Novel C5-Substituted 2′-Deoxyuridine Derivatives Bearing Amino-Linker Arms: Synthesis, Incorporation into Oligodeoxyribonucleotides, and Their Hybridization Properties

Hiroaki Ozaki, Akiko Nakamura, Midori Arai, Masakazu Endo, and Hiroaki Sawai*

Department of Chemistry, Faculty of Engineering, Gunma University, 1-5-1 Tenjin-cho, Kiryu, Gunma 376

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2′-Deoxyuridine derivatives bearing several kinds of amino-linker arms at C5 position were synthesized from 5-(methoxycarbonylmethyl)-2′-deoxyuridine and ethylenediamine, 1,6-hexanediamine, or tris(2-aminoethyl)amine. The modified nucleosides were incorporated into oligodeoxyribonucleotides at one or three positions in place of thymidine residues. The thermal stability of the duplexes was investigated. Three incorporations of ethylenediamine or tris(2-aminoethyl)amine at the C5-position increase the duplex stability. The amino-linker arm affected the stability of the duplexes depending on the number of amino groups in the linker arm and the length of the arm. The linker arm improved the nuclease resistance at 5′-side phosphodiester linkage of the modified nucleoside in oligodeoxyribonucleotides.

Modified oligonucleotides have become an important tool as antisense agents and nucleic acid hybridization probes for diagnostic and therapeutic applications and for genetic uses.[1] Their modifications have been introduced at the base, sugar, and phosphate moieties to improve nuclease resistance, thermal stability of duplex or triplex form, and cellular uptake.[2] The nucleoside analogs have an advantage of multiple modifications at any position in oligonucleotides in comparison with only 5′- or 3′-end modification. C5-position of pyrimidine is an attractive position for the modification because C5 is located at the major groove surface in the duplex form and will not directly inhibit the hydrogen bond in an A : T base pair.

But it has been reported that the incorporation of some alkyl groups destabilized the duplex form while that of others stabilized.[3—6] For example, the incorporation of 5-[*N*-(2-aminoethyl)propionamido]-2′-deoxyuridine results in a destabilization of duplex formation by ca. 1 kcal mol^{-1} in $\Delta G°$ for an octanucleotide.[3] Hashimoto et al. have reported that a hexyl group at C5-position of 2′-deoxyuridine caused a duplex destabilization (4.5 °C decrease in Tm for undecamer containing one hexyl group) but a 6-aminohexyl group had little effect on duplex stability.[4] On the other hand, it has been reported that the incorporation of 5-[[*N*-(2-aminoethyl)]carbamoyl]-2′-deoxyuridine and 5-[[*N*-(6-aminohexyl)]carbamoyl]-2′-deoxyuridine into hexadecamers composed of 5-methyl-2′-deoxycytidine and thymidine led to a stabilization of duplex formations by 4—6 °C in Tm.[5] Interestingly, a pentadecadeoxyribonucleotide containing 5-(1-propynyl)-2′-deoxyuridine increased the duplex stability with the complementary oligoribonucleotide by 1.7 °C/substitution in Tm.[6]

Recently, we developed a new and simple synthetic route to novel C5-substituted pyrimidine nucleosides.[7] 5-(Methoxycarbonylmethyl)-2′-deoxyuridine, one of the products obtained from our synthetic method, is a useful intermediate to introduce a linker arm at the C5-position through amide linkage. Here we report the synthesis of 2′-deoxyuridine derivatives bearing several kinds of amino-linker arms, which derived from ethylenediamine, 1,6-hexanediamine, and tris(2-aminoethyl)amine, and their incorporation into oligodeoxyribonucleotides. The amino-linker arm affected the stability of the duplexes depending on the number of substitutions, the number of amino groups, and the length of its arm.

Experimental

Materials. Thin-layer chromatography (TLC) was performed on Kiesel gel 60F$_{254}$ (Art. 5554, E. Merck). Silica-gel column chromatography was performed on Wako gel C-200 (Wako Pure Chemical Industries Ltd. (Wako)). High-performance liquid chromatography (HPLC) was carried out on Wakosil 5C18 columns (4 mmϕ×250 mm length or 10 mmϕ×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 kindly measured by a staff member of the Research Institute of Kirin Brewery Co. with a Hitachi M-80-B instrument in FD mode. Oligodeoxyribonucleotides were synthesized by a phosphoramidite chemistry on an Applied Biosystems 381A DNA synthesizer. Normal nucleoside phosphoramidites were purchased from Applied Biosystems or Wako. Dichloro(2-cyanoethoxy)phosphine was prepared by the described method.[8] 3′,5′-Di-*O*-acetyl-5-(methoxycarbonylmethyl)-2′-deoxyuridine (**1**) was prepared by the method described previously.[7] Snake venom phosphodiesterase (SVPD) was purchased from Worthinton. Nuclease P1 was 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 by the usual manner.

