Highly Cooperative DNA Dialkylation by the Homodimer of Imidazole−Pyrrole Diamide−CPI Conjugate with Vinyl Linker

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Abstract: We synthesized new type of diamide−CPI conjugate possessing a vinyl linker, **7**. Sequence-selective alkylation of double-stranded DNA by **7** was investigated by high-resolution denaturing gel electrophoresis using ∼400 bp DNA fragments. Highly efficient alkylation predominantly occurs simultaneously at the purines of 5′-PyG(A/T)CPu-3′ site on both strands at a nanomolar concentration of **7**. These results suggest that the homodimer of conjugate **7** dialkylates both strands according to Dervan's pairing rule together with a new mode of recognition in which the Im-vinyl linker (L) pair targets G/C base pairs. In addition to the major dialkylation sites, a minor alkylation site was also observed at 5′-GT(A/T)GC-3′. This alkylation can be explained by an analogous slipped homodimer recognition mode in which the L−L pair recognizes the A/T base pair. Efficient dialkylation by the homodimer of **7** was further confirmed using oligonucleotides (ODNs). HPLC analysis revealed that the conjugate **7** simultaneously alkylates GN3/AN3 of the target sequences on both strands of ODNs.

Introduction

Sequence-specific DNA alkylation has significant potential for use in molecular biology and human medicine.[1,2] Although, many antitumor agents alkylate DNA, most have only limited sequence specificity and often exhibit severe toxicity to normal tissues. Rational design of alkylating agents targeting specific sequences in the human genome may provide useful molecules for various applications, including gene-targeted drugs.[3] The sequence specificities induced by DNA alkylators are usually the result of a combination of their inherent reactivities toward purine bases and the binding selectivities to DNA. While alkylation of predetermined sequences has reportedly been achieved by the triple helix formation through tethering the alkylating group to polypyrimidine oligonucleotides,[4] the triple helix approach is associated with poor cellular uptake and is limited to purine tracks, a fact which severely restricts the availability of target sites. Minor-groove binding polyamides that contain *N*-methylimidazole (Im)−*N*-methylpyrrole (Py)−hydroxylpyrrole (Hp),[5] which uniquely recognize each of the four Watson−Crick base pairs, have potential as novel recognition parts of sequence-specific DNA alkylating agents. Indeed, we recently demonstrated that hybrid molecules between segment A of duocarmycin A and Py−Im diamides alkylate a predetermined sequence in the presence of distamycin A.[6] More recently, the alkylation of predetermined seven-base pair sequences has been achieved by duocarmycin A derivatives bearing six-ring Py−Im hairpin polyamides.[7] However, these molecules only alkylate one strand of the DNA helix, and the double-strand alkylation at predetermined mixture DNA sequences still remains a challenge.[8] Molecular modeling suggested that the insertion of a vinyl linker (L) between polyamide and CPI adjusts the location of the cyclopropane ring of the conjugate and in this way improves the alkylation efficacy of

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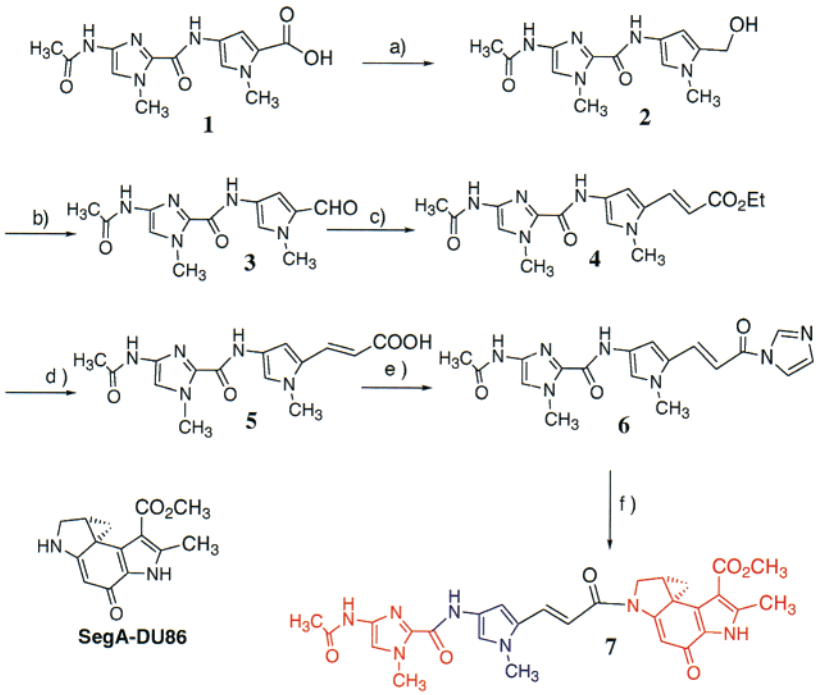
Scheme 1[a]



[a] (a) Benzotriazole-1-yloxytris (dimethylamino)phosphonium hexafluorophosphate (BOP), THF, then $NaBH_4$; (b) MnO_2, THF; (c) triethyl phosphonoacetate, NaH, THF; (d) $NaOH/H_2O$/MeOH; (e) 1,1′-carbonyldiimidazole, DMF; (f) NaH, DMF, segment A of DU86.

the conjugates. In fact, Lown et al. demonstrated that incorporation of a vinyl linker dramatically enhances the efficiency of DNA alkylation as well as cytotoxicity. However, their molecules are limited to the recognition of A−T rich sequences.[9] Thus, diamide−CPI conjugate **7** was designed, synthesized, and evaluated as a novel sequence-specific DNA alkylating agent. We found that **7** alkylates double-stranded DNA at predetermined DNA sequences through highly cooperative homodimer formation.

