J|A|C|S
ARTICLES
Published on Web 03/01/2003

Highly Efficient Sequence-Specific DNA Interstrand Cross-Linking by Pyrrole/Imidazole CPI Conjugates

Toshikazu Bando,[†] Akihiko Narita,[†] Isao Saito,[‡] and Hiroshi Sugiyama*,[†]

Contribution from the Division of Biofunctional Molecules, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Surugadai, Kanda, Chiyoda, Tokyo 101-0062, Japan, and Department of Synthetic Chemistry and Biological Chemistry, Faculty of Engineering, Kyoto University, Yoshida, Sakyo, and Kyoto 606-8501, Japan, SORST, Japan Science and Technology Corporation (JST)

Received September 8, 2002; E-mail: sugiyama.chem@tmd.ac.jp

Abstract: We have developed a novel type of DNA interstrand cross-linking agent by synthesizing dimers of a pyrrole (Py)/imidazole (Im)-diamide-CPI conjugate, ImPyLDu86 (**1**), connected using seven different linkers. The tetramethylene linker compound, **7b**, efficiently produces DNA interstrand cross-links at the nine-base-pair sequence, 5′-PyGGC(T/A)GCC**Pu**-3′, only in the presence of a partner triamide, **ImImPy**. For efficient cross-linking by **7b** with **ImImPy**, one A·T base pair between two recognition sites was required to accommodate the linker region. Elimination of the A·T base pair and insertion of an additional A·T base pair and substitution with a G·C base pair significantly reduced the degree of cross-linking. The sequence specificity of the interstrand cross-linking by **7b** was also examined in the presence of various triamides. The presence of **ImImIm** slightly reduced the formation of a cross-linked product compared to **ImImPy**. The mismatch partners, **ImPyPy** and **PyImPy**, did not produce an interstrand cross-link product with **7b**, whereas **ImPyPy** and **PyImPy** induced efficient alkylation at their matching site with **7b**. The interstrand cross-linking abilities of **7b** were further examined using denaturing polyacrylamide gel electrophoresis with 5′-Texas Red-labeled 400- and 67-bp DNA fragments. The sequencing gel analysis of the 400-bp DNA fragment with **ImImPy** demonstrated that **7b** alkylates several sites on the top and bottom strands, including one interstrand cross-linking match site, 5′-PyGGC(T/A)GCC**Pu**-3′. To obtain direct evidence of interstrand cross-linkages on longer DNA fragments, a simple method using biotin-labeled complementary strands was developed, which produced a band corresponding to the interstrand cross-linked site on both top and bottom strands. Densitometric analysis indicated that the contribution of the interstrand cross-link in the observed alkylation bands was approximately 40%. This compound efficiently cross-linked both strands at the target sequence. The present system consisted of a 1:2 complex of the alkylating agent and its partner **ImImPy** and caused an interstrand cross-linking in a sequence-specific fashion according to the base-pair recognition rule of Py-Im polyamides.

Introduction

DNA interstrand cross-linking agents effectively inhibit both DNA replication and gene expression by preventing the melting of the two strands, and therefore, they have considerable potential in molecular biology and human medicine.[1] Thus, the mechanism of the action of interstrand cross-linking agents and the design of new types of cross-linking agents has been extensively investigated. Cross-linking agents, including antitumor antibiotics, such as mitomycin C, carzinophilin A, and synthetic antitumor agents, such as bizelesin and nitrogen mustard derivatives, show intrinsic sequence-selectivity in the formation of interstrand cross-links.[2] However, interstrand cross-linking agents that can target a predetermined sequence are quite rare.[3] By utilizing the sequence-specific recognition for triple helix formation from the major groove of DNA, Kutyavin et al. have demonstrated that a polypyrimidine 19-mer possessing a chlorambucil moiety at both the 5′ and the 3′ ends can alkylate several sites on both DNA strands in a sequence-specific manner. However, the target sequence was limited to a polypurine sequence, and the efficiency of the interstrand cross-linking was not high.[4]

Dickerson and colleagues have demonstrated using X-ray crystallography that the natural product, netropsin, containing two *N*-methylpyrrole (Py) groups forms a 1:1 complex in the minor groove with an A·T rich sequence.[5] Dickerson and Lown suggested that the conversion of Py to an imidazole (Im) in the

[†] Tokyo Medical and Dental University.
[‡] Kyoto University.

(1) Rajski, S. R.; Williams, R. M. *Chem. Rev.* **1998**, *98*, 2723.
(2) (a) Norman, D.; Live, D.; Sastry, M.; Lipman, R.; Hingerty, B. E.; Tomasz, M.; Broyde, S.; Patel, D. J. *Biochemistry* **1990**, *29*, 2861. (b) Fujiwara, T.; Saito, I.; Sugiyama, H. *Tetrahedron Lett.* **1999**, *40*, 315. (c) Hodgkinson, T. J.; Shipman, M. *Tetrahedron* **2001**, *57*, 4467. (d) Seaman, F. C.; Chu, J.; Hurley, L. *J. Am. Chem. Soc.* **1996**, *118*, 5383. (e) Lawley, P. D. *BioEssays* **1995**, *17*, 561. (f) Hopkins, P. B.; Millard, J. T.; Woo, J.; Weidner, M. F.; Kirchner, J. J.; Sigurdsson, S. T.; Raucher, S. *Tetrahedron* **1991**, *47*, 2475.
(3) (a) Sigurdsson, S. T.; Rink, S. M.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 12633. (b) Zhou, Q.; Duan, W.; Simmons, D.; Shayo, Y.; Raymond, M. A.; Dorr, R. T.; Hurley, L. H. *J. Am. Chem. Soc.* **2001**, *123*, 4865.
(4) Kutyavin, I. V.; Gamper, H. B.; Gall, A. A.; Meyer, R. B., Jr. *J. Am. Chem. Soc.* **1993**, *115*, 9303.

10.1021/ja028459b CCC: $25.00

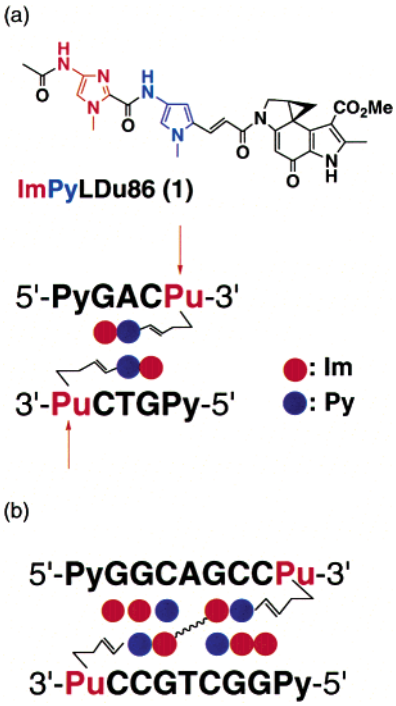
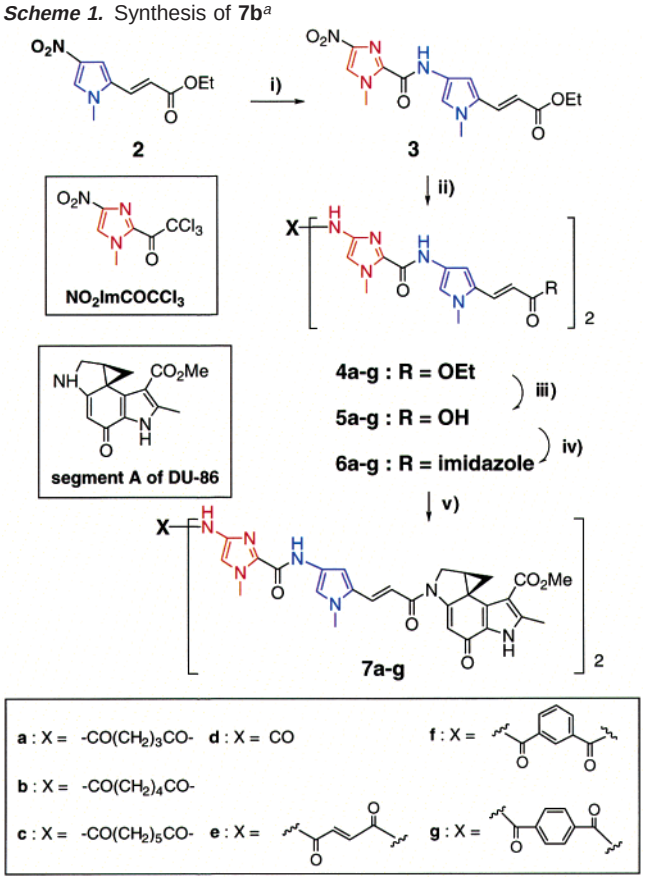


Figure 1. (a) The chemical structure of ImPyLDu86 (**1**) and a schematic representation of the recognition of the 5′-PyGACPu-3′ sequence by the homodimer of **1**. The arrows indicate the site where alkylation takes place. (b) A putative binding mode of the 1:2 complex of covalent dimer of **1**, **7b**, with **ImImPy** to 5′-PyGGCAGCCPu-3′ sequence.

A·T binders should lead to G·C specificity through the introduction of a new hydrogen bond acceptor for the protruding 2-amino group of guanine.[6] When these substitutions were made in netropsin and distamycin A, rather complicated results were obtained. Specific G·C binding was achieved using the idea of the 2:1 ligand binding motif to optimize contacts to the minor groove.[7] On the basis of these contributions to the concept of minor groove sequence information readout, Dervan et al. have designed and synthesized minor groove-binding Py-Im hairpin polyamides that uniquely recognize each of the four Watson−Crick base pairs.[8] Sequence-specific DNA recognition in the minor groove depends on the sequence of side-by-side pairings oriented in the amino-carboxyl (N−C) direction with respect to the 5′−3′ direction of the DNA helix. Antiparallel pairing of Im opposite a Py group (Im/Py) recognizes a G·C base pair, whereas a Py/Py pair recognizes A·T or T·A base pairs.[9] Hydroxypyrrole-Py pairing (Hp/Py) distinguishes the T·A from the A·T base pairs.[10] These Py-Im hairpin polyamides have a binding affinity and sequence-specificity comparable to those of transcription factors.[11]

Scheme 1. Synthesis of **7b**[a]

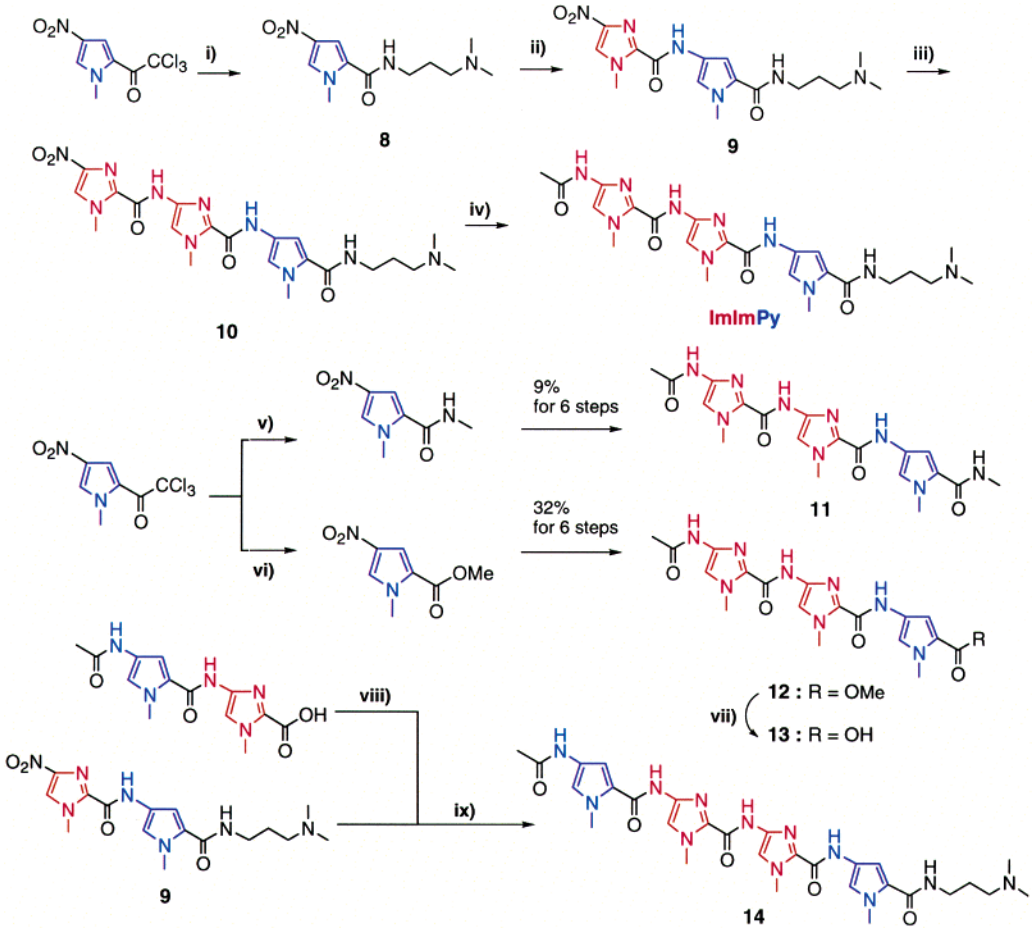


[a] Conditions: i) Pd−C, $NaBH_4$,MeOH then $NO_2ImCOCCl_3$, $^iPr_2NEt$, CH_2Cl_2, 52%; ii) Pd−C, $NaBH_4$, MeOH-AcOEt, then adipoyl chloride, CH_2Cl_2, $^iPr_2NEt$, 36%; iii) DBU−H_2O (1:1) then 1% aqueous HCl, 57%; iv) CDI, DMF, 99%; v) segment A of DU-86, NaH, DMF, 61%.