Synthesis of 5-(*N*-Substituted Carbamoylmethyl)-2′-deoxyuridine (3a′—c′). 5-[*N*-(2-Trifluoroacetylaminoethyl)]carbamoylmethyl-2′-deoxyuridine (3a′): A mixture containing **1** (0.568 g, 1.48 mmol), ethylenediamine (1.19 mL, 17.7 mmol), and 4-dimethylaminopyridine (0.01 g, 0.082 mmol) in methanol (2 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 ether or benzene to precipitate 5-[*N*-(2-aminoethyl)]carbamoylmethyl-2′-deoxyuridine (**2a**) as an oily residue. Then the terminal amino group of **2a** was protected by trifluoroaçetyl group without further purification. Ethyl trifluoroacetate (0.5 mL, 4.18 mmol) was added dropwise to a solution of the crude product (**2a**) in methanol (2 mL) containing 4-dimethylaminopyridine (0.012 g, 0.098 mmol) and the reaction mixture was stirred at room temperature for 2 d. The resulting white precipitates were collected by filtration. Further, the purified product was obtained by silica-gel column chromatography using 15% methanol in dichloromethane as an eluent. Yield of **3a′** was 92.5% (0.581 g) from **1**. High purity samples for the analyses were obtained from recrystallization in ethanol. Mp >220 °C (decomp). ^{1}H NMR (D_2O) δ=7.82 (s, 1H, H6), 6.31 (t, 1H, J=6.6 Hz, H1′), 4.48 (m, 1H, H3′), 4.06 (m, 1H, H4′), 3.81 (m, 2H, H5′), 3.44 (m, 4H, –CH_2CH_2–), 3.30 (s, 2H, C5–CH_2–), 2.42 (m, 2H, H2′); UV (H_2O, pH 7.0) λ_{max} 266.5 nm (ε 8900 $M^{-1}cm^{-1}$) (M=mol dm^{-3}); MS Found: m/z 425. Calcd for $C_{15}H_{20}F_3N_4O_7$: MH^+, 425.34. Anal. Found: C, 41.33; H, 4.47; N, 12.54%. Calcd for $C_{15}H_{19}F_3N_4O_7 \cdot 1/2H_2O$: C, 41.58; H, 4.65; N, 12.93%.

5-[*N*-(6-Trifluoroacetylaminohexyl)]carbamoylmethyl-2′-deoxyuridine (3b′): **3b′** was similarly prepared from **1** and 1,6-hexanediamine. Yield of **3b′** was 90% from **1**. High purity samples for the analyses were obtained from recrystallization in ethanol–methanol. Mp 158—159 °C. ^{1}H NMR (CD_3OD) δ=7.91 (S, 1H, H6), 6.29 (t, 1H, J=6.6 Hz, H1′), 4.41 (m, 1H, H3′), 3.91 (m, 1H, H4′), 3.76 (m, 2H, H5′), 3.33—3.12 (M, 6H, C5–CH_2– and –$NHCH_2$– ×2), 2.27 (m, 2H, H2′), 1.60—1.29 (m, 8H, –$(CH_2)_4$–); UV (H_2O, pH 7.0) λ_{max} 265.6 nm (ε 10000 $M^{-1}cm^{-1}$); MS Found: m/z 481. Calcd for $C_{19}H_{28}F_3N_4O_7$: MH^+, 481.45. Anal. Found: C, 47.46; H, 5.80; N, 11.67%. Calcd for $C_{19}H_{27}F_3N_4O_7$: C, 47.50; H, 5.66; N, 11.66%.

