Chemical Synthesis and Properties of an Oligodeoxyribonucleotide Containing a 2-Deoxyribosylformamide Residue

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To elucidate the conformational properties of DNA with a 2-deoxyribosylformamide residue (dF), an oligodeoxyribonucleotide containing this abasic residue in a specific position of the nucleotide sequence was synthesized by the standard solid-phase phosphotriester method. Deprotection of the synthesized oligonucleotide was performed under routine alkaline and acidic conditions. The presence of a dF residue in the oligomer was confirmed by ion-spray mass spectrometry. A dF residue was found to affect considerably the stability of the DNA duplex, as determined from the melting behavior of the dF-containing duplex.

Keywords oligodeoxyribonucleotide; 2-deoxyribosylformamide; ion-spray mass spectrometry; thermal denaturation

In living cells, exposure of nucleic acids to environmental agents such as ionizing radiation,[1] ultraviolet (UV) light,[2] and a variety of chemical agents[3] leads to modification of their constituents and conformational alterations in the DNA. In particular, the base degradations induced in DNA are mostly noncoding lesions and are thought to be directly responsible for cellular lethality, mutagenesis, and carcinogenesis.[4] A major portion of DNA damage results from free radicals and the consequent base damage results in a loss of base-pairing abilities. 2-Deoxy-β-D-ribofuranosyl-*N*-formamide (2-deoxyribosylformamide, dF) is a major oxidative product formed from T in the DNA by ionizing radiation.[5] The formation of dF may involve the attack of the hydroxy radical on the C5–C6 double bond of the pyrimidine ring,[6] followed by hydrolytic cleavage of the hydroxy–hydroperoxy intermediates. A similar oxidative product was found among the degradation products of d(CpG) by a 1,10-phenanthroline–copper ion complex.[7] Since the modification was derived from the dG residue, the first step in the production of dF may be oxidation of the guanine residue at the C4–C5 double bond.[8] Recently, attempts to synthesize oligonucleotides containing various types of thymine damage at a defined position have been made.[9]

We are interested in the influence of the dF residue in the DNA chain, and its structural and biochemical consequences. However, a wide variety of types of base damage by free radicals makes it extremely difficult to prepare oligonucleotides which contain only one type of damage at a defined site with free radicals. In this paper, we report on the synthesis of an oligodeoxyribonucleotide containing a dF at a defined site, and on the thermal denaturation of a duplex containing this abasic damage.

Experimental

Materials and General Procedures Silica gel column chromatography was conducted with Merck Silica gel 60. Reverse-phase silica gel chromatography was conducted with a Waters preparative C18 (mesh 55—105 μm). Thin-layer chromatography (TLC) was performed on Merck Silica gel $60F_{254}$ using a $CHCl_3$–MeOH mixture. Nuclease P1 was obtained from Yamasa Shoyu Co. and incubation was conducted in 0.05 M ammonium acetate (pH 5) at 37 °C for 2 h with the enzyme (5 μg/ml).

UV absorption spectra were recorded on a Shimadzu UV-250 spectrophotometer. For melting temperature measurement, a Shimadzu UV-3100 spectrophotometer equipped with a SPR-5 Shimadzu temperature programmer was used. Circular dichroism (CD) spectra were recorded on a JASCO J-600 spectropolarimeter. For temperature variation experiments, a thermo-jacketed cell and a circulating bath were used. The molar absorption coefficient, ε, and the molar ellipticity, [θ], are presented in terms of per base residue values. An ion-spray mass spectrum was taken on a PE SCIEX API-III mass spectrometer equipped with an atmospheric pressure ion source in the negative ion mode. Synthesized oligonucleotide was dissolved in a CH_3OH–H_2O (1 : 1) mixture containing 0.1% HCOOH, 1 mM NH_4OAc and continuously injected at a rate of 5 μl/min (approximately 100 pmol/μl). ^{1}H-NMR spectra were recorded on a Bruker AC250 (250 MHz) spectrometer with tetramethylsilane (TMS) as an internal standard. HPLC was conducted with a Shimadzu LC-6A pump and an SPD-6A UV monitor. The reverse-phase column used was a Wakopac WS-DNA (250 × 4.6 mm i.d.).

2-Deoxyribosylformamide (dF, **1**) was synthesized according to the published procedure[10] (TLC ($CHCl_3$–MeOH, 10 : 1): Rf = 0.08, (ethyl acetate–acetone–water, 5 : 10 : 1): Rf = 0.50).