Results and Discussion

Synthesis. Although Lown et al. reported on the importance of the vinyl linker for biological activity, their molecules are limited to pyrrole triamide, and no detailed synthetic procedures have yet been reported.[9] We developed an alternative synthetic methodology, as shown in Scheme 1. The imidazole moiety was incorporated into the novel conjugate for the first time. Hydroxybenzotriazolyl ester, formed in situ from carboxylic acid **1** and a BOP reagent, reacts with sodium borohydride in THF to give alcohol **2** at a moderate yield.[10] Oxidation of **2** quantitatively produces aldehyde **3**. The key intermediate **4** was obtained through *Wadsworth−Emmons reaction*[11] at a good yield. Hydrolysis and activation were performed as previously described[6,7] to give activated amide **6**. As the alkylating moiety, segment A of DU86 was employed due to its superior chemical stability compared to that of duocarmycin A, which was synthesized through five steps according to reported procedures.[12] Finally, the coupling reaction between **6** and segment A of DU86 accomplished the synthesis of **7** at an excellent yield.

Highly Cooperative Double-Strand Alkylation within over 400-Base Pair DNA Fragments. Sequence-selective alkylation by **7** was investigated on both strands of 5′-end Texas Red-labeled DNA of three sets of ∼400 bp DNA fragments using a DNA sequencer as previously described.[5f−7] Surprisingly, in all three sets of DNA fragments most of the alkylation occurred in close proximity of both strands. A typical sequencing analysis of the alkylated fragment after heat treatment is shown in Figure 1. Under the present experimental conditions, alkylation by **7** *occurred predominantly at the purines of sequence 5′-PyG(A/T)CPu-3′ of both strands (sites 1−3) with reasonable efficiency at a nanomolar concentration within a 450 bp DNA fragment.* Dialkylation at sites 1−3 can be explained by the mode of recognition shown in Figure 2, in which the Im−L pair targets the G/C base pair. In addition to the major dialkylation sites, minor alkylation was also observed at sequence 5′-GT(A/T)-GC-3′ of site 4. This alkylation can be explained by an analogous slipped homodimer recognition mode in which the L−L pair recognizes the A/T base pair (Figure 2). It is important to note that almost all alkylation on the two strand occurred simultaneously. These results clearly indicate that conjugate **7** dialkylates both strands through cooperative homodimer formation. Densitometric analysis of the cleavage bands indicated that the efficiency of alkylation by **7**, which is the amount of DNA cleavage divided by that of the agent approached 69%, confirming the unusually high efficiency of dialkylation.

Highly Cooperative Double-Strand Alkylation Confirmed at the Oligonucleotide Level Using HPLC Product Analysis. To clarify the mechanism of DNA alkylation by **7**, we investigated the alkylation of two oligonucleotides, ODN 1 and 2, both of which were designed according to the gel experiments described above. To obtain better separation of the two strands of ODNs when using our HPLC separation conditions, we added two T residues to the 3′-end of the lower strand (ODN 2). HPLC

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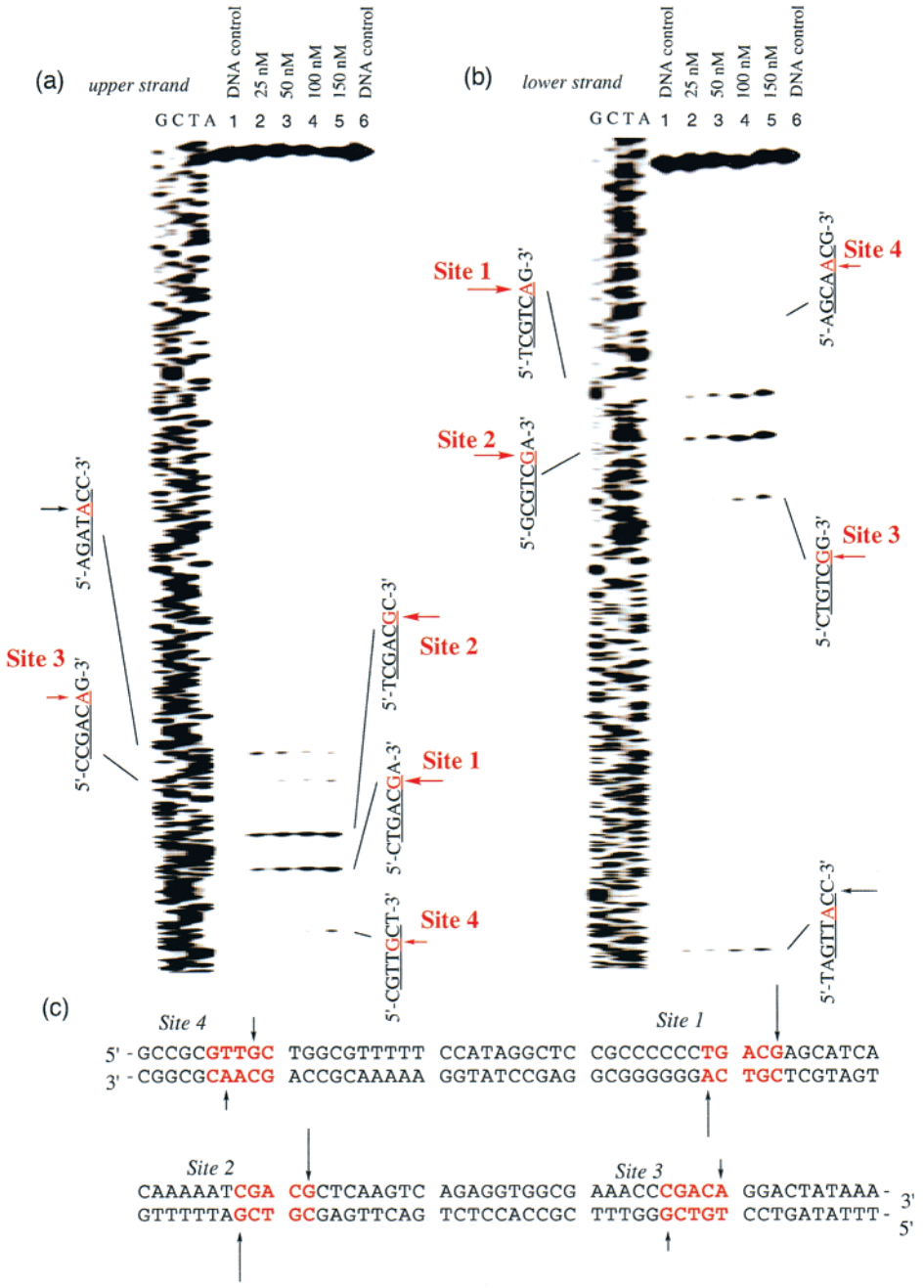