5-[*N*-[2-[*N*,*N*-Bis(2-trifluoroacetylaminoethyl)-amino]ethyl]carbamoylmethyl-2′-deoxyuridine (3c′): **3c′** was also prepared from **1** and tris(2-aminoethyl)amine in a similar manner. After purification by silica-gel column chromatography using 15% methanol in dichloromethane as an eluent, coevaporation of the pure fraction with CH_2Cl_2 gave pure **3c′** as a foam. Yield 70% from **1**; ^{1}H NMR (D_2O) δ=7.74 (s, 1H, H6), 6.23 (t, 1H, J=6.7 Hz, H1′), 4.39 (m, 1H, H3′), 3.99 (m, 1H, H4′), 3.75 (m, 2H, H5′), 3.39—3.21 (m, 8H, C5–CH_2– and $NHCH_2$– ×3), 2.73—2.61 (m, 6H, $N(CH_2-)_3$), 2.34 (m, 2H, H2′); UV (H_2O, pH 7.0) λ_{max} 265.9 nm (ε 8600 $M^{-1}cm^{-1}$); MS Found: m/z 607. Calcd for $C_{21}H_{29}F_6N_6O_8$: MH^+, 607.49. Anal. Found: C, 38.15; H, 4.31; N, 12.20%. Calcd for $C_{21}H_{28}F_6N_6O_8 \cdot CH_2Cl_2$: C, 38.22; H, 4.37; N, 12.15%.

Synthesis of 5′-DMTr-Nucleoside Derivatives (4a′—c′). 5′-*O*-(4,4′-Dimethoxytrityl)-5-[*N*-(2-trifluoroacetylaminoethyl)]carbamoylmethyl-2′-deoxyuridine (4a′): **3a′** (0.4 g, 0.943 mmol) was reacted with 4,4′-dimethoxytrityl chloride (DMTr-Cl, 0.50 g, 1.48 mmol) in pyridine (1.5 mL) containing 4-dimethylaminopyridine (10 mg, 0.082 mmol) overnight at room temperature. 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 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, **4a′**. Yield of **4a′** was 60.4% (0.414 g). ^{1}H NMR ($CDCl_3$) δ=7.94 (s, 1H, imido-NH), 7.81 (s, 1H, H6), 7.39—6.81 (m, 13H, Ar), 6.09 (t, 1H, H1′), 4.56 (m, 1H, H3′), 3.94 (m, 1H, H4′), 3.78 (s, 6H, CH_3O–), 3.43—3.29 (m, 8H, H5′, –CH_2CH_2–, and C5–CH_2–), 2.64 and 2.33 (m, 2H, H2′).

5′-*O*-(4,4′-Dimethoxytrityl)-5-[*N*-(6-trifluoroacetylaminohexyl)]carbamoylmethyl-2′-deoxyuridine (4b′): Protection of the 5′-OH of **3b′** with 4,4′-dimethoxytrityl group was carried out in a similar manner. Yield 51%; ^{1}H NMR ($CDCl_3$) δ=7.75 (s, 1H, H6), 7.40—6.80 (m, 13H, Ar), 6.21 (t, 1H, H1′), 4.53 (m, 1H, H3′), 4.00 (m, 1H, H4′), 3.76 (s, 6H, CH_3O–), 3.37—3.08 (m, 8H, H5′, –$NHCH_2$– ×2, and C5–CH_2–), 2.33 (m, 2H, H2′), 1.57—1.30 (m, 8H, –$(CH_2)_4$–).

5′-*O*-(4,4′-Dimethoxytrityl)-5-[*N*-[2-[*N*,*N*-bis-(2-trifluoroacetylaminoethyl)amino]ethyl]carbamoylmethyl-2′-deoxyuridine (4c′): Protection of the 5′-OH of **3c′** with 4,4′-dimethoxytrityl group was carried out in a similar manner. Yield 66%; ^{1}H NMR ($CDCl_3$) δ=7.80 (s, 1H, H6), 7.39—6.82 (m, 13H, Ar), 6.25 (t, 1H, H1′), 4.57 (m, 1H, H3′), 4.06 (m, 1H, H4′), 3.78 (s, 6H, CH_3O–), 3.45—3.16 (m, 8H, H5′, –$NHCH_2$– ×2, and C5–CH_2–), 2.35 (m, 2H, H2′), 2.72—2.49 (m, 6H, $N(CH_2)_3$–).

