Molecular Design of a Pyrrole–Imidazole Hairpin Polyamides for Effective DNA Alkylation

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Abstract: New hairpin polyamide–CPI (CPI = cyclopropylpyrroloindole) conjugates, compounds **12**–**14**, were synthesized and their DNA-alkylating activities compared with the previously prepared hairpin polyamide, compound **1**, by high-resolution denaturing gel electrophoresis with 450 base pair (bp) DNA fragments and by HPLC product analysis of the synthetic decanucleotide. In accord with our previous results, alkylation by compound **1** occurred predominantly at the G moiety of the sequence 5′-AGTCA*G*-3′ (site 3). However, compound **12**, in which the structure of the alkylating moiety of compound **1** is replaced with segment A of duocarmycin A DU-86 (CPI), did not show any DNA alkylating activity. In clear contrast, the hairpin CPI conjugate **13**, which differs from compound **1** in that it lacks one Py unit and possesses a vinyl linker, alkylated the A of 5′-AGTC*A*G-3′ (site 3) efficiently at nanomolar concentrations. Alkylation by compound **14**, which has a vinyl linker, occurred at the A of 5′-AGTCC*A*-3′ (site 6) and at several minor alkylation sites, including mismatch alkylation at A of 5′-TCACA*A*-3′ (site 2). The significantly different reactivity of the alkylating hairpin polyamides **1**, **12**, **13**, and **14** was further confirmed by HPLC product analysis by using a synthetic decanucleotide. The results suggest that hairpin polyamide–CPI conjugate **13** alkylates effectively according to Dervan's pairing rule, and with a new mode of recognition in which the Im–vinyl linker (L) pair targets G–C base pairs. These results demonstrate that incorporation of the vinyl-linker pairing with Im dramatically improves the reactivity of hairpin polyamide–CPI conjugates.

Keywords: antitumor agents · DNA cleavage · DNA recognition · molecular recognition · nucleic acid

Introduction

Dervan and colleagues have developed minor groove-binding hairpin molecules that uniquely recognize each of the four Watson–Crick base pairs.[1] Sequence-specific DNA recognition 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. 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.[2] A hydroxypyrrole–Py pairing (Hp/Py) distinguishes T-A from A-T base pairs.[3] These Py–Im hairpin polyamides have binding affinities and sequence-specificity comparable to those of transcription factors.[4] Therefore, Py–Im hairpin polyamides can potentially control DNA replication and gene expression. In fact, synthetic Py–Im polyamides that target promoter regions have been shown to inhibit transcription by preventing specific binding by transcription factors.[5] However, polyamides targeting coding regions do not inhibit transcription.[6] Therefore, the target sequence of a nonreactive hairpin polyamide is limited to the promoter region. On the other hand, sequence-specific DNA alkylating polyamides can potentially control gene expression by targeting not only the promoter region, but also the coding sequence.[7]

We recently demonstrated that molecule **1**, a hybrid between segment A of duocarmycin A (Du)[8] and a Py–Im hairpin polyamide, selectively alkylates one of the matched sequences within a 450 base pair (bp) DNA fragment (Figure 1).[9] Sequence-specific alkylation was observed even at the 50 nanomolar concentration region of the alkylating agent. However, the efficiency of alkylation (i.e., the amount of cleavage divided by the amount of alkylating agent) was 7.4%. Alkylation in this system was rather slow, and was observed to continue even after one week.[9]

In trying to improve the alkylating activity of the polyamide, we found that insertion of a vinyl linker (L) between

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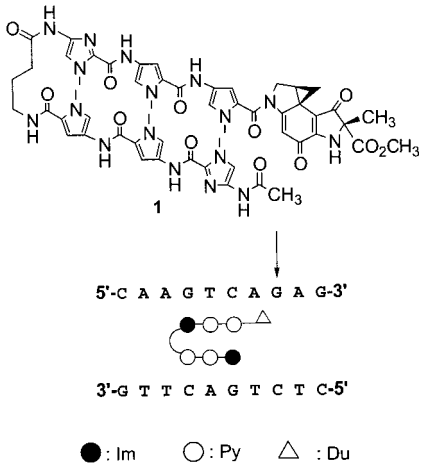