Duocarmycin A (Duo) is a highly potent antitumor antibiotic,[12] which binds to AT-rich sequences and selectively alkylates the N3 site of adenine (A) at the 3′ end of three or more consecutive A·T base pairs in DNA.[13] 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.[14]

(5) Kopka, M. L.; Yoon, C.; Goodsell, D.; Pjura, P.; Dickerson, R. E. *J. Mol. Biol.* **1985**, *183*, 553.
(6) (a) Kopka, M. L.; Yoon, C.; Goodsell, D.; Pjura, P.; Dickerson, R. E. *Proc. Natl. Acad. Sci. U.S.A.*, **1985**, *82*, 1376. (b) Lown, J. W.; Krowicki, K.; Bhat, U. G.; Skorobogaty, A.; Ward, B.; Dabrowiak, J. C. *Biochemistry* **1986**, *25*, 7408.
(7) (a) Wade, W. S.; Mrksich, M.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 8783. (b) Dwyer, T. J.; Geierstanger, B. H.; Bathini, Y.; Lown, J. W.; Wemmer, D. E. *J. Am. Chem. Soc.* **1992**, *114*, 5911.
(8) (a) For recent review, see: Dervan, P. B. *Bioorg. Med. Chem.* **2001**, *9*, 2215. (b) Wemmer, D. E.; Dervan, P. B. *Curr. Opin. Struct. Biol.* **1997**, *7*, 355. (c) Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Nature* **1996**, *382*, 559. (d) Turner, J. M.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 7636. (e) Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 6953. (f) Swalley, S. E.; Baird, E. E.; Dervan, P. B. *Chem. Eur. J.* **1997**, *3*, 1600. (g) Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Angew. Chem., Int. Ed.* **1998**, *37*, 1421.
(9) (a) White, S.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 8756. (b) Dervan, P. B.; Burli, R. W. *Curr. Opin. Chem. Biol.* **1999**, *3*, 688. (c) Kielkopf, C. L.; Baird, E. E.; Dervan, P. B.; Rees, D. C. *Nat. Struct. Biol.* **1998**, *5*, 104.
(10) (a) Kielkopf, C. L.; White, S.; Szewczyk, J. W.; Turner, J. M.; Baird, E. E.; Dervan, P. B.; Rees, D. C. *Science* **1998**, *282*, 111. (b) White, S.; Szewczyk, J. W.; Turner, J. M.; Baird, E. E.; Dervan, P. B. *Nature* **1998**, *391*, 468.
(11) (a) Trauger, J. W.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1998**, *120*, 3534. (b) Turner, J. M.; Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1998**, *120*, 6219. (c) Bremer, R. E.; Baird, E. E.; Dervan, P. B. *Chem. Biol.* **1998**, *5*, 119.
(12) (a) For recent review, see: Boger, D. L.; Boyce, C. W.; Garbaccio, R. M.; Goldberg, J. *Chem. Rev.* **1997**, *97*, 787. (b) Takahashi, I.; Takahashi, K.; Ichimura, M.; Morimoto, M.; Asano, K.; Kawamoto, I.; Tomita, F.; Nakano, H. *J. Antibiot.* **1988**, *41*, 1915. (c) Ichimura, M.; Muroi, K.; Asano, K.; Kawamoto, I.; Tomita, F.; Morimoto, M.; Nakano, H. *J. Antibiot.* **1988**, *41*, 1285. (d) Yasuzawa, T.; Iida, K.; Muroi, K.; Ichimura, M.; Takahashi, K.; Sano, H.; *Chem. Pharm. Bull.* **1988**, *36*, 3728. (e) Asai, A.; Nagamura, S.; Saito, H. *J. Am. Chem. Soc.* **1994**, *116*, 6, 4171. (f) Boger, D. L.; McKie, J. A.; Nishi, T.; Ogiku, T. *J. Am. Chem. Soc.* **1997**, *119*, 311.
(13) (a) Sugiyama, H.; Hosoda, M.; Saito, I.; Asai, A.; Saito, H. *Tetrahedron Lett.* **1990**, *31*, 7197. (b) Boger, D. L.; Ishizaki, T.; Zarrinmayeh, H. *J. Org. Chem.* **1990**, *55*, 4499. (c) Boger, D. L.; Ishizaki, T.; Zarrinmayeh, H.; Kitos, P. A.; Suntornwat, O. *J. Am. Chem. Soc.* **1990**, *112*, 2, 8961. (d) Boger, D. L.; Ishizaki, T.; Zarrinmayeh, H. *J. Am. Chem. Soc.* **1991**, *113*, 3, 6645. (e) Boger, D. L. *Acc. Chem. Res.* **1995**, *28*, 8, 20. (f) Boger, D. L.; Yun, W. Y.; Han, N. H. *Bioorg. Med. Chem.* **1995**, *3*, 1429. (g) Boger, D. L.; Johnson, D. S. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1438. (h) Schnell, J. R.; Ketchem, R. R.; Boger, D. L.; Chazin, W. J. *J. Am. Chem. Soc.* **1999**, *121*, 5645.

Scheme 2 [a]



[a] Conditions: i) $H_2NCH_2CH_2CH_2NMe_2$, 97%; ii) Pd−C, H_2, MeOH−AcOEt then $O_2NImCOCCl_3$, $^iPr_2NEt$, CH_2Cl_2, 86%; iii) Pd−C, H_2, MeOH−AcOEt then $O_2NImCOCCl_3$, $^iPr_2NEt$, CH_2Cl_2, 44%; iv) Pd−C, $NaBH_4$, MeOH then Ac_2O, $^iPr_2NEt$, 47%; v) $MeNH_2$, $^iPr_2NEt$, , 87%; vi) NaH, MeOH, 96%; vii) NaOH, 79%; viii) DCC, HOBt; ix) Pd−C, H_2, MeOH−AcOEt then $^iPr_2NEt$, DMF, 55%.

The NMR-refined structure of a Duo-Dist-d(CAGGTGGT)/d(ACCACCTG) complex demonstrated that the three Py residues of Dist recognize the complementary strand of the reacting octamer, similar to the base-pair recognition rule of Py-Im polyamides. In fact, the addition of Py-Im triamides to the Duo reaction can effectively change the sequence-specificity of Duo in a predictable manner.[15] These results suggest that Py-Im polyamides can be used as versatile recognition components of sequence-specific DNA alkylating agents. Thus, we synthesized hybrid molecules constructed from the A segment of Duo and from Py-Im polyamides specifically alkylated at predetermined sequences.[16] The alkylating Py-Im hairpin polyamides selectively alkylated one of the matching sites within a 400-bp DNA fragment. Sequence-specific alkylation was observed even at 50 nM concentrations of the alkylating agent. Other groups also synthesized analogous CC-1065 and duocarmycin A related cyclopropapyrroloindole (CPI) or cyclopropabenzoindole (CBI) Py-Im conjugates and examined DNA alkylating activities.[17] Lown and colleagues have found that insertion of a trans vinyl linker (L) between the Py and CPI groups greatly enhanced the alkylating activity, as well as the cytotoxicity.[17a] We also demonstrated that the Py-Im diamide-CPI conjugates with the vinyl linker, ImPyLDu86 (**1**), and alkylates double-stranded DNA at predetermined sequences through the formation of a highly cooperative homodimer, as shown in Figure 1a.[18] We found that the efficiency of alkylation by **1**, which is the degree of DNA alkylation divided by that of the agent, was 69%, thus confirming the unusually high efficiency of dialkylation.

We extended our concept to synthesize the alkylating Py-Im triamides, ImPyImLDu86 and ImImPyLDu86, which sequence-specifically alkylate their target sequences in supercoiled plasmid DNA.[19] More recently, we have demonstrated that incorporation of the vinyl linker pairing with Im dramatically improves the reactivity of hairpin polyamide-CPI conjugates.[20] These results encouraged us to design sequence-specific interstrand cross-linking agents,[21] because these agents require a high efficiency of alkylation on both sides, and the Py-Im polyamide CPI

(14) (a) Yamamoto, K.; Sugiyama, H.; Kawanishi, S. *Biochemistry* **1993**, *32*, 1059. (b) Sugiyama, H.; Lian, C.; Isomura, M.; Saito, I.; Wang, A. H.-J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 14405.
(15) Fujiwara, T.; Tao, Z.-F.; Ozeki, Y.; Saito, I.; Wang, A. H.-J.; Lee, M.; Sugiyama, H. *J. Am. Chem. Soc.* **1999**, *121*, 7706.
(16) (a) Tao, Z.-F.; Fujiwara, T.; Saito, I.; Sugiyama, H. *Angew. Chem., Int. Ed.* **1999**, *38*, 650. (b) Tao, Z.-F.; Fujiwara, T.; Saito, I.; Sugiyama, H. *J. Am. Chem. Soc.* **1999**, *121*, 4961.
(17) (a) Wang, Y.; Gupta, R.; Huang, L.; Luo, W.; Lown, J. W. *Anticancer Drug Des.* **1996**, *11*, 15. (b) Jia, G.; Iida, H.; Lown, J. W. *Heterocycl. Commun.* **1998**, *4*, 557. (c) Boger, D. L.; Han, N. *Bioorg. Med. Chem.* **1997**, *233*, 5. (d) Wurtz, N. R.; Dervan, P. B. *Chem. Biol.* **2000**, *7*, 154. (e) Chang, A. Y.; Dervan, P. B. *J. Am. Chem. Soc.* **2000**, *122*, 4856.
(18) Tao, Z.-F.; Saito, I.; Sugiyama, H. *J. Am. Chem. Soc.* **2000**, *122*, 1602.
(19) Fujimoto, K.; Iida, H.; Kawakami, M.; Bando, T.; Tao, Z.-F.; Sugiyama, H. *Nucleic Acids Res.* **2002**, *30*, 3748.
(20) Bando, T.; Narita, A.; Saito, I.; Sugiyama, H. *Chem. Eur. J.* **2002**, *8*, 4781.
(21) Bando, T.; Iida, H.; Saito, I.; Sugiyama, H. *J. Am. Chem. Soc.* **2001**, *123*, 5158.

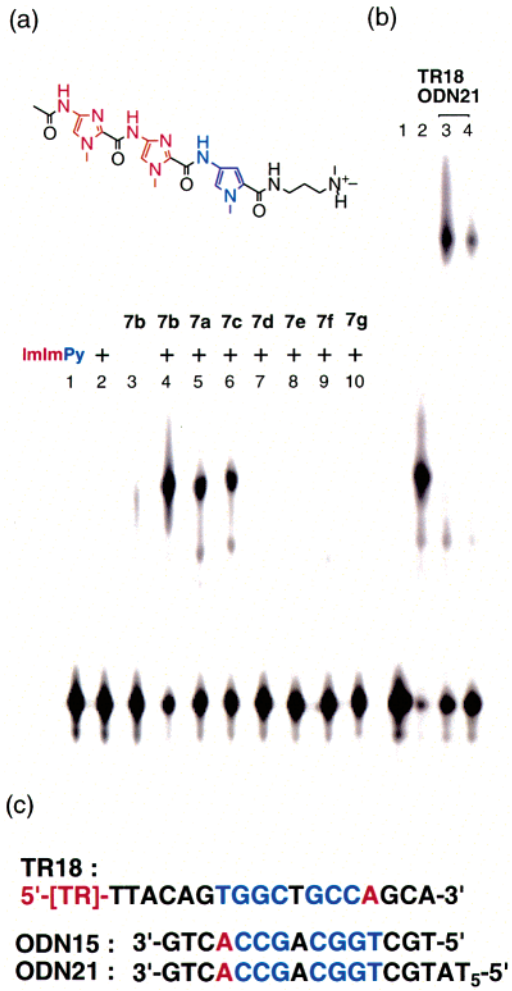


Figure 2. (a) The denaturing polyacrylamide gel electrophoresis of 5′-[TR]-TTACAGTGGCTGCCAGCA-3′ (TR18) and 5′-TGCTGGCAGC-CACTG-3′ (ODN15) cross-linked by **7a**−**g** in the presence and absence of **ImImPy**: lane 1 = DNA control; lane 2 = 30 μM **ImImPy**; lane 3 = 24 μM **7b**; and lanes 4−10 = 30 μM **ImImPy** and 24 μM with **7a**−**g**, respectively. (b) The denaturing polyacrylamide gel electrophoresis of TR18 cross-linked by **7b** and **ImImPy** in the presence of different lengths of complementary strand: lane 1 = DNA control; lane 2 = **7b** (24 μM), **ImImPy** (30 μM), and ODN15; lane 3 = **7b** (50 μM), **ImImPy** (100 μM), and 5′-TTTTTATGCTGGCAGCCACTG-3′ (ODN21); lane 4 = **7b** (24 μM), **ImImPy** (30 μM), and ODN21. (c) Sequences of TR18, ODN15, and ODN21. The alkylated bases and recognition sequences are represented in red and blue, respectively.

conjugate with a vinyl linker fulfills this requirement. In this study, we have examined in detail the sequence-specificity of interstrand cross-linking by covalent dimers of **1** connected with seven different linkers. The results indicate that sequence-specificity of one of the dimers, **7b**, can be controlled in a predictive manner by changing the partner Py-Im triamide. Using a new detection method, we have demonstrated that sequence-specific interstrand cross-linking effectively occurs at the target sequence within 67- and 400-bp DNA fragments.

Results and Discussion

Synthesis. To date, various types of CPI or CBI dimers, including alternative points of attachment to CPI moiety, have been synthesized.[22] However, control of the sequence specificity of the cross-linking agent is very difficult to achieve. We have designed an interstrand cross-linking system by forming a 1:2 complex of an alkylating dimer component with a partner Py-Im triamide, as shown in Figure 1b. To achieve efficient interstrand cross-linking, we synthesized dimers of **1** possessing various linkers (**7a**−**g**). Synthetic routes for compounds **7a**−**g** are summarized in Scheme 1. The dimeric unit **4a** was synthesized by the reduction of **3** with $NaBH_4$, Pd−C, and H_2 followed by coupling with glutaric dibenzotriazole ester, whereas linkers **4b**−**g** were prepared by coupling with the corresponding linker dicarbonyl chlorides. The alkylating moiety, segment A of DU-86 (CPI), was prepared via a five-step procedure according to literature reports.[23] After subsequent hydrolysis with DBU, the carboxyl groups were activated by CDI to form linkers **6a**−**g**. The activated amides **6a**−**g** were coupled with CPI to give compounds **7a**−**g** in a moderate yield. After purification by high-performance liquid chromatography (HPLC), the polyamide-CPI conjugates **7a**−**g** were used in the DNA alkylation experiments. Partner Py-Im triamides and tetraamides with various N- and C-terminal groups **10**−**14** were synthesized as shown in Scheme 2.

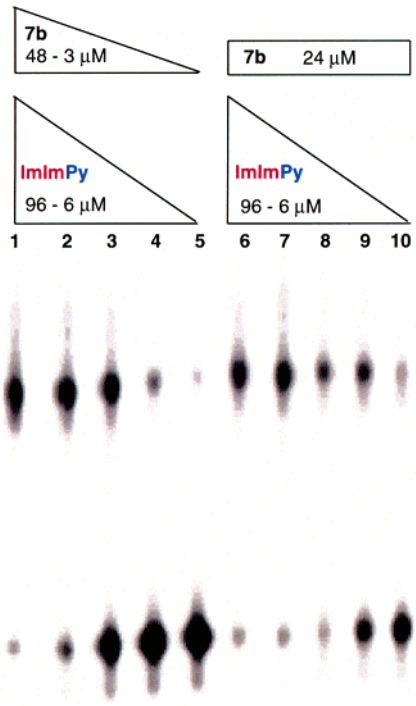


Figure 3. Denaturing polyacrylamide gel electrophoresis of TR18/ODN15 cross-linked by **7b** with various concentrations of **7b** with **ImImPy**: lane 1 = 96 μM **ImImPy** and 48 μM **7b**; lane 2 = 48 μM **ImImPy** and 24 μM **7b**; lane 3 = 24 μM **ImImPy** and 12 μM **7b**; lane 4 = 12 μM **ImImPy** and 6 μM **7b**; lane 5 = 6 μM **ImImPy** and 3 μM **7b**; lanes 6−10 = 24 μM **7b** and 96, 48, 24, 12, and 6 μM **ImImPy**, respectively.

