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Mechanistic Studies on Photoinduced Cross-Linking and Specific Cleavage at Guanine by Dibenzoyldiazomethane-Oligodeoxynucleotide Conjugate

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Photoirradiation of dibenzoyldiazomethane-oligodeoxynucleotide (DBDM-ODN) conjugate, 5′-DBDM-$(CH_2)_6$-d(AAAAAGGGAAGGAAA)-3′ **3**, in the presence of 23-mer ODN, 5′-d(TTTCCTTCCCTTTTTG$_{16}$TTG$_{19}$TG$_{21}$TT)-3′ **ODN1**, having a complementary sequence, induced cross-links of both oligomers. By hot piperidine treatment, the specific cleavage of target **ODN1** at G$_{16}$ was observed. The photo-cross-linked ODNs were purified by electrophoresis on polyacrylamide gel. Upon hot piperidine treatment of the isolated cross-linked ODNs, both the G$_{16}$ cleavage band and the band of reverted **ODN1** were observed.

INTRODUCTION

Molecular design of sequence-specific DNA-cleaving agents is a formidable challenge and such molecules would have broad applications as artificial restriction enzymes, anticancer agents, and drugs for gene therapy (*1–10*). Among various approaches for such sequence-specific DNA cleavage, DNA alkylation by electrophilic species tethered to oligodeoxynucleotide (ODN) usually induces highly selective DNA cleavage at target sequence (*11–20*) due to the intrinsic nucleophilic reactivity of guanine N7 in the major groove or in some cases adenine N3 in the minor groove. However, it has also been pointed out that such electrophile-ODN conjugates tend to self-decompose gradually by reaction with internal nucleophiles or to undergo nonspecific alkylation before duplex formation. Thus, the postactivation of an electrophile precursor by a phototriggered process would be highly attractive for sequence-specific DNA alkylation by designed ODN[2] conjugates.

Toward this end, we have designed a novel photoinducible DNA-alkylating agent

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[2] Abbreviations used: BK, benzoyl ketene; DBDM, dibenzoyldiazomethane; ODN, oligodeoxynucleotide.

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that can generate a strong electrophile after complexation with the target sequence triggered by photoirradiation and alkylate guanine base at the close vicinity (*21–23*). Our attention has been focused on the compounds having dibenzoyldiazomethane (DBDM) **1a** as a chromophore, which produces highly electrophilic benzoyl ketene (BK) **2** via photochemical Wolff rearrangement (*21–23*). We previously observed that DNA cleavage occurred selectively at guanine (G) residues when DNA was photoirradiated in the presence of water-soluble monomer DBDM **1b** followed by hot piperidine treatment (*21,22*). G-selective DNA cleavage may also occur by singlet oxygen mediated mechanism (*24–27*) or by one-electron oxidation of DNA (*28–35*). The experiment on the DNA cleavage by **1b** in the presence of singlet oxygen quencher indicated that the G cleavage mediated by singlet oxygen, if it occurs, is only a minor process in the present case (*22*). The observed equal G cleavage of 5′GG3′ sequence is also inconsistent with the one-electron oxidation mechanism, since 5′-G of 5′GG3′ sequence is known to be the most readily oxidizable site in DNA one-electron oxidation (*29–35*). Photoirradiation of a conjugate of ODN and DBDM, in which DBDM was tethered to the 5′-end of ODN through hexamethylene aminoalkyl linker, in the presence of target ODN-induced cross-links of both oligomers, and the highly selective cleavage at the G residue that is most proximal to the chromophore was observed by hot piperidine treatment (*22*). Thus, previous results strongly support that photogenerated BK **2** is indeed a reactive species that is responsible for the cross-linking and subsequent G cleavage.

We herein describe that (i) photoinduced cleavage at a guanine residue by DBDM-ODN conjugate actually proceeds via a covalent modification of the G residue and (ii) the major cross-linking reaction between these two oligomers is the acylation of the N-2 amino group of guanine with BK. Moreover, we also report that the cross-linking is a reversible process (Fig. 1).

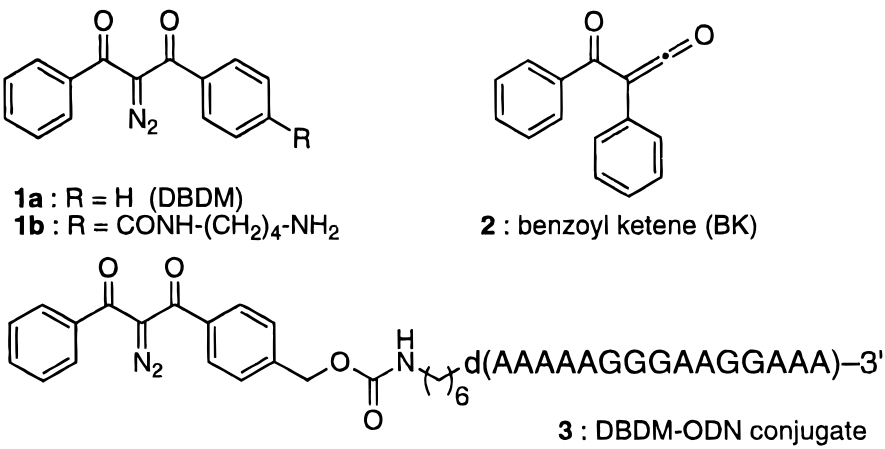


FIG. 1. Structures of DBDM derivatives.

