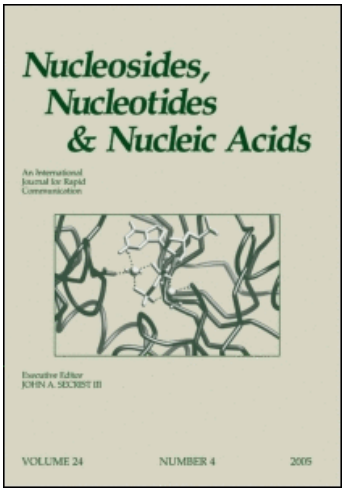

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Effect of Imino Group of a Linker Arm at the C5 Position of a Pyrimidine Nucleoside on the Thermal Stabilities of DNA/DNA and DNA/RNA Duplexes†

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ABSTRACT

The modified ODN's bearing C5-substituted 2′-deoxyuridine derivative were synthesized by a post-synthetic modification with an unsymmetrical triamine. The effect of the C5-substituent on the duplex formation with complementary DNA or RNA differed with the position of an imino group in the linker-arms.

Key Words: Pyrimidine; ODNs; *N*-(2-aminoethyl)-1,3-propanediamine.

INTRODUCTION

C5-position of a pyrimidine is well utilized as a modification site of oligodeoxyribonucleotides (ODNs) and the ODNs are applied to a DNA probe and an antisense molecule.[1–5] The substituent at C5 of a pyrimidine does not inhibit the base pairing with the complementary strand, but the thermal stability of the duplex

†In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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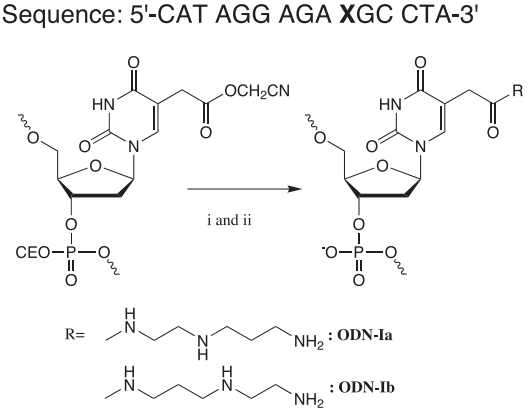
depends on the kind of tethers and functional groups. We have studied the effect of a linker at C5-position of a pyrimidine nucleoside on a duplex stability. In these studies, we found that the length and functional groups of the linker arms affected on the thermal stability of the duplexes.[6–8] In this study, we have synthesizied the modified oligodeoxyribonucleotides containing C5-substituted 2′-deoxyuridine bearing an unsymmetric triamine, *N*-(2-aminoethyl)-1,3-propanediamine.

RESULTS AND DISCUSSION

The amine-modified ODNs were synthesized from the modified ODN containing 5-cyanomethoxycarbonylmethyl-2′-deoxyuridine by a post-synthetic modification method, as shown in Scheme 1. 15mer ODN (5′-d(CATAGGAGAXGCCTA)-3′, X = 5-cyanomethoxycarbonylmethyl-2′-deoxyuridine) containing 5-cyanomethoxycarbonylmethyl-2′-deoxyuridine was synthesized by previous described method.[9,10] After assembling 15mer ODN on a CPG support, its CPG support was treated with 1 M *N*-(2-aminoethyl)-1,3-propanediamine in THF at r. t. for 18 h followed by 28% aq. ammonia at 55°C for 12 h. After 5′-DMTr-protected 15mer ODN was purified by reversed-phase HPLC and subsequent deprotection of 5′-DMTr group, the 15mer ODN could be separated into two isomers as shown in Figure 1a. The fast isomer of 15mer ODN was eluted at 15.0 min and the slow one at 15.3 min. Parts of these two products were digested by nucleases (snake venom phosphodiesterase, nuclease P1, and alkaline phosphatase) and analyzed by reversed-phase HPLC (Figure 1b and 1c). The peaks of the modified nucleosides were present at 4 and 5 min. For the identification of the products, C5-substituted 2′-deoxyuridine (**1a** and **1b**) were prepared from the reaction of C5-methoxycarbonylmethyl-2′-deoxyuridine with *N*-(2-aminoethyl)-1,3-propanediamine. Both isomers (**1a** and **1b**) were separated by reversed-phase HPLC and analyzed by ESI-MS and ^{1}H NMR. From these ^{1}H NMR, it was found that the amido bond for both isomers was formed with each terminal amino group of *N*-(2-



Scheme 1. i) 1 M *N*-(2-aminoethyl)-1,3-propanediamine in THF, r.t., 18 h; ii) 28% aq. ammonia, 55°C, 12 h.

