaveMatrics, Inc.) and a Power-Macintosh 8500 computer (Apple Computer, Inc.).

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