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DNA Alkylation by Pyrrole−Imidazole *seco*-CBI Conjugates with an Indole Linker: Sequence-Specific DNA Alkylation with 10-Base-Pair Recognition through Heterodimer Formation

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Abstract: The sequence-specific DNA alkylation by conjugates **4** and **5**, which consist of *N*-methylpyrrole (Py)−*N*-methylimidazole (Im) polyamides and 1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (*seco*-CBI) linked with an indole linker, was investigated in the absence or presence of partner Py−Im polyamide **6**. High-resolution denaturing polyacrylamide gel electrophoresis revealed that conjugate **4** alkylates DNA at the sequences 5′-(A/T)GCCT**A**-3′ through hairpin formation, and alkylates 5′-GGAAA-GAAA**A**-3′ through an extended binding mode. However, in the presence of partner Py−Im polyamide **6**, conjugate **4** alkylates DNA at a completely different sequence, 5′-AGGTTGTCC**A**-3′. Alkylation of **4** in the presence of **6** was effectively inhibited by a competitor **7**. Surface plasmon resonance (SPR) results indicated that conjugate **4** does not bind to 5′-AGGTTGTCC**A**-3′, whereas **6** binds tightly to this sequence. The results suggest that alkylation proceeds through heterodimer formation, indicating that this is a general way to expand the recognition sequence for DNA alkylation by Py−Im *seco*-CBI conjugates.

Introduction

Duocarmycin A (Duo) is a highly potent antitumor antibiotic that selectively alkylates the N3 of adenine (A) at the 3′ end of three or more consecutive AT base pairs in DNA. We discovered that the addition of distamycin A (Dist) markedly modulates the alkylation sites, primarily at the G residues in GC-rich sequences, by forming a cooperative heterodimer between Duo and Dist in a minor groove.[1] We also found that the addition of *N*-methylpyrrole (Py)−*N*-methylimidazole (Im) triamides to the Duo reaction effectively changed the sequence specificity of the Duo in a predictive manner according to the sequence recognition rule of Py−Im polyamides.[2] Py−Im polyamides are synthetic minor groove-binding molecules that can recognize predetermined DNA sequences.[3] These results suggest that sequence-specific DNA alkylation can be achieved by heterodimer formation between alkylating agents and partner molecules in the minor groove. Thus, we have designed and synthesized various types of sequence-specific alkylating agents by conjugating alkylating agents and Py−Im polyamides and have investigated their DNA alkylating activity and biological functions.[4] Importantly, the insertion of a vinyl linker between segment A of DU-86[5] and the Py−Im polyamides dramatically enhances DNA alkylating reactivity[6] and cytotoxicity against human cancer cell lines by more than 100-fold as compared to compounds with no vinyl linker.[7] We demonstrated that alkylation of the coding region of the template strand by Py−Im polyamides with vinyl linkers effectively terminates transcription, producing truncated mRNA.[8] We have also demonstrated that alkylating Py−Im polyamides causes silencing of luciferase genes in mammalian cells.[9]

Recently, we have developed the alkylating Py−Im polyamides, using 1,2,9,9*a*-tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]-indol-4-one (CBI)[10] as an alkylating moiety[11] and 5-amino-1*H*-indole 2-carbonyl (indole) as a linker. New conjugates can readily be synthesized by Fmoc solid-phase synthesis and

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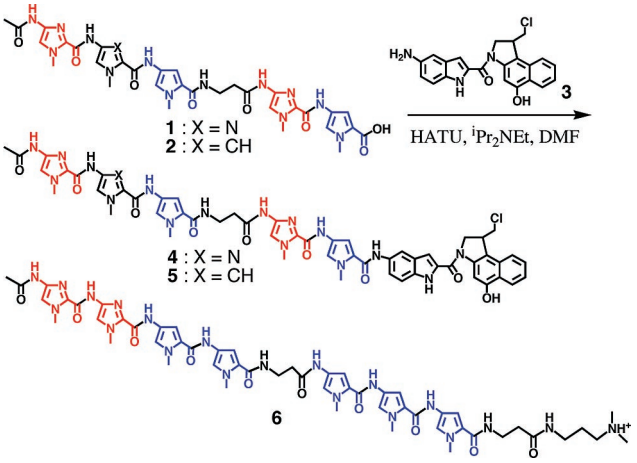

Figure 1. Synthesis scheme for the preparation of *seco*-CBI conjugates **4** and **5** with indole linker, and the chemical structure of the partner Py−Im polyamide **6**.

subsequent liquid-phase coupling of CBI with indole. Introduction of automated synthesis speeds up the synthesis of the alkylating Py−Im polyamides, and we confirmed that this new type of alkylating polyamide can silence the GFP gene and shows cytotoxicity comparable to or better than that of human cancer cell lines.[12] Moreover, we recently developed hairpin polyamides with nine-base-pair recognition sequences.[13] Further expanding the sequence specificity of alkylating agents will be useful for silencing specific gene expression.

In this study, Py−Im polyamide *seco*-CBI conjugates **4** and **5** were designed, which sequence-specifically alkylate at the target sequences in the presence of partner molecule **6**. We demonstrated that the combination of *seco*-CBI conjugate **4** and partner molecule **6** specifically alkylates DNA at a predetermined 10 bp sequence through heterodimer formation.

Results and Discussion

Synthesis. We synthesized Py−Im *seco*-CBI conjugates **4** and **5** and partner Py−Im polyamide **6** as shown in Figure 1. Py−Im polyamides **1** and **2**, processing carboxylic acid terminals, were synthesized by Fmoc solid-phase synthesis using an oxime resin and subsequent NaOH detaching treatment.[14] The indole linker and DNA alkylating units, *seco*-CBI, were introduced to **1** and **2** by coupling with conjugate **3** using HATU. This coupling with conjugate **3** was applicable to various carboxylic acids in the Py−Im polyamides. Partner polyamide **6** was also synthesized by the Fmoc solid-phase synthesis procedure using a CLEAR resin and subsequent 3-(dimethylamino)-1-propylamine (Dp) treatment. After reverse-phase HPLC purification, *seco*-CBI conjugates **4** and **5** were employed in the alkylation of DNA.