Phosphitylation of 5′-DMTr-Nucleoside Derivatives (5a′—c′). 3′-*O*-[(2-Cyanoethyl)(diisopropylamino)]phosphino-5′-*O*-(4,4′-dimethoxytrityl)-5-[*N*-(2-trifluoroacetylaminoethyl)]carbamoylmethyl-2′-deoxyuridine (5a′): Phosphitylating reagent, chloro-(2-cyanoethoxy)diisopropylaminophosphine, was prepared in situ immediately before use from dichloro-(2-cyanoethoxy)phosphine and diisopropylamine. Diisopropylamine

(154 μL, 1.10 mmol) was added dropwise into a solution of dichloro-(2-cyanoethoxy)phosphine (70 μL, 0.55 mmol) in dry dichloromethane (5 mL) under N_2 atmosphere at 0 °C and the solution was stirred at room temperature for 1 h. Some (3.75 mL) of this reaction solution was added dropwise to a solution of **4a′** (0.1 g) in dry dichloromethane (1 mL) containing *N*-ethyl-*N*,*N*-diisopropylamine (192 μL, 1.10 mmol) under N_2 atmosphere at room temperature. After this mixture was stirred for 1 h, 20 μL of dry methanol was added to the reaction mixture and the reaction mixture was poured into cold ethyl acetate (30 mL, pre-washed with 5% aq sodium hydrogencarbonate solution). The solution was washed once with 5% aq sodium hydrogencarbonate solution, dried with anhydrous sodium sulfate, and evaporated to dryness. The crude product was purified by silica-gel column chromatography using ethyl acetate–methanol–triethylamine (89/1/10, v/v/v) as an eluent. The appropriate fractions were collected, evaporated, and precipitated with a small amount of dichloromethane into hexane to give white precipitates. Yield of **5a′** was 81.3% (0.143 g). ^{31}P NMR ($CDCl_3$) δ=149.51 and 149.68 ppm.

3′-*O*-[(2-Cyanoethyl)(diisopropylamino)]phosphino-5′-*O*-(4,4′-dimethoxytrityl)-5-[*N*-(6-trifluoroacetylaminohexyl)]carbamoylmethyl-2′-deoxyuridine (5b′): The phosphitylation of **4b′** was carried out in the same method as described above. Yield 77%; ^{31}P NMR ($CDCl_3$) δ=149.18 and 149.35 ppm.

3′-*O*-[(2-Cyanoethyl)(diisopropylamino)]phosphino-5′-*O*-(4,4′-dimethoxytrityl)-5-[*N*-[2-[*N*,*N*-bis(2-trifluoroacetylaminoethyl)amino]ethyl]carbamoylmethyl-2′-deoxyuridine (5c′): The phosphitylations of **4c′** was carried out in the same method as described above. Yield 76%; ^{31}P NMR ($CDCl_3$) δ=149.46 and 149.51 ppm.

Oligodeoxyribonucleotide Synthesis. Oligodeoxyribonucleotides and their analogs were prepared using normal phosphoramidite coupling procedure on a DNA synthesizer. The oligodeoxyribonucleotides (25mer's) bearing the C5-substituted 2′-deoxyuridine in place of one or three thymidines were synthesized along with the normal and complementary oligodeoxyribonucleotides. For the synthesis of oligodeoxyribonucleotides containing the modified base, **5a′**, **5b′**, or **5c′** was incorporated into the oligodeoxyribonucleotides at the appropriate position by using the normal synthetic cycle, except for the reaction time for the coupling step. The coupling times were 3, 1.5, and 5 min for **5a′**, **5b′**, and **5c′**, respectively. After the normal deprotection and cleavage from CPG support with concd aq ammonia solution, the modified oligodeoxyribonucleotides with 5′-(4,4′-dimethoxytrityl) group were isolated by reversed-phase HPLC on a Wakosil 5C18 column (10 mmϕ×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 80% acetic acid by the usual procedure to remove a 4,4′-dimethoxytrityl group, followed by desalting on a Sephadex G-25 column. **S25-a** and **c**, and **T25-a** and **c** were further purified by reversed-phase HPLC after deprotection of 5′-(4,4′-dimethoxytrityl) group. Isolated yields: **S25a**, 27%; **S25b**, 30%; **S25c**, 20%; **T25a**, 20%; **T25b**, 5%; **T25c**, 16%; **N25**, 6%, **c25**, 22%.

Nuclease Digestion of Oligodeoxyribonucleotides Containing the Modified Base. Digestion with Snake Venom Phosphodiesterase and Alkaline Phosphatase: 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. The reaction mixtures were analyzed by reversed-phase HPLC as shown in Figs. 1a and 1b for **S25a** and **T25a**.