2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl-*N*-formamide (2) After co-evaporation with dry pyridine, dF (1.0 mmol) was dissolved in pyridine (3 ml) and 4,4′-dimethoxytrityl chloride (1.2 mmol) was added. The mixture was stirred at room temperature for 2 h. Then 50% aqueous pyridine was added and the product was extracted with $CHCl_3$ (30 ml). The organic layer was washed with saturated $NaHCO_3$ (20 ml × 2) and water (20 ml × 2) and dried with Na_2SO_4. The solvent was evaporated off, and the residue was co-evaporated with toluene. The residue was dissolved in $CHCl_3$ and chromatographed on a column of Silica gel 60. Stepwise elution with $CHCl_3$–MeOH mixtures was performed. The fractions containing the product were combined and concentrated. The residue was co-evaporated with pyridine and dissolved in $CHCl_3$. Compound **2** was isolated by precipitation from hexane. The yield was 217 mg (0.47 mmol, 47% from **1**). TLC ($CHCl_3$–MeOH, 10 : 1): Rf = 0.51. UV_{max} (95% EtOH): 268 (shoulder), 273, 281. ^{1}H-NMR (in $CDCl_3 + D_2O$, two conformers) δ: 2.0—2.3 (m, 2H, H-2, 2′), 3.1—3.4 (m, 2H, H-5, 5′), 3.79 (s, 6H, CH_3O), 3.9—4.0 (m, 1H, H-4), 4.3—4.4 and 4.5—4.6 (m, 1H, H-3), 5.9—6.1 (m, 1H, H-1), 6.8—7.4 (m, 13H, H-aromatic), 8.19 and 8.23 (s, 1H, H–C=O). *Anal.* Calcd for $C_{27}H_{29}NO_6$: C, 69.96; H, 6.31; N, 3.02. Found: C, 69.86; H, 6.30; N, 3.29.

2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl-*N*-formamide-3-*O*-[(2-chlorophenyl)-(2-cyanoethyl)-phosphate] (3) 2-Chlorophenylphosphoro dichloridate (1 mmol) was added to a solution of triazole (2.2 mmol) and triethylamine (2.2 mmol) in dioxane and stirred at 0 °C under a dry N_2 atmosphere. The precipitated salt was filtered off and the filtrate was added to a solution of **2** (232 mg, 0.5 mmol) in dry pyridine (1 ml) at room temperature. After completion of the reaction (1 h, TLC analysis), ethylene cyanohydrin (1.75 mmol) was added to the mixture together with *N*-methylimidazole (2 mmol) at room temperature with stirring. After 2 h, 50% aqueous pyridine (0.5 ml) was added to the reaction mixture. The solvent was evaporated and the residue was dissolved in $CHCl_3$ (30 ml). The organic layer was washed with water (30 ml × 2) and 0.1 M $Et_3NH_2CO_3$ (30 ml × 2) and evaporated. The residue was co-evaporated with pyridine and toluene. The resulting gum was dissolved in $CHCl_3$ and chromatographed on a column of Silica gel 60. Elution was carried out with $CHCl_3$–MeOH mixtures. The fractions containing the product were combined and concentrated. The residue was co-evaporated with pyri-

dine and toluene, and the resultant gum was dissolved in $CHCl_3$. Compound **3** was isolated by precipitation from hexane. The yield was 189 mg (0.27 mmol, 54% from **2**). TLC ($CHCl_3$–MeOH, 10:1): $Rf=0.59$. UV_{max} (95% EtOH): 267.5 (shoulder), 274.5, 281 (shoulder). ^{1}H-NMR (in $CDCl_3+D_2O$, four conformers) δ: 8.22, 8.20, 8.14, and 8.11 (s, 1H, H–C=O), 6.8—7.46 (m, 17H, H-aromatic). *Anal.* Calcd for $C_{36}H_{36}ClN_2O_9P$: C, 61.15; H, 5.13; N, 3.96. Found: C, 60.92; H, 5.35; N, 4.15.

Triethylammonium[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl-*N*-formamide-3-*O*-(2-chlorophenyl phosphate)] (4) Compound **3** (183 mg, 0.26 mmol) was dissolved in a mixture of pyridine, triethylamine, and water (3:1:1, 5 ml). The solution was kept at room temperature for 15 min, and checked for disappearance of **3** by TLC on a silica gel plate. The solvent was evaporated off, and the residue was co-evaporated with pyridine and toluene and dissolved in $CHCl_3$. Compound **4** was precipitated as a gum from hexane and was dried *in vacuo*, giving a foam. The yield was 177 mg (0.24 mmol, 91%). TLC ($CHCl_3$–MeOH, 10:1): $Rf=0.13$, (ethyl acetate–acetone–water, 5:10:1): $Rf=0.20$.

