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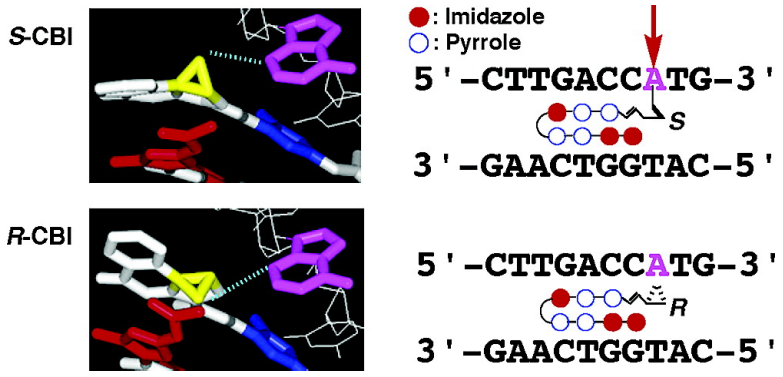
*J. Am. Chem. Soc.*, **2004**, 126 (29), 8948-8955• DOI: 10.1021/ja049398f • Publication Date (Web): 02 July 2004



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# Enantioselective DNA Alkylation by a Pyrrole−Imidazole *S*-CBI Conjugate

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***Abstract:*** Conjugates **12S** and **12R** of *N*-methylpyrrole (Py)−*N*-methylimidazole (Im) seven-ringed hairpin polyamide with both enantiomers of 1,2,9,9a-tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (CBI) were synthesized, and their DNA alkylating activity was examined. High-resolution denaturing gel electrophoresis revealed that **12S** selectively and efficiently alkylated at one match sequence, 5′-TGACC***A***-3′, in 450-bp DNA fragments. The selectivity and efficiency of the DNA alkylation by **12S** were higher than those of the corresponding cyclopropapyrroloindole (CPI) conjugate, **11**. In sharp contrast, another enantiomer, **12R**, showed very weak DNA alkylating activity. Product analysis of the synthetic decanucleotide confirmed that the alkylating activity of **12S** was comparable with **11** and that **12S** had a significantly higher reactivity than **12R**. The enantioselective reactivity of **12S** and **12R** is assumed to be due to the location of the alkylating cyclopropane ring of the CBI unit in the minor groove of the DNA duplex. Since the CBI unit can be synthesized from commercially available 1,3-naphthalenediol, the present results open up the possibility of large-scale synthesis of alkylating Py−Im polyamides for facilitating their use in future animal studies.

## Introduction

On the basis of previous efforts over a period of four decades on minor groove sequence information readout by small molecules,[1] Dervan's group has developed minor groove-binding Py−Im hairpin polyamides that precisely recognize each of the four Watson−Crick base pairs according to the binding rule of Py−Im polyamides.[2] Antiparallel pairing of Im opposite Py (Im/Py) recognizes a G−C base pair, whereas a Py/Py pair recognizes A−T or T−A base pairs.[3] Sequence-specific DNA recognition of Py−Im hairpin polyamides in the minor groove depends on the sequence of side-by-side aromatic amino acid pairing oriented in the amino-carboxyl (N-C) direction with respect to the 5′-3′ direction of the DNA helix. These Py−Im hairpin polyamides have a high binding affinity and a sequence specificity comparable to those of transcription factors.[4]

Duocarmycin A (Duo) is a highly potent antitumor antibiotic, which alkylates the N3 site of adenine (A) at the 3′ end of three or more consecutive A−T base pairs in DNA. We found that, in the presence of distamycin A (Dist), Duo efficiently alkylates completely different sequences, such as the 3′ end of G in the 5′-AGGTG-3′ sequence. NMR structural analysis has demonstrated that the efficient reaction proceeds by forming a cooperative heterodimer between the Duo and Dist. Interestingly, the replacement of Dist with various Py−Im triamides changes the sequence-specific alkylation by Duo in a predictive manner, analogous to the recognition rule of Py−Im polyamides.[5] These results suggest that Py−Im polyamides and Duo can be used as versatile recognition and alkylating moieties, respectively, for the design of sequence-specific alkylating agents. Thus, we have synthesized various types of conjugate between segment A of Duo or DU-86 and Py−Im polyamides.[6] We have demonstrated that conjugate **1**, a combined segment A of DU-86[7] with Py−Im hairpin polyamides, effectively alkylates the N3 position of adenine in a targeted match sequence within a 450-bp DNA fragment in nanomolar concentrations (Figure 1).[8] Importantly, the insertion of a vinyl linker between segment A

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**10.1021/ja049398f CCC: $27.50** 

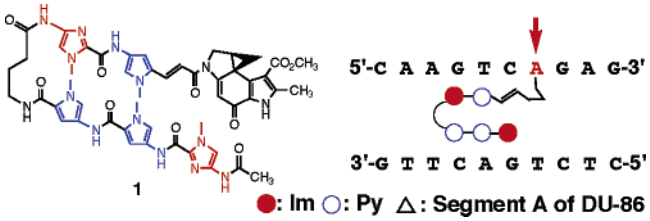


***Figure 1.*** Chemical structure of compound **1** and a schematic representation of the specific alkylation in the 5′-AGTCA-3′ sequence by **1**. The arrow indicates the site of the alkylation.

of DU-86 and the Py−Im polyamides dramatically enhances the DNA alkylating reactivity, as well as the cytotoxicity against human cancer cell lines, more than 100-fold compared to the case with no vinyl linker.[9] We demonstrated that an alkylating Py−Im polyamide, which recognizes specific sites on the template strand of a coding region in a green fluorescent protein, effectively inhibits transcription in an in vitro transcription system. The results indicate that the compound can target the promoter region and also the coding region, which dramatically increases its choice of target sequence as an antigene agent.[10] Recently, we found that alkylating Py−Im polyamides that differ only in that the C−H bond is substituted by an N atom in the second ring showed significantly different cytotoxicities in 39 human cancer cell lines.[11] More recently, we demonstrated that alkylating Py−Im polyamide induces sequence-specific gene silencing in human cell lines.[12] These results suggest the intriguing possibility that DNA alkylating agents that recognize longer base-pair sequences may provide a promising approach for developing new types of antitumor agent.

In the next step toward the goal of developing antitumor agents, some of the alkylating Py−Im polyamides need to be examined in animal studies, such as xenografts possessing human cancers. However, it is not practical for us to synthesize sufficient quantities of Py−Im CPI conjugates for animal studies, because segment A of DU-86 is prepared in five steps in an overall 25% yield from the natural antitumor antibiotic, duocarmycin $B_2$.[7] To overcome this problem, we searched for an alternative alkylating moiety that was equivalent to segment A of DU-86 in terms of its DNA alkylation and that could be synthesized from commercially available starting materials using a general synthetic methodology. We selected 1,2,9,9a-tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (CBI) as an alkylating unit, because Boger's group has already established an efficient synthetic route for CBI,[13] and they have demonstrated that CBI derivatives show better reactivity to DNA and are more stable under neutral and acidic conditions relative to the corresponding CPI derivatives.[14] In our preliminary experiments, we confirmed that the half-lives of ImPyDu86 and ImPyCBI in a pH = 7.0 solution at 37 °C are 53 h and more than 10 d, respectively. Thus, the choice of CBI as an alkylating unit for Py−Im hairpin polyamides would improve their stability and reactivity in an aqueous solution. In fact, Chang and Dervan have already synthesized different types of Py−Im polyamide−CBI conjugate, **13S** and **13R**, and have examined their DNA alkylating activities.[15]

