Nucleosides and Nucleotides. 131. Synthesis and Properties of Oligonucleotides Containing 5-Formyl-2′-deoxyuridine[1]

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Received April 27, 1994; accepted June 27, 1994

Thymidine was converted into 5-formyl-2′-deoxyuridine (1), which was incorporated into oligonucleotides, 5′d(GGAGA1CTCC)3′ (I-1) and 5′d(GCTGC1GCGAAAGCTG)3′ (II-1). To avoid side-reactions and degradation, protection of the formyl group of 1 using a newly developed protecting group, *N,N*-di-(3,5-dichlorophenyl)ethylenediamine, was necessary. Compound 1 was unstable under the conditions employed for enzymatic complete digestion of oligonucleotides, so that a peak corresponding to 1 was not detected clearly by HPLC analysis of a nucleoside mixture obtained by complete hydrolysis of I-1. Therefore, the oligonucleotide I-1 was treated with cyanomethylenetriphenylphosphorane to give an oligonucleotide containing (*E*) and (*Z*)-5-(2-cyanovinyl)-2′-deoxyuridine, which was then hydrolyzed, and the newly generated nucleosides were detected by HPLC analysis. The T_m of the self-complementary oligonucleotide I-1 (40 °C) was higher than that of the parent oligonucleotide, 5′d(GGAGATCTCC)3′, (31 °C) in a buffer containing 0.01 M sodium phosphate (pH 7.0) and 0.1 M NaCl. DNA replication study on a template-primer system [primer, 5′d(^{32}P-CAGCTTTCGC)3′; template, 3′d(GTCGAAAGCGXCGTCG)5′ (X=1 or T)] showed that dATP was incorporated into the DNA strand at a site opposite to 1 by Klenow DNA polymerase, but with a reduced rate. The formyl group of 1 in the oligonucleotides reacted with amines to give Schiff base derivatives.

Keywords γ-irradiation; mutation; oligonucleotide synthesis; 5-formyluracil

5-Formyl-2′-deoxyuridine (**1**) (Chart 1), one of the modified nucleosides isolated from γ-irradiated calf thymus DNA in aqueous solution, was found to be mutagenic to *Salmonella typhimurium* TA102.[3] Also, **1** was supposed to be involved in DNA–protein cross-links[4] by forming Schiff bases composed of the formyl group of **1** and certain amino groups in a protein such as the ε-amino group of lysine.[3] However, the properties of **1** in DNA strands in living cells such as repair of **1**, mismatched base-pair formation on replication, DNA–protein cross-link formation, degradation, *etc.* have not been studied.

Therefore, it is important to synthesize DNA oligomers containing **1** at a distinct position and examine the properties of the oligomers. However, some difficulties in chemical synthesis of oligonucleotides containing **1** were expected. For instance, Armstrong *et al.* reported that 5-formyluridine was transformed into a mixture of α- and β-anomers along with unknown compounds in aqueous alkaline solution.[5] Instability of **1** under alkaline conditions at even pH 8 was observed in our experiments.[6] Furthermore, Park *et al.* reported that treatment of **1** with phosphorus oxychloride did not give the desired 5′-phosphate derivative but gave unknown compounds.[7] Consequently, the formyl group of **1** should be protected during synthesis of oligonucleotides. The protecting group must be stable not only under basic conditions, such as NH_4OH treatment used for deprotection of the acyl groups of exocyclic amines and β-cyanoethyl groups, but also under acidic conditions used for deprotection of 4,4′-dimethoxytrityl (DMTr) groups in cycles of DNA synthesis on a DNA synthesizer. Moreover, the protecting group must be removed by the treatment with aqueous acetic acid used for final deprotection of the DMTr group.

We recently synthesized a short oligonucleotide containing **1**, 5′d(TT1TTT)3′ using a substituted diphenylethylenediamine protecting group. The existence of **1** in the oligonucleotide was confirmed by chemical conversion of **1** into 5-hydroxymethyl-2′-deoxyuridine, which was detected by HPLC after enzymatic complete digestion of the oligonucleotide.[8] However, incorporation of **1** into longer oligonucleotides with various sequences and confirmation of the existence of **1** in the oligonucleotides were unsuccessful. In this report, we introduced **1** into oligonucleotides, 5′d(GGAGA1CTCC)3′ (I-**1**) and 5′d(GCTGC1GCGAAAGCTG)3′ (II-**1**), using a new protecting group, *N,N*′-di-(3,5-dichlorophenyl)ethylenediamine (diCPhEDA), for the protection of the formyl group of **1**. Substitution of the protons of the phenyl group with electron-withdrawing groups could stabilize the imidazolidine groups under acidic conditions. The oligonucleotides were used for a thermal denaturation study and a study of DNA–protein cross-links.

Results and Discussion

Synthesis of Oligonucleotides 3′,5′-Di-*O*-acetylthymidine (**2**) was converted into a 5-formyl derivative **3** by the reported method with several modifications (Chart 1).[9] Then, **3** was treated with diCPhEDA (**4**) in the presence of D-camphorsulfonic acid as an acid catalyst to give the *N,N*′-di-(3,5-dichlorophenyl)imidazolidine derivative **5** in 72% yield. Deacetylation of **5** with NaOMe gave **6** as crystals, and this product was converted into a corresponding nucleoside 3′-phosphoramidite.[10]

Oligonucleotides, 5′d(GGAGA1CTCC)3′ (I-**1**) and 5′d(GCTGC1GCGAAAGCTG)3′ (II-**1**), were synthesized on a DNA synthesizer by the phosphoramidite method.[11] The average coupling yield of **8** was 82%, but this was not optimized and could probably be increased as in the case of the similar amidite unit.[8] The fully protected oligonucleotides were treated with concentrated NH_4OH to give oligonucleotides protected by DMTr at