Oligonucleotide Synthesis and Purification Oligodeoxyribonucleotides were synthesized by the standard solid-phase phosphotriester method.[11] The manual synthesizer was charged with nucleoside-loaded 1% polystyrene resin (10 μmol). After the chain assembly was completed, the resultant oligomer was treated with 1 M tetramethylguanidine *syn*-*p*-nitrobenzaldoximate in dioxane–water (0.87:1.00, v/v) for 48 h and filtered. The filtrate was dried *in vacuo*, followed by treatment with concentrated NH_4OH at 60 °C for 6 h. The dimethoxytrityl (DMTr)-containing oligomer was purified on a Sephadex 25 column and on a reverse-phase C_{18} column. Detritylation was performed with 80% AcOH for 15 min at room temperature. The product was purified by DEAE-cellulose (DE 52, Whatman) chromatography. The oligomer was eluted at 0.27 M NaCl concentration (0.02 M Tris–HCl (pH 8.0)–7 M urea). The main peak was further purified by reverse-phase high-performance liquid chromatography (HPLC). The purity of the final product was checked by HPLC and found to be greater than 97%.

Results and Discussion

Synthesis of Oligodeoxyribonucleotide Containing the 2-Deoxyribosylformamide Residue Solid-phase synthesis of the oligodeoxyribonucleotides used in this study, d(CGCFCGCGTGCG), d(CGCACGCGTGCG), d(CGCACGCGAGCG), and d(CGCCCGCGTGCG), was performed by the phosphotriester method.[11] The preparation of the protected 2-deoxyribosylformamide phosphodiester block **4** is shown in Chart 1. Compound **1** was not decomposed under the conditions of deprotection of normally synthesized oligodeoxyribonucleotide, namely 2% benzenesulfonic acid in $CHCl_3$–MeOH (3:1), 80% AcOH, and concentrated NH_4OH at 60 °C (6 h) (data not shown). Consequently, DMTr was chosen as the protecting group for the 5-*O*-hydroxy group of the deoxyribose residue and *o*-chlorophenyl was chosen as the protecting group for the internal phosphotriester group. Compound **2** was prepared from **1** by using DMTrCl. The structure of the purified **2** was confirmed by ^{1}H-NMR, which revealed the presence of two conformers due to the energy barrier to amide bond rotation of the formamide group (Fig. 1). The *cis-trans* conformers of compound **1** are also detectable by ^{1}H-NMR.[10] Compound **3** was derived from **2** in order to purify the phosphorylated product by silica gel column chromatography. The ^{1}H-NMR spectrum of compound **3** showed the presence of four conformers due to the amide bond rotation and phosphate isomer, as detected by the well-separated formyl proton resonances at low field (8.22—8.11 ppm). Compound **4** was derived from **3** by treatment with pyridine–triethylamine–water (3:1:1) for 15 min at room temperature. The well-dried foam of compound **4** was used without further purification for the synthesis of an oligodeoxyribonucleotide containing a dF residue, on a 1% polystyrene support. The condensation reactions were carried out with 2,4,6-trimethylbenzenesulfonyl-3-nitrotriazolide (MSNT). Compound **4** was inserted with 88% coupling yield, as calculated from the amount of released trityl cation. The fully protected oligonucleotide containing a dF residue was deprotected and purified by the same procedures as used for deprotection and purification of normal oligodeoxyribonucleotides. The purity of the final products used in this study was checked by reverse-phase HPLC (Fig. 2). The retention time of the normal oligonucleotide (12 mer) was approximately 28 min (Fig. 2, C, D). In the case of d(CGCACGCGTGCG), as this oligomer is self-complementary, it was eluted at 28 and 48 min as the single helix and duplex, respectively. The peak of the duplex disappeared when the column was heated at 50 °C. The elution profile of the oligomer containing a dF residue was unique (Fig. 2, B). The retention time of this oligomer was approximately 22 min, which is 6 min faster than that of the normal 12 mer. Furthermore, the top of the peak of this oligomer was divided into two. This phenomenon seems to be attributable to the *cis-trans* conformers of the dF residue in the oligomer. By means of molecular weight measurement of the oligodeoxyribonucleotide containing a dF residue by ion-spray mass spectrometry in the negative ion mode,[12] the presence of a dF residue was confirmed (Fig. 3). The formation of