Figure 1. Thermally induced strand cleavage of 5′-end Texas Red-labeled 450-base pair (bp) DNA fragments by conjugate **7**. Results using 5′-end labeled upper strand (pUC 18 F 780−1229) (a) and 5′-end labeled lower strand (pUC 18 R 1459−1908) (b) DNA fragments are shown. These two DNA fragments are complementary. Lanes 1 and 6, DNA control; G, C, T, A are Saenger sequencing standard. Drug concentrations are indicated in lanes 2−5. Sequences containing dialkylation sites 1−4 are represented (c). The arrows indicate the site of alkylation.

analysis of the reaction mixture of ODN 1 and 2 with **7** revealed that conjugate **7** simultaneously alkylated GN3 of both ODN 1 and 2 at the target sequences and thus produced a dialkylated product (Figure 3). It is noteworthy that the complex of ODN 1−ODN 2−**7** in HPLC profile consistently showed one peak, indicating the strong stability of the complex. Direct observation of molecular ions (−5, −6, −7, −8, and −9) for the ODN 1−ODN 2−**7** complex by electron-spray MS further confirmed that double alkylation by **7** greatly stabilizes duplex DNA (Figure 4a). Similarly, ODNs 3 and 4 were used to investigate the alkylation of slipped homodimer recognition mode at site 4 of the gel results. The alkylation of ODNs 3 and 4 by **7** exhibited a moderate efficiency and produced different kinetics compared with that of ODNs 1 and 2, which is consistent with the DNA alkylation efficiency demonstrated by the high-resolution gel experiments (Figure 4b inset). As before, the complex ODN 3, 4 −**7** showed high stability and this was confirmed by electron spray MS (Figure 4b), suggesting cooperative dialkylation.

High Stability of Oligonucleotide−Drug Dialkylation Complex Confirmed by Melting Temperature Experiment. To determine how much stability the drug gives to the DNA duplex, a UV-monitoring T_m experiment was performed. Under the present experimental conditions, duplex 5′-GATCGACGCTC-3′/5′-GAGCGTCGATC-3′ has T_m = 38 °C, and 5′-GATC-GACGCTC-3′/5′-GAGCGTCGATC-3′−**7** dialkylation complex does not start to decompose until it reaches a temperature of 90 °C, which we refer to as the decomposing temperature (T_d) for the complex, suggesting that **7** stabilizes the DNA duplex