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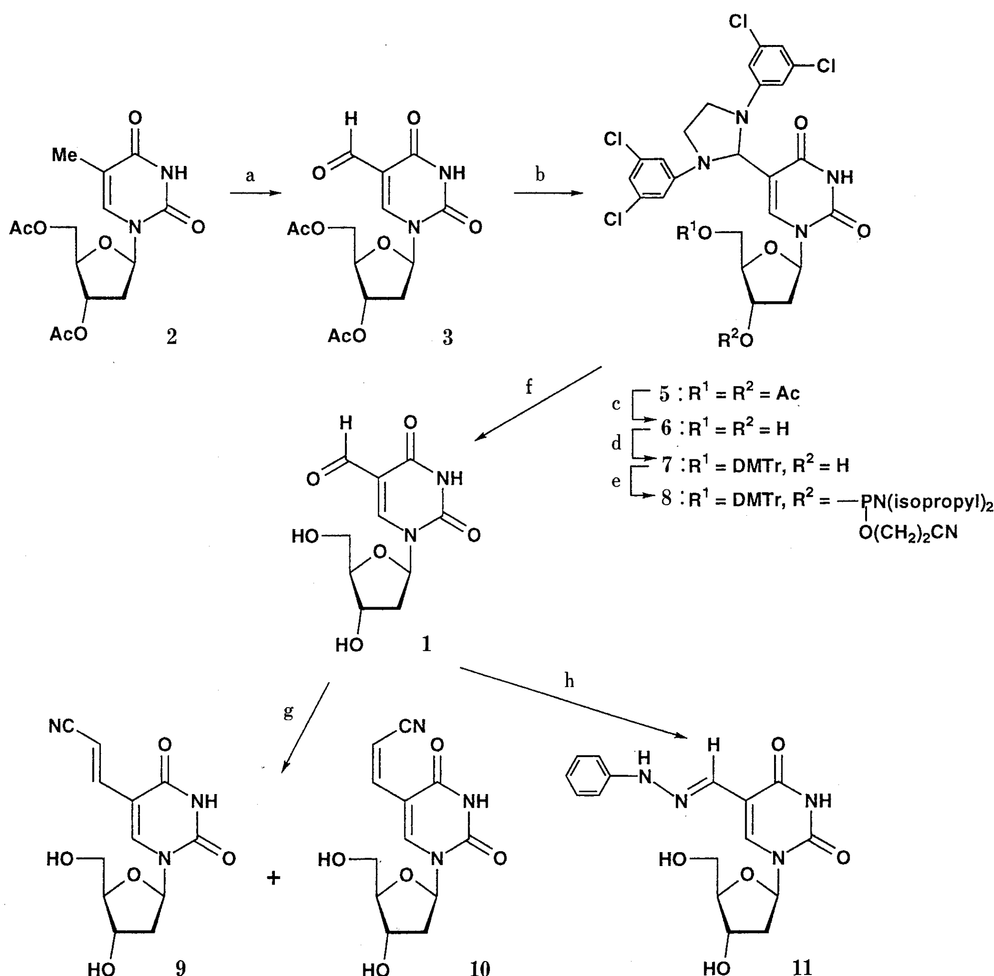

Chart 1. Synthesis of Nucleosides

a; $K_2S_2O_8$, $CuSO_4$, 2,6-lutidine, aqueous CH_3CN, 65 °C, 2 h. 52%. b; 1) diCPhEDA (**4**), D-camphorsulfonic acid, DMF, room temperature, 18 h. 72%. c; NaOMe, room temperature, 1 h. 98%. d; DMTrCl, pyridine, room temperature, 3 h. 60%. e; 2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, *N*,*N*-diisopropylethylamine, CH_2Cl_2, room temperature, 54%. f; Aqueous AcOH, room temperature, overnight, 98%. g; Cyanomethylenetriphenylphosphorane, room temperature, overnight, 93%. h; Phenylhydrazine, aqueous EtOH, room temperature, 1 h, 84%.

the 5′-end and by diCPhEDA at the formyl group, and these products were purified on a C-18 silica gel column with a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (TEAA) buffer. An example is shown in Fig. 1. The main UV absorbing peak (fractions 30—35) contained the desired partially protected oligonucleotide, which was sufficiently pure according to HPLC analysis with a C-18 column (Fig. 2a). HPLC analysis of the smaller peak (fractions 25—29) showed that the fractions contained a complicated mixture of oligonucleotides (data not shown), which might have been generated by loss of the diCPhEDA group followed by degradation of the 5-formyl-2′-deoxyuridine residue. The main UV-absorbing fractions were combined and desalted by passing them through a short C-18 column. This desalting step was indispensable since direct concentration of the oligonucleotide solution in TEAA buffer causes partial deprotection of DMTr and diCPhEDA groups, probably due to lowering of the pH of the solution, and then the deprotected oligonucleotide is degraded to give the complicated mixture detected by HPLC (data not shown).

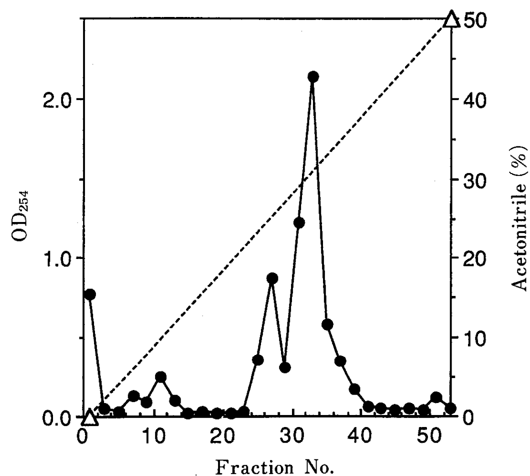

Fig. 1. Elution Profile of **I-1** Protected with DMTr at the 5′-End and with diCPhEDA at the Formyl Group on the C-18 Column

The oligomer was eluted by a linear gradient of CH_3CN from 0 to 50% in 0.1 M TEAA buffer (pH 7.0) (200 ml).