Figure 1. Chemical structure of compound **1** and a schematic representation of the recognition of the AGTCAG sequence by **1**. The arrow indicates the site of alkylation.

the polyamide and cyclopropylpyrroloindole (CPI) adjusted the location of the reactive cyclopropane ring, enhancing its reactivity. As a result, highly sequence-specific cooperative double-strand alkylation of DNA is facilitated through highly cooperative homodimer formation.[10] Quantitative analysis indicated that the efficiency of this system reached 69%. In these studies, we changed the alkylating moiety to segment A of DU-86 (CPI) because it is more chemically stable under the conditions in which it is coupled to Py/Im polyamides that have L linkers. The highly efficient DNA alkylation facilitated by the incorporation of L allowed us to develop a new type of sequence-specific DNA interstrand cross-linking agent that cross-links double strands only in the presence of ImImPy, at the 9-bp sequence 5′-PyGGC(T/A)GCC*Pu*-3′.[11] In developing an efficient sequence-specific alkylating hairpin polyamide, the choice of alkylating moiety and its location in the minor groove is very important. We describe here a comparative study of DNA alkylation by four different alkylating Py/Im hairpin polyamides, three of which were newly synthesized.

Results and Discussion

Synthesis: We synthesized hairpin polyamide-CPI conjugates **12**–**14** 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** with a good yield. Hydrolysis of **4** gave the key carboxylic acid, compound **5**, which was used as the common N-terminal half of conjugates **12**–**14**. Compounds **8a** and **8b** were prepared by previously reported procedures.[9–11] Compound **8c** was synthesized by the reduction of compound **6** with Pd/C and $NaBH_4$, followed by coupling with compound **7** by using FDPP. Reduction of **8a**–**c**, followed by coupling with compound **5** by using FDPP, produced **9a**–**c**. After subsequent deprotection with 1,8-diazabicyclo[4.3.0]undec-7-ene (DBU), the carboxylic acids **10a**–**c** were activated with 1,1′-carbonylimidazole (CDI), as previously described[10] to give **11a**–**c**. The alkylating moiety, segment A of DU-86 (CPI), was prepared through five steps according to the reported procedures.[12] Finally, the synthesis of conjugates **12**–**14** was accomplished by coupling **11a**–**c** and CPI, with a moderate yield. After purification by HPLC, the hairpin polyamide–CPI conjugates **12**–**14** were used for DNA alkylation experiments.