Modulation of DNA Alkylation by Conjugate 4 by Partner Polyamide 6. Sequence-selective DNA alkylation by *seco*-CBI conjugate **4** in the absence or presence of the partner polyamide **6** was investigated using a 5′-Texas Red-labeled 727 bp DNA fragment and automated DNA sequencer as previously described.[2,4a,b] Alkylation was carried out at 23 °C for 15 h, followed by quenching by the addition of calf thymus DNA. The samples were heated at 94 °C under neutral conditions for 20 min. The alkylation sites were visualized by thermal cleavage of the DNA strand. Under these heating conditions, all of the purine N3 alkylated sites in the DNA produced cleavage bands almost quantitatively on the gel.

Sequencing gel analysis of the DNA fragments alkylated by conjugate **4** with the increase of the partner polyamide **6** after heat treatment is shown in Figure 2. It was revealed that DNA alkylation by **4** occurred mainly at six sites, 5′-TACCA**A**-3′ (site 1), 5′-TGCCC**A**-3′ (site 2), 5′-TGCCT**A**-3′ (site 3), 5′-TGGGAAAAT**A**-3′ (site 4), 5′-AGCCT**A**-3′ (site 5), and 5′-GGAAAGAAA**A**-3′ (site 6). DNA alkylation at sites 1, 2, 3, and 5 can be explained by hairpin formation. Although γ-aminobutyric acid has been used as a turn in the hairpin Py−Im polyamides, these results indicate that β-alanine can also be used as a turn in hairpin polyamides. These results are consistent with the previous observation that polyamides with β-alanine bind to the target sequence as a hairpin.[15] The alkylation at sites 4 and 6 can be explained by an extended form with one or two base mismatch recognition. It was reported that Py−Im polyamides can bind to the minor groove of DNA in a monomeric mode.[16]

The addition of the partner polyamide **6** gradually changed the site of DNA alkylation. At a low concentration of **6** (1−5-fold greater than conjugate **4**), DNA alkylation occurred at the site 5′-GGGAAAATAGACC**A**-3′ (site 9), with the disappearance of alkylation at site 4 (lanes 3−5). The alkylation at site 9 can be explained by simultaneous hairpin formation by **4** and **6** at the proximity region. At higher concentrations of **6** (more than 10-fold greater than conjugate **4**), DNA alkylation occurred mainly at the site 5′-AGGTTGTCC**A**-3′ (site 10), with decreased alkylation at the other alkylation sites (lanes 6−10). Alkylation at site 10 can be explained by the formation of heterodimer of conjugate **4** and partner molecule **6**, as shown in Figure 2b.

DNA Alkylating Activities of Conjugate 4. We found that the addition of **6** dramatically modulates sequence-specific alkylation by **4** through heterodimer formation. We then examined alkylation by different concentrations of conjugate **4** in the absence and presence of 500 nM of **6** using a 5′-Texas Red-labeled 727 bp DNA fragment. Sequencing gel analysis of the alkylated DNA fragment after heat treatment is shown in Figure 3. It was found that DNA alkylation by **4** occurred mainly at three sites (sites 3, 5, and 6) in the absence of **6**, and at one site (site 10) in the presence of **6**. Under these conditions, modulation of the site of DNA alkylation is clearly observed. Alkylation activities by **4** in the presence of **6** were comparable to those of **4**, which clearly indicates that the addition of 500 nM of **6** affects alkylation selectivity without the loss of alkylating activity. Similar modulation of alkylation by another conjugate, **5**, through heterodimer formation also was observed in the presence of **6** (see Supporting Information), which

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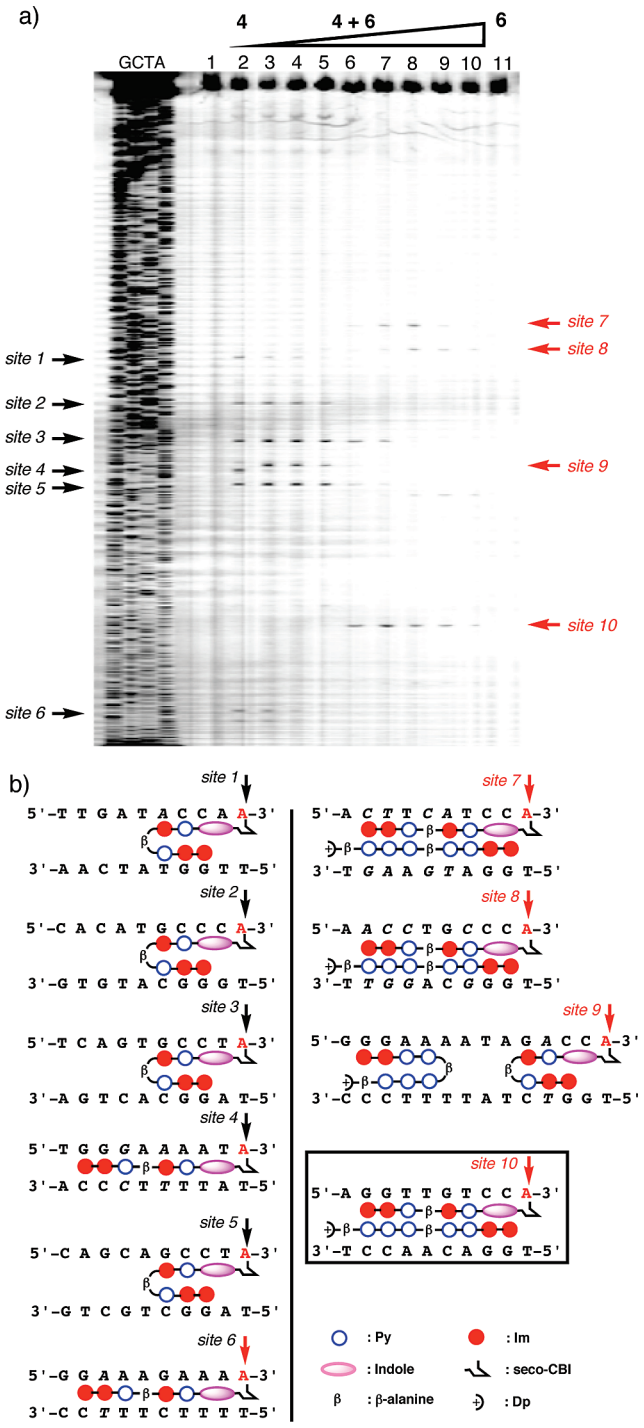

Figure 2. (a) Thermally induced strand cleavage of the 5′-Texas Red-labeled 727 bp DNA fragment by conjugate **4** at 23 °C for 15 h. Lane 1, DNA control; lane 2, 50 nM of **4** in the absence of **6**; lanes 3−10, 50 nM of **4** in the presence of 50, 100, 200, 500 nM, 1, 2, 5, 10 μM of **6**; lane 11, 10 μM of **6**. (b) Schematic representation of the recognition of match and mismatch sequences by **4**. The arrows indicate the site of alkylation by **4**. The alkylating base is shown in bold, and the mismatch-binding base is shown in italics.

supports the generality of sequence-specific alkylation through heterodimer formation.