Each oligonucleotide was treated with 80% acetic acid overnight at room temperature to remove DMTr and diCPhEDA groups, then the solution was concentrated and the residue was coevaporated with water. The residue was dissolved in water and the solution was passed through

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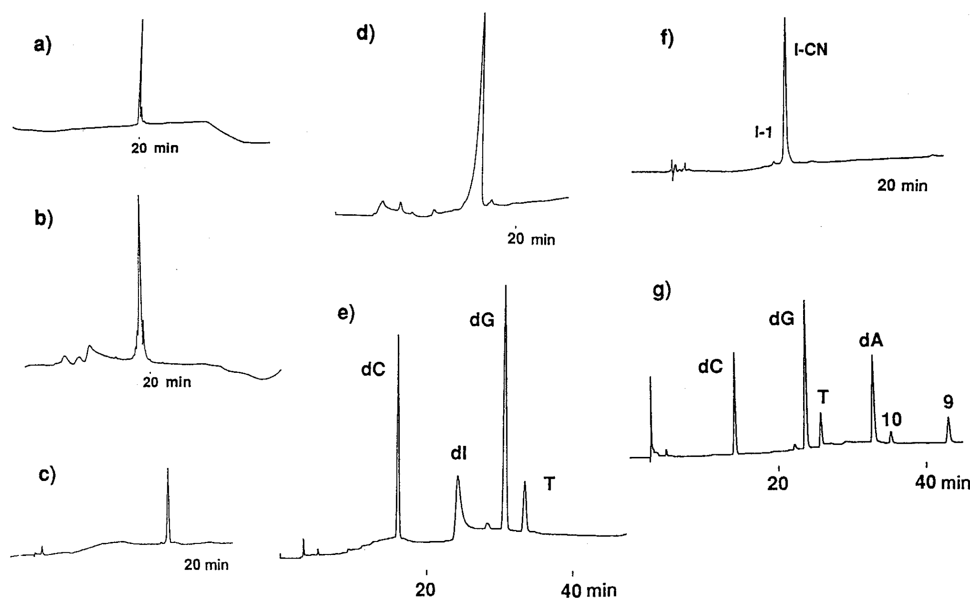

Fig. 2. HPLC Profiles

The peaks were observed at 260 nm. a) Fraction 33 (Fig. 1), a C-18 column (Inertsil ODS-2), a linear gradient of CH_3CN from 18 to 45% (20 min) in 0.1 M TEAA buffer (pH 6.8). b) I-1, (after deprotection), the C-18 column, a linear gradient of CH_3CN from 2.5 to 18% (20 min) in 0.1 M TEAA buffer (pH 7.0). c) I-1 (after HPLC purification), same condition as the profile b. d) I-1 (after HPLC purification), TSK gel DEAE, a linear gradient of ammonium formate from 0.3 to 0.8 M (20 min) in 20% aqueous CH_3CN. e) The nucleoside mixture obtained by hydrolysis of I-1 with nuclease P1 and acid phosphatase, the C-18 column, a linear gradient of MeOH from 0 to 25% (40 min) in 10 mM sodium phosphate (pH 7.2) with the C-18 column. f) I-CN, the C-18 column, a linear gradient of CH_3CN from 10 to 23% (20 min) in 0.1 M TEAA buffer (pH 7.0) with the C-18 column. g) A nucleoside mixture obtained by hydrolysis of I-CN with venom phosphodiesterase and alkaline phosphatase, the C-18 column, a linear gradient of MeOH from 0 to 25% (40 min) to 25% (10 min) in 10 mM sodium phosphate (pH 7.2).

the short C-18 column to remove DMTrOH and diCPhEDA. Even though great care was taken with the above procedures such as deprotection, work up, and desalting steps, the samples of the fully deprotected oligonucleotides contained many minor peaks beside the main peaks. An example is shown in Fig. 2b. The samples were purified by HPLC with the C-18 silica gel column, then the purified oligonucleotide was desalted as described above, and 8 optical density (OD) units (at 254 nm) of **I-1** and 4.5 OD units of **II-1** were obtained starting from the protected nucleosides (1 μmol) bound to a controlled glass support. The oligonucleotides showed single peaks on HPLC with the C-18 column and on a DEAE column (TSK gel DEAE, Toso). Examples are shown in Fig. 2c, d.

We previously reported that **1** was not detected in a nucleoside mixture obtained by enzymatic complete hydrolysis of 5′d(TT1TTT)3′ with a mixture of venom phosphodiesterase and alkaline phosphatase.[8] Therefore, we chemically converted **1** into 5-hydroxymethyl-2′-deoxyuridine, which was detected after complete hydrolysis.[8] However, reduction of the formyl group of **1** in **I-1** was unsuccessful since the reactivity of the formyl group appeared to depend on sequences around **1**. Therefore, we tried to hydrolyze **I-1** under acidic conditions. Namely, the oligonucleotide was completely hydrolyzed by using a mixture of nuclease P1 and acid phosphatase,[12] then the nucleoside composition was analyzed by HPLC. However, no sharp peak corresponding to **1** was observed even though the composition of other nucleosides was similar to the theoretical value (G : I : T : C = 3 : 2 : 1 : 3, I = deoxyinosine)[13] (Fig. 2e). The result was similar to that obtained using a mixture of venom phosphodiesterase and alkaline phosphatase. Therefore, we tried to develop a new method to confirm the existence of **1** in the oligonucleotides and found that treatment of oligonucleotide **I-1** with cyanomethylenetriphenylphosphorane in an aqueous MeOH solution gave an oligonucleotide (I-CN) (Fig. 2f) containing (*E*)- and (*Z*)-5-(2-cyanovinyl)-2′-deoxyuridine (**9, 10**) (Chart 1). Then, I-CN was completely hydrolyzed by using the mixture of venom phosphodiesterase and alkaline phosphatase and the nucleoside composition was analyzed by HPLC (Fig. 2g). Peaks corresponding to **9** and **10**, which were confirmed by co-elution with authentic samples synthesized as illustrated in Chart 1, were observed. The result clearly indicated that **1** was successfully incorporated into the oligonucleotides by using the diCPhEDA group. Even though the protecting group was not completely stable and was partially deprotected in the synthesizer, it was useful for synthesis of small or medium-size oligonucleotides, which can be used for biochemical studies. One of the difficulties of this synthesis is the instability of **1** in the oligonucleotides after removal of the diCPhEDA group, which caused low yields of final purified oligonucleotides. Instability of **1** in the oligonucleotides was also observed in a gel electrophoresis experiment in which each oligonucleotide phosphorylated with ^{32}P at the 5′-end showed complicated spots on polyacrylamide gel electrophoresis under denaturing conditions using a Tris–borate buffer[14] (data not shown), even though each oligonucleotide gave a single peak by HPLC analysis with an anion exchange column (Fig. 2d). The amino group in the Tris–borate buffer used in the gel electrophoresis experiments could react with the formyl group. However, the oligonucleotides **I-1** and **II-1** can be stored at $-30\,°C$ for at least 6 months in neutral solutions.