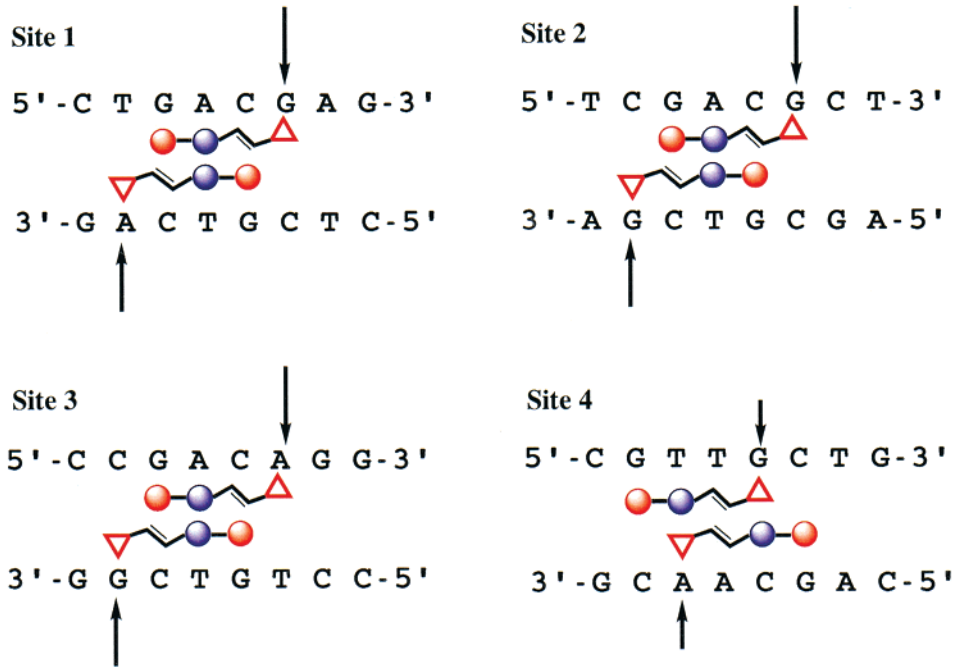


Figure 2. Schematic representation of conjugate **7**-DNA interacting modes. The arrows represent the site of alkylation. The imidazole and pyrrole rings are represented by red and blue circles, respectively; the red triangle represents segment A of DU86.

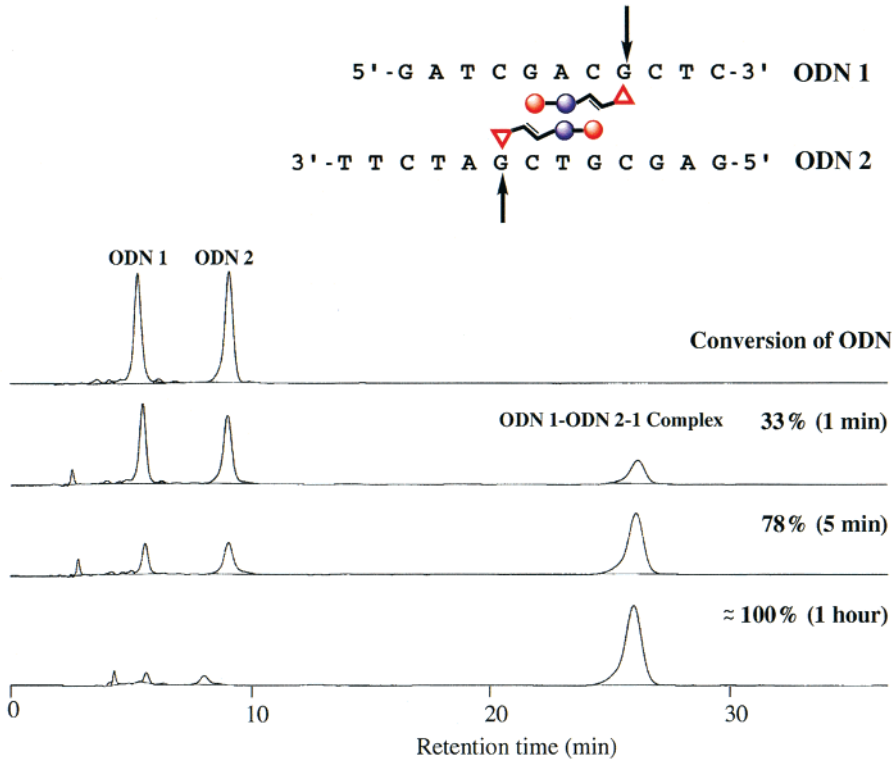


Figure 3. HPLCprofiles of alkylation of ODN1, 2 by conjugate **7** after the indicated incubation periods. The arrows represent the site of alkylation.

by at least 52 °C ($T_d - T_m$). Similarly, **7** stabilizes the ODN 3 and 4 duplex at least by 42 °C [T_d (80 °C) − T_m (38 °C)] (data not shown).

Characterization of the Dialkylation Complex by Thermal Degradation. Although gel and oligonucleotide experiments demonstrated that **7** shows a high efficiency in alkylating both strands of DNA, one may argue that alkylation comes from one of the two monomers in the dimer and possibly never occurs on both strands at the same time. Therefore, unambigous characterization of the dialkylation complex by thermal degradation was performed. Despite of the fact that complex ODN−**7** is too stable to be denatured, it is well-known that covalently alkylated duocarmycin-oligonucleotide adducts are easily converted to abasic site-containing oligonucleotides, and ultimately