Further Digestion with Nuclease P1: To the reaction mixtures treated with snake venom phosphodiesterase and alkaline phosphatase, nuclease P1 (ca. 4 units) and 0.1 M sodium acetate (pH 4.75) were added, then the reaction mixtures were incubated overnight at 37 °C. The reaction mixtures were analyzed by reversed-phase HPLC as shown in Figs. 1c and 1d for **S25a** and **T25a**. The nucleoside composition ratios were calculated from areas of the peaks in the HPLC chart. The extinction coefficients (at 260 nm) of natural nucleosides used for the calculation were as follows: dC, 7400; dG, 11500; dT, 8700; dA, 15400. The extinction coefficients (at 260 nm) of the modified nucleoside were as follows: **2a**, 8000; **2b**, 9300; **2c**, 7900. As these values for the modified nucleosides were calculated from absorption spectra of **3a′**—**c′**, they are not exact values but are sufficient for the calculation of the nucleoside composition analysis. The ratio of each nucleoside from the HPLC analysis was as follows: A : G : C : T : T (T represents C-5 modified nucleoside) : 9 : 6 : 5 : 4 : 1 for **S25a**—**c** (9 : 6 : 5 : 4 : 1 calcd for **S25a**—**c**); 9 : 6 : 3 : 4 : 3 for **T25a**—**c** (9 : 6 : 3 : 4 : 3 calcd for **T25a**—**c**).

Tm Measurements. UV absorbance was measured with a Hitachi Model 200-10 spectrophotometer equipped with an EYELA NCB-221 (Tokyo Rikaki Co.) water bath thermocontroller. The solution temperature in a cuvette was measured directly with a thermocouple. Absorbance and temperature data were recorded on a multi-pen recorder (Rikadenki Co.). The rate of heating was 0.5 °C min^{-1} or 0.25 °C min^{-1}. T_m values were obtained in 10 mM sodium phosphate buffer (pH 7.0) containing 50 mM sodium chloride at a duplex concentration of 2×10^{-6} M. Tm values were determined from the first differentials of the absorbance vs. temperature plot using an Igor graphing and data analysis program (WaveMatrics, Inc.) and a Macintosh IIci computer (Apple computer, Inc.).

Results and Discussion

The synthetic route of the modified nucleosides is shown in Scheme 1. The nucleoside, **1**, was easily reacted with alkylene diamine or trisamine to introduce an amine-linker at C5-position of 2′-deoxyuridine. The terminal amino group of the compound was protected by trifluoroacetyl group without further purification after removal of excess amine and solvent. Each nucleoside analog was obtained in a sufficient yield and was identified by ^{1}H NMR, mass spectroscopy, and elemental analysis. These modified nucleosides were converted to the protected nucleoside phosphoramidites by the usual method for oligodeoxyribonucleotide synthesis.[9] Oligodeoxyribonucleotides containing modified base were synthesized on a DNA synthesizer by using commercially available normal nucleoside phosphoramidites and the protected C5-substituted 2′-deoxyuridine phosphoramidites. Their reactiv-