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Thermal Denaturation of Duplexes Profiles of thermal denaturation of the self-complementary duplexes, 5′d(GGAGA1CTCC)3′ (I-**1**) and a control 5′d(GGAGATCTCC) (I-T), showed single transitions corresponding to a helix-to-coil transition (Fig. 3). The T_m of I-**1** (40 °C) was much higher than that of the control (31 °C). It is well known that hydrophobic substituents, such as methyl, Br, *etc.*, at the 5-position of the uracil moiety stabilize duplex formation.[15] The formyl group, which stabilized duplex formation in our study, has similar bulkiness to a methyl group, though it is hydrophilic, not hydrophobic properties.[16] Thus, our result indicated that a hydrophilic substituent, such as the formyl group, could also stabilize duplex formation. Probably duplex formation was stabilized by the electron-withdrawing effect of the formyl group, which would increase the acidity of the N^3-imino proton.

Deoxynucleoside 5′-Triphoephate (dNTP) Incorporation by Klenow DNA Polymerase Mispair formation of **1** in DNA strands with incoming dNTP substrates during replication of DNA could be an important step for mutation. To discover which nucleotide can be incorporated into DNA strands at the site opposite to **1**, the template, 5′d(GCTGCXGCGAAAGCTG)3′ (X=**1** or T), and a primer, 5′d(^{32}P-CAGCTTTCGC)3′, were incubated with Klenow DNA polymerase in the presence of each dNTP, and the reaction products were analyzed by polyacrylamide gel electrophoresis under denaturing conditions[14] (Fig 4a). Spots due to newly generated 5′d(^{32}P-CAGCTTTCGCA)3′, in which dATP was incorporated at the 3′-end of the primer, were observed in the reactions using II-**1** and II-T as the templates. Incorporation of dGTP at the 3′-end was also observed. The **1**:G mismatch base-pair was formed like the T:G mismatch base-pair, which had been observed under similar conditions.[17] In contrast to purine nucleoside triphosphates being incorporated into the DNA strands, pyrimidine nucleoside triphosphates, TTP and dCTP, were not incorporated.

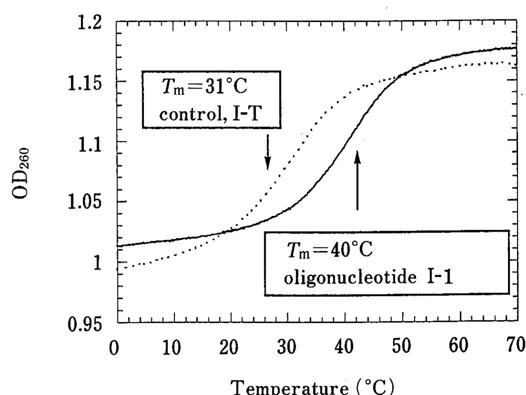
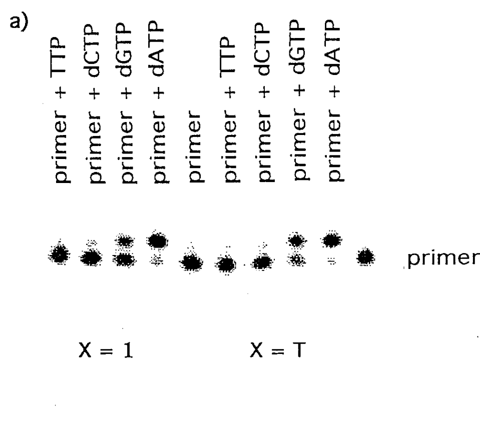


Fig. 3. Thermal Denaturation Profiles

Conditions are described in Experimental.

The elongation rate of DNA strands in the presence of dATP, dGTP, and dCTP was next examined (Fig. 4b). Under conditions such that the intact primer was almost consumed after 40 min with II-T as a template, the intact primer still remained after 90 min with II-**1**. DNA strand elongation was retarded by the presence of **1**, since incorporation of dATP at the site opposite to **1** was much slower than that of T. But once dATP was incorporated at the site opposite to **1**, the DNA strand seemed to be elongated smoothly, since a full-length strand was already observed after 20 min.

Schiff Base Formation of 1 with Amines It was reported that **1** and its sugar-protected derivatives formed Schiff bases with various amines such as ethylamine, methylamine, lysine, *etc.*[18] We also observed that **1** and ethanolamine formed a Schiff base at physiological pH (pH 7—8) by a spectroscopic study (data not shown). To confirm the Schiff base formation of **1** in DNA strands under physiological conditions, I-**1** was treated with ethanolamine and the reaction mixture was analyzed by HPLC. However, a newly generated oligonucleotide containing Schiff base residue could not be clearly detected by HPLC (data not shown). Therefore, we studied optical properties of several Schiff base derivatives and found that the phenylhydrazone derivative **11** (Chart 1) has a characteristic absorbance at longer wavelengths. Also,



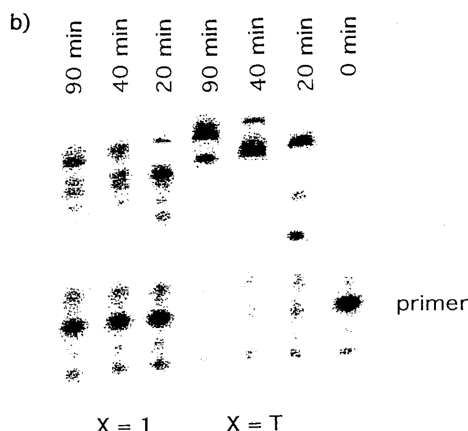


Fig. 4. a) Radioactivity of the Polyacrylamide Gel Showing Incorporation of Each dNTP into the DNA Strand at the Site Opposite to X b) Profiles of DNA Elongation in the Presence of dNTPs

a) The natural template II-T (right) or the modified template II-**1** (left) and the primer were incubated with Klenow DNA polymerase in the presence of dATP, dGTP, dCTP, TTP or no nucleoside triphosphate. b) The natural template II-T (right) or the modified template II-**1** (left) and the primer were incubated with Klenow DNA polymerase in the presence of nucleoside triphosphates (dATP dGTP and dCTP) or no nucleotide triphosphate. The reaction mixture was incubated for 20, 40, and 90 min.