In the present study, we have synthesized the enantiomer of CBI tethered to a Py−Im hairpin with a vinyl linker to construct two new conjugates, **12S** and **12R**, and have examined their sequence-specific DNA alkylating activity and compared them with that of CPI conjugate, **11**.[8]

## Results and Discussion

**Synthesis.** We synthesized the hairpin polyamide−CBI conjugates **12S** and **12R**, as shown in Scheme 1. The reduction of compound **2** by Pd−C and $H_2$, followed by coupling with compound **3** using pentafluorophenyl diphenylphosphinate (FDPP), produced compound **4** in a good yield. Hydrolysis of **4** gave the key carboxylic acid, compound **5**, which was used as the common *N*-terminal half of conjugates **12S** and **12R**. Compound **8** was synthesized by the reduction of compound **6**[8] with Pd−C and $NaBH_4$, followed by coupling with compound **5** using FDPP. After subsequent deprotection using DBU, the carboxylic acid **9** was activated with CDI to give **10**. The alkylating moieties, enantiomerically pure *S*- and *R*-CBI, were prepared according to literature procedures.[13] Finally, the synthesis of conjugates **12S** and **12R** was accomplished by coupling **10** with *S*- and *R*-CBI using NaH. After purification using HPLC, the hairpin polyamide−CBI conjugates **12S** and **12R** were employed in the DNA alkylation experiments. Conjugate **11** was synthesized according to the reported procedures.[8]

**Evaluation of the DNA Alkylating Activity Using 450-bp DNA Fragments.** Sequence-selective alkylation by compounds **11**, **12S**, and **12R** was investigated using the two complementary 5′-Texas Red-labeled 450-bp DNA fragments, pUC18 R1861*-2310 and pUC18 F328*-777, employing an automated DNA sequencer, as has been described in a previous article.[6b] The asterisk indicates Texas Red modification, and the nucleotide numbering starts with the replication site. Alkylation was carried out at 23 °C for 3 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 at the alkylated sites. Under these heating conditions, all the purine N3-alkylated sites in the DNA produced cleavage bands almost quantitatively on the gel.[6b] Sequencing analysis of the alkylated DNA fragment, pUC18 R1861*-2310, after heat treatment is shown in Figure 2. Alkylation by the Py−Im hairpin polyamide−CPI conjugate **11** occurred predominantly at the A site of the match sequence of 5′-TGACC*A*-3′ (site 1), together with

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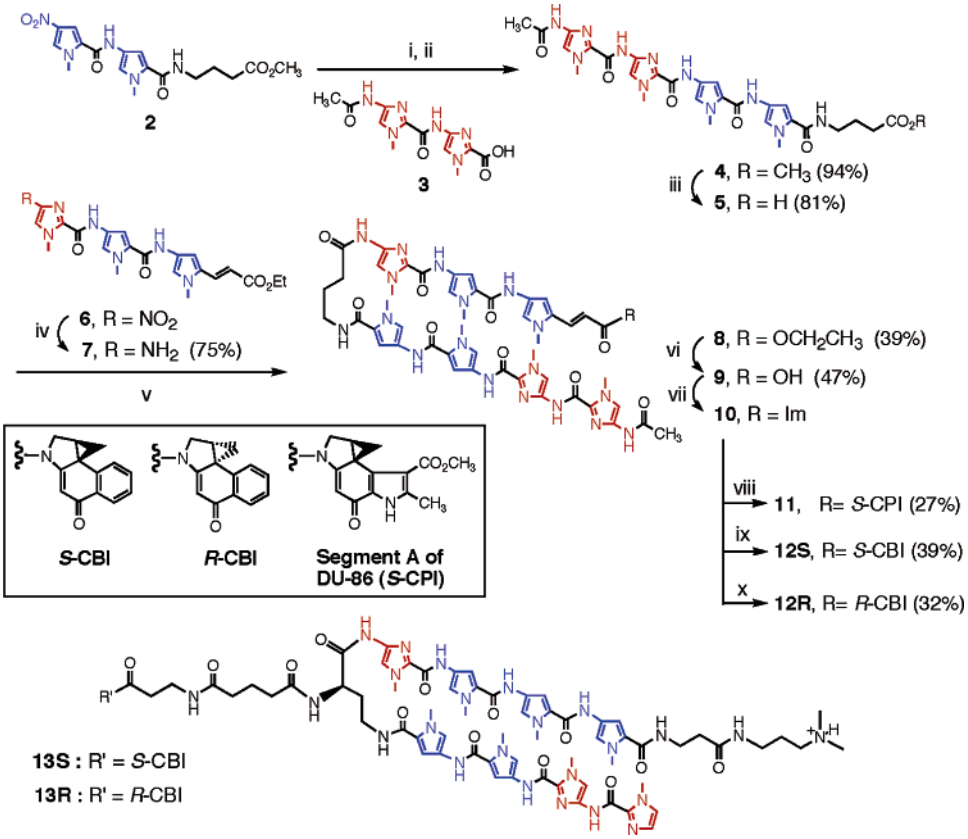
***Scheme 1*** [a]




[a] Reaction conditions: (i) Pd−C, $H_2$, MeOH−AcOEt. (ii) **3**, FDPP, $^{i}Pr_2NEt$, DMF. (iii) NaOH, $H_2O$. (iv) Pd−C, $NaBH_4$, MeOH−AcOEt. (v) **7**, FDPP, $^{i}Pr_2NEt$, DMF. (vi) DBU, $H_2O$. (vii) 1,1′-Carbonyldiimidazole, DMF. (viii) Segment A of DU-86, NaH, DMF. (ix) *S*-CBI, NaH, DMF. (x) *R*-CBI, NaH, DMF.

a minor mismatch alkylation at the G site of 5′-T*T*ACC*G*-3′ (site 2) (lanes 2−6). Similar mismatch alkylations by the Im−Py pair at the sites adjacent to the $\gamma$-turn have been observed previously.[15]

Interestingly, efficient alkylation by the Py−Im hairpin polyamide−CBI conjugate **12S** occurred exclusively at site 1 at a lower concentration compared to those for compound **11** (lanes 7−11) without any alkylation at site 2, indicating a superior reactivity as well as a better recognition ability of **12S** compared to **11**. In clear contrast to the significant reactivity of **12S**, alkylation by **12R** was observed as a very faint band only at a concentration of 40 nM (lane 12). These results clearly indicate that **12S**, which has a cyclopropane ring in a natural configuration, has at least a 20-fold higher reactivity compared to **12R**. These results are consistent with previous observations by Boger et al. that the natural *S*-enantiomers of CC-1065 and other analogues are about 10 times more reactive than unnatural *R*-enantiomers.[16]

In the complementary DNA fragment, pUC18 F328*-777, DNA alkylation was not observed for compounds **11**, **12S**, and **12R** at a concentration of 40 nM (Figure 1S, Supporting Information). Since this fragment does not contain a match sequence, 5′-(T/A)G(T/A)CCPu-3′, for these agents, the results are consistent with the sequence specificity of the agents. It is important to note that this fragment contains a reversed match site, 5′-PuCC(T/A)G(T/A)-3′. It has been reported that the reversed binding orientation of Py−Im polyamides, the N−C direction with respect to the 3′-5′ direction, is sometimes predominant in some cases.[3a] The lack of DNA alkylation on the complementary strand clearly indicates that these agents, especially **12R**, do not alkylate DNA in a reversed binding orientation under the conditions used.