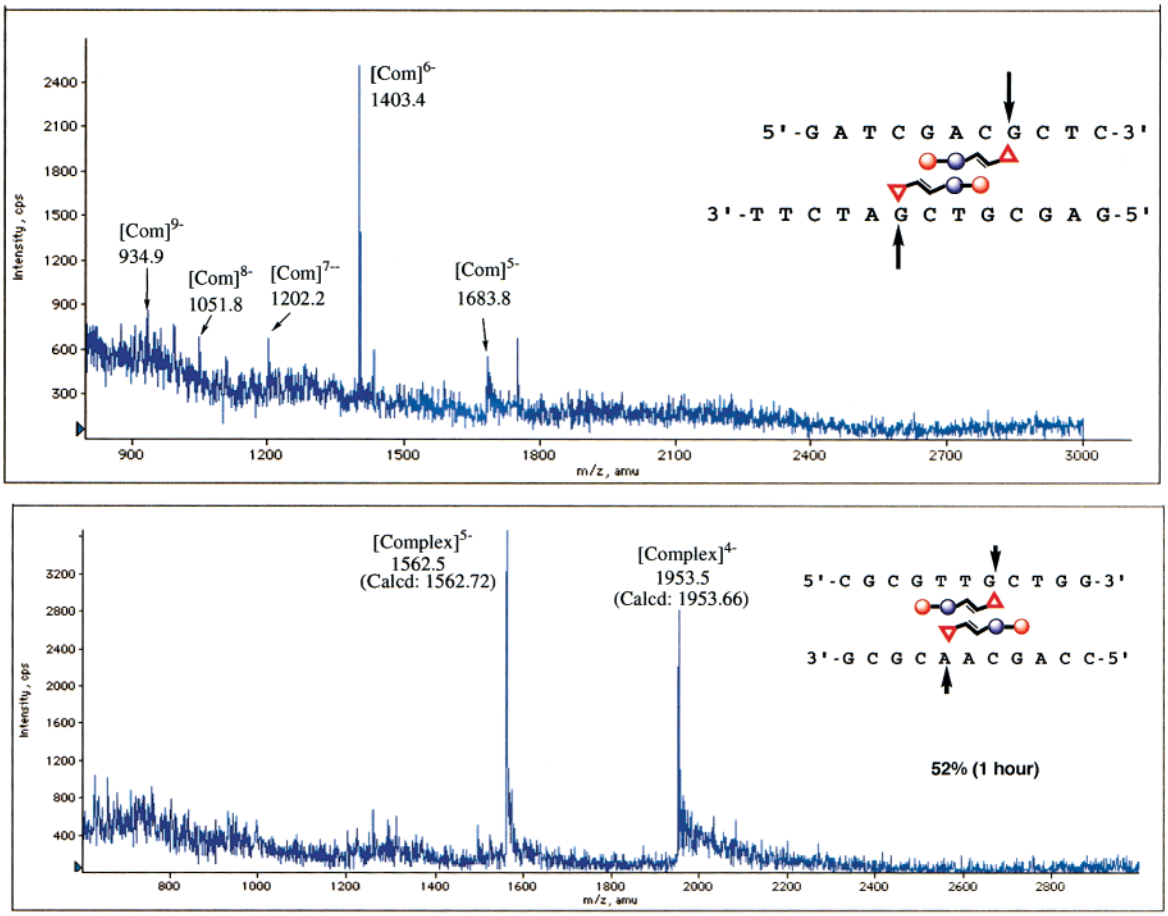


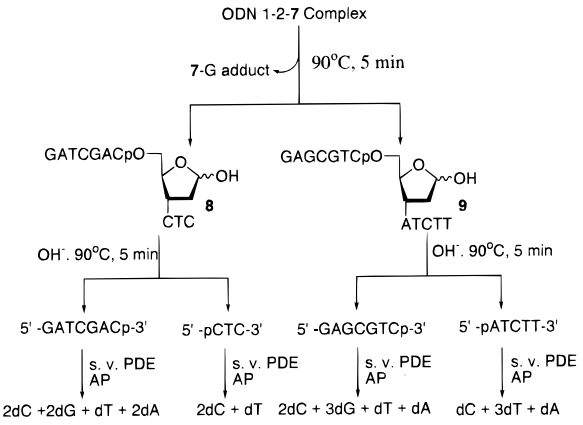
Figure 4. Electron spray mass spectra of oligonucleotide-**7** dialkylation complex in solution. The calculated *m*/*z* value is shown in the parenthesis. *y*-axis indicates relative peak intensity. (a) ODN1,2-**7** dialkylation complex. (b) ODN 3,4-**7** dialkylation complex; inset, interaction mode of ODN3,4-conjugate **7** and the alkylation efficiency after various indicated incubation time at 0 °C is also included.

give rise to the cleavage of the DNA strand upon heating. The characterization of the latter two processes has been well documented.[13] The ODN 1− and 2−**7** complex in Figure 3 was thus collected and heated at 90 °C for 5 min, and two abasic site-containing oligonucleotides **8** and **9** were immediately produced at a high yield without producing any intact oligonucleotide (Scheme 2 and Figure 5a). Cleavage of the abasic site in **8** and **9** with hot alkali (0.1 N NaOH, 90 °C, 5 min) produced the corresponding cleavage oligonucleotides (Scheme 2 and Figure 5b), whose compositions were unambiguously confirmed by enzymatic digestion with snake venom phosphodiesterase and alkaline phosphatase (Scheme 2). It is worth mentioning that even when the concentration ratio of the ODN 1 and 2 duplex and **7** is 1:1, the drug was almost quantitatively utilized to alkylate both strands at the same time without producing any monoalkylation (data not shown). The ODN 3− and 4−**7** dialkylation complex was similarly characterized (data not shown). The unambiguous characterization of oligonucleotides−**7** dialkylation complex provides further evidence that conjugate **7** highly cooperatively, efficiently, and simultaneously alkylates both strands of DNA in a predictable manner.