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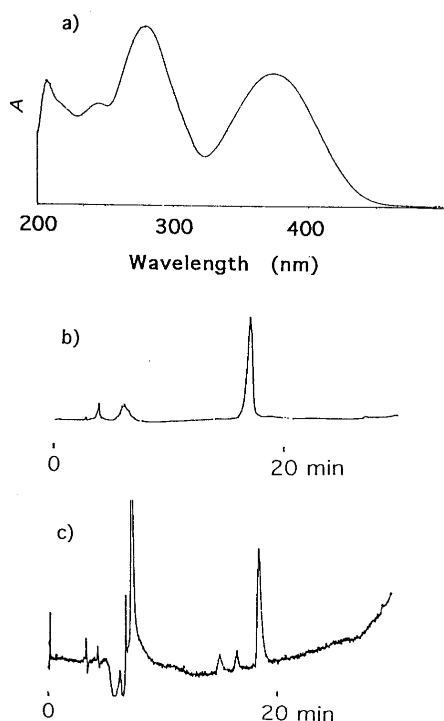

Fig. 5. a) A VIS and UV Absorption Profile of **12** in MeOH b) HPLC Profile of I-1, a Linear Gradient of CH_3CN from 2.5 to 18% (20 min) in 0.1 M TEAA Buffer (pH 7.0) with the C-18 Column. c) HPLC Profile of I-1 in the Presence of Phenylhydrazine, a Linear Gradient of CH_3CN from 2.5 to 18% (20 min) in 0.1 M TEAA Buffer (pH 7.0) with the C-18 Column

b) Peaks were observed at 260 nm. c) Peaks were observed at 420 nm.

formation of the phenylhydrazone derivative was observed immediately and quantitatively on incubation of the oligonucleotides with phenylhydrazine. For example, incubation of **1** with phenylhydrazine gave the hydrazone derivative **11** (Chart 1), of which the UV and VIS absorbance profiles are shown in Fig. 5a. Oligonucleotide I-1 was incubated with phenylhydrazine and the reaction mixture was analyzed by HPLC. A peak which has a longer retention time than I-1 (Fig. 5b) was observed at 420 nm (Fig. 5c). Since I-1 and phenylhydrazine do not have optical absorption at 420 nm, the peak must be due to the oligonucleotide containing the phenylhydrazone derivative **11**. The result may indicate the Schiff base formation of the formyl group in DNA with certain amine residues. Further studies on DNA–protein cross-links will be of interest.

Conclusion

A new protecting group of the formyl group in 5-formyl-2′-deoxyuridine (**1**) was developed. Using the protecting group, **1** was incorporated into DNA oligomers. From a thermal denaturation study, it was shown that the formyl group of **1** could stabilize duplex formation. Mispair formation of **1** may not be a major contributor to mutagenesis since a DNA replication study showed that dATP was preferentially incorporated into the DNA strand at the site opposite to **1** and no gross mismatch base-pair formation was observed. However, DNA replication was delayed by the presence of **1** in the DNA strand and this phenomenon could damage cells. It was also shown that the formyl group was reactive with Wittig reagent or alkylamines in DNA. Therefore, the formyl group may participate in DNA–protein cross-link formation through a covalent bond, such as Schiff base formation between the formyl group and some amine residues of a protein.

Experimental

Melting points were measured on a Yanagimoto MP-3 micromelting point apparatus (Yanagimoto, Japan) and are uncorrected. Mass spectra (MS) were measured on a JEOL JMS-DX303 spectrometer at an ionizing voltage of 70 eV. Field desorption mass spectra (FD-MS) and fast atom bombardment mass spectra (FAB-MS) were measured on a JEOL JMS-HX110 at an ionizing voltage of 70 eV. The ^1H-NMR spectra were recorded on a JEOL JNM-FCC 100FT (100 MHz), a JEOL JNM-GX 270 (270 MHz) or a Bruker ARX500 (500 MHz) spectrometer with tetramethylsilane as an internal standard. Chemical shifts are reported in parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), m (multiplet), or br (broad). All exchangeable protons were detected by disappearance of the signal on the addition of D_2O. TLC was done on Merck Kieselgel F_{254} precoated plates (Merck). The silica gel, neutralized silica gel, or silanized silica gel used for column chromatography were YMC gel 60A (70—230 mesh) (YMC Co., Ltd.), ICN silica 60A (ICN Biochemicals), or Preparative C18 125 Å (Waters), respectively. UV absorption spectra were recorded with a Shimadzu UV-240 spectrophotometer (Shimadzu Co.).

3′,5′-Di-*O*-acetyl-5-formyl-2′-deoxyuridine (3) A solution of $K_2S_2O_8$ (8.3 g, 30.6 mmol) and $CuSO_4 \cdot 5H_2O$ (1.5 g, 6.1 mmol)[19] in H_2O (60 ml) was added to a CH_3CN solution (60 ml) containing **2** (5.0 g, 15.3 mmol) and 2,6-lutidine (6.1 ml, 53.0 mmol), and the reaction mixture was stirred for 2 h at 65 °C. It was concentrated to half the initial volume and the remaining solution was extracted with EtOAc. The organic layer was successively washed with H_2O, then aqueous 5% EDTA (pH 4). The H_2O layers were combined and back-extracted with $CHCl_3$, then the organic layers were combined, dried over Na_2SO_4, and concentrated. The residue was chromatographed on a silica gel column (3.8 × 15 cm) with a mixture of EtOAc and hexane (1 : 3, v/v) as an eluent. The fractions were concentrated and the residue was crystallized from EtOAc to give **3** as white crystals (2.5 g, 52%): mp 160—161 °C. EI-MS *m/z*: 340 (M^+). UV λ_{max} nm (MeOH): 289. ^1H-NMR (100 MHz, $CDCl_3$): 10.02 (1 H, s, CHO), 8.50 (1 H, br s, NH), 8.49 (1 H, s, H-6), 6.33 (1 H, dd, $J_{1',2'a}=8.0$, $J_{1',2'b}=5.8$ Hz, H-1′), 5.30 (1 H, m, H-3′), 4.30 (3H, m, H-4′, H-5′a, b), 2.80—2.20 (2 H, m, H-2′a, b), 2.22 (3 H, s, Ac), 2.12 (3H, s, Ac). *Anal.* Calcd for $C_{14}H_{16}N_2O_8$: C, 49.42; H, 4.74; N, 8.23. Found: C, 49.01; H, 4.73; N, 7.95.