Chang and Dervan reported that both *S*- and *R*-CBI Py−Im hairpins, **13S** and **13R**, become alkylated at adenines in the neighborhood of their targeting match sites and show no enantioselective alkylation.[15] Although comparative studies have not been done, the enantioselective alkylating activity of **12S** and **12R** is attributed to the more accurate position of the cyclopropane ring of CBI in a fixed geometry in the DNA minor groove compared with that of **13S** and **13R**, which have a flexible 4-(2-carboxy-ethylcarbamoyl)-butyric acid linker between the $\gamma$-turn and the CBI unit.

To evaluate the rate of DNA alkylation by conjugates **12S** and **11**, DNA alkylation was examined over a short incubation period (less than 1 h), as shown in Figure 3. Quantitative analyses of the alkylation are summarized in Figure 3b. It was found that DNA alkylation by **12S** at site 1 was observed at concentrations of 40 and 20 nM, even after 5 min; after 1 h incubation, almost all the DNA was consumed to provide an alkylating product at a concentration of 40 nM. However, DNA alkylation by **11** was inefficient, and after 1 h incubation, only

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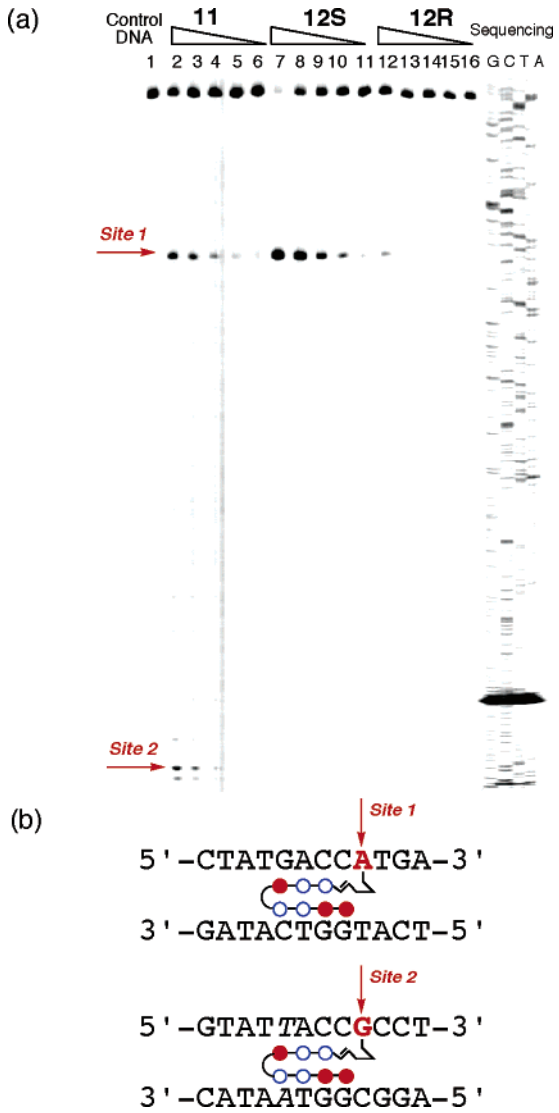


***Figure 2.*** (a) Thermally induced strand cleavage of the 5′-Texas Red-labeled 450-bp DNA fragment, pUC18 R1861*-2310, by conjugates **11**, **12S**, and **12R** at 23 °C for 3 h. Lane 1 = DNA control. Lanes 2−6 = 40, 20, 8, 4, and 2 nM of **11**. Lanes 7−11 = 40, 20, 8, 4, and 2 nM of **12S**. Lanes 12−16 = 40, 20, 8, 4, and 2 nM of **12R**. (b) Schematic representation of the recognition in the 5′-TGACCA-3′ (match) and 5′-T*T*ACCG-3′ (mismatch) sequences by **11** and **12S**. The arrows indicate the site of alkylation by **11** and **12S**. The alkylating base is shown in red bold, and the mismatch-binding base is shown in italics.

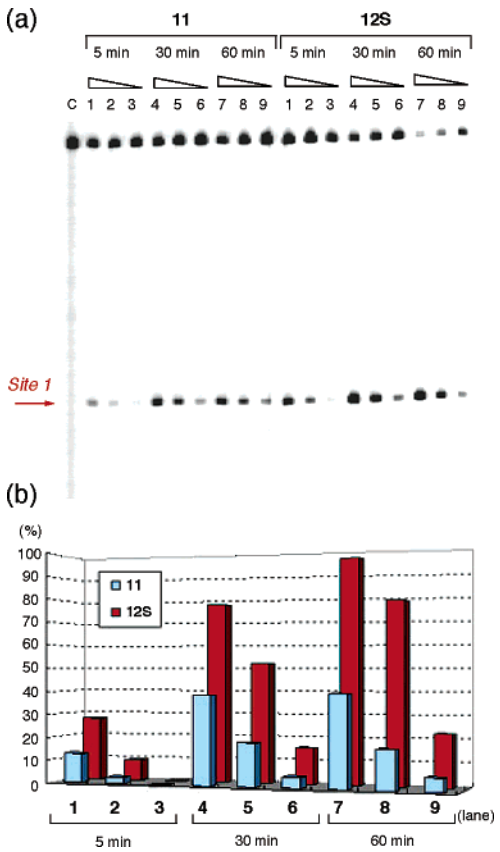


***Figure 3.*** (a) Thermally induced strand cleavage of 5′-Texas Red-labeled 450-bp DNA fragment, pUC18 R1861*-2310, by conjugates **11** and **12S**. Lane C = DNA control. Lanes 1−3 = 40, 20, and 8 nM of **11** and **12S**, 5 min incubation. Lanes 4−6 = 40, 20, and 8 nM of **11** and **12S**, 30 min incubation. and Lanes 7−9 = 40, 20, and 8 nM of **11** and **12S**, 60 min incubation. (b) The percentage of alkylated DNA at the 5′-TGACC*A*-3′ fragment under same conditions, using **11** and **12S** summarized graphically.

38% of the DNA was alkylated at site 1 at a concentration of 40 nM of **11** together with a mismatch alkylation at site 2. These results clearly demonstrate that the *S*-CBI unit is a better DNA alkylating moiety than segment A of DU-86, and they indicate that the DNA hairpin CBI conjugates, such as compound **12S**, are appropriate candidates for further biological study.