Alkylation of DNA Not Including the Sites Alkylated by Conjugate 4 Alone. Although the modulated DNA alkylation shown in Figures 2 and 3 suggests that it is caused by the formation of a heterodimer of **4** and **6**, there is an alternative explanation: that partner **6** is covering up the sites that **4** could

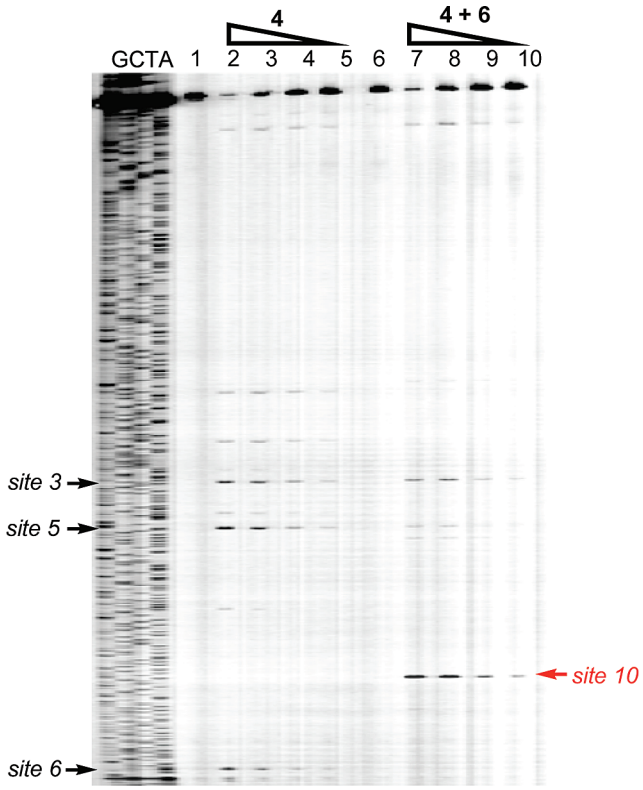

Figure 3. Thermally induced strand cleavage of the 5′-Texas Red-labeled 727 bp DNA fragment by conjugate **4** in the absence and presence of **6** at 23 °C for 15 h. Lanes 1 and 6, DNA control; lanes 2−5, 50, 25, 5, and 2.5 nM of **4**; lanes 7−10, 50, 25, 5, and 2.5 nM of **4** in the presence of 500 nM of **6**.

originally bind to and that **4** is displaced to other sites on the fragment. To test this possibility, we examined alkylation of a 317 bp DNA fragment (Supporting Information) that does not contain a match site for the hairpin and extended forms of **4**. Sequencing gel analysis of the alkylated DNA after heat treatment is shown in Figure 4. It was found that treatment of this DNA with **4** alone did not produce apparent alkylation bands (lane 2). Densitometric analysis indicated that DNA was not consumed at all (<0.1%, Figure 4c). The results clearly indicate that **4** itself does not have good binding sites in this DNA fragment. However, the addition of polyamide **6** initiated DNA alkylation at sites 1 and 2 (lanes 3−7). In the presence of 250 nM of **6**, alkylation by **4** occurred at sites 1 (5.1%) and 2 (1.4%), with the consumption of DNA (6.5%). The alkylation can be explained by the formation of a heterodimer with fully match (site 1) and one base mismatch (site 2) recognition. These results strongly suggest that the alkylation is caused by a heterodimer of **4** and **6**, not by a simple mass action effect.

Inhibition of DNA Alkylation by 4 and 6 by a Competitor, Py−Im Polyamide 7. To verify the formation of heterodimer between **4** and **6**, inhibition of DNA alkylation by **4** and **6** was investigated using Py−Im polyamide **7**, which recognizes the same sequence as **4** and serves as a competitor (Figure 5a). Upon addition of **7** to the reaction mixture, alkylation at site 10 was effectively inhibited (Figure 5b−d). At 50 nM, **7** inhibited the alkylation by more than >70%, and at 200 nM, almost complete inhibition of alkylation was observed. These results further support DNA alkylation through heterodimer formation.

Proposed Mechanism of Alkylation by 4 in the Presence of 6 at a Target Sequence. To obtain further insight into the