Reaction of 5′-[TR]-TTACAGTGGCTGCCAGCA-3′-(TR18)/5′-TGCTGGCAGCCACTG-3′ (ODN15) with 7a−g in the presence of ImImPy. The interstrand cross-linking abilities of **7a**−**g** were investigated using 5′-Texas Red-labeled-TTACAGTGGCTGCC**A**GCA-3′ (TR18) and the complementary 5′-TGCTGGCAGCC**A**CTG-3′ (ODN15). Alkylation was carried out at 37 °C for 18 h, and the reaction was quenched by the addition of calf thymus DNA. The reaction mixture was

(22) (a) Kumar, R.; Lown, J. W. *Org. Lett.* **2002**, *11*, 1851. (b) Jia, G.; Iida, H.; Lown, J. W. *Heterocycl. Commun.* **1998**, *4*, 557. (c) Reddy, B. S.; Sharma, S. K.; Lown, J. W. *Curr. Med. Chem.* **2001**, *8*, 475. (d) Boger, D. L.; Searcey, M.; Tse, W. C.; Jin, Q. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 495.
(23) Nagamura, S.; Asai, A.; Kanda, Y.; Kobayashi, E.; Gomi, K.; Saito, H. *Chem. Pharm. Bull.* **1996**, *44*, 1723.

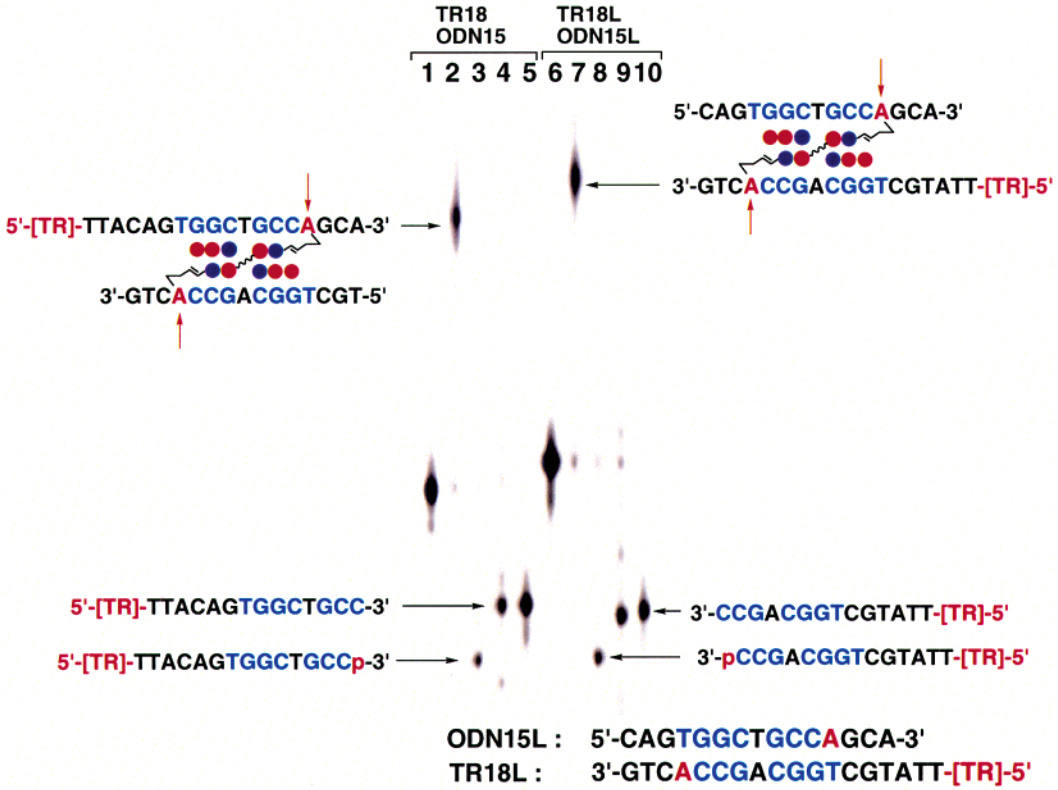


Figure 4. Denaturing polyacrylamide gel electrophoresis of 5′-Texas Red-labeled oligonucleotides. Lanes 1−5 and lanes 6−10 used TR18/ODN15 and 5′-[TR]-TTATGCTGGCAGCCACTG-3′ (TR18L)/5′-CAGTGGCTGCCAGCA-3′ (ODN15L), respectively. Lanes 1 and 6 = DNA controls; lanes 2 and 7 = reaction mixtures; lanes 3 and 8 = reaction mixtures after exposure to heat and hot piperidine treatment; lanes 4 and 9 = samples from lanes 3 and 8 after exposure to alkaline phosphatase; lanes 5 and 10 = 5′-[TR]-TTACAGTGGCTGCC-3′ and 5′-[TR]-TTATGCTGGCAGCC-3′, respectively.

concentrated at room temperature to avoid any depurination of the alkylated bases, and the product was directly analyzed by denaturing polyacrylamide gel electrophoresis using an automated DNA sequencer. As shown in Figure 2a (see lanes 4−6), the compounds **7a**−**c** generated slow-migrating bands in the presence of **ImImPy**, while the other compounds, such as **7d**, **e**, and **g**, did not generate such slow migrating bands under the same conditions (see lanes 7−10). When a longer complementary 5′-TTTTTATGCTGGCAGCC**A**CTG-3′ (ODN21) was employed in the same reaction, a further shifted slower-migrating band appeared (Figure 2b, lanes 3 and 4), indicating that these slow-migrating bands are corresponding cross-linked products. In the absence of **ImImPy**, compound **7b** showed only a trace of a slow-migrating band, indicating the indispensable role of the partner in the formation of an interstrand cross-link (Figure 2a, lane 3). It is speculated that a trace of a slow-migrating band may be due to alkylation products derived from the 1:1 monomeric binding of Py-Im polyamide.[24] Interestingly, the rigid linker compounds, **7d**, **e**, and **g** did not produce any cross-linked products, and only the flexible methylene linker compounds, **7a**−**c** caused cross-linking. The two faint bands produced by **7f** with **ImImPy** are assumed to be cross-linked and monoalkylation products, respectively. Further identification was not successful due to the low efficiency of the formations. These results clearly indicate the importance of flexibility in the linker region between two recognition moieties. These results clearly demonstrate that the combination of the flexible methylene linker compounds, **7a**−**c** and **ImImPy** efficiently produce DNA interstrand cross-links.

(24) Urbach, A. R.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4343.

To obtain the optimum conditions for efficient cross-linking, the degree of interstrand cross-linking was monitored under various concentrations of **7b** and **ImImPy**, as shown in Figure 3. It was found that interstrand cross-linking largely depended on the concentrations of **7b** and **ImImPy**, and that 24 μM of **7b** and 48 μM of **ImImPy** were required for the efficient cross-linking of 3 μM of **TR18/ODN15** (see lane 2)**.** With 24 μM of **7b**, more than 1 equiv of **ImImPy** was required for efficient cross-linking (see lanes 6−8). Thus, the following alkylation experiments were mostly performed under the standard conditions of 24 μM of **7b** and 30 μM of **ImImPy**.

Chemical Elucidation of Interstrand Cross-Linked Sites in TR18/ODN15. To study the interstrand cross-linking bases in TR18/ODN15, we analyzed the degradation products of interstrand cross-links in detail, as shown in Figure 4. The reaction mixture of TR18/ODN15 with **7b** and **ImImPy** containing a slow-migrating product (lane 2) was heated to 90 °C for 5 min and then subjected to a hot piperidine treatment (90 °C, 20 min). Such a treatment without using heating at neutral pH did not cause efficient strand cleavage at the adenine alkylation sites. Using this treatment, the slow-migrating band was converted to a faster migrating product (lane 3). The faster migrating band was converted into a slightly slower migrating band by dephosphorylation with alkaline phosphatase (lane 4), the mobility of which was identical to that of the authentic fragments of 5′-[TR]-TTACAGTGGCTGCC-3′ (lane 5). These results clearly demonstrate that interstrand cross-linking selectively occurred at the A_{15} position of TR18. Similar experiments using labeled lower-strand 5′-[TR]-TTATGCTGGCAGCC**A**CTG-3′ (TR18L) and 5′-CAGTGGCTGCC**A**GCA-3′ (ODN15L)

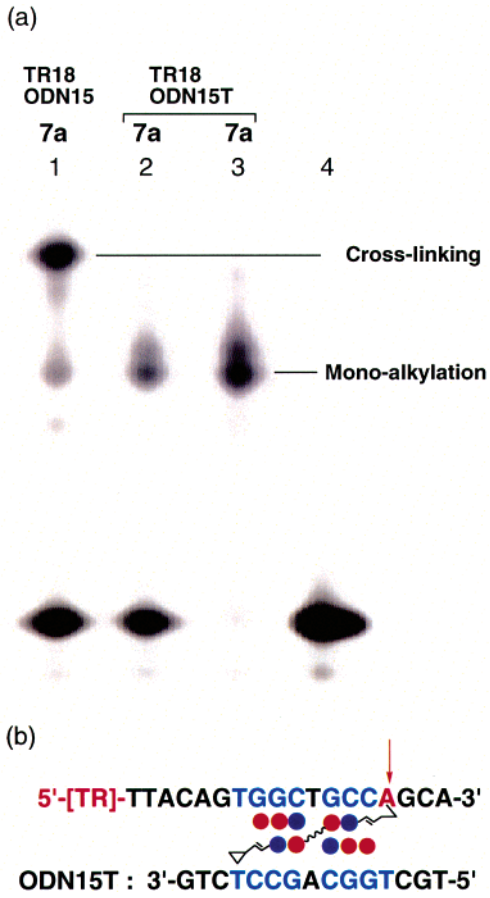


Figure 5. Denaturing polyacrylamide gel electrophoresis of TR18 cross-linked and monoalkylated by **7a** with **ImImPy** in the presence of a different complementary strand. (a) Lane 1 = **7a** (24 μM), **ImImPy** (24 μM), and ODN15; lane 2 = **7a** (24 μM), **ImImPy** (24 μM), and 5′-TGCTGGCAGC-CTCTG-3′ (ODN15T); lane 3 = **7a** (48 μM), **ImImPy** (48 μM), and ODN15T; and lane 4 = DNA control. (b) A schematic representation of the monoalkylation of TR18/ODN15T by **7a** with **ImImPy**.

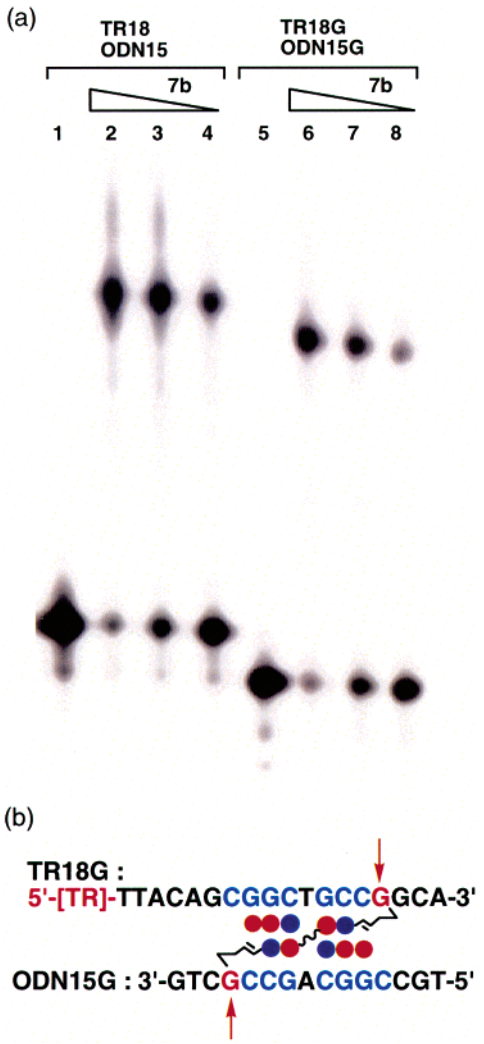


Figure 6. Denaturing polyacrylamide gel electrophoresis of 5′-Texas Red-labeled DNA fragments cross-linked by **7b** and **ImImPy**. Lanes 1−4 employed TR18/ODN15, and lanes 5−8 employed 5′-[TR]-TTACAGCG-GCTGCC**G**GCA-3′ (TR18G)/5′-TGCCGGCAGCC**G**CTG-3′ (ODN15G). (a) Lanes 1 and 5 = DNA control; lanes 2 and 6 = **7b** (24 μM) and **ImImPy** (30 μM); lanes 3 and 7 = **7b** (12 μM) and **ImImPy** (30 μM); lanes 4 and 8 = **7b** (6 μM) and **ImImPy** (30 μM). (b) A schematic representation of the interstrand cross-linking of TR18G/ODN15G by **7b** with **ImImPy**.

produced an analogous slow-migrating band of the cross-link product (lane 7). Heating and a hot piperidine treatment yielded a faster migrating band (lane 8). This fragment was converted by dephosphorylation to a slightly faster migrating band (lane 9), which comigrated with authentic 5′-[TR]-TTATGCTG-GCAGCC-3′. These results clearly demonstrate that interstrand cross-linking selectively occurred at the A_{15} position of TR18L.

The compounds with tri- and pentamethylene linker compounds, **7a** and **7c**, gave fewer cross-linked products, indicating that the tetramethylene linker is an optimal spacer for the formation of interstrand cross-linking. The exact reason for the efficient cross-linking by **7b** is not known. However, flexibility and an appropriate length of the linker would be important factors for the cross-linking reaction. The band that appeared slightly faster than the cross-linking product, observed in the case of **7a** with **ImImPy**, was assumed to be a monoalkylated product. This is because this band was predominantly formed when the same reaction using **7a** was carried out with TR18/5′-TGCTGGCAGCC**T**CTG-3′ (ODN15T) in which Target A, in the lower strand, was substituted with Target T (Figure 5, lanes 2 and 3).

It has been demonstrated that alkylating Py-Im polyamide can alkylate adenine and guanine,[16,17a,18] whereas Duo mainly alkylates adenine in AT-rich sequences.[13] To test whether this type of interstrand cross-link also occurs in G residues, the reactions of 5′-[TR]-TTACAGTGGCTGCC**G**GCA-3′ (TR18G) and 5′-TGCTGGCAGCC**G**CTG-3′ (ODN15G) with **7b** in the presence of 30 μM of **ImImPy** were examined. It was found that three different concentrations of **7b** cross-linked TR18G and ODN15G in the presence of **ImImPy** with a similar efficiency as that of TR18/ODN15 (Figure 6). These results clearly indicate that the interstrand cross-link system of **7b** with **ImImPy** can alkylate either A or G at matching sites, i.e. a 5′-PyGGC(T/A)GCC**Pu**-3′ sequence.

Base-Pair Preference of Linker Regions. As the γ-turn and β-tail of hairpin polyamides[25] and the linker region of tandem hairpins recognize A·T base pairs,[26] we expected that one A·T base pair between two recognition moieties was required to accommodate the linker region to enable efficient cross-linking. In fact, deletion of the A·T base pair of 5′-[TR]-TTACAGTG-GCGCCAGCA-3′ (TR17) and 5′-TGCTGGCGCCACTG-3′

(25) Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1999**, *121*, 1113.
(26) Herman, D. M.; Baird, E. E.; Dervan, P. B. *Chem. Eur. J.* **1999**, *5*, 975.

(ODN14), and the insertion of two A·T base pairs of 5′-[TR]-TTACAGTGGC*TT*GCCAGCA-3′ (TR19) and 5′-TGCTG-GC*AA*GCCACTG-3′ (ODN16) significantly reduced the cross-linking efficiency of **7a**−**c** in the presence of **ImImPy** compared with TR18/ODN15 (Figure 7a). Substitution of the A·T linker with a G·C base pair, 5′-[TR]-TTACAGTGGC*C*GCCAGCA-3′ (TR18CG) and 5′-TGCTGGC*G*GCCACTG-3′ (ODN15CG), yielded several slow-migrating bands with some tailing present. Some of these bands may be due to monoalkylation, which includes newly produced half-match sites produced by the incorporation of the C·G base pair (Figure 7b). These results clearly demonstrate that the linker region of the present cross-linked system prefers single A·T base pairs.