**HPLC Analysis of DNA Alkylation by Conjugate 12S.** To clarify the mechanism of DNA alkylation by conjugate **12S**, we investigated the alkylation of the duplex decanucleotide, 5′-CTTGACCATG-3′ (ODN1)/5′-CATGGTCAAG-3′ (ODN2), which was designed according to the results of the gel electrophoresis experiments described above. HPLC analysis of the reaction mixtures containing ODN1/ODN2 and **12S** after 5 min revealed that conjugate **12S** and ODN1 containing the target sequence were consumed and yielded a new peak (retention time = 19.4 min) (Figure 4b). The new peak was ascribed to the ODN1−**12S** alkylation complex using electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS), which was used to observe the −3 and −4 molecular ions from the ODN1−**12S**, as shown in Figure 5. Quantitative HPLC analysis indicated that the yield of the ODN1−**12S** adduct was 56 and 93% after 5 min and 1 h, respectively. HPLC analysis of the reaction mixture containing ODN1/ODN2 and **11** showed a very similar profile (Figure 4c), and the alkylation proceeded to 57 and 81% yields after 5 min and 1 h incubation, respectively. The results indicate that **12S** and **11** have similar reactivity toward ODN1/ODN2. The reason for the discrepancy between the results obtained from gel electrophoresis and HPLC product analysis is not clear; however, this can be explained by the nature of ODN1/ODN2, which does not possess the competitive alkylation site 2. In contrast, conjugate **12R** exhibited a very small peak for the adduct, even after 1 h incubation, confirming the significantly lower reactivity of **12R** compared with that of **12S** (Figure 4d). These results clearly indicate that **12S** and **11** have almost comparable reactivity. The alkylating site in ODN1 was determined using HPLC product analysis, employing a previously reported procedure.[8,17] When the ODN1−**12S** adduct was heated to 90 °C for 10 min,

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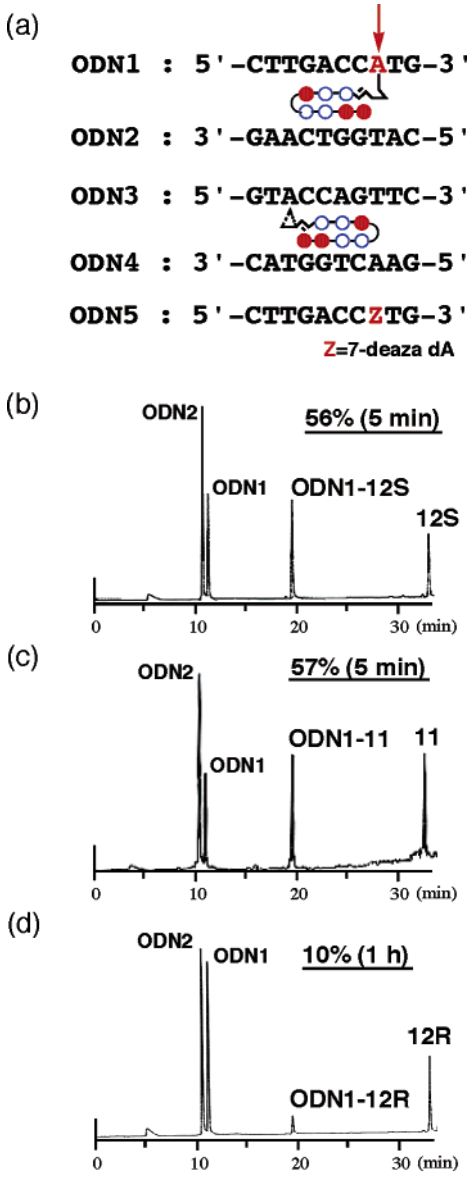


***Figure 4.*** (a) Schematic representation of ODN1/ODN2 alkylation by **12S**, ODN3/ODN4 alkylation by **12R**, and sequence of ODN5. HPLC analysis of ODN1/ODN2 with (b) **12S**, (c) **11**, and (d) **12R**.

formation of an abasic site containing ODN1 and **12S**-A adduct was observed. ESI-TOF mass spectrometry of these products is shown in Figure 2S. The cleavage of the abasic site containing ODN1 with hot alkali (0.1 N NaOH, 90 °C, 10 min) and enzymatic dephospholylation with alkaline phosphatase produced the corresponding cleaved oligonucleotides, 5′-TG-3′ and 5′-CTTGACC-3′, which had retention times identical to those of the authentic oligomers. These results strongly suggest that sequence-specific N3 alkylation by **12S** efficiently occurred at the $A_8$ position of ODN1.

To confirm that DNA alkylation occurs at the N3 position, alkylation of the 7-deazaadenine-(*Z*)-containing decamer, ODN5/ODN2, by **12S** was examined using a method analogous to that reported previously.[8] HPLC analysis of the reaction mixture indicated that the formation of the corresponding adduct and the yield of the ODN5−**12S** adduct was 60% after 5 min (Figure 3S, Supporting Information). The structure of the ODN5−**12S** alkylation complex was confirmed by ESI-TOF mass spectrometry. The results clearly indicate that **12S** alkylates at the N3 position of adenine to produce the corresponding alkylated products.

To evaluate the possibility of alkylation by the reversed orientation of **12R**, the reaction of ODN3/ODN4 was also examined. HPLC analysis of the reaction mixture indicated that neither the formation of the adduct nor the consumption of ODN3 was observed, confirming the absence of any reactivity of **12R** in the reversed binding orientation (data not shown).

**Interpretation of Enantioselectivity Variation by Molecular Modeling.** To gain an insight into the molecular basis of the enantioselectivity of the hairpin polyamides **12S** and **12R**, we constructed a model of a hairpin polyamide−decamer complex based on the $^1$H NMR structure of the DNA binding hairpin polyamide and the Duo-Dist−octamer complex.[18,19] Because Boger et al. have already demonstrated that nucleophilic attack exclusively occurs at the C9 position in the case of CBI,[20] covalent bond formation between N3 of A and C9 of CBI unit was considered. The energy-minimized structure of the ODN1/ODN2−**12S** complex suggests that the cyclopropane subunit of *S*-CBI is located in close proximity (2.94 Å), and in a reasonable direction, to the nucleophilic N3 group of the $A_8$ residues (the angle of the N3−C9−C8b bond = 144.4°). The energy-minimized structure also suggests that the *S*-CBI unit stacks well with the N-terminal Im residue (Figure 6a,c). In sharp contrast, in the energy-minimized ODN1/ODN2−**12R** complex, the cyclopropane subunit of *R*-CBI is far from the nucleophilic N3 group of the $A_8$ residues (4.00 Å with an angle of the N3−C9−C8b bond = 106.9°). The different binding orientation of the *S*- and *R*-CBI units explains well the significantly enantioselective reactivity of **12S** and **12R**.

## Conclusions

We have successfully synthesized both enantiomers of the Py−Im hairpin polyamide−CBI conjugates, **12S** and **12R**, and the corresponding CPI conjugate, **11**. We have evaluated these DNA alkylating polyamides in detail using high-resolution denaturing gel electrophoresis employing 450-bp DNA fragments, and from HPLC product analysis using decanucleotides. The results clearly indicate that Py−Im CBI conjugate **12S**, possessing cyclopropane in a natural configuration, effectively and efficiently alkylates at one match sequence, 5′-TGACCA-3′. The selectivity and the efficiency of DNA alkylation by **12S** were higher than that of the corresponding CPI conjugate, **11**. Our preliminary study has demonstrated that, in a screening panel of 39 human cancer cell lines, the deacetylamino derivative of racemic **12** is 1 order of magnitude more potent compared to the deacetylamino **11** (Figure 4S, Supporting Information). Since the CBI unit can be synthesized from commercially available 1,3-naphthalenediol, the present results open up the possibility of synthesizing alkylating Py−Im polyamides on a large scale, which allows for future animal studies in the development of antitumor agents.