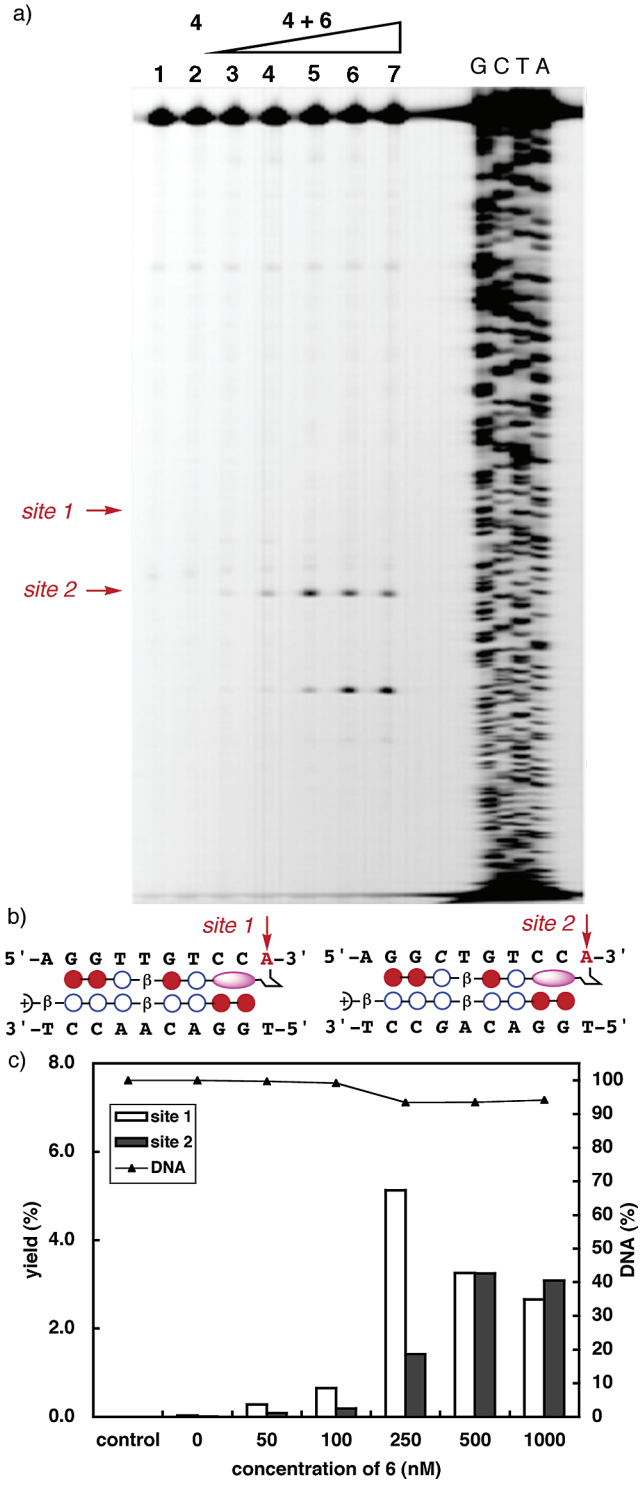

Figure 4. (a) Thermally induced strand cleavage of the 5′-Texas Red-labeled 317 bp DNA fragment by conjugate **4** in the absence and presence of **6** at 23 °C for 15 h. Lane 1, DNA control; lane 2, 50 nM of **4**; lanes 3−7, 50 nM of **4** in the presence of 50, 100, 250, 500, and 1000 nM of **6**. (b) Schematic representation of the recognition of match and mismatch sequences by **4**. The arrows indicate the site of alkylation by **4**. The alkylating base is shown in red, and the mismatch-binding base is shown in italics. (c) Alkylation yield at site 1 (white bars) and site 2 (gray bars), and consumption of substrate DNA (black line) calculated from densitometric analyses of lanes 1−7.

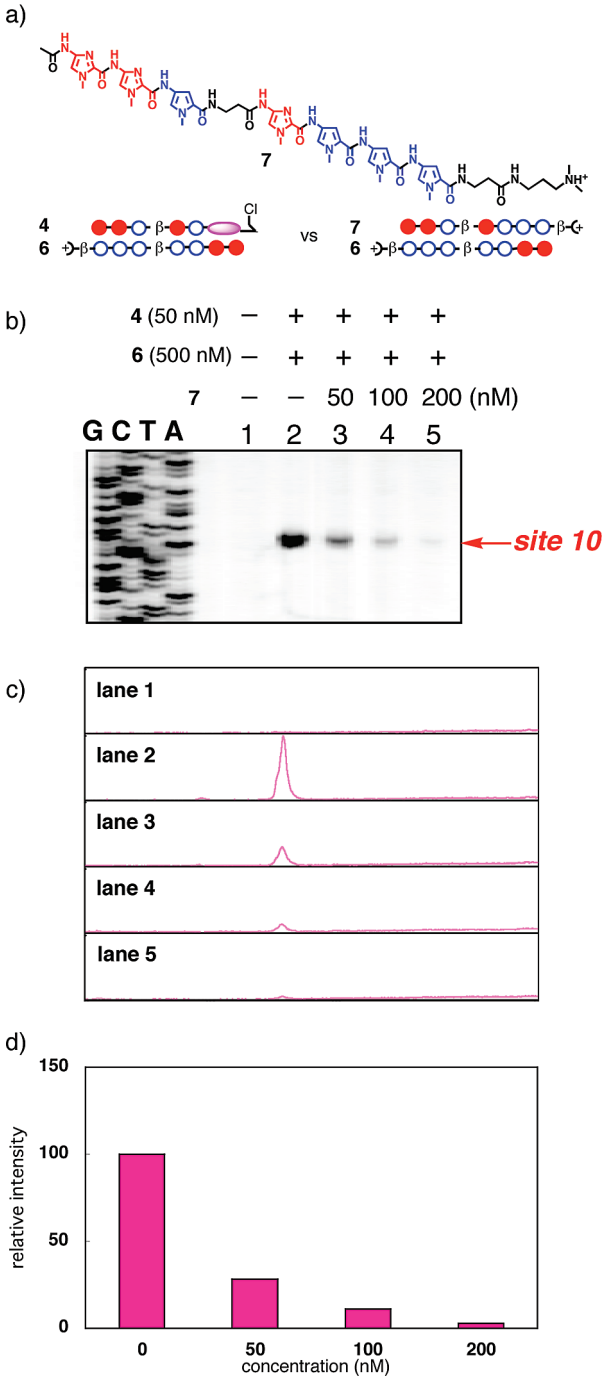

Figure 5. Competitive alkylation assay. (a) Chemical structure and schematic representation of competitor **7** for conjugate **4** (the same recognition sequence). (b) Thermally induced strand cleavage of the 5′-Texas Red-labeled 727 bp DNA (expansion of site 10 in Figure 2) by conjugate **4** in the presence of partner **6** and competitor **7** at 23 °C for 15 h. Lane 1, DNA control; lane 2, 50 nM of **4** in the presence of 500 nM of **6**; lanes 3−5, 50 nM of **4** in the presence of 500 nM of **6** and 50, 100, and 200 nM of competitor **7**. (c) Densitometric analyses of lanes 1−5. (d) Alkylation activity at site 10 calculated from (c) with the increase of competitor **7**.

mechanism of DNA alkylation through heterodimer, the binding affinity of conjugate **4** and partner polyamide **6** was examined using the surface plasmon resonance (SPR) technique with biotinylated hairpin DNA. For the measurement of conjugate **4**, G/C-substituted hairpin was used to avoid DNA alkylation at adenine.[11b] Dissociation equilibrium constants (K_D) were obtained by fitting the resulting sensorgrams (Figure 6) to a theoretical model, providing the affinity of binding for **4** and **6**