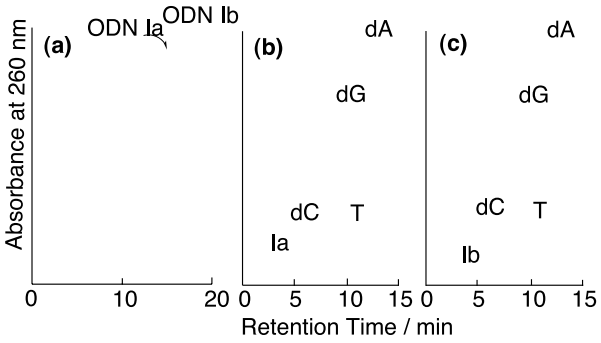


Figure 1. HPLC profiles of crude ODN 15mer (a) and the hydrorized ODN-1a (b) and ODN-1b (c) by nuclease. HPLC condition: column, Wakosil 5C18 (4 mmϕ × 250 mm); eluent, linear gradient of acetonitrile conc. in 50 mM TEAA (pH 7.0) in 35 min; flow rate, 1 mL/min.

aminoethyl)-1,3-propanediamine, and the fast eluting isomer of the modified nucleoside was identified as a compound **1a** and the slow eluting one as a compound **1b**. ODN containing **1a** named **ODN-Ia** and ODN containing **1b** named **ODN-Ib**.

Figure 2 shows the CD spectra for the unmodified (**N-ODN**) and modified ODNs (**ODN-Ia** and **-Ib**) duplexes formed with DNA and RNA. Similar CD spectra between **N-ODN**/DNA, **ODN-Ia**/DNA, and **ODN-Ib** indicate that the conformations of these duplexes are B-form and the modification of ODN with the linker had slight effect on a global conformation. The CD spectra of **N-ODN**/RNA and modified ODN/RNA closely resemble the spectrum of an A-form RNA/RNA duplex and this structure is commonly described as an ''A-form-like'' conformation. Also it was suggested that the modification of ODN had little effect on a global conformation of the ODN/RNA duplexes.

Table 1 shows the melting temperatures and the thermodynamic parameters of the ODN/DNA or ODN/RNA duplexes with previously reported values for aminoalkyl modification.[6–8] The thermodynamic parameters for the duplex formation were estimated from the curve fitting for each melting curve.[a] For ODN/DNA duplexes, **ODN-Ia** shows higher Tm value than **ODN-Ib**, and **ODN-Ib** shows same Tm value as the normal ODN (**N-ODN**). In the thermodynamic parameter, both **ODN-Ia**/DNA and **ODN-Ib**/DNA duplex were stabilized by entropic effect compared to **N-ODN**/DNA duplex. This is in contrast to **ODN-2N**, which has an amino group at the same position as the imino group of **ODN-Ia**, and **ODN-6N**, which doesn't have an imino group in the linker arm. It is considered that the imino group doesn't completely have a positive charge to promote an electrostatic interaction under the experimental condition since the pKa of the imino group in *N*-(2-aminoethyl)-1,3-propanediamine is 6.18.[12]

For ODN/RNA, the effect of the C5-substituent of **ODN-Ia** and **-Ib** on the duplex formation is smaller than that for ODN/DNA. The duplex formations of **ODN-Ia** , **-Ib**, and **-6N** with RNA showed more negative ΔS than that of **ODN-2N**. This suggests that

[a]The calculation was performed by a nonlinear, least squares calculation program ''TMSPEC,'' which was developed by Kodama et al.: Ref. [11].

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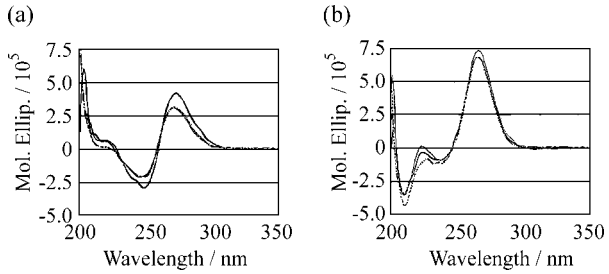


Figure 2. CD sprctra of ODN/DNA (a) or ODN/RNA(b) duplexes. Sample concentrations and buffer solutions were the same as in Table 1. The line represented for **N-ODN** (plain), **ODN-Ia** (bold), and **ODN-Ib** (gray).

a long linker-arm even containing a polar imino group at C5-position is unfavorable for duplex formation. Also, the duplex of **ODN-Ia**, **-Ib**, and **-6N** with RNA are not so much stabilized in terms of enthalpy compared with **ODN-6N**/DNA duplex. The conformation of the ODN/RNA duplex is A-form-like helix conformation as indicated from the CD spectra. The major groove of A-form helix (width and depth of major groove in A-DNA are 2.7 Å and 13.5 Å, respectively)[13] is narrower and deeper than that of B-form helix (width and depth of major groove in B-DNA are 11.7 Å and 8.5 Å, respectively).[13] Therefore, the terminal amino group of the linker arm in the ODN/RNA duplex may not effectively interact with moieties of the complementary strand since the freedom of the linker arm is limited.