## Experimental Section

**General Methods.** Reagents and solvents were purchased from standard suppliers and used without further purification. Abbreviations of some reagents: DBU, 1,8-diazabicyclo[4.3.0]undec-7-ene; $^i$Pr$_2$NEt,

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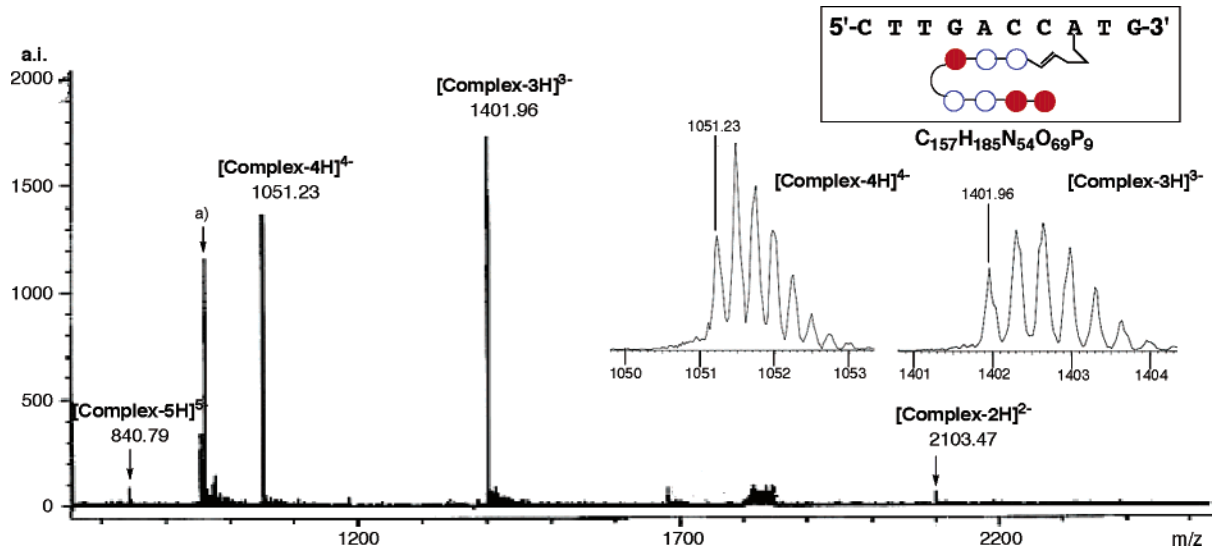


***Figure 5.*** Electrospray ionization time-of-flight mass spectrometry of ODN1−**12S** alkylation complex. (a) Abasic site containing ODN1, [5′-CTTGACC-(deoxyribose)-TG-3′-3H]$^{3-}$: 960.48 (calcd, 960.49).

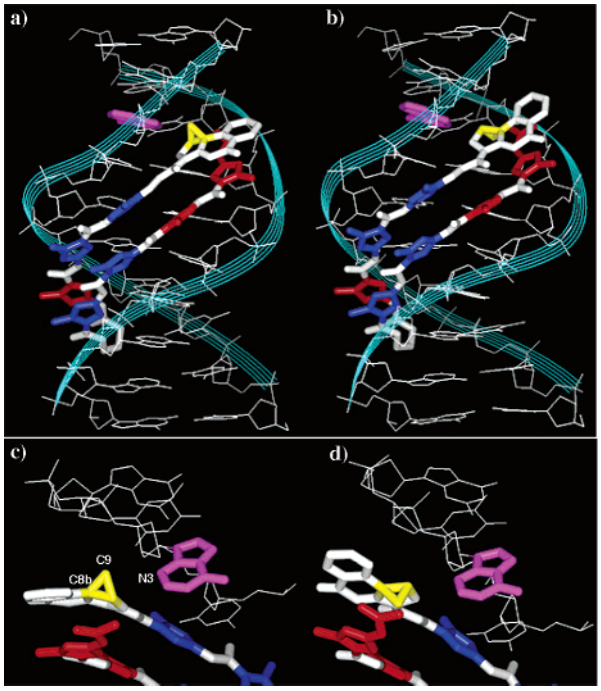


***Figure 6.*** The energy-minimized structure of d(CTTGACCATG) ODN1/d(CATGGTCAAG) ODN2−**12S** (a) and −**12R** (b) complexes. Minimization was performed in the presence of 18 sodium cations and a 10-Å layer of $H_2O$ using the CFF force field. For simplicity, the sodium ions and water molecules are not represented. Each strand of DNA is drawn in white, and the ribbon representation is drawn in light blue. Py and Im are drawn in blue and red, respectively, with the reacting A bases drawn in purple. The cyclopropane units in the CBI moiety of the hybrids are drawn in yellow. Plots of the side views of hairpin polyamides, **12S** and **12R**, are shown in (c) and (d), respectively.

*N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; FDPP, pentafluorophenyl diphenylphosphinate. Reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm silica gel 60 plates impregnated with 254-nm fluorescent indicator (from Merck). Plates were visualized by UV illumination. NMR spectra were recorded with a JEOL JNM-A 500 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), and br (broad). Electrospray ionization mass spectrometry (ESMS) and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) were produced on a API 150 (PE SCIEX) and BioTOF II (Bruker Daltonics) mass spectrometer. Polyacrylamide gel electrophoresis was performed on a HITACHI 5500-S DNA sequencer. Ex Taq DNA polymerase and Suprec-02 purification cartridges were purchased from Takara Co., the Thermo Sequenase core sequencing kit and loading dye (dimethylformamide with fuschin red) were purchased from Amersham Co. Ltd, 5′-Texas-Red-modified DNA oligomer (18-mer) was purchased from Kurabo Co. Ltd, and 50% Long Ranger gel solution was purchased from FMC Bioproducts. P1 nuclease and calf intestine alkaline phosphatase (AP, 1000 units/mL) were purchased from Roche Diagnostics. Both enantiomers of 1,2,9,9a-tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (***S*-CBI** and ***R*-CBI**) were synthesized and separated by published methods.[13] The purities of 5-(benzyloxy)-3-(*tert*-butyloxycarbonyl)-1-(hydroxymethyl)-1,2-dihydro-3H-benz[*e*]indole, (*R*)-*O*-acethyl mandelate ester [(1*R*,2′*R*)], and (1*S*,2′*R*) were more than 99%, as determined by HPLC. The following precursors were prepared by the reported procedures.[6]

**$NO_2$PyPy-γ-$CO_2CH_3$ (2).** $^1$H NMR (500 MHz, $CDCl_3$): δ 7.58 (s, 1H; CH), 7.53 (brs, 1H; NH), 7.16 (s, 1H; CH), 7.15 (s, 1H; CH), 6.51 (s, 1H; CH), 6.16 (brs, 1H; NH), 4.01 (s, 3H; $NCH_3$), 3.91 (s, 3H; $NCH_3$), 3.67 (s, 3H; $OCH_3$), 3.41 (q, $J$ = 6.5 Hz, 2H; $CH_2$), 2.42 (t, $J$ = 7.0 Hz, 2H; $CH_2$), 1.91 (qu, $J$ = 7.0 Hz, 2H; $CH_2$). ESMS *m*/*e* calcd for $C_{17}H_{22}N_5O_6$ [M + H]$^+$, 392.2; found, 392.1. **AcImImCO$_2$H (3):** $^1$H NMR (500 MHz, [$D_6$]DMSO): δ 10.37 (s, 1H; NH), 9.60 (s, 1H; NH), 7.63 (s, 1H; CH), 7.48 (s, 1H; CH), 3.96 (s, 3H; $NCH_3$), 3.93 (s, 3H; $NCH_3$), 2.03 (s, 3H; $COCH_3$). ESMS *m*/*e* calcd for $C_{12}H_{15}N_6O_4$ [M + H]$^+$, 307.1; found, 307.3.