diCPhEDA (4)[20] A dimethylformamide (DMF) solution (10 ml) containing 3,5-dichloroaniline (11 g, 69 mmol) and ethylene dibromide (2.1 g, 11 mmol) was stirred at 130 °C for 6 h, then the mixture was concentrated and the residue was extracted with $CHCl_3$. The organic layer was successively washed with H_2O, then 1 N HCl solution (twice), dried over Na_2SO_4, and concentrated. The residue was chromatographed on a silica gel column (5.0 × 15 cm) with a mixture of EtOAc and hexane (1 : 9, v/v) as an eluent. The fractions were concentrated, then the residue was dissolved in hexane and crystals were collected to give **4** (2.3 g, 50%): mp 127—129 °C. FAB-MS *m/z*: 350 (M^+). ^1H-NMR (100 MHz, DMSO-d_6): 6.60—6.24 (6H, m, Ph), 3.42—3.24 (4H, m, PhHNC$\underline{H}_2$-C$\underline{H}_2$NHPh). *Anal.* Calcd for $C_{14}H_{12}Cl_4N_2$: C, 48.03; H, 3.46; Cl, 40.51; N, 8.00. Found: C, 48.01; H, 3.41; Cl, 40.32; N, 7.90.

3′,5′-Di-*O*-acetyl-5-[N^1,N^3-di-(3,5-dichlorophenyl)imidazolidino-2-]-2′-deoxyuridine (5) D-Camphorsulfonic acid (7 mg, 0.03 mmol) was added to a DMF solution (10 ml) containing **3** (500 mg, 1.5 mmol) and **4** (770 mg, 2.2 mmol), and the reaction mixture was stirred for 18 h at room temperature. The reaction mixture was neutralized by adding saturated aqueous $NaHCO_3$, and the precipitates were extracted with EtOAc. The organic layer was successively washed with saturated aqueous $NaHCO_3$, then H_2O, dried over Na_2SO_4, and concentrated. The residue was chromatographed on a silica gel column (3.8 × 12.5 cm) with a mixture of EtOAc and hexane (2 : 3, v/v) as an eluent. The fractions were concentrated and the residue was crystallized from a mixture of EtOAc and hexane to give **5** (710 mg, 72%): mp 266—267 °C. FAB-MS *m/z*: 673 ($M^+ + 1$). ^1H-NMR (270 MHz, $CDCl_3$): 11.52 (1H, s, NH), 8.41 (1H, s, H-6), 6.79 (2H, s, dichlorophenyl), 6.69 (4H, s,

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dichlorophenyl), 6.15—6.09 [2H, m, H-1′, C<u>H</u>(NPhCH$_2$)$_2$], 5.12 (1H, s, 3′-OH), 4.30 (1H, s, 5′-OH), 3.90—3.80 (3H, m, H-4′, H-5′), 3.71—3.66 (4H, m, PhNC<u>H</u>$_2$C<u>H</u>$_2$NPh), 2.13—2.00 (2H, m, H-2′). *Anal.* Calcd for $C_{28}H_{26}Cl_4N_4O_7$: C, 50.02; H, 3.90; Cl, 21.09; N, 8.33. Found: C, 50.23; H, 4.02; Cl, 20.79; N, 8.13.

5-[N^1,N^3-Di-(3,5-dichlorophenyl)imidazolidino-2-]-2′-deoxyuridine (6) Compound **5** (200 mg, 0.3 mmol) was dissolved in a 0.2 M solution of NaOMe in MeOH (3.5 ml) and the mixture was stirred at room temperature. After 1 h, EtOAc and saturated aqueous NaH_2PO_4 were added to the reaction mixture and the organic layer was separated, washed with H_2O, dried over Na_2SO_4, and concentrated. The residue was chromatographed on a silica gel column (2.3 × 10 cm) with 6% EtOH in $CHCl_3$ as an eluent. The fractions were concentrated to give **6** as white crystals (171 mg, 98%): mp 151—158 °C. FAB-MS *m/z*: 589 (M^+ + 1). ^{1}H-NMR (270 MHz, $CDCl_3$): 11.52 (1H, s, NH), 8.41 (1H, s, H-6), 6.79 (2H, s, dichlorophenyl), 6.69 (4H, s, dichlorophenyl), 6.15—6.09 [2H, m, H-1′, -C<u>H</u>(NPhCH$_2$)$_2$], 5.12 (1H, s, 3′-OH), 4.30 (1H, s, 5′-OH), 3.90—3.80 (3H, m, H-4′, H-5′a, b), 3.71—3.66 (4H, m, PhNC<u>H</u>$_2$C<u>H</u>$_2$NPh), 2.13—2.00 (2H, m, H-2′a, b). *Anal.* Calcd for $C_{24}H_{22}Cl_4N_4O_5$: C, 49.00; H, 3.77; Cl, 24.11; N, 9.52. Found: C, 49.23; H, 3.91; Cl, 24.06; N, 9.41.

5′-*O*-Dimethoxytrityl-5-[N^1,N^3-di-(3,5-dichlorophenyl)imidazolidino-2-]-2′-deoxyuridine (7) DMTrCl (570 mg, 1.7 mmol) was added to a pyridine solution (5 ml) containing **6** (550 mg, 0.94 mmol) and the whole was stirred at room temperature. After 3 h, EtOH (6.5 ml) was added to the reaction mixture and the solution was concentrated. The residue was dissolved in $CHCl_3$ and the solution was washed three times with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, and concentrated. The residue was coevaporated with toluene and chromatographed on a neutralized silica gel column (2.8 × 14 cm) with 1% EtOH in $CHCl_3$ as an eluent. The fractions were concentrated and the residue was dissolved in a small volume of $CHCl_3$. The solution was added dropwise to hexane (500 ml) and the resulting precipitates were collected to give **7** (280 mg, 34%). The filtrate was concentrated to give **7** as a foam (46 mg, 6%): FAB-MS *m/z*: 891 (M^+ + 1). ^{1}H-NMR (270 MHz, DMSO-d_6): 11.49 (1H, s, NH), 8.10 (1H, s, H-6), 7.29—7.20 (9H, m, DMTr), 6.87—6.60 (10H, m, DMTr, dichlorophenyl), 6.07—6.04 [2H, m, H-1′, 5-C<u>H</u>(NPhCH$_2$)$_2$], 5.33 (1H, br s, 3′-OH), 4.23 (1H, m, H-3′). 3.72, (6H, s, methoxy groups of DMTr), 3.37—3.32 (4H, m, PhNC<u>H</u>$_2$C<u>H</u>$_2$NPh), 3.25—3.22 (2H, m, H-5′), 2.51—2.15 (2H, m, H-2′). *Anal.* Calcd for $C_{45}H_{40}Cl_4N_4O_7$: C, 60.69; H, 4.53; Cl, 15.92; N, 6.29. Found: C, 60.25; H, 4.52; Cl, 15.68; N, 6.19.