Evaluation of DNA alkylation using 450-bp DNA fragments: Sequence-selective alkylation by compounds **1**, **12**, **13**, and **14** was investigated on 5′-Texas-Red-labeled 450-bp DNA fragments, prepared by using an automated DNA sequencer, as previously described.[9, 10, 13] Alkylation was carried out at 23 °C for 24 h and quenched by the addition of calf thymus DNA. Samples were heated at 94 °C under neutral conditions for 20 min. The sites of alkylation were visualized by thermal cleavage of the DNA strand at the alkylated sites. Under these heating conditions, all purine N3 alkylated sites in the DNA produced cleavage bands almost quantitatively on the gel. Subsequent hot piperidine treatment (0.1M, 90 °C, 20 min) did not further enhance the cleavage bands, indicating that the neutral heating conditions used (94 °C, 20 min) were sufficient to cleave all the DNA alkylated sites. Sequencing analysis of the alkylated DNA fragments after heat treatment is shown in Figure 2. As previously reported, alkylation by hairpin polyamide **1** occurred predominantly at the G moiety of sequence 5′-AGTCA*G*-3′ (site 3) at nanomolar concentrations (lanes 1–4). In contrast to the reactivity of **1**, alkylation by **12** was not observed at concentrations of 12.5–100 nM of the alkylating agent (lanes 5–8). In separate experiments, DNA alkylation was not even observed at a concentration of 10 μM of **12** (data not shown). These results clearly indicate that substitution of the alkylating moiety of **1** with CPI completely abolishes the DNA alkylating activity. The dramatically different reactivities of **1** and **12** could be attributed to the reactivity of the alkylating moieties in a fixed geometry in the DNA minor groove. On the other hand, conjugate **13**, which differs from **1** in that it lacks one Py unit and possesses a vinyl linker, alkylated the A of 5′-AGTC*A*G-3′ (site 3) and G of 5′-AGTC*G*-3′ (site 8) at a concentration of 12.5 nM of **13**, and most of the DNA fragment was consumed by the alkylation at a concentration of 100 nM of **13** (lane 9). However, conjugate **14** has an additional vinyl linker with respect to **12** and showed a level of DNA alkylating activity intermediate between those of **1** and **13**. Conjugate **14** mainly alkylates the A of 5′-AGTCC*A*-3′ (site 6), but also binds at several minor alkylation sites, including mismatch alkylation at the A of 5′-TCACA*A*-3′ (site 2) and 5′-TGAGA*A*-3′ (site 10). These results demonstrate that incorporation of a vinyl linker paired with Im dramatically improves the reactivity of hairpin polyamide–CPI conjugates. These results suggest that the hairpin polyamide–CPI conjugate **13** alkylates effectively according to Dervan's pairing rule, and exhibits a new mode of recognition in which the Im/vinyl linker (L) pair targets G–C base pairs.

To evaluate the improved reactivity of conjugate **13** relative to **1**, DNA alkylation was examined over a short incubation period (<1 h). Surprisingly, alkylation by **13** was observed even after 5 min, as shown in Figure 3. In clear contrast,