**AcImImPyPy-γ-$CO_2CH_3$ (4).** To a solution of compound **2** (0.5 g, 1.28 mmol) in MeOH−AcOEt (1:1, 10 mL) was added 10% Pd−C (100 mg), and the reaction mixture was stirred for 2 h at room temperature under an $H_2$ atmosphere. The catalyst was removed by filtration through Celite. The filtrate was concentrated in vacuo to produce crude amine (396 mg), which was used in the next step without further purification. To the solution of crude amine (396 mg, 1.09 mmol) in 8 mL of DMF was added compound **3** (334 mg, 1.09 mmol) and FDPP (1.26 g, 3.28 mmol), followed by $^i$Pr$_2$NEt (1.15 mL, 6.60 mmol). The solution was stirred for 18 h and concentrated to a residue, which was subjected to column chromatography (silica gel, 0−5% MeOH in $CHCl_3$, gradient elution) to produce compound **4** (0.78 g, 94% yield) as a yellow powder. $^1$H NMR (500 MHz, [$D_6$]DMSO): δ 10.33 (s, 1H; NH), 10.32 (s, 1H; NH), 9.92 (s, 1H; NH), 9.34 (s, 1H; NH), 8.03 (brt, 1H; NH), 7.57 (s, 1H; CH), 7.51 (s, 1H; CH), 7.28 (d, $J$ = 1.5

Hz, 1H; CH), 7.18 (d, $J$ = 1.5 Hz, 1H; CH), 7.15 (d, $J$ = 1.5 Hz, 1H; CH), 6.88 (d, $J$ = 1.5 Hz, 1H; CH), 4.02 (s, 3H; $NCH_3$), 3.99 (s, 3H; $NCH_3$), 3.85 (s, 3H; $NCH_3$), 3.80 (s, 3H; $NCH_3$), 3.59 (s, 3H; $OCH_3$), 3.19 (q, $J$ = 6.5 Hz, 2H; $CH_2$), 2.35 (t, $J$ = 7.5 Hz, 2H; $CH_2$), 2.05 (s, 3H; $COCH_3$), 1.75 (qu, $J$ = 7.5 Hz, 2H; $CH_2$). ESMS *m*/*e* calcd for $C_{29}H_{36}N_{11}O_7$ $[M + H]^+$, 650.3; found, 650.3.

**AcImImPyPy-γ-$CO_2$H (5).** To a suspension of compound **4** (0.78 g, 1.20 mmol) in 80 mL of $H_2O$ was added NaOH (1 g, 25 mmol). The solution was stirred for 15 h at room temperature, and the aqueous solution was acidified to pH 2 at 0 °C. The precipitate was collected by filtration, washed with water, and dried to produce compound **5** (0.62 g, 81% yield) as a white powder. $^1$H NMR (500 MHz, [$D_6$]DMSO): $\delta$ 10.31 (s, 1H; NH), 10.29 (s, 1H; NH), 9.90 (s, 1H; NH), 9.32 (s, 1H; NH), 8.01 (brt, 1H; NH), 7.56 (s, 1H; CH), 7.50 (s, 1H; CH), 7.27 (d, $J$ = 2.0 Hz, 1H; CH), 7.17 (d, $J$ = 2.0 Hz, 1H; CH), 7.14 (d, $J$ = 2.0 Hz, 1H; CH), 6.87 (d, $J$ = 2.0 Hz, 1H; CH), 4.01 (s, 3H; $NCH_3$), 3.98 (s, 3H; $NCH_3$), 3.84 (s, 3H; $NCH_3$), 3.79 (s, 3H; $NCH_3$), 3.18 (q, $J$ = 6.5 Hz, 2H; $CH_2$), 2.24 (t, $J$ = 7.5 Hz, 2H; $CH_2$), 2.04 (s, 3H; $COCH_3$), 1.70 (qu, $J$ = 7.5 Hz, 2H; $CH_2$). ESMS *m*/*e* calcd for $C_{28}H_{34}N_{11}O_7$ $[M + H]^+$, 636.3; found, 636.2.

**AcImImPyPy-γ-ImPyPyLC$O_2$Et (8).** To a solution of compound **6** (81 mg, 0.173 mmol) in MeOH−AcOEt (1:1, 3 mL) was added 10% Pd−C (40 mg). After $NaBH_4$ (12 mg, 0.302 mmol) in $H_2O$ (0.2 mL) was added dropwise at 0 °C, the reaction mixture was stirred for 30 min at room temperature. The catalyst was removed by filtration through silica gel. The filtrate was concentrated in vacuo to produce crude amine **7** (57 mg, 75%), which was used in the next step without further purification. To a solution of crude amine **7** (57 mg, 0.13 mmol) in 0.5 mL DMF was added compound **5** (82 mg, 0.13 mmol) and FDPP (150 mg, 0.39 mmol), followed by $^i$Pr$_2$NEt (136 μL, 0.78 mmol). The solution was stirred for 16 h and concentrated to a residue, which was subjected to column chromatography (silica gel, 0−10% MeOH in $CHCl_3$, gradient elution) to produce **8** (53 mg, 39% yield) as a brown powder. $^1$H NMR (500 MHz, [$D_6$]DMSO): $\delta$ 10.30 (s, 1H; NH), 10.28 (s, 1H; NH), 10.23 (s, 1H; NH), 9.94 (s, 1H; NH), 9.92 (s, 1H; NH), 9.90 (s, 1H; NH), 9.35 (s, 1H; NH), 8.00 (brt, 1H; NH), 7.56 (s, 1H; CH), 7.50 (d, $J$ = 15.0 Hz, 1H; C*H*═CHCO), 7.49 (s, 1H; CH), 7.45 (s, 1H; CH), 7.40 (s, 1H; CH), 7.27 (s, 1H; CH), 7.26 (s, 1H; CH), 7.17 (s, 1H; CH), 7.15 (s, 1H; CH), 7.14 (s, 1H; CH), 6.89 (s, 1H; CH), 6.74 (s, 1H; CH), 6.06 (d, $J$ = 15.0 Hz, 1H; CH═C*H*CO), 4.14 (q, $J$ = 7.0 Hz, 2H; $CH_2$), 4.00 (s, 3H; $NCH_3$), 3.98 (s, 3H; $NCH_3$), 3.95 (s, 3H; $NCH_3$), 3.85 (s, 6H; $NCH_3$), 3.80 (s, 3H; $NCH_3$), 3.68 (s, 3H; $NCH_3$), 3.42 (m, 2H; $CH_2$), 2.36 (m, 2H; $CH_2$), 2.03 (s, 3H; $COCH_3$), 1.79 (m, 2H; $CH_2$), 1.23 (t, $J$ = 7.0 Hz, 3H; $CH_3$). ESMS *m*/*e* calcd for $C_{49}H_{57}N_{18}O_{10}$ $[M + H]^+$, 1057.4; found, 1057.3.