Conclusions

The present study is the first report of sequence-specific cooperative double-strand alkylation of DNA. The development of sequence-specific DNA cleaving molecules that go beyond the specificities of the natural enzymes is a long-term dream at the interface of chemistry and biology. It has been reported that

Scheme 2



purine alkylation sites in the DNA target give dsDNA fragments with residual phosphates that can be used as substrates for ligase.[8a] In this sense, conjugate **7** is the first example of an artificial "restriction enzyme". Recently, synthetic Py−Im polyamides have been shown to disrupt specific transcription factor−DNA interaction and to inhibit basal and activated transcription from various RNA polymerase II and III promo-

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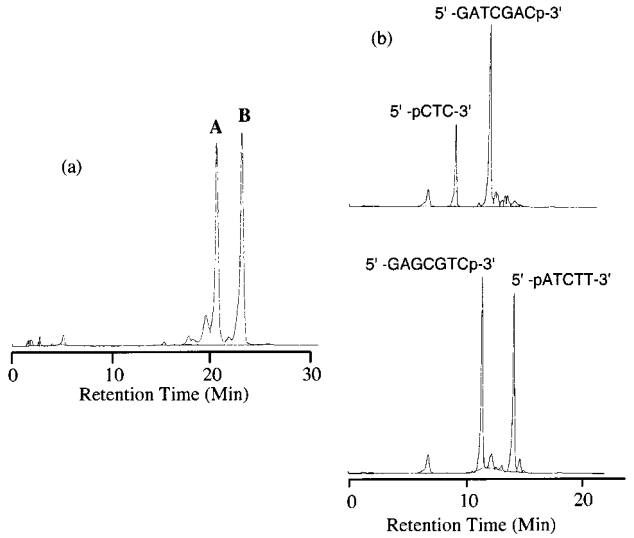

Figure 5. HPLC profiles of characterization of the dialkylation complex. (a) abasic site-containing oligonucleotides; (b) cleavage products at the abasic site; (c) enzymatic digestion analysis of the cleavage products.

ters.[14] More recently, Py−Im polyamides have also been used to activate transcription by blocking the DNA-binding activity of a repressor protein.[15] The stability of the polyamide−DNA complex is one of the key factors necessary for efficient regulation of specific genes. Anchoring the polyamides at both strands of predetermined sequences dramatically stabilizes the drug−DNA complex, which would in turn be expected to enhance the gene-regulating capacity. In addition, conjugate **7** would also benefit from the inherent DNA damage by alkylation compared with general polyamides in terms of biological activity. The new DNA dialkylating agent developed in the present investigation may provide a promising approach for developing new types of sequence-specific DNA dialkylating and cross-linking agents. Further studies on the specificity, optimization, and applicability of this new class of DNA dialkylating agents are currently in progress.

Experimental Section

General Methods. Reagents and solvents were purchased from standard suppliers without further purification. Abbreviations of some reagents are the following: DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; BOP, benzotriazole-1-yloxytris (dimethylamino)-phosphonium hexafluorophosphate. Reactions were monitored by thin-layer chromatography (TLC) using 0.25 mm silica gel 60 plates impregnated with 254 nm fluorescent indicator (purchased from Merck). Plates were visualized by UV light. NMR spectra were recorded on a JEOL JNM-A 500 magnetic resonance spectrometer, and tetramethylsilane was used as the internal standard. Proton NMR spectra were recorded in parts per million (ppm) downfield relative to a tertramethylsilane. The following abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), b (broad). Electron impact (EI) mass spectra were recorded on a JNM-AX 505 mass spectrometer, electrospray ionization mass spectra (ESIMS) on a PE SCIEX API 165 mass spectrometer and high-resolution fast atom bombardment mass spectra (FABMS) on a JEOL-JMS-SX102A mass spectrometer. Polyacrylamide gel electrophoresis was performed on a HITACHI 5500-S DNA sequencer. Ex Taq DNA polymerase and filter tube (Suprec-02) were purchased from Takara Co., thermo sequenase core sequencing kit and loading dye (dimethylformamide with fushin red) from Amersham Co., Ltd, 5′-end Texas Red-modified DNA oligomer (18mer) from Kurabo Co., Ltd, and 50% Long Ranger gel solution from FMC Bioproducts. Calf intestine alkaline phosphatase (AP, 1000 units/mL) and snake venom phosphodiesterase (s.v. PDE, 3 units/mL) were purchased from Boehringer Mannheim.

4-[[4-(Acetylamino)-1-methylimidazol-2-yl]carbonylamino]-2-hydroxylmethyl-1-methylpyrrole (2). To a mixture of 205 mg (0.67 mmol) of **1**, 326 mg (0.74 mmol) of BOP, and 170 μL of DIEA in 30 mL of THF was added 98 mg (2.59 mmol) of $NaBH_4$. The mixture was stirred for 3 h at room temperature, and the solvent was evaporated to give a residue, to which was added 20 mL of CH_3OH and 5 mL H_2O. The resulting mixture was stirred for 1 h to produce a clear solution. Removal of the solvents under reduced pressure gave a light yellow residue, which was subjected to flash chromatography using a mixture of CH_3OH and CH_2Cl_2 as eluent to afford 92.6 mg of **2** as a white solid in 47.4% yield. ^{1}H NMR (DMSO-d_6) δ 10.24 (s, 1H), 9.62 (s, 1H), 7.38 (s, 1H), 7.10 (d, J = 2.0 Hz, 1H), 6.09 (d, J = 2.0 Hz, 1H), 4.86 (t, J = 5.5 Hz, 1H), 4.34 (d, J = 5.5 Hz, 2H), 3.93 (s, 3H), 3.54 (s, 3H), 2.01 (s, 3H). HREIMS *m*/*e* calcd for $C_{13}H_{17}N_5O_3$ 291.1331, found 291.0987.