Optimal Partner Triamide for Interstrand Cross-Linkage of TR18/ODN15 by 7b. We examined the interstrand cross-linking by **7b** in the presence of four different Py/Im triamides at concentrations of 15 and 30 μM. The results of denaturing polyacrylamide gel electrophoresis are shown in Figure 8. In the case of **ImImIm** (lanes 3 and 7), the reduction of the formation of a cross-linked product relative to **ImImPy** (lanes 2 and 6) was observed: 44−19% and 98−81% at 15 and 30 μM of triamide, respectively, indicating that the Im/Im pair binds moderately to the G·C base pairs to facilitate the interstrand cross-link. These results indicate that the Py/Im pairing rule does not strictly operate on the third residue of the triamides in the present interstrand cross-linking system. A similar ambiguity in base-pair recognition by Im/Im and Py/Py pairs of the polyamides of the G·C base pair has been previously reported.[27] In clear contrast, the formation of the cross-linked product was significantly reduced in the presence of **ImPyPy** or **PyImPy** (lanes 4, 5, and 8). However, at 30 μM of **PyImPy**, a significant DNA consumption was observed (lane 9), presumably due to the monoalkylation at the half-matched site 5′-GCTG-3′. These results indicate that the Py/Im pairing rule operates for the first and second residues of the Py-Im triamides.

Optimal Terminal Group of Partner Triamide for Interstrand Cross-Link of TR18/ODN15 by 7b. We then examined the interstrand cross-linking by **7b** in the presence of a **ImImPy** derivative with different terminal groups (**10**−**14**) at concentrations of 15 and 30 μM. Substitution of the C-terminal diaminopropyl group with methylamine (**11**), methyl ester (**12**), or carboxylic acid (**13**) decreased the efficacy of cross-linking (Figure 9, lanes 1−3 and lanes 7−9). In addition, modification to the N-terminal group of **ImImPy** (**10** and **14**) reduced the activity (lanes 4, 5, 10, and 11). Densitometric analysis of the gel electrophoresis indicated that the cross-linking efficiency when compounds **11**−**14** were employed was less than 10% of that of **ImImPy** at 15 μM. Although 30 μM of **11** yielded several slow-migrating bands, the cross-linking efficiency by compound **11** was apparently lower than that of **ImImPy** under the same conditions. These results indicate that interstrand cross-linking proceeds efficiently with **ImImPy** according to the base-pair recognition rule of Py-Im polyamides.[8] It is important to note that **7b** with the tetraamide, **PyImImPy** (**14**), did not yield a cross-linked product (lanes 4 and 10). These results suggest that steric hindrance between CPI and the N-terminal Py inhibits an efficient cross-linking reaction.

(27) (a) Yang, X.-L.; Kaenzig, C.; Lee, M.; Wang, A. H.-J. *Eur. J. Biochem.* **1999**, *263*, 646. (b) Weyermann, P.; Dervan, P. B. *J. Am. Chem. Soc.* **2002**, *124*, 6872.

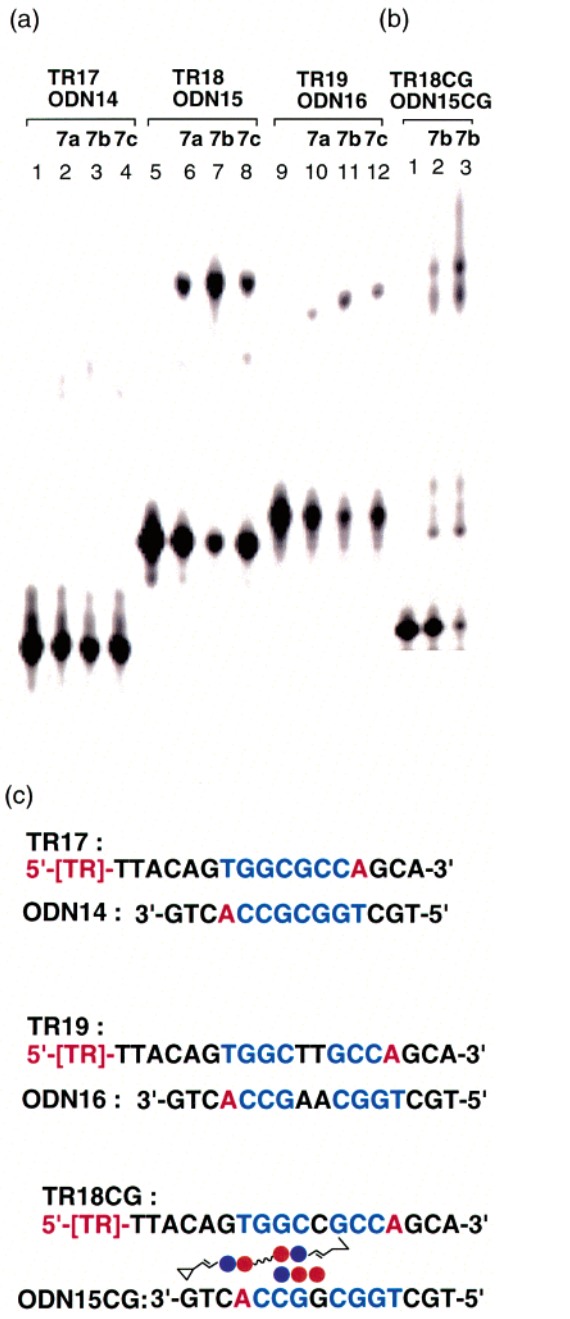


Figure 7. (a) Denaturing polyacrylamide gel electrophoresis of 5′-Texas Red-labeled DNA fragments treated by **7a**−**c** in the presence of **ImImPy**. Lanes 1−4 = 5′-[TR]-TTACAGTGGCGCCAGCA-3′ (TR17)/5′-TGCTG-GCGCCACTG-3′ (ODN14); lanes 5−8 = TR18/ODN15; lanes 9−12 = 5′-[TR]-TTACAGTGGC*TT*GCCAGCA-3′ (ODN19)/5′-TGCTGGC*AA*GC-CACTG-3′ (ODN16). Lane 1 = DNA control; lanes 2−4 = 30 μM **ImImPy** and 24 μM **7a**−**c**, respectively; lane 5 = DNA control; lanes 6−8 = 30 μM **ImImPy** and 24 μM **7a**−**c**, respectively; lane 9 = DNA control; lanes 10−12 = 30 μM **ImImPy** and 24 μM **7a**−**c**, respectively. (b) Denaturing polyacrylamide gel electrophoresis of 5′-Texas Red-labeled DNA fragments 5′-[TR]-TTACAGTGGCCGCCAGCA-3′ (TR18CG)/5′-TGCTGGC*G*GC-CACTG-3′ (ODN15CG) treated by **7b** in the presence of **ImImPy**. Lane 1 = DNA control; lane 2 = 15 μM **ImImPy** and 24 μM **7b**; and lane 3 = 30 μM **ImImPy** and 24 μM **7b**. (c) Sequences of TR17, ODN14, TR19, ODN16, TR18CG, and ODN15CG. The target bases and recognition sequences are represented in red and blue, respectively. A schematic representation of the monoalkylation of TR18CG/ODN15CG by **7b** with **ImImPy**.

Control of Sequence Specificity by Changing the Py-Im Triamide. We examined the sequence specificity of alkylation

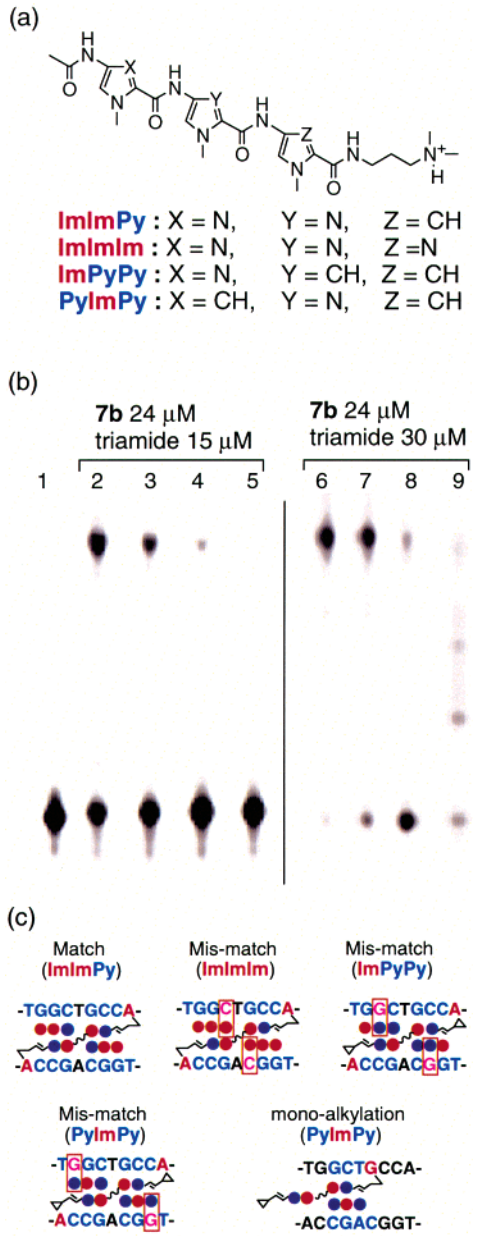


Figure 8. Denaturing polyacrylamide gel electrophoresis of TR18/ODN15 cross-linked by **7b** (24 μM) with Py-Im triamide (15 μM or 30 μM). (a) Chemical structures of Py-Im triamides. (b) Lane 1 = DNA control; lane 2 = 15 μM **ImImPy**; lane 3 = 15 μM **ImImIm**; lane 4 = 15 μM **ImPyPy**; lane 5 = 15 μM **PyImPy**; lane 6 = 30 μM **ImImPy**; lane 7 = 30 μM **ImImIm**; lane 8 = 30 μM **ImPyPy**; and lane 9 = 30 μM **PyImPy**. (c) A schematic representation of the interstrand cross-linking and monoalkylation of TR18/ODN15 by **7b** with triamide.

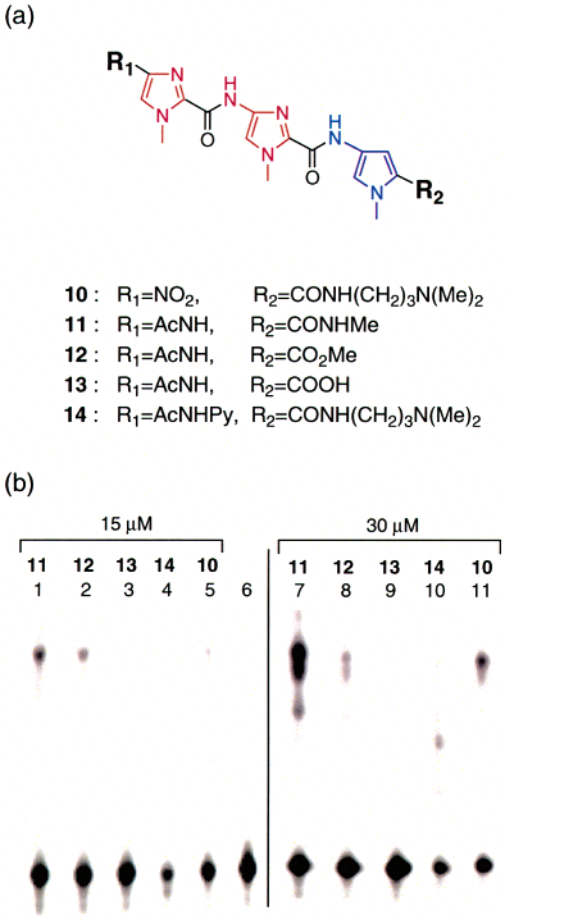


Figure 9. Denaturing polyacrylamide gel electrophoresis of TR18/ODN15 cross-linked by **7b** (24 μM) with the **ImImPy** derivatives, **10**−**14** (15 μM or 30 μM). (a) Chemical structures of compounds **10**−**14**. (b) Lane 1 = 15 μM **11**; lane 2 = 15 μM **12**; lane 3 = 15 μM **13**; lane 4 = 15 μM **14**; lane 5 = 15 μM **10**; lane 6 = DNA control; lane 7 = 30 μM **11**; lane 8 = 30 μM **12**; lane 9 = 30 μM **13**; lane 10 = 30 μM **14**; and lane 11 = 30 μM **10**.

by changing the partner Py-Im triamides. We designed a 67-bp DNA sequence including three matching sites for **7b** with the partner molecules **ImPyPy**, **ImImPy**, and **PyImPy**. If **7b** and each triamide were cooperatively bound to the minor groove of DNA according to the base-pair recognition rule of Py-Im polyamides, then sequence-specific interstrand cross-linking should be observed for each matching site. We used two sets of duplex 67-mers (TRF-67/R-67 and F-67/TRR-67), in which the one of the strands was labeled using 5′-Texas Red, as shown in Figure 10. In all three cases, sequence-selective alkylations at their cross-linked matching sites, 5′-TGAC(A/T)GTCA-3′ (sites 1 and 5), 5′-TGGC(A/T)GCCA-3′ (sites 2 and 7), and 5′-TAGC(A/T)GCTA-3′ (sites 4 and 8), on both DNA strands were observed in the presence of triamides (1 μM). However, alkylations at 5′-TGACA-3′ (site 6) and 5′-TAGCTG-3′ (site 3) were also observed under these conditions. Alkylation at sites 3 and 6 can be explained by the binding of a 1:1 complex of **7b** and triamide in which the alkylation part recognizes the base sequence by a pair of Py/Im polyamides, the tetramethylene linker region recognizes one or two AT base pairs, and the rest of the molecule recognizes a base pair by the monomeric recognition mode (Figure 10c).[24] These results demonstrate that sequence-specificity of DNA alkylation can be reasonably controlled by changing the partner Py-Im triamide according to the base recognition rule of Py-Im polyamides.