**AcImImPyPy-γ-ImPyPyLC$O_2$H (9).** To a suspension of compound **8** (53 mg, 0.05 mmol) in 0.2 mL $H_2O$ was added DBU (0.1 mL, 0.67 mmol). The solution was stirred for 18 h and concentrated to a residue, which was subjected to trituration with $Et_2O$ and AcOEt. After column chromatography (silica gel, 0−20% MeOH in $CHCl_3$, gradient elution), the crude was acidified with 1% HCl. The precipitate was collected by filtration, washed with water, and dried to produce **9** (24 mg, 47% yield) as a brown powder. $^1$H NMR (500 MHz, [$D_6$]DMSO): $\delta$ 10.30 (s, 1H; NH), 10.28 (s, 1H; NH), 10.24 (s, 1H; NH), 9.94 (s, 1H; NH), 9.90 (s, 2H; NH), 9.36 (s, 1H; NH), 8.00 (brt, 1H; NH), 7.56 (s, 1H; CH), 7.50 (s, 1H; CH), 7.46 (d, $J$ = 15.0 Hz, 1H; C*H*═CHCO), 7.45 (s, 1H; CH), 7.37 (s, 1H; CH), 7.27 (s, 1H; CH), 7.26 (s, 1H; CH), 7.17 (s, 1H; CH), 7.15 (s, 1H; CH), 7.14 (s, 1H; CH), 6.89 (s, 1H; CH), 6.71 (s, 1H; CH), 5.98 (d, $J$ = 15.0 Hz, 1H; CH═C*H*CO), 4.01 (s, 3H; $NCH_3$), 3.98 (s, 3H; $NCH_3$), 3.94 (s, 3H; $NCH_3$), 3.85 (s, 6H; $NCH_3$), 3.80 (s, 3H; $NCH_3$), 3.66 (s, 3H; $NCH_3$), 3.42 (m, 2H; $CH_2$), 2.37 (m, 2H; $CH_2$), 2.03 (s, 3H; $COCH_3$), 1.79 (m, 2H; $CH_2$). ESMS *m*/*e* calcd for $C_{47}H_{53}N_{18}O_{10}$ $[M + H]^+$, 1029.4; found, 1029.3.

**AcImImPyPy-γ-ImPyPyLCPI (11).** To a solution of compound **9** (15.0 mg, 14.6 μmol) in DMF (0.2 mL) was added 1,1′-carbonyldiimidazole (7.0 mg, 44.0 μmol). The mixture was stirred for 18 h at room temperature. Evaporation of the solvent gave a yellow residue, which was triturated with ethyl ether (5 mL × 3) to produce **10** (14 mg) as a yellow powder, which was used in the next step without further purification. To a solution of sodium hydride (5.0 mg, 0.13 mmol, 60% oil suspension) in DMF (0.1 mL) was added segment A of DU-86 (5 mg, 19.5 μmol) in DMF (0.1 mL). Crude **10** (14 mg) in DMF (0.1 mL) was added at 0 °C, and the reaction mixture was then stirred for 30 min at 0 °C. The reaction mixture was quenched by the addition of 50 mM sodium phosphate buffer (2 mL, pH 6.86) at 0 °C. Evaporation of the solvent gave a yellow residue, which was subjected to column chromatography (silica gel, 0−5% MeOH in $CHCl_3$, gradient elution) to produce compound **11** (5.0 mg, 27% yield for two steps) as a yellow powder. After further purification by HPLC using a Chemcobond 5-ODS-H column (0.1% AcOH/$CH_3$CN 0−50% linear gradient, 0−40 min, 254 nm), **11** (retention time: 34.1 min) was used in the DNA alkylation reaction. $^1$H NMR (500 MHz, [$D_6$]DMSO): $\delta$ 11.80 (brs, 1H; NH), 10.30 (s, 1H; NH), 10.27 (s, 1H; NH), 10.24 (s, 1H; NH), 9.94 (s, 1H; NH), 9.90 (s, 2H; NH), 9.32 (s, 1H; NH), 8.00 (brt, 1H; NH), 7.57 (d, $J$ = 14.5 Hz, 1H; C*H*═CHCO), 7.56 (s, 1H; CH), 7.50 (s, 1H; CH), 7.45 (s, 1H; CH), 7.37 (s, 1H; CH), 7.27 (s, 2H; CH), 7.17 (s, 1H; CH), 7.15 (s, 2H; CH), 6.89 (s, 2H; CH), 6.55 (d, $J$ = 14.5 Hz, 1H; CH═C*H*CO), 6.48 (s, 1H; CH), 4.27 (m, 1H; NCH*H*), 4.19 (m, 1H; NC*H*H), 4.03 (s, 3H; $NCH_3$), 4.00 (s, 3H; $NCH_3$), 3.97 (s, 3H; $NCH_3$), 3.85 (s, 6H; $NCH_3$), 3.80 (s, 3H; $NCH_3$), 3.72 (s, 3H; $OCH_3$), 3.70 (s, 3H; $NCH_3$), 3.50 (m, 1H; CH), 3.16 (m, 2H; $CH_2$), 2.47 (s, 3H; $CH_3$), 2.35 (m, 2H; $CH_2$), 2.14 (m, 1H; CH*H*), 2.03 (s, 3H; $COCH_3$), 1.75 (m, 2H; $CH_2$), 1.29 (m, 1H; C*H*H). ESI-TOF-MS (monoisotopic) $[M + H]^+$ 1269.5132 (calcd 1269.5091 for $C_{61}H_{65}N_{20}O_{12}$).

**AcImImPyPy-γ-ImPyPyLCBI(*S*) (12S).** To a solution of compound **9** (8.0 mg, 7.7 μmol) in DMF (0.2 mL) was added 1,1′-carbonyldiimidazole (3.5 mg, 22.0 μmol). The mixture was stirred for 18 h at room temperature. Evaporation of the solvent gave a yellow residue, which was triturated with ethyl ether (5 mL × 3) to produce **10** (8 mg) as a yellow powder, which was used in the next step without further purification. To a solution of sodium hydride (4.0 mg, 0.1 mmol, 60% oil suspension) in DMF (0.1 mL) was added *S*-CBI (4.6 mg, 23.3 μmol) in DMF (0.1 mL). Crude **10** (8 mg) in DMF (0.1 mL) was added at 0 °C, and the reaction mixture was then stirred for 30 min at 0 °C. The reaction mixture was quenched by the addition of 50 mM sodium phosphate buffer (2 mL, pH 6.86) at 0 °C. Evaporation of the solvent gave a yellow residue, which was subjected to column chromatography (silica gel, 0−5% MeOH in $CHCl_3$, gradient elution) to produce compound **12S** (3.6 mg, 39% yield for two steps) as a yellow powder. After further purification by HPLC using a Chemcobond 5-ODS-H column (0.1% AcOH/$CH_3$CN 0−50% linear gradient, 0−40 min, 254 nm), **12S** (retention time: 35.6 min) was used in the DNA alkylation reaction. $^1$H NMR (500 MHz, [$D_6$]DMSO): $\delta$ 10.32 (s, 1H; NH), 10.29 (s, 1H; NH), 10.25 (s, 1H; NH), 9.99 (s, 1H; NH), 9.98 (s, 1H; NH), 9.92 (s, 1H; NH), 9.33 (s, 1H; NH), 8.01 (brt, 1H; NH), 7.98 (d, $J$ = 8.0 Hz, 1H; ArH), 7.60 (d, $J$ = 15.0 Hz, 1H; C*H*═CHCO), 7.56 (s, 1H; CH), 7.50 (s, 1H; CH), 7.45 (s, 1H; CH), 7.42 (m, 1H; ArH), 7.39 (s, 1H; CH), 7.36 (m, 1H; ArH), 7.27 (s, 2H; CH), 7.19 (d, $J$ = 8.0 Hz, 1H; ArH), 7.17 (s, 1H; CH), 7.15 (s, 1H; CH), 6.93 (s, 1H; ArH), 6.89 (s, 1H; CH), 6.57 (d, $J$ = 15.0 Hz, 1H; CH═C*H*CO), 4.33 (m, 1H; NCH*H*), 4.27 (m, 1H; NC*H*H), 4.00 (s, 3H; $NCH_3$), 3.97 (s, 3H; $NCH_3$), 3.95 (s, 3H; $NCH_3$), 3.84 (s, 6H; $NCH_3$), 3.79 (s, 3H; $NCH_3$), 3.72 (s, 3H; $NCH_3$), 3.19 (m, 3H; $CH_2$), 2.36 (m, 2H; $CH_2$), 2.03 (s, 3H; $COCH_3$), 1.78 (m, 2H; $CH_2$), 1.71 (m, 1H; C*H*H), 1.55 (m, 1H; C*H*H). ESI-TOF-MS (monoisotopic) $[M + H]^+$ 1208.4929 (calcd 1208.4927 for $C_{60}H_{62}N_{19}O_{10}$).