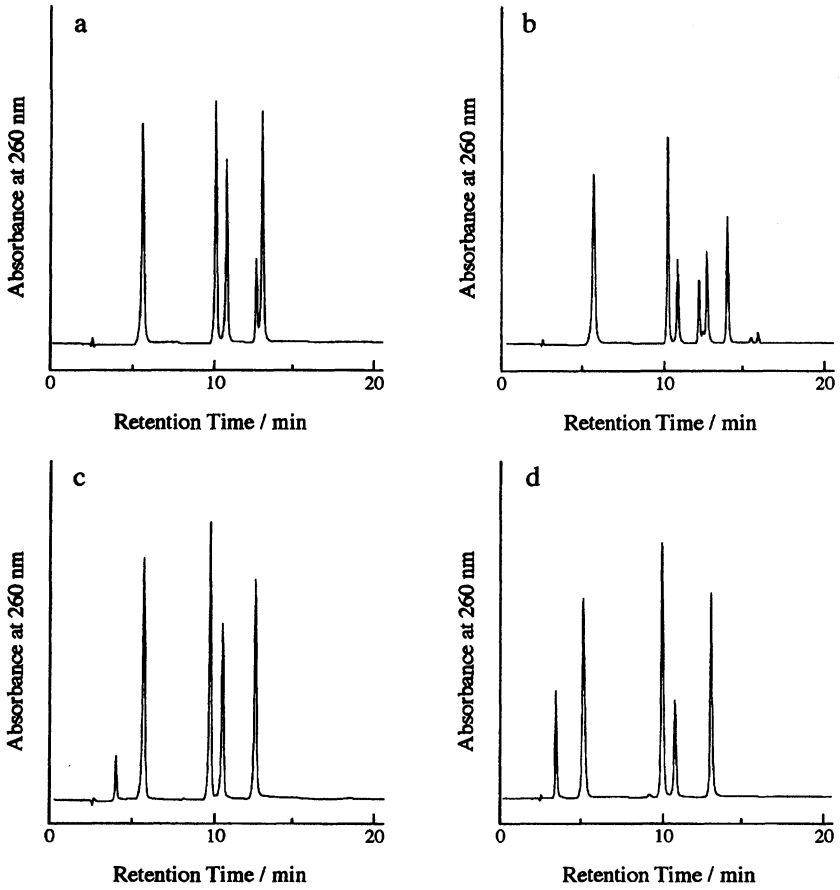
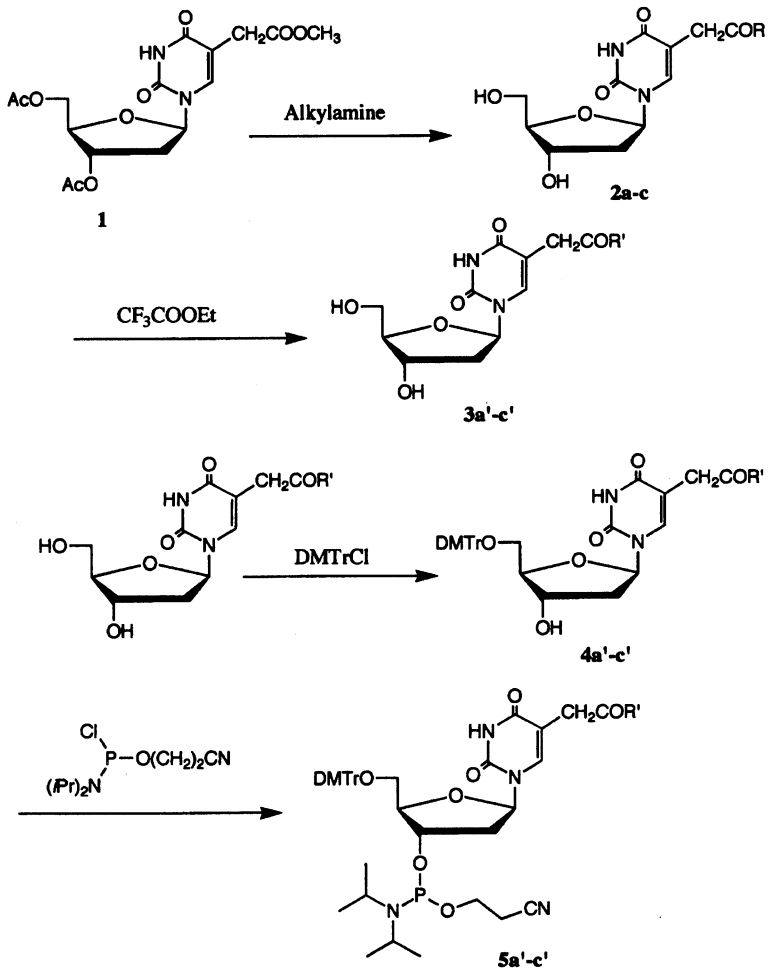


Fig. 1. HPLC analyses of the hydrolysate produced from the treatment of **S25a** and **T25a** with snake venom phosphodiesterase (SVPD), alkaline phosphatase (AP), and nuclease P1. (a) **S25a** treated with SVPD and AP. (b) **T25a** treated with SVPD and AP. (c) **S25a** treated with SVPD, AP, and nuclease P1. (d) **T25a** treated with SVPD, AP, and nuclease P1. HPLC condition: column, Wakosil 5C18 (4 mmϕ×250 mm length); eluent, 2.1—30.1% acetonitrile (in 28 min) in 50 mM TEAA (pH 7.0); flow rate, 1.0 mL min^{-1}. Retention times; 3.7 min, **2a**; 5.6 min, dC; 10.1 min, dG; 10.8 min, T; 12.6 min, d(GpT)*; 12.9 min, dA; 14.3 min, d(ApT)* (*; these compounds were considered as these dimers, see text).