Plots of ΔS versus ΔH for both ODN/DNA and ODN/RNA gave a linear relationship as shown in Figure 3. Such enthalpy–entropy compensation was related to the properties of bulk water and difference in hydration states of the single-strand form and the double-stranded form.[14]

In conclusion, we obtained the linkage isomers, which were synthesized from the modified ODN bearing C5-substituted 2′-deoxyuridine derivative with an unsymmetrical triamine by a post-synthetic modification method. The effect of the C5-substituent on the duplex formation with the complementary DNA and RNA differed with the position of an imino group in linker-arms. These knowledges are useful for the design of a linker at C5-position of pyrimidine nucleoside in duplexes.

EXPERIMENTAL

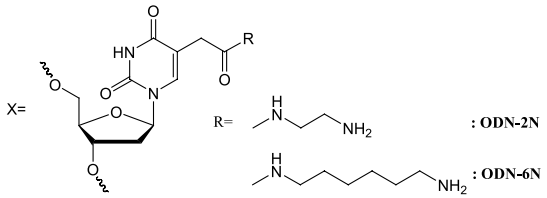
General. Thin-layer chromatography (TLC) was performed on Kieselgel $60F_{254}$ (Art. 5554, E. Merck). Silica–gel column chromatography was performed on Wako gel C-200 (Wako Pure Chemical Industries Ltd. (Wako)), Silica gel 60 (63–200 μm or 40–63 μm, Merck). High-performance liquid chromatography (HPLC) 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 were obtained with a JEOL α-500 spectrometer. ^{1}H NMR spectra were recorded

Table 1. Melting temperatures and thermodynamic parameters.

Abbr.	Tm/°C	ΔH^0/kJ mol^{-1}	ΔS^0/J mol^{-1} K^{-1}	ΔG_{37}/kJ mol^{-1}
DNA				
N-ODN	50.6	− 496	− 1420	− 55.5
ODN-Ia	53.6	− 428	− 1200	− 55.8
ODN-Ib	50.6	− 418	− 1180	− 52.0
ODN-2N*	53.7	− 516	− 1460	− 63.1
ODN-6N*	47.9	− 559	− 1630	− 53.4
RNA				
N-ODN	51.2	− 400	− 1120	− 52.6
ODN-Ia	51.7	− 426	− 1200	− 53.8
ODN-Ib	50.0	− 421	− 1190	− 51.9
ODN-2N	55.9	− 397	− 1090	− 58.9
ODN-6N	50.7	− 421	− 1180	− 55.0

Condition: Conc. of duplexes, 2 μM; buffer, 150 mM sodium chloride/10 mM sodium phosphate (pH 7.0) for ODN/DNA, 150 mM sodium chloride/10 mM sodium phosphate (pH 7.0)/10 μM EDTA for ODN/RNA; increasing or decreasing rate of temperature, 0.5°C/min. The thermodynamic parameters for the duplex formation were estimated from the curve fitting for each melting curve. **ODN-2N** has a same sequence as **ODN-Ia** and **-Ib** except for the containing *N*-(2-aminoethyl)carbamoylmethyl group at C5-position of the modified nucleoside. **ODN-6N** has a same sequence as **ODN-Ia** and **-Ib** except for the containing *N*-(6-aminohexyl)carbamoylmethyl group at C5-position of the modified nucleoside.
*The data reported previously.

ODN-2N and -6N: 5'-CAT AGG AGA **X**GC CTA-3'



relative to internal tetramethylsilane. Mass spectra were measured by a Applied Biosystems/MDS-Sciex API-100 instrument in ESI mode. CD spectra were recorded on a JASCO J-720 spectropolarimeter equipped with a PTC-343 temperature controller, using the same solution for the Tm measurements at 20°C. Oligodeoxyribonucleotides were synthesized by a phosphoramidite chemistry on an Applied Biosystems 381A DNA synthesizer. 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.