**AcImImPyPy-γ-ImPyPyLCBI(*R*) (12R).** A synthetic procedure similar to that used for the preparation of compound **12S** was followed to prepare compound **12R**, with a yield of 32% for two steps from **9**. After further purification by HPLC using a Chemcobond 5-ODS-H column (0.1% AcOH/$CH_3$CN 0−50% linear gradient, 0−40 min, 254 nm), **12R** (retention time: 35.6 min) was used in the DNA alkylation

reaction. ESI-TOF-MS (monoisotopic) $[M + H]^+$ 1208.4905 (calcd 1208.4927 for $C_{60}H_{62}N_{19}O_{10}$).

**Preparation of 5′-Texas Red-Modified 450-bp DNA Fragments.** The 5′-Texas Red-modified 450-bp DNA fragments pUC18 F328*-777 and pUC18 R1861*-2310 (these two fragments are complementary) were prepared by polymerase chain reaction with 5′-Texas Red-modified 21-mer primers: 5′-TGTAAAACGACGGCCAGTGCC-3′ (pUC18 forward, 328−349) and 5′-TGCTGGCCTTTTGCTCACATG-3′ (pUC18 reverse, 1861−1872). Fragments were purified by filtration using Suprec-02, 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 **11**, **12S**, **12R** in 10 $\mu$L of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 23 °C. The reaction was quenched by the addition of calf thymus DNA (1 mM, 1 $\mu$L) and heating for 5 min at 90 °C. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in 8 $\mu$L of loading dye (formamide with fuschin red), heated at 94 °C for 20 min, and then immediately cooled to 0 °C. A 2-$\mu$L aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a Hitachi 5500-S DNA sequencer.

**Alkylation of Oligonucleotides by Conjugates 12S as Monitored by HPLC.** Oligonucleotides were synthesized on an automated DNA synthesizer. A reaction mixture (50 $\mu$L) containing conjugates **12S** (150 $\mu$M) and the duplex oligonucleotide (100 $\mu$M duplex concentration) in 50 mM sodium cacodylate buffer (pH 7.0) was incubated at 23 °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 with 50 mM ammonium formate and a 0−50% acetonitrile linear gradient (0−40 min) at a flow rate of 1.0 mL/min at 254 nm.

ODN1−**12S** complex was detected at 19.4 min. ESI-TOF-MS (monoisotopic) $[M - 3H]^{3-}$ 1401.96 (calcd: 1402.00) and $[M - 4H]^{4-}$ 1051.23 (calcd: 1051.25). ODN5-**12S** complex was also detected at 19.4 min. ESI-TOF-MS (monoisotopic) $[M - 3H]^{3-}$ 1401.66 (calcd: 1401.67), $[M - 4H]^{4-}$ 1050.99 (calcd: 1051.00) and $[M - 5H]^{5-}$ 840.60 (calcd: 840.60).

**Characterization of ODN1−12S Alkylation Complexes.** The ODN1−**12S** complex was collected by HPLC (elution with 50 mM ammonium formate and a 0−50% acetonitrile linear gradient [0−40 min] at a flow rate of 1.0 mL/min). The collected fraction (ODN1−**12S**) was frozen and dried, and then heated at 90 °C for 10 min. The degradation products were collected by HPLC (elution with 50 mM ammonium formate and a 0−50% acetonitrile linear gradient [0−40 min] at a flow rate of 1.0 mL/min at 254 nm).

Abasic site containing ODN1, 5′-CTTGACC-(deoxyribose)-TG-3′, was detected at 10.9 min. ESI-TOF-MS (monoisotopic) $[M - 3H]^{3-}$ 960.51 (calcd: 960.49) and $[M - 4H]^{4-}$ 720.13 (calcd: 720.12). **12S**-A was detected at 30.6 min. ESI-TOF-MS (monoisotopic) $[M + H]^+$ 1343.55 (calcd: 1343.55 for $C_{65}H_{67}N_{24}O_{10}$) and $[M + 2H]^{2+}$ 672.28 (calcd: 672.28).

The collected fraction (abasic site containing ODN1) was frozen and dried, and then in the presence of 0.1 N NaOH at 90 °C for 10 min. After neutralization, oligonucleotides were digested with AP (5 units/mL) in 5 mM Na cacodylate buffer (pH 7.0) at 37 °C for 30 min. The composition of the cleaved oligonucleotides was analyzed by HPLC (elution with 50 mM ammonium formate and a 0−15% acetonitrile linear gradient [0−20 min] at a flow rate of 1.0 mL/min at 254 nm) and confirmed 5′-TG-3′ and 5′-CTTGACC-3′ by comparing authentic oligonucleotides.

**Molecular Modeling Studies.** Minimizations were performed with the Discover (MSI, San Diego, CA) program using CFF force-field parameters. The starting structure was built on the basis of the NMR structure of the ImPyPy-$\gamma$-PyPyPy-d(CGCTAACAGGC)/d(GCCTGTTAGCG) complex[18] and the Duo-Dist−octamer complex.[19] The connecting parts between them were built using standard bond lengths and angles. The CBI unit of the assembled initial structure was energy-minimized using a distance-dependent dielectric constant of $\epsilon = 4r$ ($r$ stands for the distance between atoms i and j) and with convergence criteria having an RMS gradient of less than 0.001 kcal/mol Å. Eighteen Na cations were placed at the bifurcating position of the O−P−O angle at a distance of 2.51 Å from the phosphorus atom. The resulting complex was soaked in a 10-Å layer of water. The whole system was minimized without any constraint, to the stage where the RMS was less than 0.001 kcal/mol Å.

**Acknowledgment.** This work was supported by a Grant-in-Aid for Priority Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

**Supporting Information Available:** Sequencing gel analysis of **11**-, **12S**-, and **12R**-treated pUC18 F328*-777 (Figure 1S), ESI-TOF-MS of an abasic site containing ODN1 and **12S**-A adduct (Figure 2S), HPLC analysis of ODN5/ODN2 and **12S** (Figure 3S), and human cancer cell line screening panels of deacetylamino-**11** and **12** (Figure 4S). This material is available free of charge via the Internet at http://pubs.acs.org.

JA049398F