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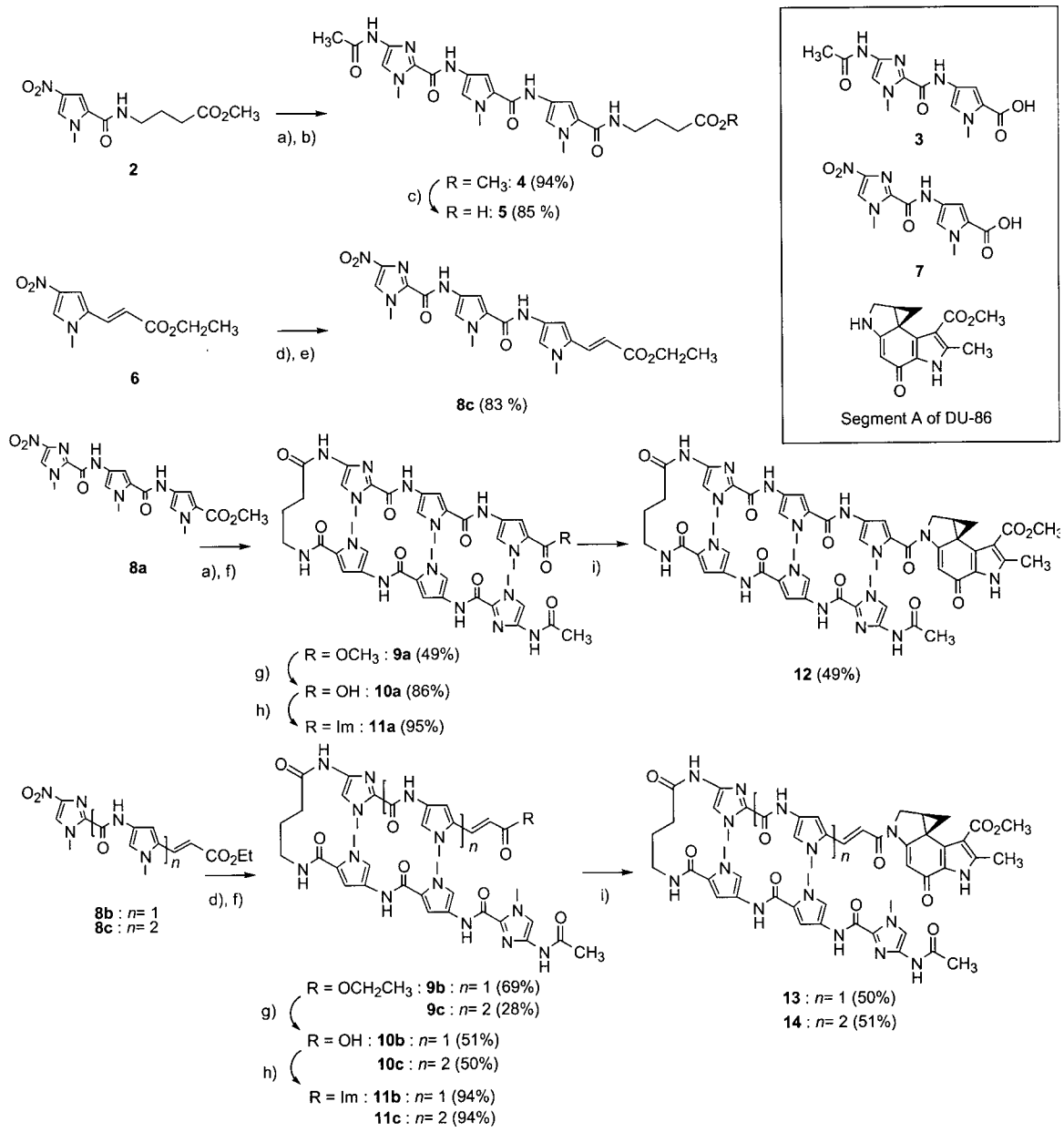

Scheme 1. a) Pd/C, H_2, MeOH/AcOEt; b) **3**, FDPP, iPr$_2$NEt, DMF; c) NaOH, H_2O; d) Pd/C, $NaBH_4$, MeOH/AcOEt; e) **7**, FDPP, iPr$_2$NEt, DMF; f) **5**, FDPP, iPr$_2$NEt, DMF; g) DBU, H_2O; h) CDI, DMF; i) segment A of DU-86, NaH, DMF.

alkylation by **1** was not observed after 1 h of incubation. These results clearly indicate that the DNA alkylating activity of hairpin polyamide–CPI conjugate **13** was dramatically improved relative to the activity of **1**. Densitometric analysis of the alkylated DNA by **1** and **13** is summarized at the bottom of Figure 3. The half-lives of DNA were roughly estimated to be 1 h for compound **13**, and 4 d for **1**. Therefore, the rate acceleration due to the incorporation of the linker is roughly estimated to be 100-fold.

Efficient DNA alkylation by conjugate 13 was further confirmed at the oligonucleotide level by using HPLC product analysis: To clarify the sites of DNA alkylation by conjugates **1**, **12**, **13**, and **14**, we investigated the alkylation of the duplex decanucleotide, 5′-CAAGTCAGAG-3′ (ODN1)/5′-CTCTGACTTG-3′ (ODN2), which was designed according to the results of the gel electrophoresis experiments described above. HPLC analysis of the reaction mixtures combining the decanucleotide with **1**, **12**, **13**, or **14** revealed that these conjugates alkylated at the A or G of the target sequence to produce the corresponding alkylated products, although the reactivities of these compounds varied, as summarized in Figure 4. These alkylating sites were determined by HPLC product analysis, by using a previously reported procedure.[14]