4-[[4-(Acetylamino)-1-methylimidazol-2-yl]carbonylamino]-1-methylpyrrole-2-aldehyde (3). A mixture of 85 mg (0.29 mmol) of **2** and 550 mg of activated MnO_2 (85%) in 30 mL of THF was stirred for 1.5 h at room temperature, and the solid was removed by filtration. After removal of the solvent, crude product was obtained. ^{1}H NMR analysis indicated that the purity of the product was >95%, and therefore it was directly used for the next reaction without further purification. ^{1}H NMR (DMSO-d_6) δ 10.21 (s, 1H), 10.18 (s, 1H), 9.50 (s, 1H), 7.63 (s, 1H), 7.43 (s, 1H), 7.10 (d, J = 2.0 Hz, 1H), 3.94 (s, 3H), 2.84 (s, 3H), 2.02 (s, 3H). HREIMS *m*/*e* calcd for $C_{13}H_{15}N_5O_3$ 289.1175, found 289.1204.

Ethyl 4-[[4-(Acetylamino)-1-methylimidazol-2-yl]carbonylamino]-1-methylpyrrole-2-acrylate (4). To a suspension of 23 mg (0.58 mmol) of NaH (60%) in 6 mL of THF in an ice bath was added 116 mL of triethyl phosphonoacetate. The mixture was stirred for 5 min and **3** (all) in 25 mL of THF was added. The resulting mixture was stirred overnight. Evaporation of THF gave a residue, which was subjected to flash chromatography using ethyl acetate as eluent to afford 88.5 mg of **4** as a yellow solid in 84% yield (two steps based on **2**). ^{1}H NMR (DMSO-d_6) δ 10.25 (s, 1H), 9.87 (s, 1H), 7.51 (d, J = 15.9 Hz, 1H), 7.44 (d, J = 1.8 Hz, 1H), 7.42 (s, 1H), 6.84 (d, J = 1.8 Hz, 1H), 6.11 (d, J = 15.9 Hz, 1H), 4.16 (q, J = 7.0 Hz, 2H), 4.13 (s, 3H), 3.70 (s, 3H), 2.02 (s, 3H), 1.24 (t, J = 7.0 Hz, 3H). HREIMS *m*/*e* calcd for $C_{17}H_{21}N_5O_4$ 359.1593, found 359.1602.

4-[[4-(Acetylamino)-1-methylimidazol-2-yl]carbonylamino]-1-methylpyrrole-2-acrylic acid (5). To a solution of 70 mg (0.2 mmol) of **4** in 5 mL of CH_3OH was added 1.5 mL of 2N aqueous NaOH and 3 mL of H_2O. The mixture was stirred for 4.5 h at room temperature. The solvent was removed by evaporation under reduced pressure, and 20 mL of H_2O was added. The resulting mixture was filtered, and the filtrate was acidified with 2N HCl to pH 2−3. The gel-like precipitate was collected by filtration and dried to give 43 mg of **5** in 67% yield. ^{1}H NMR (DMSO-d_6) δ 10.24 (s, 1H), 9.84 (s, 1H), 7.43 (d, J = 15.0 Hz, 1H), 7.41 (s, 1H), 7.40 (s, 1H), 6.78 (s, 1H), 6.03 (d, J = 15.0 Hz, 1H), 3.94 (s, 3H), 3.67 (s, 3H), 3.86 (s, 3H); ESIMS *m*/*e* calcd for $C_{15}H_{16}N_5O_4$ (M − H) 330.3, found 330.2.

AcImPyLCOIm (6). To a solution of 26.4 mg (0.08 mmol) of **5** in 2 mL of DMF was added 49.9 mg (0.31 mmol) of 1,1′-carboxyldiimidazole. The mixture was stirred overnight at room temperature, and 20 mL of H_2O was added. The yellow precipitate was collected by filtration to afford 20.5 mg of **6** in 68% yield. ^{1}H NMR (DMSO-d_6) δ 10.23 (s, 1H), 10.04 (s, 1H), 8.67 (s, 1H), 7.90 (d, J = 1.0 Hz, 1H), 7.88 (d, J = 15.5 Hz, 1H), 7.50 (d, J = 2.0 Hz, 1H), 7.44 (s, 1H), 7.32 (d, J = 2.0 Hz, 1H), 7.16 (d, J = 15.5 Hz, 1H), 7.10 (S, 1H), 3.96 (s, 3H), 3.79 (s, 3H), 2.03 (s, 3H); $C_{18}H_{18}N_7O_3$ (M − H) 380.4, found 380.4.