3′-*O*-[2-Cyanoethyl(*N,N*-diisopropyl)phosphinyl]-5′-*O*-dimethoxytrityl-5-[N^1,N^3-di-(3,5-dichlorophenyl)imidazolidino]-2′-deoxyuridine (8) After it was dried by coevaporation twice with anhydrous pyridine, **7** (250 mg, 0.28 mmol) was dissolved in CH_2Cl_2 (5 ml). *N,N*-Diisopropylethylamine (98 μl, 2 eq) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (94 μl, 1.5 eq)[10] were added to the solution and the mixture was stirred for 1 h at room temperature. $CHCl_3$ and saturated aqueous $NaHCO_3$ were added to the reaction mixture and the organic layer was washed twice with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, and concentrated. The residue was chromatographed on a neutralized silica gel column (2.3 × 10 cm) with a mixture of EtOAc and hexane (1 : 2, v/v) as an eluent. The fractions were concentrated to give **8** as a foam (185 mg, 54%): FAB-MS *m/z*: 1091 (M^+ + 1).

2′-Deoxy-5-formyluridine (1) Compound **6** (200 mg, 0.34 mmol) was dissolved in a mixture of AcOH (5 ml) and H_2O (0.5 ml) and the solution was stirred at 50 °C. After 16 h, the solvents were evaporated and the residue was coevaporated three times with a mixture of EtOH and H_2O (1 : 1, v/v). Et_2O and H_2O were added to the residue and the H_2O layer was extracted three times with Et_2O. The H_2O layer was concentrated and the residue was crystallized from MeOH to give **1** (85 mg, 98%): mp 165—168 °C. ^{1}H-NMR (270 MHz, DMSO-d_6): 11.74 (1H, br s, NH), 9.76 (1H, s, CHO), 8.71 (1H, s, H-6), 6.09 (1H, t, J = 6.4 Hz, H-1′), 5.27 (1H, d, 3′-OH), 5.11 (1H, t, 5′-OH), 4.24 (1H, m, H-3′), 3.86 (1H, m, H-4′), 3.62 (2H, d, H-5′a, b), 2.23 (2H, m, H-2′a, b). *Anal.* Calcd for $C_{10}H_{12}N_2O_6$: C, 46.88; H, 4.72; N, 10.93. Found: C, 46.59; H, 4.74; N, 10.74.

(*E*)- and (*Z*)-5-(2-Cyanovinyl)-2′-deoxyuridine (9, 10) Compound **1** (50 mg, 0.2 mmol) and cyanomethylenetriphenylphosphorane (65 mg, 1.1 eq) were dissolved in a mixture of H_2O (0.5 ml) and MeOH (1 ml) and the reaction mixture was kept at room temperature overnight. The solvents were evaporated and the residue was chromatographed on a silica gel column (1.5 × 5 cm) with 15% MeOH in $CHCl_3$ as an eluent. Fractions were concentrated to give a mixture of **9** and **10** as a solid (50 mg, 93%). A part of the mixture was separated by HPLC using a C-18 column (YMC D-ODS-5, 20 × 250 mm) with aqueous 40% MeOH at a flow rate of 5 ml/min. Peaks at the retention times of 15.6 and 20.3 min, respectively, were detected at 275 nm and collected. The solvent was removed and the well-dried solid samples were analyzed by ^{1}H-NMR and MS spectroscopy. Physical data for (*E*)-5-(2-cyanovinyl)-2′-deoxyuridine (**9**, the retention time: 15.6 min); ^{1}H-NMR (500 MHz, DMSO-d_6): 11.69 (1H, br s, NH), 8.59 (1H, s, H-6), 7.14 (1H, d, J = 12.1 Hz, vinylic), 6.17 (1H, t, J = 6.6 Hz, H-1′), 5.72 (1H, d, J = 12.1 Hz, vinylic), 5.28 (1H, d, 3′-OH), 4.91 (1H, t, 5′-OH), 4.25 (1H, br s, H-3′), 3.82 (1H, dd, H-4′), 3.57 (2H, d, H-5′a, b), 2.10—2.25 (2H, m, H-2′a, b). EI-HR-MS *m/z* Calcd for $C_{12}H_{13}N_3O_5$: 279.0855. Found: 279.0833. Physical data for (*Z*)-5-(2-cyanovinyl)-2′-deoxyuridine (**10**, the retention time: 20.3 min); ^{1}H-NMR (500 MHz, DMSO-d_6): 11.68 (1H, br s, NH), 8.35 (1H, s, H-6), 7.23 (1H, d, J = 16.3 Hz, vinylic), 6.52 (1H, d, J = 16.3 Hz, vinylic), 6.09 (1H, t, J = 6.3 Hz, H-1′), 5.18 (1H, br s, OH), 5.13 (1H, br s, OH), 4.24 (1H, dd, J = 4.3 Hz, J = 9.1 Hz, H-3′), 3.82 (1H, dd, J = 3.6 Hz, J = 7.2 Hz, H-4′), 3.65 (1H, dd, $J_{4',5'a}$ = 3.6 Hz, J_{gem} = 11.9 Hz, H-5′a), 3.58 (1H, dd, $J_{4',5'b}$ = 3.7 Hz, J_{gem} = 11.9 Hz, H-5′b), 2.12—2.21 (2H, m, H-2′a, b). EI-HR-MS *m/z* Calcd for $C_{12}H_{13}N_3O_5$: 279.0855. Found: 279.0832.