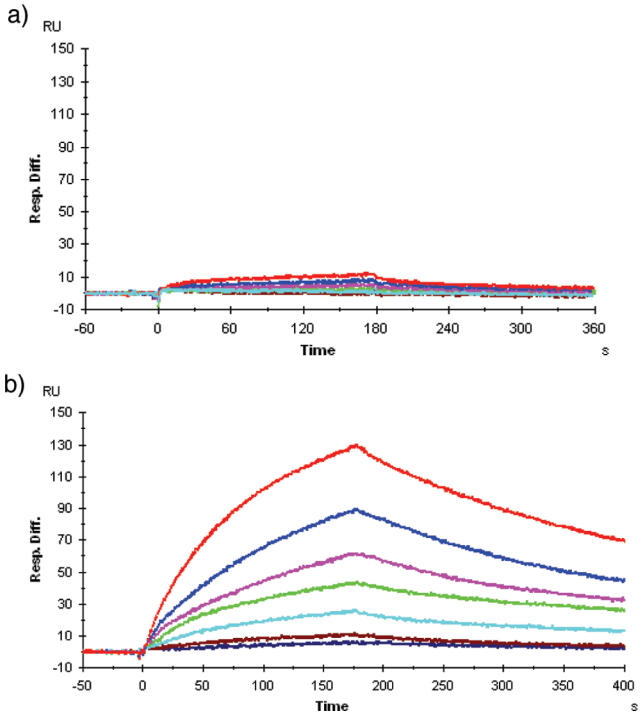

Figure 6. SPR sensorgrams for the interaction of conjugate **4** (a) and polyamides **6** (b) with the site 10 sequence, 5′-AGGTTGTCC(G/A)-3′, at 25 °C. (a) Conjugate **4** for the cognate sequences of site 10; 5′-AGGTTGTCCG-3′ at the concentration from 0.1 to 10 μM in HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) with 0.5% DMSO (v/v). (b) Polyamide **6** for the cognate sequences of site 10, 5′-AGGTTGTCCA-3′, at concentrations from 25 to 1600 nM in HBS-EP buffer with 0.1% DMSO.

according to previous studies.[17,18] The K_D value for **4** was not determined accurately because of its very low affinity to this hairpin, and it was estimated to be greater than 1×10^{-5} M. The results are consistent with the observation that **4** itself did not alkylate at site 10. In clear contrast, the K_D value for **6** was determined as 2.96 ($\pm$0.04) $\times 10^{-7}$ M for **6**, which is a significantly higher binding affinity for this sequence than that for **4**. The proposed scheme of the alkylation by conjugate **4** in the presence of the partner polyamide **6** is shown in Figure 7. First, partner **6** binds at the target sequence as a monomer. Conjugate **4** then binds to the monomeric binding complex of **6** to form the heterodimer of **4** and **6** in the minor groove and subsequently alkylates DNA at the target site (the proposed binding model of **4** and **6** to target oligonucleotides is in the Supporting Information). The initial binding of **6** would create a good binding site for conjugate **4**, which explains the results of efficient competitive inhibition by **7** (Figure 5). Moreover, the retardation of alkylation was observed in the presence of higher concentrations of **6** (lanes 9 and 10 in Figure 2), which is fully consistent with the interference of heterodimer formation by the homodimer of **6**. The reaction mechanisms are similar to those of previously observed efficient DNA alkylation by duocarmycin A and distamycin A.[1]

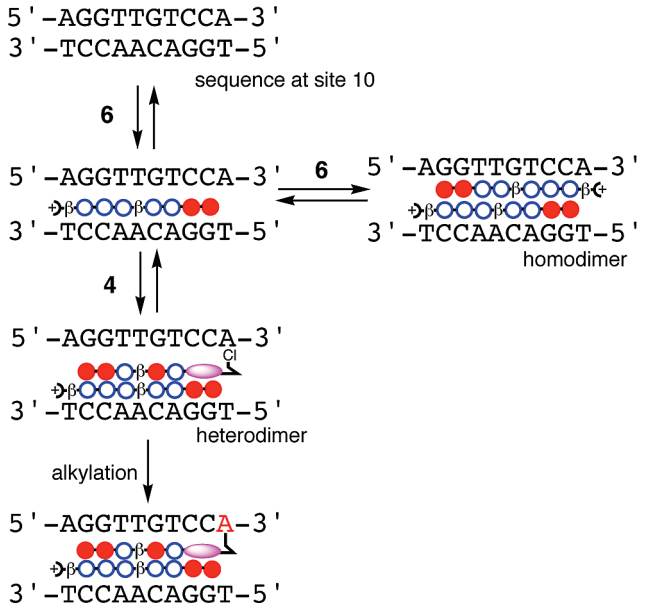

Figure 7. The proposed scheme of the alkylation by conjugate **4** in the presence of the partner polyamide **6**.

Conclusions

Sequence-specific alkylating agents have significant potential for use in molecular biology and human medicine. Rational design of alkylating agents targeting specific sequences in the human genome would provide useful molecules for various applications, including gene-targeted drugs. We have synthesized the linear Py−Im polyamide *seco*-CBI conjugates **4** and **5** and the partner polyamide, **6**. High-resolution denaturing polyacrylamide gel electrophoresis clearly demonstrated that partner **6** controls the site of DNA alkylation by Py−Im *seco*-CBI conjugate **4**. Py−Im−CBI conjugate and partner Py−Im polyamide formed a heterodimer and efficiently alkylated at the 10 bp DNA match sequences 5′-AGGTTGTCC**A**-3′. To the best of our knowledge, this is the first demonstration of sequence-specific DNA alkylation by Py−Im polyamides with 10 bp recognition. In nature, a lot of DNA binding proteins form cooperative dimers, recognize specific sites of DNA, and regulate transcription.[19] These results indicate that heterodimer formation is a general way to expand the recognition sequence for DNA alkylation by Py−Im *seco*-CBI conjugates.