The ODN1-**13** and ODN1-**14** alkylation complexes in Figure 4 were collected and heated at 90 °C for 10 min, and the cleavage of the abasic site with hot alkali (0.1 N NaOH, 90 °C, 10 min) produced the corresponding cleaved oligonucleotides. The compositions of these oligonucleotides were unambiguously confirmed by enzymatic digestion with nucle-

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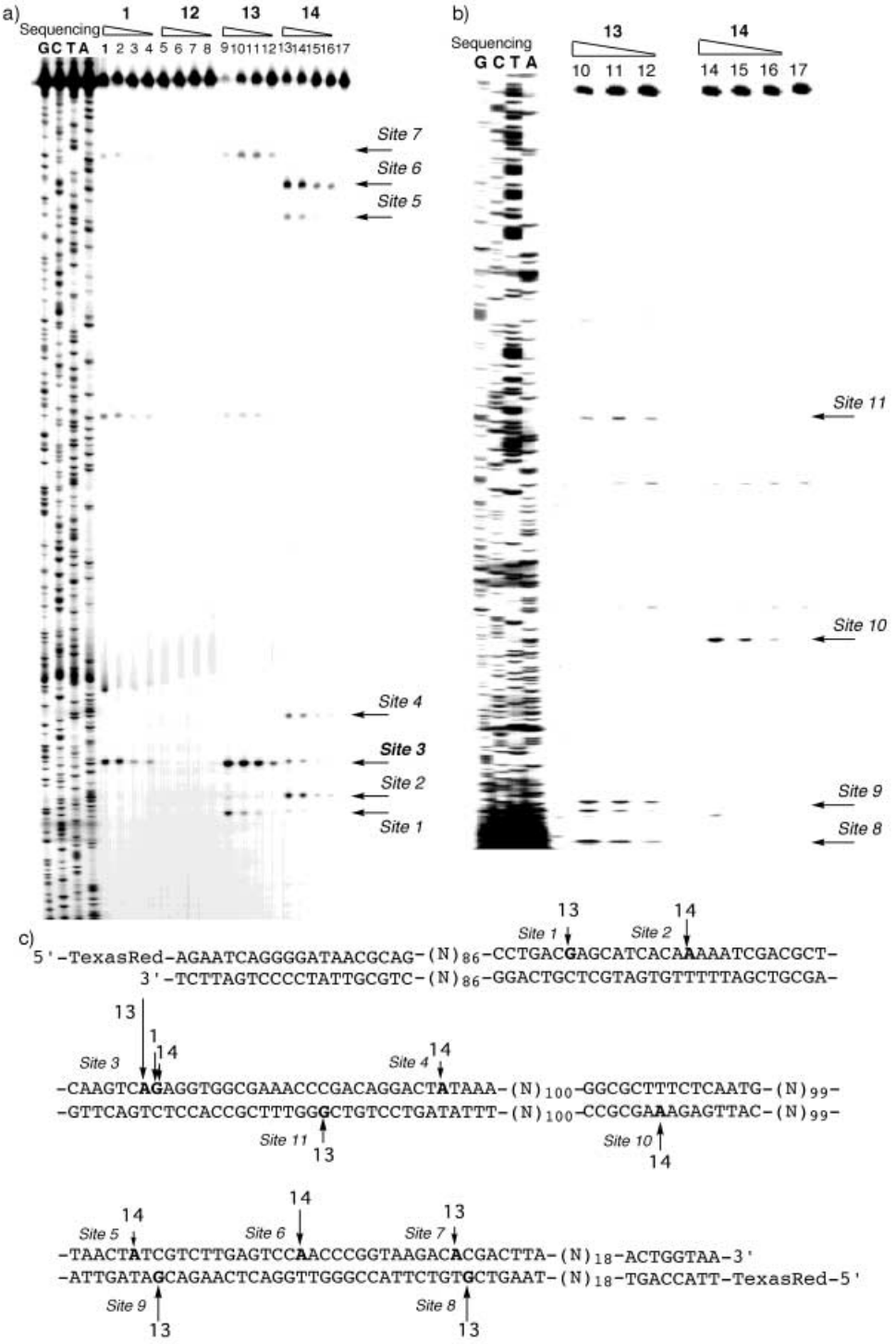