MATERIALS AND METHODS

General Methods and Materials

^{1}H and ^{13}C NMR spectra were measured with JEOL JNM α-400 (400 MHz) spectrometer. Coupling constants (J values) are represented in Hz. Mass spectra were recorded on a JEOL JMS HX-110 spectrometer. Photoirradiation at 366 nm was carried out using a Funakoshi TEL-33 transilluminator. Gel electrophoresis was carried out on a Gibco BRL Model S2 apparatus. Densitometric analysis of the gel was carried out using Bio-Rad GS-700 imaging densitometer with analytical software Molecular Analyst (Version 2.1). Wakogel C-200 was used for silica gel flash chromatography. Precoated TLC plates Merck silica gel 60 F_{254} was used for monitoring reactions. HPLC was performed on cosmosil $5C_{18}$AR column with Gilson Chromatography Model 305 using UV detector Model 118 at 254 nm. All enzymes used in the studies were from commercial sources. [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from Amersham. All DNA oligomers were purchased from Greiner Japan.

*Preparation of DBDM-ODN Conjugate **3***

To an acetonitrile solution (20 μl) of 4-(2-diazo-3-phenyl-1,3-propanedion-1-*yl*)-phenylmethyl *N*-hydroxysuccinimidyl carbonate **4** (42 μg, 100 nmol) was added ODN possessing hexamethylene aminoalkyl linker (0.5 mM, 20 μl), purchased from Greiner Japan, in the presence of saturated $NaHCO_3$ (10 μl) and the mixture was kept at ambient temperature for about 15 min. The reaction mixture was purified by HPLC on a cosmosil $5C_{18}$AR (4.6 $\times$ 150 mm, elution with a solvent mixture of 0.1 M triethylamine acetate, pH 7.0, linear gradient over 30 min from 5 to 50% acetonitrile at a flow rate of 1.0 ml/min) to give **3** (t = 25.8 min). The concentration of **3** was determined by comparison of the peak areas of four nucleosides obtained by enzymatic digestion.

*Measurement of Thermal Melting Temperature (Tm) for Duplex **ODN1-3***

ODN1 (60 μM, final base concentration) and **3** (40 μM, final base concentration) were taken in a buffer containing sodium cacodylate (10 mM, pH 7.0) and NaCl (100 mM). Thermal denaturation profile was obtained with a Jasco V-550 spectrometer equipped with a Peltier temperature controller. The absorbance of the sample was monitored at 260 nm from 2 to 80°C with a heating rate of 1°C/min. The T_m value was determined from a plot of absorbance (A_{260}) *versus* temperature and assigned as the temperature at 1/2 (ΔA_{260}).

*Photoinduced Cleavage of ^{32}P-5′-End-Labeled **ODN1** by DBDM-ODN Conjugate **3***

Photoinduced cleavage of 23-mer **ODN1** by **3** was carried out in a 50 μl total volume containing sonicated calf thymus DNA (10μM base pair concentration), 5×10^4 cpm ^{32}P-5′-end-labeled **ODN1**, NaCl (100 mM), and DBDM-ODN conjugate **3** (1 μM) in 10 mM sodium cacodylate buffer at pH 7.0. The reaction mixture was kept at 4°C for 12 h and irradiated with transilluminator (366 nm) at a distance of 10 cm at 0°C for 1 h. After irradiation, the reaction mixture was ethanol precipitated

by addition of 10 μl of 3 M sodium acetate and 900 μl of ethanol. The precipitated DNA was washed with 100 μl of 80% cold ethanol and dried *in vacuo*. The precipitated DNA was dissolved in 100 μl of 10% piperidine (v/v) and heated at 90°C for 30 min. The solution was concentrated to dryness using vacuum rotary evaporator and resuspended in 5 μl of 80% formamide loading buffer (a solution of 80% v/v formamide, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). All DNA samples obtained above and Maxam-Gilbert A+G and C+T sequencing markers (*36*) were heat denatured at 90°C for 1 min and quick-chilled on ice. The samples (1 μl, 1×10^4 cpm) were loaded onto 15% (19:1) polyacrylamide and 7 M urea sequencing gel and electrophoresed at 1900 V for approximately 2 h. The gel was dried and exposed to X-ray film with intensifying sheet at −70°C.