Sequence-Specific Interstrand Cross-Linking within a 400-bp DNA Fragment. We evaluated the interstrand cross-linking activity of **7b** with **ImImPy** using a 5′-Texas Red-labeled 400-bp DNA fragment possessing one matching site, 5′-TG-GCAGCC**G**-3′. After DNA alkylation, the reaction mixture was heated to 94 °C for 20 min, and thermally induced cleavage fragments were analyzed using a sequencing gel. It was found that a large excess of **ImImPy** (>25 μM) relative to **7b** (100 nM) was necessary for efficient alkylation of the 400 bp DNA fragment, suggesting the presence of a nonspecific monomeric

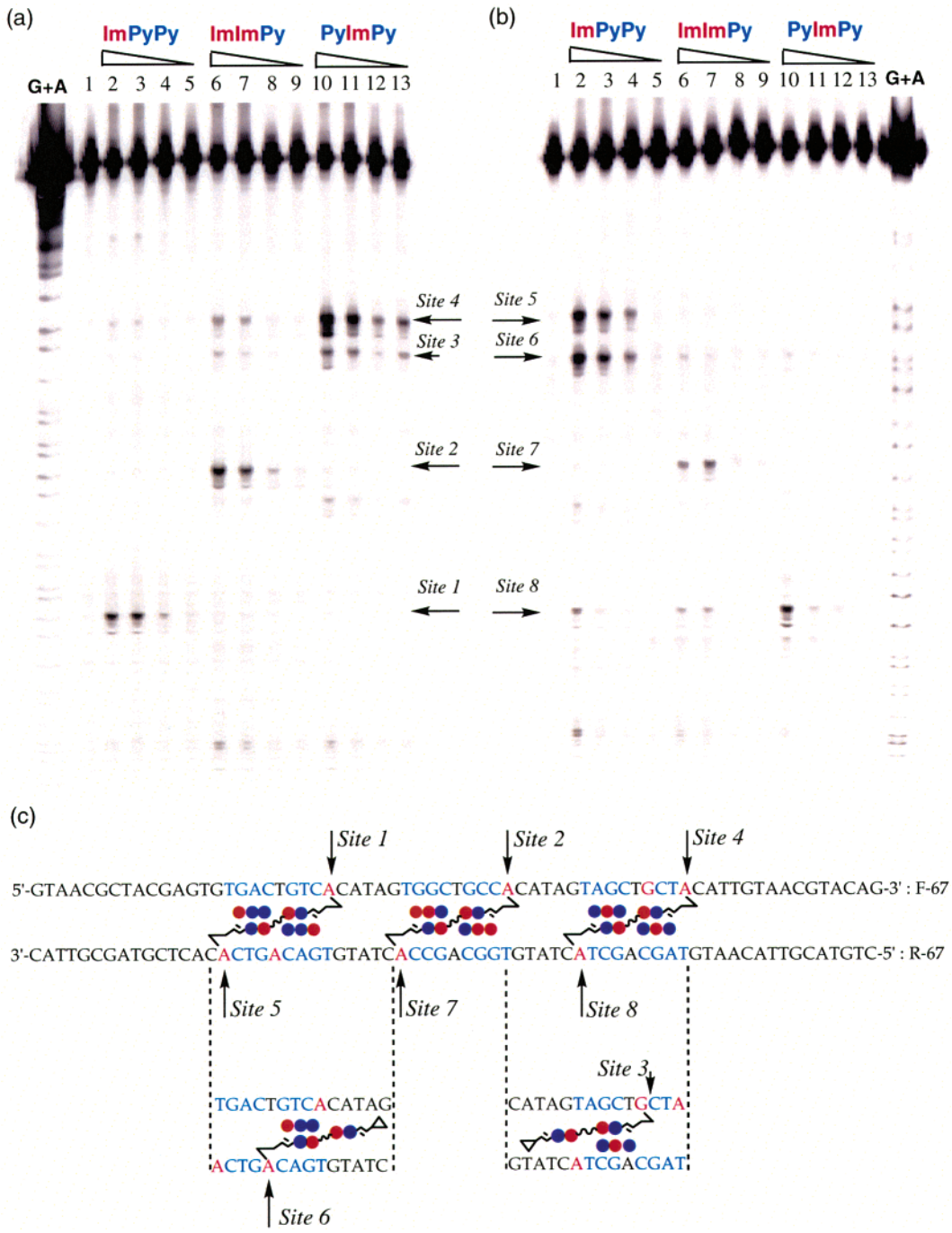


Figure 10. Sequence-specific alkylation of 5′-Texas Red-labeled: (a) TRF-67/R-67 and (b) F-67/TRR-67 DNA fragments by **7b** with Py-Im triamide. Sites of DNA alkylation were analyzed by thermal cleavage of the DNA strand at the alkylated sites using denaturing polyacrylamide gel electrophoresis. Lane 1 = DNA control; lanes 2−5 = 1 μM **ImPyPy** and 500, 250, 100, and 50 nM **7b**, respectively; lanes 6−9 = 1 μM **ImImPy** and 500, 250, 100, and 50 nM **7b**, respectively; lanes 10−13 = 1 μM **PyImPy** and 500, 250, 100, and 50 nM **7b**, respectively. (c) A schematic representation of the putative interstrand cross-linking and monoalkylation of a 67-bp DNA fragments by **7b** with **ImPyPy**, **ImImPy**, and **PyImPy**. The arrows indicate the site of alkylation.

or dimeric binding of **ImImPy** (Figure 11a). Figures 11 (b) and (c) clearly demonstrated that **7b** with 30 μM of **ImImPy** alkylates several sites (sites 1−11) on both DNA strands, including the target sequence, 5′-PyGGC(A/T)GCC**Pu**-3′ (sites 5 and 8). These results suggest that **7b** with **ImImPy** alkylates DNA in a 1:2 and a 1:1 binding mode, which is consistent with the monoalkylations shown in Figure 10.

However, the sequencing analysis used employing top and bottom strand individually labeled fragments could not distinguish between interstrand cross-linking and monoalkylation. To determine the contribution of interstrand cross-links in the observed alkylation at sites 5 and 8, a simple method using a 5′-biotin-labeled complementary strand was developed, as outlined in Scheme 3. After treatment with **7b** and **ImImPy**, the DNA fragments were collected using streptavidin-bound magnetic particles (MAGNOTEX-SA), and then the particles were washed with alkaline solution. Under these conditions, only cross-linked DNA fragments should be retained on the particles. Ensuing heat and hot piperidine treatments should provide DNA cleavage band only at the interstrand cross-linking site.

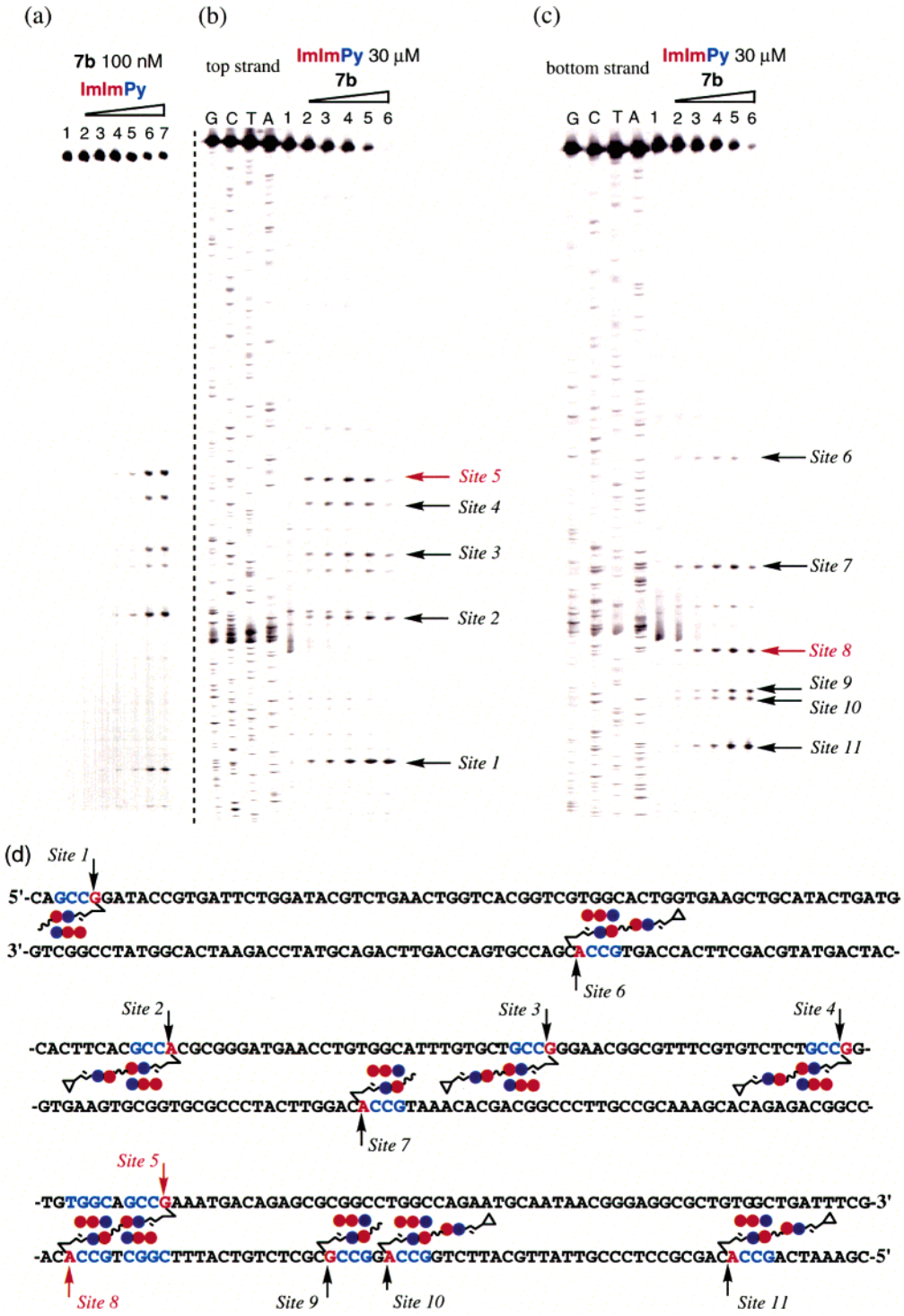


Figure 11. Sequence specific alkylation of 5′-Texas Red-labeled: (a) and (b) λDNA F 7315*-7714 (top), and (c) λDNA R 40789*-41188 (bottom) DNA fragments with a 5′-biotin-labeled complementary strand by **7b** and **ImImPy**. The sites of DNA alkylation were analyzed by denaturing polyacrylamide gel electrophoresis. (a) Lane 1 = DNA control; lanes 2−7 = 0.5, 2, 5, 10, 25, and 30 μM **ImImPy** and 100 nM **7b**; (b) and (c) lane 1 = DNA control; lanes 2−6 = 30 μM **ImImPy** and 25, 50, 100, 250, and 500 nM **7b.** The asterisk indicates 5′-Texas Red modification. (d) A schematic representation of the interstrand cross-linking and monoalkylation of a 400-bp DNA fragment by **7b** with **ImImPy**. The arrows indicate the site of alkylation.

5′-Double-labeled DNA was alkylated with **7b** under one-hit conditions in the presence of **ImImPy**. After collection using streptavidin-bound magnetic particles, the particles were washed with alkaline solution. The particles were heated in a buffer solution, and subjected to a hot piperidine treatment (90 °C, 20 min). The resulting DNA fragments were analyzed using a sequencing gel. The results of the top and bottom strand of the 400-bp DNA are shown in Figure 12, a and b. The bands corresponding to the two interstrand cross-link sites were significantly retained relative to other monomeric sites. The almost complete disappearance of the starting DNA also confirmed the reliability of the present method. Densitometric analysis demonstrated that the contribution of the interstrand cross-links of the top and bottom strand were approximately

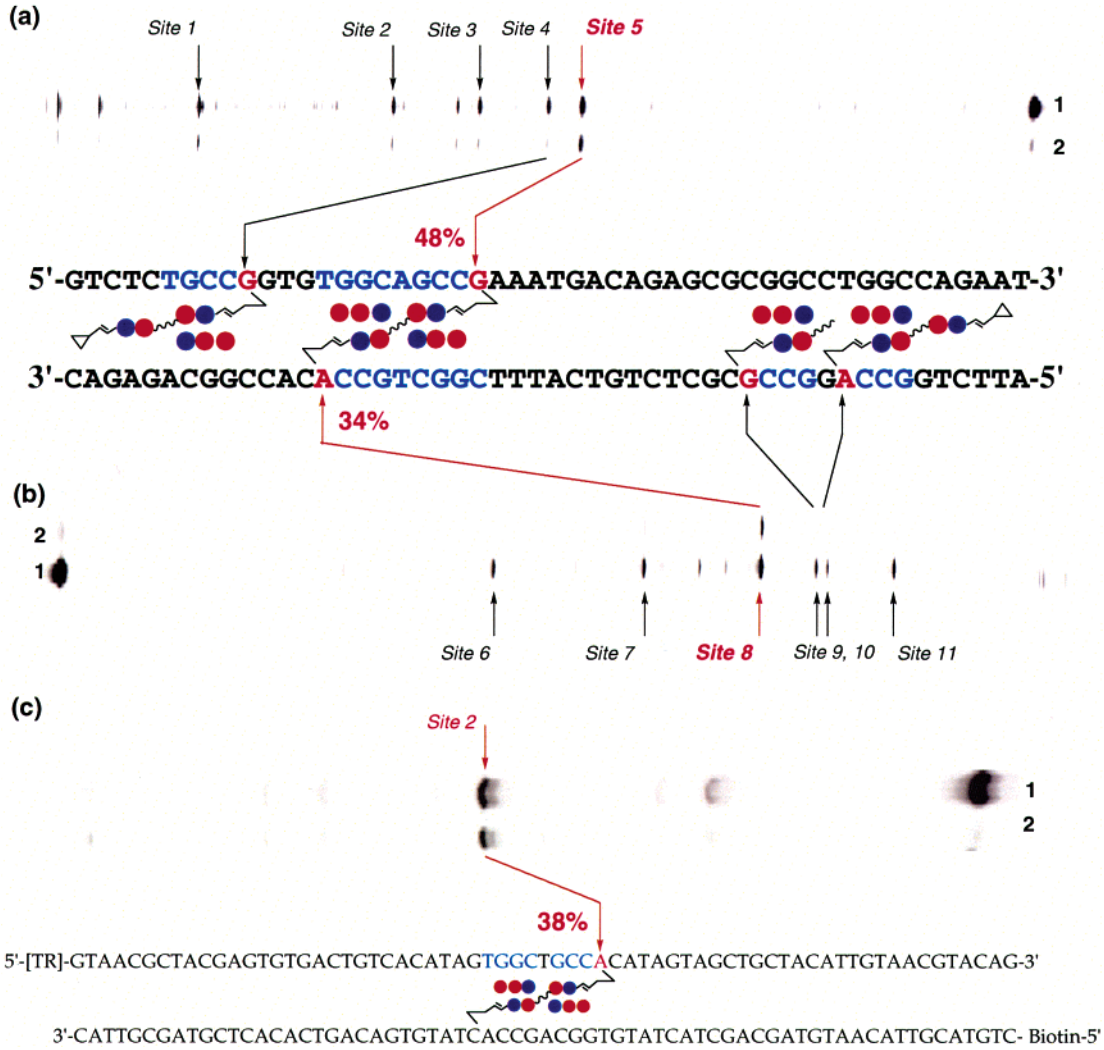


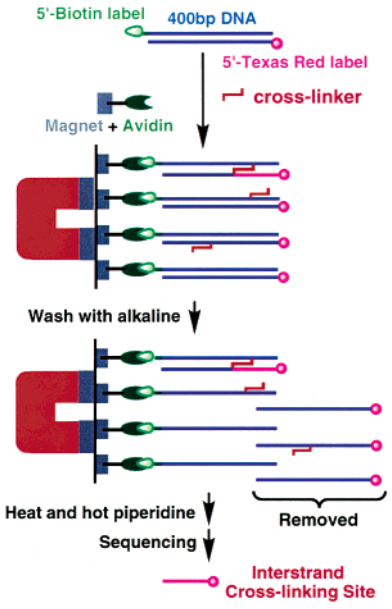
Figure 12. Interstrand cross-link of 5′-Texas Red-labeled: (a) λDNA F 7315*-7714 (top), (b) λDNA R 40789*-41188 (bottom), and (c) TRF-67 DNA fragments with a 5′-biotin-labeled complementary strand by **7b** with **ImImPy**. Sites of DNA alkylation were analyzed after heating and a hot piperidine treatment by denaturing polyacrylamide gel electrophoresis both before, and after subjecting the solution to the detection method described in Scheme 3. Lane 1 = alkylation with **7b** (a and b = 100 nM, and c = 500 nM) and **ImImPy** (a and b = 25 μM, and c = 1 μM); lane 2 = alkylated DNA of lane 1 captured by streptavidin-bound magnetic particles, and then washed with alkaline solution. The asterisk indicates 5′-Texas Red modification. Schematic representations of the interstrand cross-linking and monoalkylation by **7b** with **ImImPy** are also included. The arrows indicate the site of alkylation.