Figure 2. Thermally-induced strand cleavage of 5′-Texas-Red-labeled 450-bp DNA fragments by conjugates **1**, **12**, **13**, or **14**. Results with a) 5′-end labeled top strand (pUC 18 F780–1229) and b) 5′-end labeled bottom strand (pUC 18 R1459–1908) DNA fragments are shown. These two DNA fragments are complementary. Lanes 1–4, 100, 50, 25, 12.5 nM of **1**; lanes 5–8, 100, 50, 25, 12.5 nM of **12**; lanes 9–12, 100, 50, 25, 12.5 nM of **13**; lanes 13–16, 100, 50, 25, 12.5 nM of **14**; lane 17, DNA control. c) Sequences containing the alkylation sites. Arrows indicate the site of alkylation by **1**, **12**–**14**. Alkylated bases are shown in bold.

ase P1 and alkaline phosphatase, as shown in Scheme 2. The ODN1-**1** and ODN1-**12** alkylation complexes were similarly characterized (data not shown).

Alkylation of ODN1/ODN2 by compound **1** occurred predominantly at the G_8 of 5′-CAAGTCA*G*AG-3′, as previously reported.[9] After 5 h incubation, almost half of ODN1 was alkylated under these conditions. In contrast to the reactivity of **1**, DNA alkylation by **12** was the slowest of all the conjugates (13% completed after 17 h, Figure 4). These results clearly indicate the significantly different DNA-alkylating activities of **1** and **12**, as was observed in the alkylation of 450-bp DNA fragments. Alkylation of ODN1/

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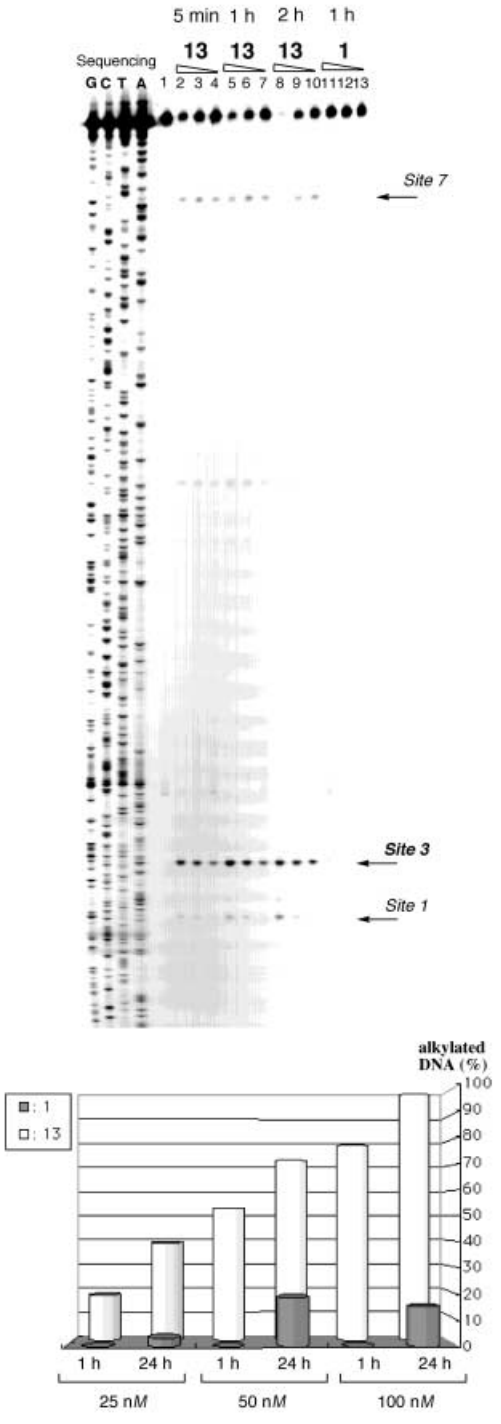