ity were checked by coupling with a thymidine-linked CPG support. The results indicated that the modified nucleoside phosphoramidite analogs were less reactive than the normal nucleoside phosphoramidite, but a longer reaction time (≤5 min) for the tetrazole-catalyzed coupling reaction gave a sufficient yield (>90%). Sequences of the synthesized oligodeoxyribonucleotides are listed in Table 1. The oligomers were purified twice by reversed-phase HPLC before and after deprotection of 5′-DMTr group. Confirmation of the oligomers was performed by nuclease digestion. When the modified oligomer was treated with snake venom phosphodiesterase (SVPD, 3′-exonuclease) and alkaline phosphatase (AP) over 12 h, four normal nucleosides and some unknown products, which did not correspond to any modified nucleoside, **2a**, **2b**, or **2c**, were detected by HPLC analysis. The HPLC profiles are shown in Fig. 1, exemplified with **S25a** and **T25a**. In the analysis of digested products of **S25a** with SVPD and AP (Fig. 1a), the composition of normal deoxynucleosides was C:G:T:A=9:5:5:4 and the unknown product (t_R=12.6 min) did not correspond to **2a**. However, successive digestion with nuclease P1, endonuclease, produced four normal deoxynucleosides and **2a**, in the ratio of C:G:T:A:**2a**=9:6:5:4:1 (Fig. 1c). After the digestion with nuclease P1, the ratio of deoxyguanosine had increased by 1 and modified nucleoside **2a** was produced. This suggests that the unknown product (t_R= 12.6 min) was composed of deoxyguanosine and **2a**. The nucleoside at the 5′-side of **2a** is deoxyguanosine in this 25mer's sequence (**S25a**, Table 1). These results indicate that the product which eluted at 12.6 min on HPLC (Fig. 1a) was d(GpT) (T represents **2a**). A similar result was obtained from the digestion of **T25a**. The digestion of **T25a** with SVPD and AP produced four deoxynucleosides (C:G:T:A=9:5:3:2) and two other products (one corresponding to the digested product of **S25a** with SVPD and AP, t_R=12.6 min and the other, t_R=14.3 min (Fig. 1b)). The successive digestion with nuclease P1 produced four deoxynucleosides and **2a** in the ratio of C:G:T:A:**2a**=9:6:3:4:3. For the other oligodeoxyribonucleotide analogs (**S25b**, **S25c**, **T25b**,



Scheme 1.

Table 1. Synthesized 25mer's Sequence

Abbreviation	Sequence
N25	5′ACATGCATCCCGTGGTCCTATCCGG3′
S25-a, b, and c	5′ACATGCATCCCGTGGTCCTATCCGG3′
T25-a, b, and c	5′ACATGCATCCCGTGGTCCTATCCGG3′
c25	5′CCGGATAGGACCACGGGATGCATGT3′

-a, T=**2a**(EDA); -b, T=**2b**(HMDA); -c, T=**2c**(TAEA).

and **T25c**), similar results were obtained. The compositions of the nucleosides after complete digestion corresponded to the desired sequences. These results suggest that 5′-side phosphodiester linkage of the modified nucleoside was not cleaved with snake venom phosphodiesterase or cleaved very slowly under the condition examined. After the complete digestion with nuclease P1, their base compositions corresponded well with the desired oligomers.

Thermal stability of duplexes of the modified oligomer with the complementary oligodeoxyribonucleotide was investigated by a UV melting experiment. Figure 2 shows typical melting curves (absorbance vs. temperature plots) of 25mer duplexes containing one modified nucleoside. This melting profile indicated that the modified 25mers formed the duplex with the complementary 25mer in a manner similar to that of the unmodified 25mer. Melting temperatures (T_m's) are summarized in Table 2. The incorporation of ethylenediamine linker or tris(2-aminoethyl)amine linker led to the stabilization of the duplex with increasing the number of the modified bases. Oligodeoxyribonucleotides possessing ethylenediamine as the linker arm showed an increase in T_m of about 3 °C/substitution, and

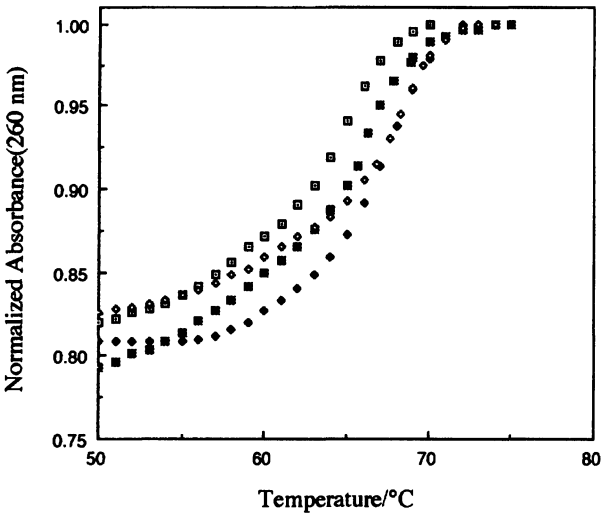


Fig. 2. Melting curves of the duplexes consisted of modified 25mers with **c25**. □, **N25**; ◆, **S25a**; ■, **S25b**; ◇, **S25c**.