48% and 34%, respectively. The results of the 64-bp DNA fragments are shown in Figure 12c, showing that the bands at the target cross-linking sites were selectively retained. The results demonstrated that 38% of the alkylation of the target sequence of the top strand was due to interstrand cross-linking. These results confirm that **7b** with **ImImPy** efficiently cross-links both strands at the target site.

These results suggest that the present system of a 1:2 complex of **7b** and **ImImPy** causes interstrand cross-linking in a sequence-specific fashion according to the base-pair recognition rule of Py-Im polyamides (Figure 13). To the best of our knowledge, this is the first demonstration of such interstrand cross-linking of a longer DNA fragment at a predetermined sequence.

In conclusion, we have developed a novel DNA interstrand cross-linking agent **7b** that cross-links double strands only in the presence of **ImImPy** in the nine-base-pair sequence, 5′-PyGGC(T/A)GCC**Pu**-3′. The present system will provide a promising approach for the design of novel sequence-specific DNA interstrand cross-linking agents. Targeting specific se-

Scheme 3



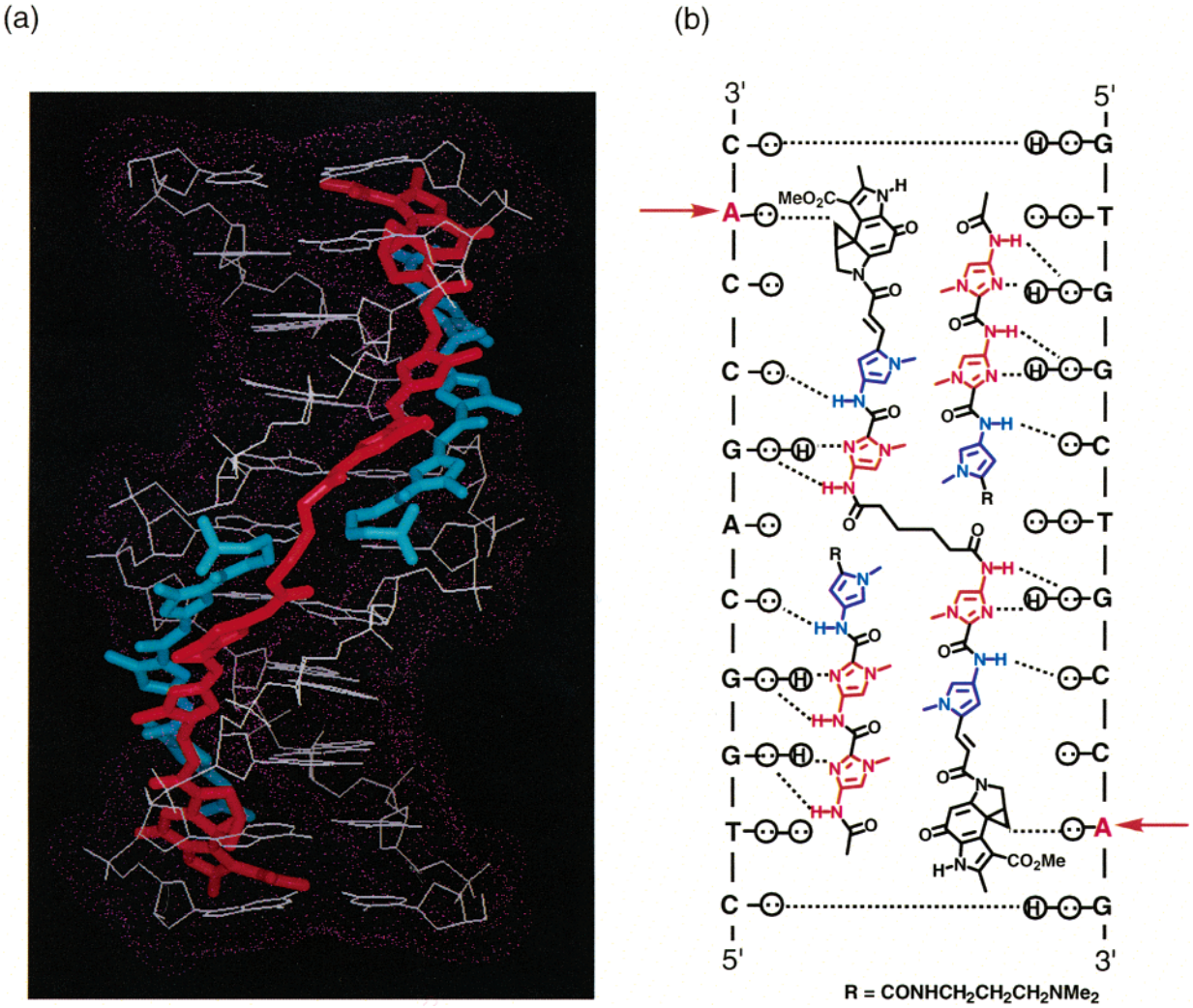


Figure 13. (a) Energy-minimized structure of **7b**-**ImImPy**$_2$-d(CTGGCTGCCAC)/ d(GTGGCAGCCAG) interstrand cross-linked complex. DNA is drawn in white. Py and Im residues of **7b** and **ImImPy** are drawn in blue and red, respectively. (b) A DNA interstrand cross-linking mode of the 1:2 complex of **7b** with **ImImPy**.

quences in the human genome by such a sequence-specific cross-linking agent would constitute a powerful gene-regulating tool.[29] Further studies on the applicability of this novel class of cross-linking agents are currently in progress.

Experimental Section

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; $^{i}Pr_2NEt$, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; FDPP, pentafluorophenyl diphenylphosphinate; CDI, 1,1′-carbonyldiimidazole. Reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm silica gel 60 plates impregnated with a 254-nm fluorescent indicator (Merck). Plates were visualized by UV illumination at 254 nm. 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 spectra (ESMS) were produced on a PE SCIEX API 165 mass spectrometer and 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. Fluorescent bands were analyzed using FRAGLYS2 (version 2.2, HITACHI) and NIH image (version 1.61) on a Power Macintosh G3. All deoxyoligonucleotides including 5′-Texas Red-labeled oligomers and 5′-biotin-labeled oligomers were purchased from JbioS. Ex *Taq* DNA polymerase and Suprec-02 purification cartridges were purchased from Takara; the Thermo Sequenase core sequencing kit and loading dye (dimethylformamide with fuchsin red) from Amersham; and 50% Long Ranger gel solution from FMC Bioproducts. P1 nuclease and calf intestine alkaline phosphatase (1000 units/mL) were purchased from Roche Diagnostics. Magnetex-SA was purchased from JSR.

O_2NPyLCO$_2$Et (2). To a solution of HNO_3 (1.5 mL, 35.7 mmol) in Ac_2O (3 mL) was slowly added 1-methyl-2-pyrrole carboxaldehyde (3.0 g, 27.5 mmol) in Ac_2O (5 mL) at −40 °C for 40 min. After the reaction mixture was stirred for 2 h at −10 °C, hexane (100 mL) was added. The precipitate was collected by filtration and washed with hexane (10 mL × 2) to afford yellow crude amine (1.43 g, 34%), which was used in the next step without further purification. To a solution of

(28) Kielkopf, C. L.; White, S.; Wzewczyk, J. W.; Turner, J. M.; Baird, E. E.; Dervan, P. B.; Rees, D. C. *Science* **1998**, *282*, 111.
(29) (a) Gottesfeld, J. M.; Neely, L.; Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Nature* **1997**, *387*, 202. (b) Henikoff, S.; Vermaak, D. *Cell* **2000**, *103*, 695. (c) Janssen, S.; Cuvier, O.; Müller, M.; Laemmli, U. K. *Mol. Cell.* **2000**, *6*, 1013. (d) Ansari, A. Z.; Mapp, A. K.; Nguyen, D. H.; Dervan, P. B.; Ptashne, M. *Chem. Biol.* **2001**, *8*, 583. (e) Ehley, J. A.; Melander, C.; Herman, D.; Baird, E. E.; Ferguson, H. A.; Goodrich, J. A.; Dervan, P. B.; Gottesfeld, J. M. *Mol. Cell. Biol.* **2002**, *22*, 1723. (f) Gottesfeld, J. M.; Belitsky, J. M.; Melander, C.; Dervan, P. B.; Luger, K. *J. Mol. Biol.* **2002**, *321*, 249.

sodium hydride (372 mg, 9.29 mmol, 60% oil suspension) in THF (5 mL) was added dropwise triethylphosphonoacetate (1.93 mL, 9.75 mmol) at 0 °C. After 1-methyl-4-nitro-2-pyrrole carboxaldehyde (1.43 g, 9.29 mmol) was added in THF (10 mL) at 0 °C, the reaction mixture was stirred for 2 h at room temperature. Following the evaporation of the solvent H_2O (10 mL) was added. The aqueous phase was extracted with EtOAc (100 mL × 2). The combined organic layers were dried (Na_2SO_4) and concentrated, and the residue was purified by column chromatography (silica gel, 25−50% EtOAc in hexane, gradient elution) to afford linker **2** (1.69 g, 81%). ^{1}H NMR ($CDCl_3$) δ 1.34 (t, J = 7.0 Hz, 3H), 3.77 (s, 3H), 4.26 (q, J = 7.0 Hz, 2H), 6.31 (d, J = 16.0 Hz, 1H), 7.11 (d, J = 1.5 Hz, 1H), 7.48 (d, J = 16.0 Hz, 1H), 7.56 (d, J = 1.5 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 14.3, 35.4, 60.8, 106.1, 118.4, 125.3, 129.8, 130.1, 136.7, 166.5. FAB MS 225 [M^+ + H].

O_2NImPyLCO$_2$Et (3). To a solution of **2** (500 mg, 2.23 mmol) in MeOH (25 mL) was added 10% Pd−C (220 mg). After $NaBH_4$ (153 mg, 4.04 mmol) in H_2O (3 mL) was added dropwise at 0 °C, the reaction mixture was stirred for 20 min at room temperature. The reaction mixture was filtered through Celite and EtOAc (500 mL) was added. The organic phase was washed with H_2O (10 mL), dried (Na_2SO_4), and concentrated in vacuo to afford 461 mg of brown crude amine, which was used in the next step without further purification. To a solution of crude amine in CH_2Cl_2 (10 mL) was added ^{i}Pr$_2$NEt (0.52 mL, 2.98 mmol). After $O_2NImCOCCl_3$ (550 mg, 2.02 mmol) was added at 0 °C, the reaction mixture was stirred for 1 h at room temperature. Evaporation of the solvent gave a brown residue, which was purified by column chromatography (silica gel, 30−50% EtOAc in hexane, gradient elution) to afford linker **3** (400 mg, 52% for two steps). ^{1}H NMR ($CDCl_3$) δ 1.33 (t, J = 7.0 Hz, 3H), 3.71 (s, 3H), 4.21 (s, 3H), 4.25 (q, J = 7.0 Hz, 2H), 6.16 (d, J = 16.0 Hz, 1H), 6.62 (d, J = 1.5 Hz, 1H), 7.32 (d, J = 1.5 Hz, 1H), 7.55 (d, J = 16.0 Hz, 1H), 7.82 (s, 1H), 8.97 (br s, 1H). ^{13}C NMR ($CDCl_3$) δ 14.3, 34.4, 37.1, 60.3, 102.5, 114.2, 117.9, 122.1, 124.4, 127.6, 131.4, 137.2, 145.3, 154.4, 167.4. FAB MS 348 [M^+ + H].

***tetra*-CH$_2$(CONHImPyLCO$_2$Et)$_2$ (4b).** To a solution of **3** (250 mg, 0.72 mmol) in MeOH−EtOAc (1:1, 10 mL) was added 10% Pd−C (120 mg). After $NaBH_4$ (54.5 mg, 1.44 mmol) in H_2O (0.5 mL) was added dropwise at 0 °C, the reaction mixture was stirred for 20 min at room temperature. The reaction mixture then subjected to flash column chromatography (silica gel, EtOAc). The solvent was then removed by evaporation under reduced pressure to give brown crude amine (141 mg), which was used in the next step without further purification. To a solution of this crude amine in CH_2Cl_2 (2 mL) was added ^{i}Pr$_2$NEt (0.25 mL, 1.33 mmol). After adipoyl chloride (32 μL, 0.22 mmol) was added dropwise to the solution at 0 °C, the reaction mixture was stirred for 14 h at room temperature. Evaporation of the solvent gave a yellow residue, which was purified by column chromatography (silica gel, 0−3% MeOH in $CHCl_3$, gradient elution) to afford **4b** (96.2 mg, 36% yield for two steps). ^{1}H NMR ($CDCl_3$) δ 1.32 (t, J = 6.5 Hz, 6H), 1.81 (s, 4H), 2.42 (s, 4H), 3.67 (s, 6H), 4.04 (s, 6H), 4.24 (q, J = 6.5 Hz, 4H), 6.10 (d, J = 15.5 Hz, 2H), 6.51 (s, 2H), 7.34 (s, 2H), 7.41 (s, 2H), 7.53 (d, J = 15.5 Hz, 2H), 8.04 (br s, 2H), 8.80 (br s, 2H). ^{13}C NMR (5% CD_3OD in $CDCl_3$) δ 14.1, 24.8, 34.1, 34.5, 34.6, 60.3, 102.3, 112.9, 114.3, 118.0, 122.8, 127.1, 131.8, 133.7, 135.8, 155.8, 168.0, 171.0. ESMS *m*/*e* calcd for $C_{36}H_{45}N_{10}O_8$ [M^+ + H] 745.3, found 745.6.

***tetra*-CH$_2$(CONHImPyLCO$_2$H)$_2$ (5b).** To a solution of **4b** (76.2 mg, 0.10 mmol) in H_2O (0.6 mL) was added DBU (0.6 mL, 4.01 mmol). The mixture was stirred for 15 h at room temperature. The solvent was removed by evaporation under reduced pressure to give a brown residue, which was triturated by Et_2O (10 mL). The resulting DBU salt was acidified with 1% aqueous HCl. The precipitate was collected by filtration and dried in vacuo to produce **5b** (40.0 mg, 57%). ^{1}H NMR (DMSO-d_6) δ 1.58 (s, 4H), 2.32 (s, 4H), 3.68 (s, 6H), 3.94 (s, 6H), 6.03 (d, J = 15.5 Hz, 2H), 6.80 (s, 2H), 7.41 (s, 2H), 7.43 (s, 2H), 7.46 (d, J = 15.5 Hz, 2H), 9.89 (br s, 2H), 10.24 (br s, 2H). ESI MS *m*/*e* calcd for $C_{32}H_{37}N_{10}O_8$ [M^+ + H] 689.3, found 689.5.