Figure 3. Thermally-induced strand cleavage of 5′-Texas-Red-labeled 450-bp DNA fragments by conjugates **13** and **1**: lane 1, DNA control; lanes 2–4, 100, 50, 25 nM of **13**, 5 min incubation; lanes 5–7, 100, 50, 25 nM of **13**, 60 min incubation; lanes 8–10, 100, 50, 25 nM of **13**, 120 min incubation; lanes 11–13, 100, 50, 25 nM of **1**, 60 min incubation. The amount of alkylated DNA under the same conditions using **1** and **13** are summarized below.

ODN2 by compound **13** proceeded dramatically faster than its alkylation by conjugate **1** (75% complete after 5 min). Furthermore, the alkylation of ODN1/ODN2 by **14** was superior to that of **1** (46% complete after 2 h), which is consistent with the results of the high-resolution gel experiments. To confirm that DNA-alkylation occurs at the N3 position, alkylation of the deazaadenine-containing decamer, 5′-CAAGTCZGAG-3′ (ODN1(Z))/ODN2 by **13** was examined. HPLC analysis of the reaction mixtures containing the ODN1(Z)/ODN2 and **13** revealed that conjugate **13** alkylates at N3 of Z_7 of ODN1(Z) to produce the corresponding alkylated products (Figure 4d and e). The reason that the observed difference in reactivity is not as dramatic as that observed in the gel experiments with ODN1/ODN2 is presumably due to the large difference in the concentrations of the decanucleotide substrate and hairpin polyamides.

To gain insight into the basis of the different reactivities of hairpin polyamides, we constructed a model of a hairpin polyamide–decamer complex based on the ^{1}H NMR structure of ImPyPyγPyPyPy-d(CGCTAACAGGC)/d(GCCTGTTAGCG).[15] The energy-minimized structure of the ODN1/ODN2-**1** complex suggests that the cyclopropane subunit of Du is located in close proximity to the nucleophilic N3 of the G_8 residues, and that one nucleotide unit (N) is probably required between the reacting base and the recognition sequence. Interestingly, the energy-minimized structure of the ODN1/ODN2-**13** complex indicates that the CPI unit stacks well with the N-terminal Im residue, whereas such stacking was not observed with the ODN1/ODN2-**1** complex. It is important to note that the stacking interaction between CPI and Im would contribute to the efficiency of DNA alkylation (Figure 5). Analogous stacking between Du and Py of distamycin A was observed in the NMR structure of a duocarmycin A/distamycin A/octamer complex in which highly efficient DNA alkylation was observed.[16] In fact, the hairpin polyamide **14**, which shows intermediate alkylation activity, does not participate in such a stacking interaction and the alkylating moiety is located in the relatively opened minor groove.

Conclusion

In conclusion, we successfully synthesized the new hairpin polyamide–CPI conjugate **13**, which has significantly improved DNA-alkylating activity over that of conjugate **1** reported previously. We evaluated the DNA alkylating capacity of **13** in detail by high-resolution denaturing gel electrophoresis with 450-bp DNA fragments and by HPLC analysis with 10-bp oligonucleotides.

The results clearly indicate that the composition of the vinyl linker dramatically affects the DNA alkylating reactivity. Furthermore, a new combination of Im and the vinyl linker effectively acted to target G–C base pairs in the sequence-specific recognition by DNA-alkylating reagents. The effective DNA-alkylating agent developed in the present investigation provides a promising approach for developing new types of biological agents to control gene transcription. We are currently investigating whether DNA-alkylating hairpin polyamides that target coding regions inhibit polymerase elongation during transcription.