Table 2. Melting Temperatures of 25-mers Bearing Linker Arms at C-5 of 2′-Deoxyuridine

Entry	5 substituted group	No. of substitution	T_m/°C
1	—	—	65.0
2	EDA	1	68.0
3		3	73.0
4	HMDA	1	66.3
5		3	62.4
6	TAEA	1	68.2
7		3	71.4

Condition: DNA concentration, each 2 μM; Buffer, 10 mM sodium phosphate containing/50 mM sodium chloride; Light pass length, 1 mm.

oligodeoxyribonucleotides possessing a tris(2-aminoethyl)amine as the linker arm also showed an increase in T_m of about 2—3 °C/substitution. On the other hand, the incorporation of three 1,6-hexanediamine linkers slightly destabilized the duplex. At pH 7.0, the primary amino group at the linker is protonated (pK_a of ethylenediamine=6.85 and 9.93, pK_a of 1,6-hexanediamine=9.83 and 10.93, and pK_a of tris(2-aminoethyl)amine=8.5, 9.6, and 10.3).[10] Such groups likely facilitate the duplex formation due to the effective charge neutralization. Also the protonated amino group could interact with the negatively charged phosphate moiety of oligonucleotide in either intra- or inter-molecular manner. On the other hand, the alkyl ether group at C5-position of pyrimidine results in some disruption of the duplex form because of the release of bound water and/or van der Waals interaction in the major groove of the double helix. The duplex stability, indicated by Tm, will depend on the balance of the two competing effects. This balanced effect was also pointed out in the case of oligodeoxyribonucleotides bearing 6-aminohexyl group at C5-position of pyrimidine.[4] It has been shown that 5-(6-aminohexyl) substituent causes a slight decrease in the duplex stability, while 5-hexyl substituent destabilizes the duplex formation to a large extent. Further, (*N*-aminoalkyl)carbamoyl and (*N*-aminoalkyl)carbamoylmethyl group at the C5-position of 2′-deoxyuridine has a different effect on the duplex stability, although the difference is only one methylene group. Ono et al.[5] explained recently that the (*N*-aminoalkyl)carbamoyl group at the C5-position of 2′-deoxyuridine stabilized the duplex form independent of the alkyl-chain length. They suggested that the increase of acidity of N^3–H and an intramolecular hydrogen bond in 5-(*N*-aminoethyl)-carbamoyl-2′-deoxyuridine affect the stability of the duplex formation. Our results show that the ethylenediamine linker and tris(2-aminoethyl)-amine linker have a large effect of a positively charged amino group which stabilizes the duplex form while hexanediamine linker has a large effect of an alkyl tether group which destabilizes the duplex form. Tris(2-aminoethyl)amine linker has more positively net charge compared with the ethylenediamine linker, although the oligodeoxyribonucleotides bearing tris(2-aminoethyl)amine linker showed the same or a little lower Tm values compared with oligodeoxyribonucleotide bearing the ethylenediamine linker. Electrostatic effect of tris-(2-aminoethyl)amine linker could compensate its steric hindrance of the branched chain for the duplex formation. These results suggest that the length of amino linker and the number of amino groups will influence the stability of the duplexes.

In conclusion, compound **1** is useful for the incorporation of several kind of linker arms at C5-position of uracil nucleoside in oligodeoxyribonucleotides. Here, we showed the incorporation of three amino-linker arms and we found that the stability of duplex is affected by the number of substitutions, the number of amino groups, and the length of its arm. The oligodeoxyribonucleotides possessing some (*N*-aminoalkyl)carbamoylmethyl linkers described here could have useful applications in studies of biological activities of oligonucleotides, as they showed the increased hybridization ability and resistance to some nucleases. In addition, the incorporated primary amino groups are useful for the attachment of reporter groups such as fluorescence groups or enzymes.

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