***tetra*-CH$_2$(CONHImPyLCOIm)$_2$ (6b).** To a solution of **5b** (30.0 mg, 43.6 μmol) in DMF (1 mL) was added CDI (42 mg, 261 μmol). The mixture was stirred overnight at room temperature. Evaporation of the solvent gave a yellow residue, which was triturated by ethyl ether (10 mL) to produce **6b** (35.7 mg, 99%). ^{1}H NMR (DMSO-d_6) δ 1.59 (s, 4H), 2.34 (s, 4H), 3.78 (s, 6H), 3.96 (s, 6H), 7.11 (s, 2H), 7.16 (d, J = 14.5 Hz, 2H), 7.32 (s, 2H), 7.45 (s, 2H), 7.49 (s, 2H), 7.87 (d, J = 14.5 Hz, 2H), 7.90 (s, 2H), 8.68 (s, 2H), 10.07 (br s, 2H), 10.23 (br s, 2H). ESMS *m*/*e* calcd for $C_{38}H_{41}N_{14}O_6$ [M^+ + H] 789.1, found 789.3.

***tetra*-CH$_2$(CONHImPyLCODu86)$_2$ (7b).** To a solution of sodium hydride (2.6 mg, 64.4 μmol, 60% oil suspension) in DMF (0.2 mL) was added segment A of DU-86[23] (12.5 mg, 48.3 μmol) in DMF (0.2 mL). After **6b** (13.6 mg, 16.5 μmol) in DMF (0.2 mL) was added at 0 °C, the reaction mixture was stirred for 4 h at 0 °C. The reaction mixture was quenched by addition of 0.01 M of sodium phosphate buffer (2 mL) at 0 °C. Evaporation of the solvent gave a yellow residue, which was washed sequentially with H_2O (10 mL), MeOH (10 mL), and ethyl ether (10 mL) to produce **7b** (12.2 mg, 61%). The purity of **7b** was more than 85% as determined by HPLC analysis. (Wakopak 5C18, 0.05 M ammonium formate containing 0−100% acetonitrile, linear gradient, 20 min, at a flow rate of 1.0 mL/min; retention time of **7b**, 14.4 min). ^{1}H NMR (DMSO-d_6) δ 1.29 (m, 2H), 1.58 (s, 4H), 2.09 (m, 2H), 2.33 (s, 4H), 2.47 (s, 6H), 3.45 (m, 2H), 3.72 (s, 6H), 3.73 (s, 6H), 3.95 (s, 6H), 4.18 (m, 2H), 4.28 (m, 2H), 6.57 (d, J = 14.5 Hz, 2H), 6.83 (br s, 2H), 6.99 (s, 2H), 7.41 (s, 2H), 7.44 (s, 2H), 7.58 (d, J = 14.5 Hz, 2H), 9.98 (s, 2H), 10.23 (s, 2H), 12.36 (br s, 2H). ESMS *m*/*e* calcd for $C_{60}H_{61}N_{14}O_{12}$ [M^+ + H] 1169.5, found 1169.6. A synthetic procedure similar to that described for compound **7b** was followed in the preparation of **7a** and **7b**−**i**.

***tri*-CH$_2$(CONHImPyLCODu86)$_2$ (7a):** ^{1}H NMR (DMSO-d_6) δ 1.28 (m, 2H), 1.86 (m, 2H), 2.07 (m, 2H), 2.35 (s, 4H), 2.45 (s, 6H), 3.44 (m, 2H), 3.71 (s, 6H), 3.72 (s, 6H), 3.94 (s, 6H), 4.18 (m, 2H), 4.27 (m, 2H), 6.57 (d, J = 15.0 Hz, 2H), 6.81 (br s, 2H), 6.97 (s, 2H), 7.40 (s, 2H), 7.44 (s, 2H), 7.56 (d, J = 15.0 Hz, 2H), 9.94 (s, 2H), 10.21 (s, 2H), 12.34 (br s, 2H). ESI MS *m*/*e* calcd for $C_{59}H_{59}N_{14}O_{12}$ [M^+ + H] 1155.4, found 1155.7.

***penta*-CH$_2$(CONHImPyLCODu86)$_2$ (7c):** ^{1}H NMR (DMSO-d_6) δ 1.27 (m, 4H), 1.56 (m, 4H), 2.07 (m, 2H), 2.30 (m, 4H), 2.46 (s, 6H), 3.55 (m, 2H), 3.70 (s, 6H), 3.71 (s, 6H), 3.93 (s, 6H), 4.18 (m, 2H), 4.27 (m, 2H), 6.56 (d, J = 15.0 Hz, 2H), 6.81 (br s, 2H), 6.96 (s, 2H), 7.39 (s, 2H), 7.42 (s, 2H), 7.56 (d, J = 15.0 Hz, 2H), 9.99 (s, 2H), 10.21 (s, 2H), 12.35 (br s, 2H). ESI MS *m*/*e* calcd for $C_{61}H_{63}N_{14}O_{12}$ [M^+ + H] 1183.5, found 1183.6.

CO (NHImPyLCODu86)$_2$ (7d): ^{1}H NMR (DMSO-d_6) δ 1.29 (m, 2H), 2.09 (m, 2H), 2.47 (s, 6H), 3.44 (m, 2H), 3.72 (s, 6H), 3.73 (s, 6H), 3.97 (s, 6H), 4.18 (m, 2H), 4.27 (m, 2H), 6.56 (d, J = 14.5 Hz, 2H), 6.84 (br s, 2H), 7.01 (s, 2H), 7.26 (s, 2H), 7.42 (s, 2H), 7.57 (d, J = 14.5 Hz, 2H), 8.82 (br s, 2H), 10.16 (s, 2H), 12.35 (br s, 2H). ESMS *m*/*e* calcd for $C_{56}H_{53}N_{14}O_{11}$ [M^+ + H] 1084.4, found 1085.5.

***trans*-CHCH(CONHImPyLCODu86)$_2$ (7e):** ^{1}H NMR (DMSO-d_6) δ 1.30 (m, 2H), 2.08 (m, 2H), 2.47 (s, 6H), 3.47 (m, 2H), 3.73 (s, 6H), 3.74 (s, 6H), 3.99 (s, 6H), 4.20 (m, 2H), 4.29 (m, 2H), 6.59 (d, J = 15.0 Hz, 2H), 6.79 (br s, 2H), 7.01 (s, 2H), 7.28 (s, 2H), 7.43 (s, 2H), 7.58 (d, J = 15.0 Hz, 2H), 7.61 (s, 2H), 10.07 (br s, 2H), 10.89 (s, 2H), 12.36 (br s, 2H). ESMS *m*/*e* calcd for $C_{58}H_{55}N_{14}O_{12}$ [M^+ + H] 1139.4, found 1139.5.

***m*-Ph(CONHImPyLCODu86)$_2$ (7f):** ^{1}H NMR (DMSO-d_6) δ 1.26 (m, 2H), 2.07 (m, 2H), 2.45 (s, 6H), 3.40 (m, 2H), 3.71 (s, 6H), 3.72 (s, 6H), 4.02 (s, 6H), 4.13 (m, 2H), 4.25 (m, 2H), 6.56 (d, J = 15.0 Hz, 2H), 6.80 (br s, 2H), 6.98 (s, 2H), 7.41 (s, 2H), 7.56 (d, J = 15.0 Hz, 2H), 7.65 (t, J = 8.0 Hz, 1H), 7.67 (s, 2H), 8.16 (d, J = 8.0 Hz, 2H), 8.59 (s, 1H), 10.01 (s, 2H), 10.82 (s, 2H), 12.33 (br s, 2H). ESMS *m*/*e* calcd for $C_{62}H_{67}N_{14}O_{12}$ [M^+ + H] 1189.4, found 1189.5.

***p*-Ph(CONHImPyLCODu86)$_2$ (7g):** ^{1}H NMR (DMSO-d_6) δ 1.30 (m, 2H), 2.09 (m, 2H), 2.47 (s, 6H), 3.46 (m, 2H), 3.74 (s, 12H), 4.02

(s, 6H), 4.20 (m, 2H), 4.29 (m, 2H), 6.60 (d, J = 15.0 Hz, 2H), 6.83 (br s, 2H), 7.01 (s, 2H), 7.43 (s, 2H), 7.58 (d, J = 15.0 Hz, 2H), 7.68 (s, 2H), 8.12 (s, 4H), 10.05 (s, 2H), 10.96 (s, 2H), 12.36 (br s, 2H). ESI MS *m*/*e* calcd for $C_{62}H_{67}N_{14}O_{12}$ [M^+ + H] 1189.4, found 1189.5.

NO_2PyCONH(CH_2)$_3$NMe_2 (8). To *N*,*N*-dimethyl-1,3-propanediamine (1 mL, 8.33 mmol) was added O_2NPyCOCCl$_3$ (500 mg, 1.84 mmol) at 0 °C. The reaction mixture was stirred for 12 h at room temperature. After the evaporation of the solvent, the residue was recrystallized from ethyl ether (3 mL) to afford **8** (460 mg, 97%) ^{1}H NMR (CDCl$_3$) δ 1.75 (t, J = 6.0 Hz, 2H), 2.34 (s, 6H), 2.54 (t, J = 6.0 Hz, 2H), 3.49 (q, J = 6.0 Hz, 2H), 4.00 (s, 3H), 6.94 (s, 1H), 7.52 (s, 1H), 8.68 (br s, 1H). FAB MS 255 [M^+ + H].

NO_2ImPyCONH(CH_2)$_3$NMe_2 (9). To a solution of **8** (460 mg, 1.81 mmol) in MeOH (3 mL) was added 10% Pd−C (120 mg), and the reaction mixture was stirred for 2 h at room temperature under H_2 gas. The reaction mixture was filtered through Celite with MeOH (50 mL), and concentrated in vacuo to afford 413 mg of yellow amine crude, which was used in the next step without further purification. To a crude solution in CH_2Cl_2 (8 mL) was added ^{i}Pr$_2$NEt (0.5 mL, 2.87 mmol). After O_2NImCOCCl$_3$ (493 mg, 1.81 mmol) was added at 0 °C, the reaction mixture was stirred for 15 h at room temperature. Evaporation of the solvent gave a yellow residue, which was purified by successively with water (30 mL) and diethyl ether (5 mL) and then dried in vacuo to produce **9** (587 mg, 86% for two steps). ^{1}H NMR (DMSO-d_6) δ 1.61 (t, J = 7.0 Hz, 2H), 2.14 (s, 6H), 2.24 (t, J = 7.0 Hz, 2H), 3.19 (q, J = 7.0 Hz, 2H), 3.81 (s, 3H), 4.04 (s, 3H), 6.97 (s, 1H), 7.27 (s, 1H), 8.14 (br s, 1H), 8.61 (s, 1H), 10.80 (br s, 1H). FAB MS 378 [M^+ + H].

NO_2ImImPyCONH(CH_2)$_3$NMe_2 (10). To a solution of **9** (100 mg, 0.27 mmol) in MeOH (3 mL) was added 10% Pd−C (50 mg), and the reaction mixture was stirred for 4 h at room temperature under H_2 gas. The reaction mixture was filtered through Celite with MeOH (50 mL), and concentrated in vacuo to afford 65.3 mg of yellow amine crude, which was used in the next step without further purification. To a crude solution of CH_2Cl_2 (2 mL) was added ^{i}Pr$_2$NEt (0.1 mL, 0.57 mmol). After O_2NImCOCCl$_3$ (72.2 mg, 0.27 mmol) was added at 0 °C, the reaction mixture was stirred for 15 h at room temperature. Evaporation of the solvent gave a yellow residue, which was purified by washing in water (10 mL) and then diethyl ether (2 mL); the residue was then dried in vacuo to produce **10** (58.2 mg, 44% for two steps). ^{1}H NMR (DMSO-d_6) δ 1.61 (t, J = 7.0 Hz, 2H), 2.15 (s, 6H), 2.25 (t, J = 7.0 Hz, 2H), 3.19 (q, J = 7.0 Hz, 2H), 3.80 (s, 3H), 4.01 (s, 3H), 4.06 (s, 3H), 6.91 (s, 1H), 7.23 (s, 1H), 7.58 (s, 1H), 8.13 (br s, 1H), 8.65 (s, 1H), 10.29 (s, 1H). FAB MS 501 [M^+ + H].

ImImPy. To a solution of **10** (48.2 mg, 96.4 μmol) in MeOH-EtOAc (2:1, 6 mL) was added 10% Pd−C (40 mg). After NaBH$_4$ (8 mg, 0.21 mmol) in H_2O (0.4 mL) was added dropwise at 0 °C, the reaction mixture was stirred for 20 min at room temperature. The reaction mixture was filtered through Celite and concentrated in vacuo to produce 38 mg of yellow amine crude, which was used in the next step without further purification. To a crude solution in ^{i}Pr$_2$NEt (0.1 mL, 0.57 mmol) was added Ac$_2$O (0.1 mL, 1.05 mmol), and the reaction mixture was stirred for 4 h at room temperature. Evaporation of the solvent gave a yellow residue, which was purified by washing with diethyl ether (2 mL) and dried in vacuo to produce **ImImPy** (23.2 mg, 47% for two steps). ^{1}H NMR (DMSO-d_6) δ 1.61 (m, 2H), 2.04 (s, 3H), 2.14 (s, 6H), 2.25 (m, 2H), 3.19 (m, 2H), 3.81 (s, 3H), 3.98 (s, 3H), 4.00 (s, 3H), 6.92 (s, 1H), 7.23 (s, 1H), 7.51 (s, 1H), 7.56 (s, 1H), 8.12 (br s, 1H), 9.34 (s, 1H), 10.27 (s, 1H), 10.30 (s, 1H). FAB MS 513 [M^+ + H]. Synthetic procedures similar to those described for compound **ImImPy** were followed in the preparation of **ImImIm**, **ImPyPy**, and **PyImPy**.

ImImIm: ^{1}H NMR (DMSO-d_6) δ 1.63 (m, 2H), 2.04 (s, 3H), 2.14 (s, 6H), 2.25 (m, 2H), 3.22 (m, 2H), 3.96 (s, 3H), 3.97 (s, 3H), 4.01 (s, 3H), 7.50 (s, 1H), 7.52 (s, 1H), 7.65 (s, 1H), 8.34 (br s, 1H), 9.62 (s, 1H), 9.64 (s, 1H), 10.38 (s, 1H). ESMS *m*/*e* calcd for $C_{22}H_{32}N_{11}O_4$ [M^+ + H] 514.3, found 514.4.

ImPyPy: ^{1}H NMR (DMSO-d_6) δ 1.60 (qu, J = 7.0 Hz, 2H), 2.01 (s, 3H), 2.12 (s, 6H), 2.23 (t, J = 7.0 Hz, 2H), 3.17 (dt, J = 5.5, 7.0 Hz, 2H), 3.79 (s, 3H), 3.84 (s, 3H), 3.94 (s, 3H), 6.82 (d, J = 1.5 Hz, 1H), 7.11 (d, J = 1.5 Hz, 1H), 7.17 (d, J = 1.5 Hz, 1H), 7.26 (d, J = 1.5 Hz, 1H), 7.41 (s, 1H), 8.04 (br t, J = 5.5 Hz, 1H), 9.87 (s, 1H), 9.94 (s, 1H), 10.22 (s, 1H). ESMS *m*/*e* calcd for $C_{24}H_{34}N_9O_4$ [M^+ + H] 512.3, found 512.2.