Phenylhydrazone Derivative 11 Compound **1** (100 mg, 0.4 mmol) was added to a solution of phenylhydrazine (55 mg, 0.55 mmol) in 50% aqueous EtOH (3 ml) and the pH of the reaction mixture was adjusted to 6 by adding acetic acid. After 1.5 h, the suspended yellow crystals were collected and washed with cold 50% aqueous EtOH to give **11** (114 mg, 84%): mp 203—206 °C. EI-MS *m/z*: 346 (M^+). ^{1}H-NMR (270 MHz, DMSO-d_6): 11.54 (1H, br s, NH), 10.23 (1H, br s, NH), 8.35 (1H, s, H-6 or 5-CH), 7.72 (1H, s, H-6 or 5-CH), 7.17 (2H, t, Ph), 7.00 (2H, d, Ph), 6.71 (1H, t, Ph), 6.22 (1H, t, J = 6.7 Hz, H-1′), 5.28 (1H, d, 3′-OH), 5.07 (1H, t, 5′-OH), 4.30 (1H, m, H-3′), 3.85 (1H, m, H-4′), 3.64 (2H, d, H-5′a, b), 2.18 (2H, m, H-2′a, b). *Anal.* Calcd. for $C_{16}H_{18}N_4O_5$: C, 55.49; H, 5.24; N, 16.16. Found: C, 55.26; H, 5.39; N, 16.01.

Synthesis of Oligonucleotides Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems Model 381A) by the phosphoramidite method.[12] A solution of 1% dichloroacetic acid in CH_2Cl_2 (1 min) was used for deprotection of the DMTr group in the DNA synthesis.[8] Synthesis was monitored by spectrophotometric measurement of released dimethoxytrityl cation at 500 nm on each addition. Each oligonucleotide linked on the resin was treated with concentrated NH_4OH for 5 h at 55 °C, then the released oligonucleotide, protected by a DMTr group at the 5′-end and diCPhEDA at the formyl group was chromatographed over the C-18 silica gel (Waters) column (1 × 10 cm) with a linear gradient of acetonitrile in 0.1 M TEAA buffer (pH 7.0). Fractions were combined and concentrated to half of the initial volume, then the concentrated solution was adsorbed on a short C-18 silica gel column (1 × 1 cm). The column was washed with water (5 ml) to remove the TEAA buffer, and the oligonucleotide was eluted with 50% acetonitrile (5 ml). The fractions were concentrated and the residue was treated with 80% acetic acid overnight at room temperature. The solution was concentrated and the residue was coevaporated with H_2O. The residue was dissolved in H_2O and the deprotected oligonucleotide was adsorbed on the C-18 silica gel column to remove DMTrOH and diCPhEDA. The column was washed with H_2O (5 ml), then the oligonucleotide was eluted with aqueous 50% CH_3CN (5 ml). The sample was purified by HPLC with the C-18 silica gel column (Inertsil ODS-2, GL Science Inc.). The fractions were desalted on the C-18 column as described above.

Complete Hydrolysis of Oligonucleotides. Method A Each oligonucleotide (0.1—0.5 OD units at 254 nm) was incubated with venom phosphodiesterase (10 μg, Boehringer Mannheim, Germany) and alkaline phosphatase (0.4 units, Takara Shuzo Co., Ltd.) in a buffer containing 0.1 M Tris–HCl (pH 8.2) and 2 mM $MgCl_2$ (total 140 μl) for 24 h at 37 °C, then cold EtOH (0 °C, 320 μl) was added to the solution. The whole was kept at −20 °C for 1 h, then the solution was centrifuged for 20 min at 0 °C (12000 rpm). The supernatant was separated and concentrated. The residue, a mixture of nucleosides, was analyzed by HPLC.

Method B Each oligonucleotide (0.1—0.5 OD units at 254 nm) was incubated with Nuclease P1 (120 ng, Yamasa Shoyu Co. Ltd.) and acid phosphatase (300 units, Sigma) in a buffer containing 0.1 M NaOAc (pH 5.5) and 2 mM $ZnCl_2$ (total 25 μl) at 37°C for 24 h, then cold EtOH (0 °C, 500 μl) was added to the solution. The whole was kept at −20 °C overnight, then the solution was centrifuged at 0 °C for

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20 min (12000 rpm). The supernatant was separated and concentrated. The residue, a mixture of nucleosides, was analyzed by HPLC.

Reaction of I with Cyanomethylenetriphenylphosphorane An MeOH solution saturated with cyanomethylenetriphenylphosphorane (50 μl) was added to a solution (50 μl) containing oligonucleotide I (0.3 OD units at 254 nm) and the mixture was incubated overnight at room temperature. H_2O (500 μl) was added to the reaction mixture and the solution was washed twice with Et_2O (500 μl). The H_2O layer was concentrated and the reagent was separated by HPLC to give I-CN (0.3 OD unit), which gave a single peak on HPLC analysis.

Thermal Denaturation The solution contained each oligonucleotide (1.0 OD unit at 260 nm) in a buffer of 0.01 M sodium phosphate and 0.1 M NaCl (pH 7.0). Thermally induced transition of each mixture was monitored at 260 nm on a Gilford Response II.

Incorporation of Each dNTP into DNA Strands by Klenow DNA Polymerase Solutions (50 μl) of a template [5′d(GCTGCXGCGAA-AGCTG)3′ (X=**1** or T) (0.16 OD units at 254 nm)], a primer [5′(^{32}P-CAGCTTTCGC)3′ (0.1 OD units at 254 nm)], each dNTP (50 nmol), and Klenow DNA polymerase (0.1 units) (Takara Shuzo, Co., Ltd.) in a buffer containing 67 mM potassium phosphate (pH 7.4), 6.7 mM $MgCl_2$, and 1 mM mercaptoethanol were incubated at 20 °C. After 1 h, the reaction mixtures were heated on a boiling water bath for 5 min, cooled and concentrated. Then, the reaction mixtures were analyzed by electrophoresis on 20% polyacrylamide gel containing 8 M urea.[14] Radioactivity on the gel was visualized by a Bio-imaging Analyzer (Bas 2000, Fuji, Co. Ltd.).

Incorporation of dNTPs into DNA Strands by Klenow DNA Polymerase The same components as above, except that the solution contained a mixture of dATP, dGTP and dCTP (each 50 nmol), were incubated at 20 °C. After 20, 40 and 90 min, aliquots of the reaction mixtures were separated and analyzed by electrophoresis on 20% polyacrylamide gel containing 8 M urea.[14] The gels were analyzed as described above.

Reaction of Oligonucleotide I with Phenylhydrazine A solution (100 μl) containing oligonucleotide **I-1** (0.1 OD units at 254 nm) and phenylhydrazine (1 μl, 10 nmol) in 0.1 M TEAA buffer (pH 6.0) was incubated at room temperature. After 2 h, an aliquot of the reaction mixture was analyzed by HPLC with the C-18 column.

Acknowledgments This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture, Japan.

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