Experimental Section

General. Reagents and solvents were purchased from standard suppliers and used without further purification. Abbreviations of some reagents: Fmoc, fluorenylmethoxycarbonyl; Boc, *tert*-butoxycarbonyl; DME, 1,2-dimethoxyethane; TFA, trifluoroacetic acid; $^{i}Pr_2NEt$, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; HATU, *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloric salt. NMR spectra were recorded with a JEOL JNM-FX 400 nuclear magnetic resonance spectrometer, and tetramethylsilane was used as the internal standard. Proton NMR spectra were recorded in parts per million (ppm) downfield relative to tetramethylsilane. The following abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), qu (quintet), m (multiplet), br (broad), dd (double doublet). Electrospray ionization mass spectrometry (ESIMS) and electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) were produced on an API 150 (PE

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SCIEX) and BioTOF II (Bruker Daltonics) mass spectrometer. Polyacrylamide gel electrophoresis was performed on a HITACHI SQ5500-S and HITACHI SQ5500-E DNA sequencer. PCR amplification was carried out with an iCycler (BIO-RAD). Ex Taq DNA polymerase was purchased from Takara Co.; the Thermo Sequenase core sequencing kit and loading dye (formamide with fuschin red) were from Amersham Co. Ltd.; 5′-Texas Red-modified DNA oligomer (20-mer) was from Proligo Co. Ltd.; and 50% Long Ranger gel solution was from FMC bioproducts. Surface plasmon resonance (SPR) assays were performed on a BIACORE X system (Sweden), and processing of data was carried out using BIAevalution version 4.1. Biotinylated hairpin DNAs were obtained from Proligo and used without further purification. HBS-EP buffer (0.01 M HEPES, pH 7.4, containing 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20) and sensor chip SA were purchased from BIAcore AB (Sweden).

Solid-Phase Synthesis of Py−Im Polyamides. AcImImPy-*β*-ImPyCO$_2$H (1). AcImImPy-*β*-ImPyCO$_2$-oxime resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol. A sample of resin was cleaved with 6 mL of 1 N aqueous NaOH/DMF (1/1, v/v) for 1 h at 55 °C, and purified by HPLC using a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 0−100% linear gradient, 0−30 min, 254 nm), to produce **1** (20.0 mg, 26.8 μmol, 27%) as a yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.30 (s, 1H; NH), 10.27 (s, 1H; NH), 10.26 (s, 1H; NH), 10.01 (s, 1H; NH), 9.32 (s, 1H; NH), 8.08 (t, J = 5.2 Hz, 1H; NH), 7.55 (s, 1H; Im−H), 7.50 (s, 1H; Im−H), 7.45 (s, 1H; Im−H), 7.44 (s, 1H; Py−H), 7.22 (s, 1H; Py−H), 6.96 (s, 1H; Py−H), 6.92 (s, 1H; Py−H), 3.99 (s, 3H; NCH_3), 3.97 (s, 3H; NCH_3), 3.93 (s, 3H; NCH_3), 3.81 (s, 3H; NCH_3), 3.80 (s, 3H; NCH_3), 3.44 (q, J = 6.4 Hz, 2H; CH_2), 2.58 (t, J = 7.2 Hz, 2H; CH_2), 2.03 (s, 3H; $COCH_3$). ESI-TOFMS m/z calcd for $C_{32}H_{36}N_{14}O_8$ $[M + H]^+$ 745.28, found 745.27.

AcImPyPy-*β*-ImPyCO$_2$H (2). A synthetic protocol similar to that used for the preparation of compound **1** was followed to prepare compound **2** (26.3 mg, 35.4 μmol, 35%), which was confirmed by HPLC and ESI-TOFMS analysis. ESI-TOFMS m/z calcd for $C_{33}H_{37}N_{13}O_8$ $[M + H]^+$ 744.29, found 744.32.

BocHN-Indole-*seco*-CBI. To a solution of H_2N-indole-CO_2H (157 mg, 0.892 mmol) in 1.6 mL of DME/aqueous $NaHCO_3$ (1/1, v/v) was added Boc_2O (553 mg, 2.54 mmol), and the reaction mixture was stirred for 10 h at room temperature. After completion of the reaction confirmed by HPLC analysis, the solution was concentrated and washed by 10% aqueous hydrochloric acid. Boc-NH-indole-CO_2H was obtained as a purple powder (271 mg, 0.98 mmol, quant). Next, to a solution of Boc-NH-indole-CO_2H in DMF was added *seco*-CBI (245 mg, 1.05 mmol) with EDCI (764 mg, 4.00 mmol) and $NaHCO_3$ (336 mg, 4.00 mmol). The reaction mixture was stirred overnight at room temperature under N_2 atmosphere. After the completion of the reaction was confirmed by HPLC analysis, the solution was concentrated and washed with 1% aqueous hydrochloric acid. The product was obtained as a brown solid (493 mg, 1.00 mmol, quant). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.63 (s, 1H; NH), 10.42 (s, 1H; OH), 9.16 (s, 1H; NH), 8.13 (d, J = 8.0 Hz, 1H; CH), 7.95 (s, 1H; CH), 7.84 (d, J = 8.0 Hz, 1H, CH), 7.79 (s, 1H; CH), 7.51 (t, J = 8.0 Hz, 1H; CH), 7.37 (s, 1H; CH), 7.34 (d, J = 8.0 Hz, 1H; CH), 7.31 (t, J = 9.2 Hz, 1H; CH), 7.10 (s, 1H; CH), 4.77 (t, J = 10.0 Hz, 1H; CH), 4.30 (d, J = 12.0 Hz, 1H; CH), 4.21 (brt, 1H; CH), 4.00 (dd, J = 8.0 Hz, 3.2 Hz, 1H; CH), 3.85 (dd, J = 7.6 Hz, 3.6 Hz, 1H; CH), 1.48 (s, 9H; CH_3). ESI-TOF-MS m/z calcd for $C_{27}H_{26}ClN_3O_4$ $[M + H]^+$ 492.16, found 492.31