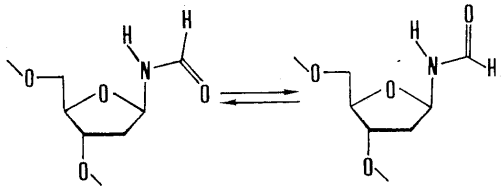
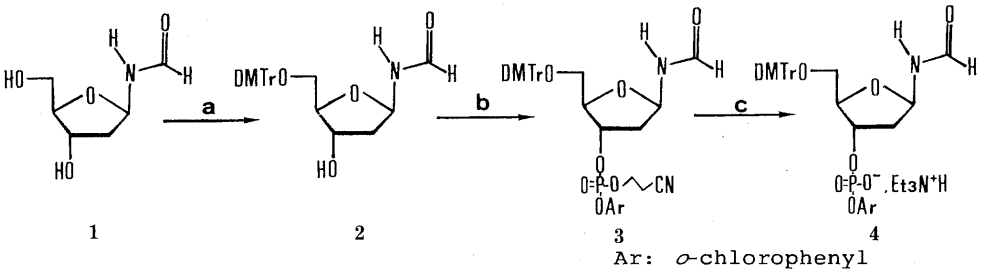


Fig. 1. *Cis* (Left) and *Trans* (Right) Conformers of dF Residue



1 2 3 4

Ar: *o*-chlorophenyl

a: DMTrCl/Py **b**: 1) *o*-chlorophenyl phosphorodi(triazolide), 2) $HOCH_2CH_2CN$/1-methylimidazole **c**: Py-Et_3N-H_2O (3:1:1)

Chart 1

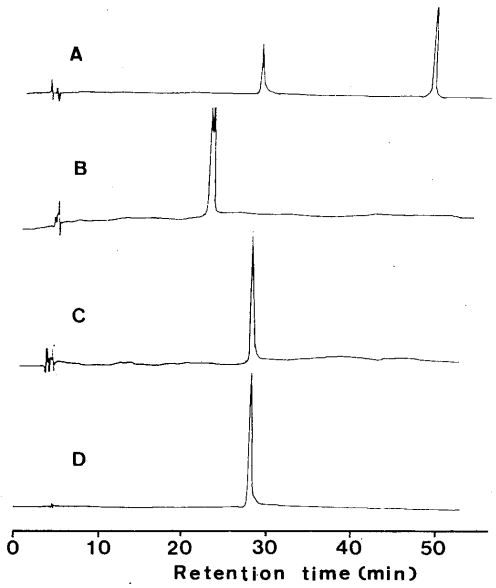


Fig. 2. HPLC Elution Profiles of the Synthesized Oligodeoxyribonucleotides

A: d(CGCACGCGTGCG); B: d(CGCFCGCGTGCG); C: d(CGCACGCGAGCG); D: d(CGCCCGCGTGCG). A linear gradient of acetonitrile from 12% to 22% in 0.1 M triethylammonium acetate (pH 7.0) in 50 min at room temperature was used. Wavelength of detection: 260 nm.

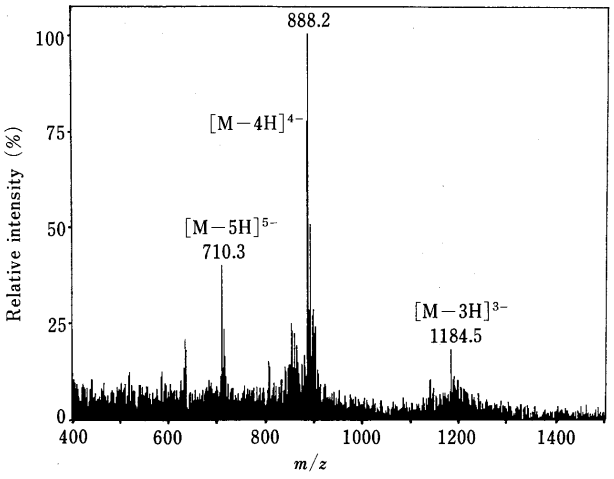


Fig. 3. Identification of d(CGCFCGCGTGCG) by Ion-Spray Mass Spectrometry

MW average = 3557.3, MW experiment = 3556.6 ± 0.2.

multiply charged ions from oligonucleotide is related to the number of phosphate residues in the molecule.[13] The mass which had the largest relative intensity, (m/z = 888.2), was the $[M-4H]^{4-}$ ion. The molecular weight of the oligomer, which was obtained by ion-spray mass spectrometry (MW = 3556.6), was in reasonable agreement with the calculated molecular weight (MW = 3557.3) and the presence of a dF residue in the oligomer was clearly confirmed.