PyImPy: ^{1}H NMR (DMSO-d_6) δ 1.85 (m, 2H), 1.96 (s, 3H), 2.46 (m, 2H), 2.76 (s, 3H), 2.77 (s, 3H), 3.24 (m, 2H), 3.81 (s, 3H), 3.82 (s, 3H), 3.96 (s, 3H), 6.92 (s, 1H), 7.01 (s, 1H), 7.22 (s, 2H), 7.52 (s, 1H), 8.18 (br s, 1H), 9.81 (s, 1H), 9.93 (s, 1H), 10.18 (s, 1H). ESMS *m*/*e* calcd for $C_{24}H_{34}N_9O_4$ [M^+ + H] 512.3, found 512.3.

AcNHImImPyCONHMe (11). To O_2NPyCOCCl$_3$ (1 g, 3.68 mmol) in THF (10 mL) was added methylamine HCl salt (0.3 g, 4.44 mmol) and ^{i}Pr$_2$NEt (2 mL, 11.48 mmol) at 0 °C. The reaction mixture was stirred for 12 h at room temperature. After evaporation of the solvent, the residue was recrystallized from ethyl ether (3 mL) to afford NO_2-PyCONHMe (586 mg, 87%, ^{1}H NMR (DMSO-d_6) δ 2.71 (d, J = 5.0 Hz, 3H), 3.89 (s, 3H), 7.34 (d, J = 2.0 Hz, 1H), 8.09 (d, J = 2.0 Hz, 1H), 8.33 (br s, 1H)). A synthetic procedure similar to that described for **ImImPy** was followed in the preparation of **11**, with a yield of 9% after six steps. ^{1}H NMR (DMSO-d_6) δ 2.03 (s, 3H), 2.68 (d, J = 4.5 Hz, 3H), 3.80 (s, 3H), 3.97 (s, 3H), 3.99 (s, 3H), 6.90 (s, 1H), 7.22 (s, 1H), 7.50 (s, 1H), 7.55 (s, 1H), 7.93 (br s, 1H), 9.32 (s, 1H), 10.24 (s, 1H), 10.28 (s, 1H). ESMS *m*/*e* calcd for $C_{19}H_{24}N_9O_4$ [M^+ + H] 442.2, found 442.1.

AcNHImImPyCO$_2$Me (12). To O_2NPyCOCCl$_3$ (1 g, 3.68 mmol) in MeOH (20 mL) was added NaH (9 mg, 0.23 mmol) at 0 °C. The reaction mixture was stirred for 1 h at room temperature. After adding *p*-TsOH (45 mg, 0.24 mmol), the solution was concentrated. The resulting white precipitate was collected by filtration, washed with water, and dried to produce NO_2PyCO$_2$Me (651 mg, 96%, ^{1}H NMR (DMSO-d_6) δ 3.79 (s, 3H), 3.92 (s, 3H), 7.31 (d, J = 2.0 Hz, 1H), 8.27 (d, J = 2.0 Hz, 1H)). A synthetic procedure similar to that described for **ImImPy** was followed in the preparation of **12**, with a yield of 32% after six steps. ^{1}H NMR (DMSO-d_6) δ 2.04 (s, 3H), 3.73 (s, 3H), 3.84 (s, 3H), 3.97 (s, 3H), 3.99 (s, 3H), 7.01 (s, 1H), 7.50 (s, 1H), 7.52 (s, 1H), 7.56 (s, 1H), 9.30 (s, 1H), 10.27 (s, 1H), 10.40 (s, 1H). ESMS *m*/*e* calcd for $C_{19}H_{23}N_8O_5$ [M^+ + H] 443.2, found 443.0.

AcNHImImPyCO$_2$H (13). To a suspension of compound **12** (536 mg, 1.21 mmol) in 20 mL H_2O was added NaOH (770 mg, 19.25 mmol). The solution was stirred for 48 h at room temperature, and the aqueous solution was acidified to pH 2 at 0 °C. The resulting precipitate was collected by filtration, washed with water, and dried to produce compound **13** (408 mg, 79% yield) as a brown powder. ^{1}H NMR (DMSO-d_6) δ 2.03 (s, 3H), 3.82 (s, 3H), 3.97 (s, 3H), 3.99 (s, 3H), 6.96 (d, J = 2.0 Hz, 1H), 7.47 (d, J = 2.0 Hz, 1H), 7.49 (s, 1H), 7.55 (s, 1H), 9.30 (s, 1H), 10.27 (s, 1H), 10.35 (s, 1H), 12.19 (br s, 1H). ESMS *m*/*e* calcd for $C_{18}H_{21}N_8O_5$ [M^+ + H] 429.2, found 429.1.

AcNHPyImImPyCONH(CH_2)$_3$NMe_2 (14). To a solution of **9** (37.7 mg, 0.1 mmol) in MeOH−EtOAc (2:1, 1.5 mL) was added 10% Pd−C (15 mg), and the reaction mixture was stirred for 18 h at room temperature under H_2 gas. The reaction mixture was filtered through Celite with MeOH (20 mL), and concentrated in vacuo to produce 29.5 mg of yellow amine crude, which was used in the next step without further purification. To a overnight-stirred solution of AcPyImCO$_2$H (30.5 mg, 0.1 mmol, ^{1}H NMR (DMSO-d_6) δ 1.95(s, 3H), 3.82 (s, 3H), 3.91 (s, 3H), 6.91 (s, 1H), 7.24 (s, 1H), 7.56 (s, 1H), 9.80 (s, 1H), 10.55 (s, 1H)), HOBt (15.3 mg, 0.1 mmol) and DCC (20.6 mg, 0.1 mmol) in DMF (0.6 mL) was added amine crude in ^{i}Pr$_2$NEt (0.1 mL, 0.57 mmol), and the reaction mixture was stirred for 3 h at room temperature. Evaporation of the solvent gave a yellow residue, which was purified by column chromatography (silica gel, 10−50% MeOH in CHCl$_3$, gradient elution) to produce **14** (34.8 mg, 55% for two steps).

^{1}H NMR (DMSO-d_6) δ 1.62 (m, 2H), 1.97 (s, 3H), 2.17 (s, 6H), 2.28 (m, 2H), 3.19 (m, 2H), 3.80 (s, 3H), 3.83 (s, 3H), 4.00 (s, 3H), 4.01 (s, 3H), 6.92 (s, 2H), 7.22 (s, 1H), 7.25 (s, 1H), 7.56 (s, 1H), 7.62 (s, 1H), 8.11 (br t, 1H), 9.38 (s, 1H), 9.83 (s, 1H), 10.23 (s, 1H), 10.36 (s, 1H). ESMS *m*/*e* calcd for $C_{29}H_{39}N_{12}O_5$ [M^+ + H] 635.3, found 635.2.

Denaturing Gel Electrophoresis of 7a−g Treated 5′-Texas Red-Labeled Oligomers. Ten microliters of a reaction mixture containing 5′-Texas Red-labeled oligonucleotide with complementary strand (3 μM duplex concentration), the desired concentration of conjugates **7a−g**, **ImImPy**, and 10% DMF in 5 mM sodium cacodylate buffer (pH = 7.0) was incubated at 37 °C for 18 h. The reaction was quenched by adding 1 μL of 1 mM calf thymus DNA, and then 110 μL of H_2O was added to this solution. A 1.5-μL aliquot was concentrated, and the resulting residue was redissolved in 8 μL of loading dye (formamide with New Fuchsin). A 2-μL aliquot was electrophoresed on a 15% denaturing polyacrylamide gel using a Hitachi 5500-S DNA sequencer.

Chemical Elucidation of Interstrand Cross-Linked Sites in TR18/ODN15. Ten microliters of a reaction mixture containing 5′-Texas Red-labeled-TTACAGTGGCTGCC**A**GCA-3′ (TR18)/5′-TGCTGGCAGC-C**A**CTG-3′ (ODN15) or 5′-[TR]-TTATGCTGGCAGCCACTG-3′ (TR18L)/5′-CAGTGGCTGCCAGCA-3′ (ODN15L) (3 μM duplex concentration), conjugate **7b** (50 μM), **ImImPy** (100 μM), and 20% DMF in 5 mM sodium cacodylate buffer (pH = 7.0) was incubated at 37 °C for 15 h. A 1-μL aliquot was diluted to a volume of 15 μL with H_2O. A 1-μL aliquot of this solution was lyophilized, and the resulting residue was redissolved in 8 μL of loading dye. A 2-μL aliquot of this solution was subjected to the denaturing gel electrophoresis process as described above. The rest of the reaction mixture was heated to 90 °C for 5 min. One microliter of piperidine was added to this solution, and the resulting solution was kept at 90 °C for 20 min. A 1-μL aliquot was then subjected to gel electrophoresis. The solution was lyophilized and then treated with calf intestine alkaline phosphatase (1 unit) in 11 μL of 5 mM sodium cacodylate buffer (pH = 7.0) at 37 °C for 2 h. A 1-μL aliquot was subjected to the gel electrophoresis process as described above.

Denaturing Gel Electrophoresis of Interstrand Cross-Link by 7b with Different Partner. Ten microliters of a reaction mixture containing TR18/ODN15 (3 μM duplex concentration), conjugate **7b** (24 μM), Py-Im partner (15 or 30 μM), and 10% DMF in 5 mM sodium cacodylate buffer (pH = 7.0) was incubated at 37 °C for 18 h. The reaction was quenched by adding 1 μL of 1 mM calf thymus DNA, and then 110 μL of H_2O was added to this solution. A 1.5-μL aliquot was concentrated, and the resulting residue was redissolved in 8 μL of loading dye. A 2-μL aliquot of this solution was subjected to the denaturing gel electrophoresis process described above.

High-Resolution Gel Electrophoresis of 7b-Triamide-Treated 67-Base Pair DNA Fragments. The top or bottom strand 5′-Texas Red-labeled 67-base pair DNA fragments (TRF-67/R-67 or F-67/TRR-67, 10 nM duplex concentration) were alkylated using the desired concentration of **7b** with Py-Im triamides (1 μM) in 10 μL of 5 mM sodium phosphate buffer (pH = 7.0) containing 10% DMF maintained at 23 °C for 21 h. The reaction was quenched by the addition of calf thymus DNA (1 mM, 1 μL), and then heated for 5 min at 90 °C. The DNA solution was concentrated under reduced pressure. The resulting pellet was dissolved in 8 μL of loading dye, heated at 94 °C for 20 min, and then immediately cooled to 0 °C. A 2-μL aliquot of the solution was electrophoresed on 15% denaturing polyacrylamide gel. The (G + A) sequencing lanes were prepared by heating the solution in 1 M piperidine at 90 °C for 10 min, according to published protocol.[30]

Preparation of 5′-Texas Red-5′−Biotin Double-Labeled 400-Base Pair DNA Fragments. The 5′-Texas Red-labeled top strand and 5′-biotin-labeled bottom strand 400-base pair DNA fragments (λDNA F7315*-7714) were prepared by PCR using 5′-Texas Red-labeled-AGAGGAGCTTGATGACACGG-3′ (λDNA forward 7315-7334), and 5′-biotin-labeled-ACTGCTCACCGGATGTAATGGTG-3′ (λDNA reverse 40789-40811) as primers, and purified by filtration using Suprec-02. Similarly, λDNA R40789*-40811 were prepared by PCR using 5′-Texas Red-labeled-ACTGCTCACCGGATGTAATGGTG-3′ (λDNA reverse 40789-40811), and 5′-biotin-labeled-AGAGGAGCTTGAT-GACACGG-3′ (λDNA forward 7315-7334) as primers. Their concentration was determined from UV absorption data. The asterisk indicates 5′-Texas Red modification, and the nucleotide numbering sequence begins with the replication site.

High-Resolution Gel Electrophoresis of 7b-ImImPy-Treated 400-Base Pair DNA Fragments. The double-labeled 400-base pair DNA fragments (9 nM duplex concentration) were alkylated by the desired concentration of **7b** and **ImImPy** in 10 μL of 5 mM sodium phosphate buffer (pH = 7.0) containing 10% DMF maintained at 23 °C for 18 h. The reaction was quenched by adding calf thymus DNA (1 mM, 1 μL) and heating for 5 min at 90 °C. The DNA solution was concentrated under reduced pressure. The resulting pellet was dissolved in 8 μL of loading dye (formamide with Fuchsin red), heated at 94 °C for 20 min, and then immediately cooled to 0 °C. A 2-μL aliquot of the resulting solution was electrophoresed on a 6% denaturing polyacrylamide gel.

Identification of Interstrand Cross-Linking Sites in 400-Base Pair DNA Fragments. The double-labeled 400-base pair DNA fragments (9 nM duplex concentration) were alkylated by **7b** (100 nM) with **ImImPy** (25 μM) in 10 μL of 5 mM sodium phosphate buffer (pH = 7.0) containing 10% DMF maintained at 23 °C for 18 h. The reaction was quenched by the addition of calf thymus DNA (1 mM, 1 μL). MAGNOTEX-SA (0.2 mg) in a 2 × binding buffer (20 mM Tris-HCl (pH = 8.0), 2 mM EDTA, 2 M NaCl, 0.2% TritonX-100) (10 μL) was added to the biotin-labeled DNA solution (10 μL). After mixing, the tube was left at room temperature for 20 min, and the supernatant was removed using a magnetic stand. A solution of 0.1 M aqueous NaOH−NaCl (10 μL) was added to the reaction tube, and the supernatant was removed. Ten microliters of H_2O was added to reaction tube, and the supernatant was removed while the tube remained on a magnetic stand. Forty microliters of H_2O was added to the reaction tube, and the solution was stirred at 90 °C for 20 min. Piperidine (1 μL) was added to the reaction tube, the solution was stirred at 90 °C for 20 min, and then the supernatant was concentrated under reduced pressure. The resulting pellet was dissolved in 8 μL of loading dye (formamide with Fuchsin red), heated at 94 °C for 20 min, and then immediately cooled to 0 °C. A 2-μL aliquot was electrophoresed on a 6% denaturing polyacrylamide gel.

Identification of Interstrand Cross-Linking Sites in 67-Base Pair DNA Fragments. The 5′-Texas Red-labeled top strand (TRF-67) and 5′-biotin-labeled bottom strand (R-67) 67-base pair DNA fragments (10 nM) were alkylated by **7b** (500 nM) with **ImImPy** (1 μM) in 20 μL of 5 mM sodium phosphate buffer (pH = 7.0) containing 10% DMF at 23 °C for 18 h. The reaction was quenched by the addition of calf thymus DNA (1 mM, 1 μL). The reaction mixture was subjected a detection method using a biotin-labeled complementary strand. The results are shown in Figure 12c.

Molecular Modeling Studies. Minimizations were performed with the Discover program (MSI, San Diego, CA) using CFF force-field parameters. The starting structure was built on the basis of the crystal structure of the $(ImHpPyPy)_2$-d$(CCAGTACTGG)_2$ complex[26] and the ^{1}H NMR structure of the Duo-Dist-d(CAGGTGGT)/d(ACCACCTG) complex.[13] The connecting parts were built using standard bond lengths and angles. The CPI moiety 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 0.1 kcal/mol Å.

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

JA028459B

(30) Maxam, A. M.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 560.