H$_2$N-Indole-*seco*-CBI (3). To a solution of BocHN-indole-CO_2H (115 mg, 0.35 mmol) in 1 mL of CH_2Cl_2 was added 1 mL of TFA/H_2O (10/1, v/v), and the reaction mixture was stirred for 1 h at room temperature. After completion of the reaction was confirmed by HPLC analysis, the solution was concentrated and washed with 10% aqueous hydrochloric acid. Compound **3** was obtained as a black powder (82 mg, 0.35 mmol, quant), which was used in the next coupling step without further purification. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.00 (s, 1H; NH), 10.46 (s, 1H; OH), 8.12 (d, J = 7.2 Hz, 1H; CH), 7.96 (s, 1H; NH), 7.85 (d, J = 8.0 Hz, 1H; CH), 7.71 (s, 1H; CH), 7.59 (d, J = 8.0 Hz, 1H; CH), 7.53 (t, J = 7.2 Hz, 1H; CH), 7.37 (t, J = 8.0 Hz, 1H; CH), 7.29 (s, 1H; NH), 7.22 (d, J = 8.0 Hz, 1H; CH), 4.80 (t, J = 10.0 Hz, 1H; CH), 4.54 (d, J = 12.0 Hz, 1H; CH), 4.24 (brt, 1H; CH), 4.01 (dd, J = 8.0 Hz, 3.2 Hz, 1H; CH), 3.87 (dd, J = 7.6 Hz, 3.6 Hz, 1H; CH), 3.33 (s, 2H; NH_2). ESI-TOF-MS m/z calcd for $C_{22}H_{18}ClN_3O_2$ $[M + H]^+$ 392.11, found 392.15.

AcImImPy-*β*-ImPy-Indole-*seco*-CBI (4). To a solution of compound **1** (5.0 mg, 6.7 μmol) in DMF (150 μL) were added ^{i}Pr$_2$NEt (2.3 μL, 1.3 μmol) and HATU (2.6 mg, 6.7 μmol). The reaction mixture was stirred for 2 h at room temperature. After the conversion from **1** to activated ester was confirmed by HPLC and ESIMS analysis, ^{i}Pr$_2$NEt (2.3 μL, 13 μmol) and **3′** (5.2 mg, 13 μmol) were added to the reaction vessel. The reaction mixture was stirred overnight at room temperature under N_2 atmosphere. Evaporation of the solvent gave a yellow residue by filtration, which was washed with chloroform (2 μL × 2) and water (2 μL × 2) and was subjected to column chromatography (silica gel 5−10% MeOH in CH_2Cl_2, gradient elution) to produce compound (3.4 mg, 3.0 μmol, 45%) as a yellow powder. **4** was used in the DNA alkylation reaction. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.67 (s, 1H; NH), 10.43 (s, 1H; OH), 10.29 (s, 2H; NH), 10.26 (s, 1H; NH), 9.96 (s, 1H; NH), 9.82 (s, 1H; NH), 9.32 (s, 1H; NH), 8.11 (d, J = 8.0 Hz, 1H; CH), 8.07 (brs, 1H; NH), 7.96 (s, 1H; CH), 7.85 (d, J = 8.0 Hz, 1H; CH), 7.55 (s, 1H; CH), 7.52 (m, 1H; CH), 7.50 (s, 2H; CH), 7.47 (s, 1H; CH), 7.43 (d, J = 9.6 Hz, 1H; CH), 7.36 (t, J = 8.0 Hz, 1H; CH), 7.32 (s, 1H; CH), 7.20 (s, 1H; CH), 7.17 (s, 1H; CH), 6.97 (s, 1H; CH), 4.80 (t, J = 10.0 Hz, 1H; CH), 4.54 (d J = 12.0 Hz, 1H; CH), 4.22 (brt, 1H; CH), 3.99 (s, 3H; CH_3), 3.97 (s, 6H; CH_3), 3.87 (s, 3H; CH_3), 3.81 (s, 3H; CH_3), 3.45 (q, J = 6.4 Hz, 2H; CH_2), 2.58 (t, J = 7.2 Hz, 2H; CH_2), 2.03 (s, 3H; $COCH_3$). ESI-TOF-MS m/z calcd for $C_{54}H_{52}ClN_{17}O_9$ $[M + H]^+$ 1118.38, found 1118.65.

A synthetic procedure similar to that used for the preparation of compound **4** was followed to prepare compound **5**.

AcImPyPy-*β*-ImPy-Indole-*seco*-CBI (5). A synthetic procedure similar to that used for the preparation of compound **4** was followed to prepare compound **7**, with a yield of 53% for one step from **2**. After purification by column chromatography (silica gel 5−10% MeOH in CH_2Cl_2, gradient elution), **5** was used in the DNA alkylation reaction. ESI-TOFMS m/z calcd for $C_{55}H_{53}ClN_{16}O_9$ $[M + H]^+$ 1117.39, found 1117.57.

AcImImPyPy-*β*-PyPyPy-*β*-Dp (6). AcImImPyPy-*β*-PyPyPy-*β*-CLEAR resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol. A sample of resin was cleaved with 3-(dimethylamino)-1-propylamine for 10 h at 55 °C, and purified by HPLC using a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 0−100% linear gradient, 0−30 min, 254 nm), to produce **6** (2.9 mg, 2.5 μmol, 2.5%) as a yellow powder.

After further purification by HPLC, **6** was used in the DNA alkylation reaction. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.32 (s, 1H; NH), 10.29 (s, 1H; NH), 9.92 (s, 1H; NH), 9.89 (s, 1H; NH), 9.88 (s, 1H; NH), 9.87 (s, 1H; NH), 9.32 (s, 1H; NH), 8.05 (t, J = 5.2 Hz, 1H; NH), 7.97 (t, J = 5.2 Hz, 1H; NH), 7.86 (t, J = 5.2 Hz, 1H; NH), 7.59 (s, 1H; Im−H), 7.50 (s, 1H; Im−H), 7.28 (s, 2H; Py−H), 7.22 (s, 1H; Py−H), 7.19 (s, 2H; Py−H), 7.14 (s, 1H; Py−H), 7.02 (s, 1H; Py−H), 6.87 (s, 1H; Py−H), 6.85 (s, 1H; Py−H), 6.82 (s, 1H; Py−H), 4.00 (s, 3H; NCH_3), 3.97 (s, 3H; NCH_3), 3.84 (s, 3H; NCH_3), 3.83 (s, 6H; NCH_3), 3.81 (s, 3H; NCH_3), 3.79 (s, 3H; NCH_3), 3.46 (m, 4H; CH_2), 3.04 (q, J = 6.4 Hz, 2H; CH_2), 2.66 (t, J = 7.2 Hz, 2H; CH_2), 2.31 (t, J = 7.2 Hz, 2H; CH_2), 2.19 (t, J = 7.2 Hz, 2H; CH_2), 2.10 (s, 6H; NCH_3), 2.04 (s, 3H; $COCH_3$) 1.51 (t, J = 7.2 Hz, 2H; CH_2). ESI-TOFMS m/z calcd for $C_{53}H_{66}N_{20}O_{10}$ $[M + H]^+$ 1143.53, found 1143.75.