Thermal Denaturation of Duplexes The stability of the duplex containing dF was examined by thermal denaturation. All the duplexes (Table I) exhibited normal helix-to-coil transition. However, the difference in melting temperature (T_m) among the three duplexes was extremely large. The T_m of the duplex containing the A–C mispair (AC duplex) was 14 degrees lower than that of the duplex

TABLE I. Melting Temperatures (T_m)[a] of DNA Duplexes[b]

DNA Duplex	T_m (°C)
5′-CGC**A**CGCGTGCG-3′ 3′-GCG**T**GCGCACGC-5′	62
5′-CGC**C**CGCGTGCG-3′ 3′-GCG**A**GCGCACGC-5′	48
5′-CGC**F**CGCGTGCG-3′ 3′-GCG**A**GCGCACGC-5′	32

a) T_m's of the duplexes (about 0.5 A_{260} unit/ml) were measured in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0). Absorbances at 260 and 270 nm were recorded and the T_m was the mean of the T_m's from both melting curves. *b*) The dots indicate hydrogen bonds and the boxed parts indicate differences among the three duplexes.

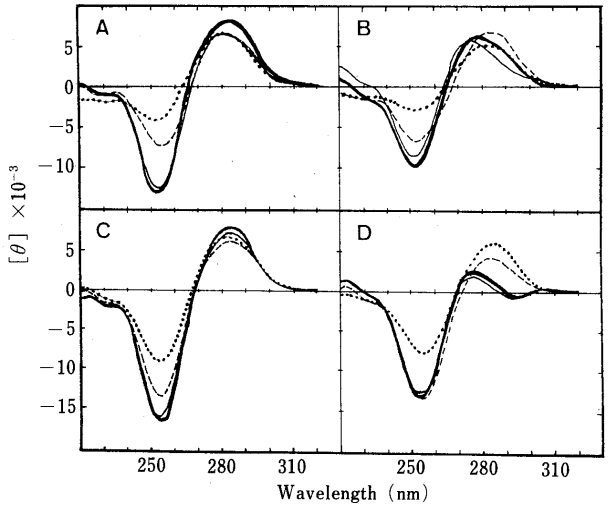


Fig. 4. CD Spectra of Duplexes, $[d(CGCACGCGTGCG)]_2$ (A, B) and d(CGCFCGCGTGCG)·d(CGCACGCGAGCG) (C, D), in Different Salt Concentrations

——, 5 °C; ——, 25 °C; – – –, 45 °C; ------, 65 °C in 0.1 M (A, C) and 4.0 M (B, D) NaCl–0.01 M sodium cacodylate (pH 7.0). Oligomer concentration: 1 A_{max} unit/ml.

containing the A : T pair (AT duplex). On the other hand, the T_m of the duplex containing an A–F mispair (AF duplex) was much lower than those of the other two duplexes. The differences in T_m between the AF duplex and the other two duplexes (AT duplex and AC duplex) were 30 and 16 degrees, respectively, possibly due to the lack of both base-pairing and base-stacking.

The CD spectra of the duplexes (AF duplex and AT duplex, 1 A_{max} unit/ml), at various temperatures in 0.1 M and 4 M NaCl are shown in Fig. 4. In 0.1 M NaCl at a low temperature, the spectral pattern is the same, but the CD magnitude of the negative band of the AF duplex at around 255 nm was smaller than that of the AT duplex (Fig. 4, A, B). This decrease in the magnitude may be due to the destacking of the AF duplex bases. In 4 M NaCl (Fig. 4, B, D), in contrast to the AF duplex, the AT duplex showed a marked change in the CD pattern at around 285 nm compared to the CD pattern of the AT duplex in 0.1 M NaCl. In other words, the conformation of the AF duplex seems to be insensitive to the change in salt concentration.

In conclusion, we have successfully introduced a dF

residue into a defined position of an oligonucleotide. The dF residue was stable enough to be used in oligodeoxyribonucleotide synthesis by the phosphotriester method. Modified oligonucleotides containing dF residues should be very useful as abasic damage models to analyze the functions of DNA repair enzymes and DNA polymerases.

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