Synthesis of modified 15mer ODN. A protected 15mer ODN (5′-d(CATAG-GAGAXGCCTA)-3′, X = 5-cyanomethoxycarbonylmethyl-2′-deoxyuridine) on CPG

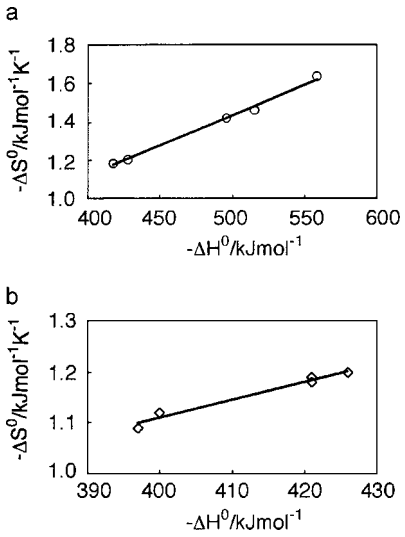


Figure 3. Plots of ΔS vs. ΔH for both ODN/DNA (a) and ODN/RNA (b).

was synthesized by previous described method. The ODN on CPG support was treated with 1 M *N*-(2-aminoethyl)-1,3-propanediamine in THF at r.t. for 18 h followed by 28% aq. ammonia at 55°C for 12 h. After the filtration to remove the CPG, the filtrate was concentrated and purified by reversed-phase HPLC. The 5′-DMTr-protected 15mer ODN was treated with 10% acetic acid aqueous solution at r.t. for 1 h to remove the 5′-DMTr group. The unprotected 15mer ODN could be separated into two isomers by reversed-phase HPLC as shown in Figure 1a. Yields of the fast isomer (**ODN-Ia**) and the slow isomer (**ODN-Ib**) were 12.8% and 13.5%, respectively.

Nuclease digestion of oligodeoxyribonucleotides containing the modified base. The modified oligomers (ca. 0.5 $OD_{260\ nm}$) were treated overnight with snake venom phosphodiesterase (0.25 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 were added nuclease P1 (ca. 4 units) and 0.1 M sodium acetate (pH 4.75), the reaction mixtures were incubated at 37°C for 3 h. The reaction mixtures were analyzed by reversed-phase HPLC. The nucleoside composition ratio was calculated from areas of the peaks in the HPLC chart.

Synthesis of compound 1a and 1b. A mixture containing 3′,5′-Di-*O*-acetyl-5-(methoxycarbonylmethyl)-2′-deoxyuridine[15] (0.200 g, 0.52 mmol), *N*-(2-aminoethyl)-1,3-propanediamine (0.81 mL, 6.24 mmol), and 4-dimethylaminopyridine (0.002 g, 0.016 mmol) in methanol (3 mL) was stirred at r.t. for 11 h. After the reaction solution was evaporated and co-evaporated with methanol, the residue was dissolved in a small amount of methanol and added dropwise to benzene to precipitate 5-substituted-2′-deoxyuridine (**1a** and **1b**) as an oily residue. Parts of both isomers (**1a** and **1b**) were separated by reversed-phase HPLC and analyzed by ESI-MS and ^{1}H NMR. Compound

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1a (fast eluted isomer): ESI-MS, calcd. for $C_{16}H_{28}N_5O_6$, 386.2; found m/z 386.3, ^{1}H-NMR in CD_3OD; 7.78 (H6, s, 1H), 6.18 (H1′, t, 2H), 4.40 (H3′, m, 1H), 3.91 (H4′, m, 1H), 3.7–3.8 (H5′, m, 2H), 3.43 (CH_2 in linker, d, 2H), 3.23 (CH_2 at C5, s, 2H), 3.02 (CH_2 in linker, t, 2H), 2.94 (CH_2 in linker, m 4H), 2.2–2.3 (H2′, m, 2H), 1.92 (CH_2 in linker, m, 2H). Compound **1b** (slow eluted isomer): ESI-MS, calcd. for $C_{16}H_{28}N_5O_6$, 386.2; found m/z 386.2,^{1}H-NMR in CD_3OD; 7.78 (H6, s, 1H), 6.18 (H1′, t, 2H), 4.40 (H3′, m, 1H), 3.91 (H4′, m, 1H), 3.7–3.8 (H5′, m, 2H), 3.30 (CH_2 in linker, m, 2H), 3.22 (CH_2 at C5, s, 2H), 3.04 (CH_2 in linker, t, 2H), 2.95 (CH_2 in linker, t, 2H), 2.79 (CH_2 in linker, t 2H), 2.2–2.3 (H2′, m, 2H), 1.76 (CH_2 in linker, m, 2H).

Tm measurements. UV absorbance was measured with a Hitachi UV-3000 spectrophotometer equipped with a Hitachi Temperature Controller SPR-10. 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 NEC personal computer PC-9821. The rate of heating or cooling was 0.5°C/min. Tm values were obtained in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride for the duplex with DNA or in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride and 0.01 mM EDTA for the duplex with RNA at a duplex concentration of 2×10^{-6} M.

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