AcImImPy-*β*-ImPyPyPy-*β*-Dp (7). AcImImPy-*β*-ImPyPyPy-*β*-CLEAR resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol. A sample of resin was cleaved with 3-(dimethylamino)-1-propylamine for 10 h at 55 °C, and purified by HPLC using a

Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 0−100% linear gradient, 0−30 min, 254 nm), to produce **7** (1.3 mg, 1.1 μmol, 1.1%) as a yellow powder. After further purification by HPLC, **7** was used in the DNA alkylation experiments. ESI-TOFMS *m/z* calcd for $C_{52}H_{65}N_{21}O_{10}$ $[M + H]^+$ 1144.53, found 1144.61.

Cloning of 317 bp DNA Fragments. All DNA fragments and primers for cloning or DNA amplification were purchased from Proligo. The 160 bp DNA fragments were prepared in a final volume of 20 μL containing 50 μM of fragment (5′-GCTTGTCACAGTGCAGCTTGA GGCTGTCCAGCTCACTCAGTGTGGCAAAGGTGCCCTTGAGGT TGTCCAGGTGAGCCAGGCCATCACTAATGTCGCGATGGA-CAGTCAGTGTGGTCTATTTCTCGGAGGCTGGACAATC-AAGGGGAAACCCTTAAGCGG-3′). Products were identified by separation in 0.5X TBE−10% native polyacrylamide gel with 0.5 μg/mL ethidium bromide using low weight DNA marker (New England BioLabs) and visualization under UV illumination. The fragments were ligated into pGEM-T Easy vectors (Promega), and *Escherichia coli* DH5α competent cells (Toyobo) were transformed and cultured on LB plates with 100 μg/mL ampicillin and 32 μg of X-gal/400 μg of IPTG overnight at 37 °C. White colonies were identified by colony direct polymerase chain reaction (PCR) in 20 μL of the reaction mixtures containing 250 nM of primer set (T7, 5′-TAATACGACTCACTAT-AGGG-3′; sp6, 5′-GATTTAGGTGACACTATAG-3′), 200 μM of deoxynucleotide triphosphates (Sigma Aldrich), 2 units taq DNA polymerase, and 1X ThermoPol Reaction Buffer (New England Bio Labs). The reaction mix was incubated at 95 °C for 5 min, then followed by 30 incubation cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s with a final extension step of 72 °C for 7 min. The appropriate colony was selected for transfer to 5 mL of LB medium with 100 μg/mL ampicillin and cultured overnight at 37 °C. The plasmids with inserts were extracted using a GenElute Plasmid Miniprep Kit (Sigma Aldrich) and identified by PCR (program and reaction mixtures same as above).

Preparation of 5′-Texas Red-Labeled DNA Fragments and High-Resolution Gel Electrophoresis. The 5′-Texas Red-modified 727 bp DNA fragments MLP (Adenovirus Major Late Promoter-Human β-globin) DNA were prepared by PCR with 5′-Texas Red-modified 20-mer primers: 5′-CCCATTCTAAACTGTACCCT-3′ (MLP DNA forward, 5943−5963) and 5′-GGCATCAAGGAAGGTGATTGG-3′ (Human β-globin DNA reverse, 705−725). The 5′-Texas Red-modified 317 bp DNA fragments containing 160 bp sequence were prepared by PCR with 5′-Texas Red-labeled T7 and sp6 promoter primer and 5′-Texas Red-labeled sp6 and T7 promoter primer from 1 ng/μL of the 160 bp fragments inserted pGEM-T Easy vector (program and other reagents same as above). Fragments were purified by GenElute PCR Cleanup Kit (Sigma Aldrich), and their concentrations were determined by UV absorption.

High-Resolution Gel Electrophoresis. The 5′-Texas Red-labeled DNA fragments (10 nM) were alkylated by various concentrations of conjugates in the absence and presence of partner polyamide **6** in 10 μL of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 23 °C. To investigate competitive inhibition by **7**, 50 nM of conjugate **4** in the presence of 500 nM of **6** and various concentrations of **7** were used. After overnight incubation, the reaction was quenched by the addition of calf thymus DNA (1 mM, 1 μL) and heating for 5 min at 90 °C. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in 5 μL of loading dye (formamide with fuschin red), heated at 94 °C for 20 min, and then immediately cooled to 0 °C. A 2 μL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a Hitachi DNA Sequencers.

Surface Plasmon Resonance (SPR) Assay. SPR experiments were performed using a BIACORE X instrument according to the previous studies.[17,18] For the measurement of conjugate **4**, biotinylated hairpin DNA 5′-biotin-GGCCGAGGTTGTCCGTGCTTTTGCACGGACAA-CCTCGGCC-3′ was immobilized to streptavidin-coated sensor chip SA at a flow rate of 5 μL/min to obtain wanted immobilization level. For the measurement of partner polyamide **6**, biotinylated hairpin DNA 5′-biotin-GGCCGAGGTTGTCCATGCTTTTGCATGGACAACCTCG-GCC-3′ was immobilized. Experiments were accomplished using HBS-EP buffer with 0.1% and 0.5% DMSO at 25 °C. The sample solutions at different concentrations ranging from 25 to 10 μM were prepared in HBS-EP buffer with 0.1% and 0.5% DMSO and injected at a flow rate of 20 μL/min. To obtain kinetics information, data processing was performed by global fitting of experimentally obtained sensorgrams to a model with 1:1 Langmuir binding using the BIAevaluation 4.1 program.

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Supporting Information Available: High-resolution denaturing polyacrylamide gel electrophoresis of conjugate **5**-treated 317 bp DNA fragment in the absence and presence of partner polyamide **6**; and the sequence of 317 bp DNA fragment and the binding model of **4** and **6** to target oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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