Piperidine-Induced Cleavage of Isolated Cross-Linked ODNs

The cross-linked ODNs obtained by photoirradiation as described above were separated from unreacted **ODN1** on a 15% denaturing polyacrylamide gel electrophoresis (7 M urea), and extracted by standard crush and soak method (*36*). The purified cross-linked ODNs (15×10^3 cpm) were dissolved in 100 μl of 10% piperidine (v/v) and heated at 90°C for 30 min. The solution was concentrated to dryness using vacuum rotary evaporator and resuspended in 5 μl of 80% formamide loading buffer. All DNA samples obtained as described above and Maxam-Gilbert A+G sequencing markers (*36*) were heat denatured at 90°C for 1 min and quick-chilled on ice. The samples (1 μl, 3×10^3 cpm) were loaded onto 15% (19:1) polyacrylamide and 7 M urea sequencing gel and electrophoresed at 1900 V for approximately 2 h. The gel was dried and exposed to X-ray film with intensifying sheet at −70°C. The gel and its densitometric data were shown in Fig. 6 and Table 1, respectively.

*Piperidine Treatment of N-2 Acylated Guanosine Derivative **6***

N-2 Acylated guanosine derivative **6** was synthesized as reported earlier (*22*). To a solution of **6** (15 mg, 0.016 mmol) in acetonitrile (0.9 ml) and H_2O (0.9 ml) was added piperidine (0.2 ml), and the reaction mixture was stirred at 90°C for 30 min.

TABLE 1

Relative Intensity of the Bands for Cross-Linked ODNs, ODN1, and G_{16} Cleavage Obtained from Densitometric Analysis of Lanes 1 and 2 in Fig. 6[a]

	Relative intensity (%)	
	Lane 1	Lane 2
Cross-linked ODNs (band X)	97	15
ODN1	3	73
G_{16} cleavage		12

[a] The relative intensity (%) of the bands for cross-linked ODNs, ODN1, and G_{16} cleavage to the total band intensity (100%) was calculated using band densities obtained by densitometric analysis of the gel shown in Fig. 6.

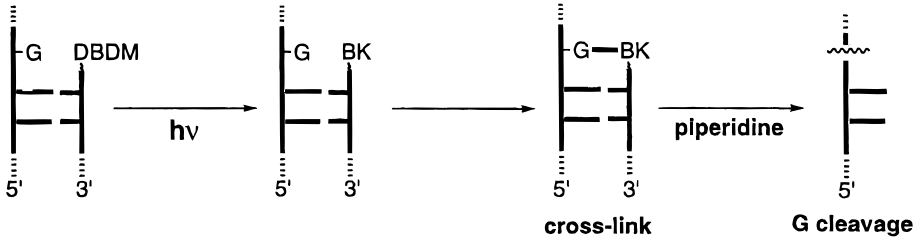
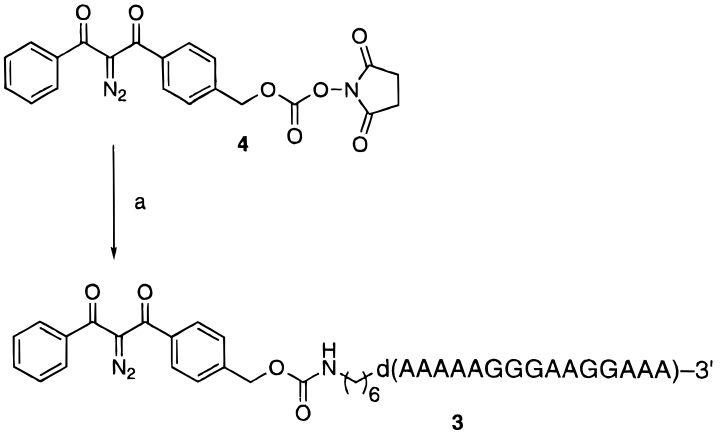


FIG. 2. Schematic illustration of the specific G cleavage of target oligodeoxynucleotide by DBDM-ODN conjugate via cross-linking.

After solvent was removed under reduced pressure, the crude product was purified by silica gel column chromatography to give **5** (9 mg, 78%) as a white solid: FABMS (NBA) (relative intensity), *m/e* 766 [(M + Na)$^+$] (50), 744 [(M + H)$^+$] (25), 197 (100, base peak); HRMS calcd for $C_{42}H_{50}O_4N_5Si_2$ [(M + H)$^+$] 744.3398, found 744.3400. The ^{1}H and ^{13}C NMR spectra were indistinguishable from those of authentic samples.

RESULTS AND DISCUSSION

In a previous DNA cleavage experiment using DBDM-ODN conjugate, we observed the cross-linking of the target ODN with DBDM-ODN conjugate together with the cleavage of the ODN at the most proximal G residue of the target sequence by piperidine treatment of the photoirradiation mixture (*22*). We proposed that this G cleavage may proceed via the cross-linking of the two oligomers at the G residue by



[a] Reagents and conditions: (a) H_2N-$(CH_2)_6$-d(AAAAAGGGAAGGAAA)-3', $NaHCO_3$, aqueous CH_3CN.

SCHEME 1