AcImPyLCOCPI (7). To a suspension of 3.2 mg (0.08 mmol) of sodium hydride (60%) in 0.3 mL of DMF at −50 °C was injected 6.1 mg (0.024 mmol) of segment A of DU86 in 0.3 mL of DMF. The

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mixture was stirred for 3 h at −50 to −40 °C. After 10.8 mg (0.028 mmol) of **6** in 1 mL of DMF was injected at −50 °C, the reaction mixture was further stirred for 5 h at −40 °C and kept at −30 °C in a refrigerator for 2 days. Next, 3 mL of 0.01 M of sodium phosphate buffer was added, and the mixture was stirred for 5 min at room temperature. The evaporation of the solvent gave a yellow residue, which was subjected to flash chromatography using a mixture of chloroform and methanol as eluent to afford 12.3 mg of **7** in 91% yield. ^{1}H NMR (DMSO-d_6) δ 12.36 (s, 1H), 10.24 (s, 1H), 9.97 (s, 1H), 7.58 (d, J = 15.0 Hz, 1H), 7.43 (s, 1H), 7.41 (d, J = 2.0 Hz, 1H), 6.99 (d, J = 2.0 Hz, 1H), 6.85 (s, br, 1 H), 6.58 (d, J = 15.0 Hz, 1H), 4.29 (d, J = 10.5 Hz, 1H), 4.19 (dd, J = 5.0 Hz and 4.5 Hz, 1H), 3.95 (s, 3H), 3.73 (s, 3H), 3.72 (s, 3H), 3.46 (m, 1H), 2.47 (s, 3H), 2.08 (m, 1H), 2.02 (s, 3H), 1.29 (t, J = 4.5 and 3.5 Hz, 1H); ESIMS *m*/*e* calcd for $C_{29}H_{28}N_7O_6$ (M − H) 570.6, found 570.4. HRFABMS *m*/*e* calcd for $C_{29}H_{30}N_7O_6$ (M + H) 572.2257, found 572.2262.

Preparation of 5′-Texas Red-End-Modified 450-Base Pair DNA Fragments. The 5′-end Texas Red-modified 450-base pair DNA fragments pUC18 F780*-1229 and pUC18 R1459*-1908 (they are complementary) were prepared by the PCR method using 5′-end Texas Red-modified 18mers 5′-AGAATCAGGGGATAACGCAG-3′ (pUC 18 forward 780−799), and 5′-TTACCAGTGGCTGCTGCCAG-3′ (pUC 18 reverse 1459−1478) as primers and purified by filtration using Suprec-02. Their concentration was determined by ethidium bromide staining. The asterisk indicates Texas Red modification, and the nucleotide numbering starts with the replication site.

High-Resolution Gel Eletrophoresis. The 5′-end Texas Red-labeled DNA fragment (75 nM) was alkylated by various concentrations of **7** in 10 μL of 10 mM Na phosphate buffer (pH 7.0) containing 10% DMF at room-temperature overnight. The reaction was quenched by the addition of calf thymus DNA (5 mM, 1 μL) and by heating for 5 min at 90 °C. DNA was collected by ethanol precipitation. The pellet was dissolved in 8 μL of loading dye (formamide with fushin red), heated at 94 °C for 30 min, and then immediately cooled to 0 °C. A 2 μL of aliquot was electrophoresed on 6% denaturing polyacrylamide gel using a Hitachi 5500-S DNA sequencer.

Alkylation of Oligonucleotides by Conjugate 7 as Monitored by HPLC. Oligonucleotides were synthesized on an automated DNA synthesizer. A reaction mixture (50 μL) containing conjugate **7** (75 μM) and the duplex oligonucleotides (25 μM duplex concentration) in 50 mM sodium cacodylate buffer (pH 7.0) was incubated at 0 °C for the indicated periods. The progress of the reaction was monitored by HPLC using a Chemcobond 5-ODS-H column (4.6 × 150 mm). Elution was performed using 0.05 M tetraethylammonium acetate (TEAA) and a 9−11% acetonitrile linear gradient (0−50 min) at a flow rate of 1.0 mL/min. Detection was performed at 254 nm. ESIMS detection of ODN−**7** dialkylation complexes. A reaction mixture (50 μL) containing conjugate **7** (75 μM) and the duplex oligonucleotides (25 μM duplex concentration) in 50 mM sodium cacodylate buffer (pH 7.0) was incubated at 0 °C for 2 days and then subjected to HPLC (Elution was performed with 0.05 M ammonium formate and a 0−50% acetonitrile linear gradient (0−40 min) at a flow rate of 1.0 mL/min.); the complex (main peak at retention time=10 min) was collected and subjected to electrospray mass spectrometer. ESMS spectra are shown in Figure 4.

Melting Temperature Experiments. Thermal denaturation profiles were obtained on a Beckman DU650 spectrophotometer equipped with a high performance temperature controller. Measurements were conducted in 50 mM sodium cacodylate buffer (pH 7.0) and the samples had a 13 μM (duplex) concentration. The absorbance of the samples was monitored at 260 nm from 4 °C to 100 °C with a heating rate of 1 °C per minute.

Characterization of ODN-7 Dialkylation Complex. The dialkylation complex with retention time at 26 min in Figure 3 was collected and then lyophilized. After TEAA was removed by another HPLC using ammonium formate−acetonitrile as eluent, the resulting complex was heated at 90 °C for 5 min and subjected to HPLC (Figure 5a). Products A and B in Figure 5a were collected and heated in the presence of 0.1 N NaOH at 90 °C for 5 min, respectively, and their cleavage products were separated by HPLC [Elution was performed with 0.05 M ammonium formate and a 0−15% acetonitrile linear gradient (0−20 min) at a flow rate of 1.0 mL/min] (Figure 5b). The composition of the cleavage oligonucleotides were confirmed by enzymatic digestion according to a previously reported procedure.[15]

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Supporting Information Available: The full sequences of pUC 18 F 780-1229 and pUC 18 R 1459-1908 DNA fragments with specific alkylation sites (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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