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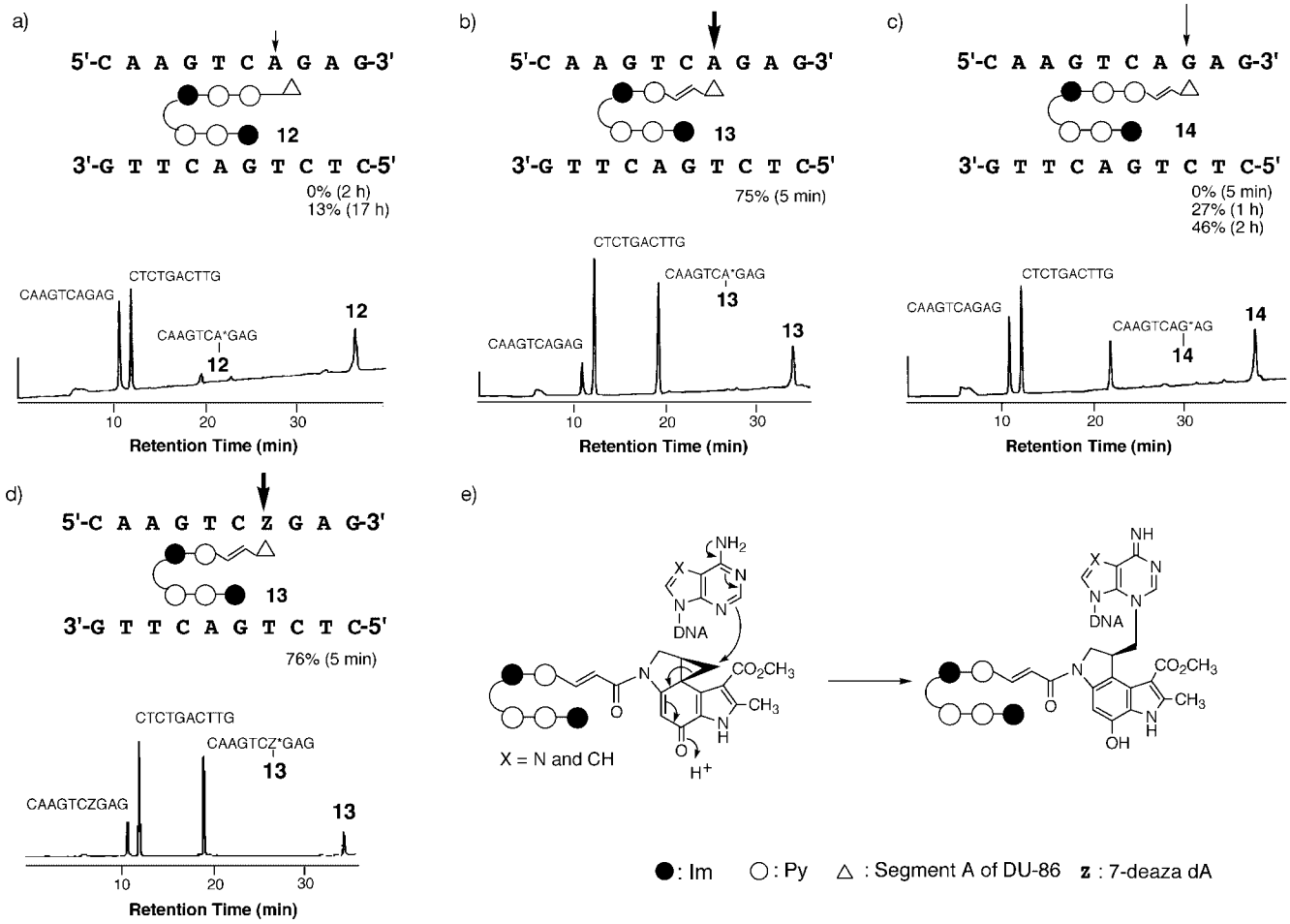
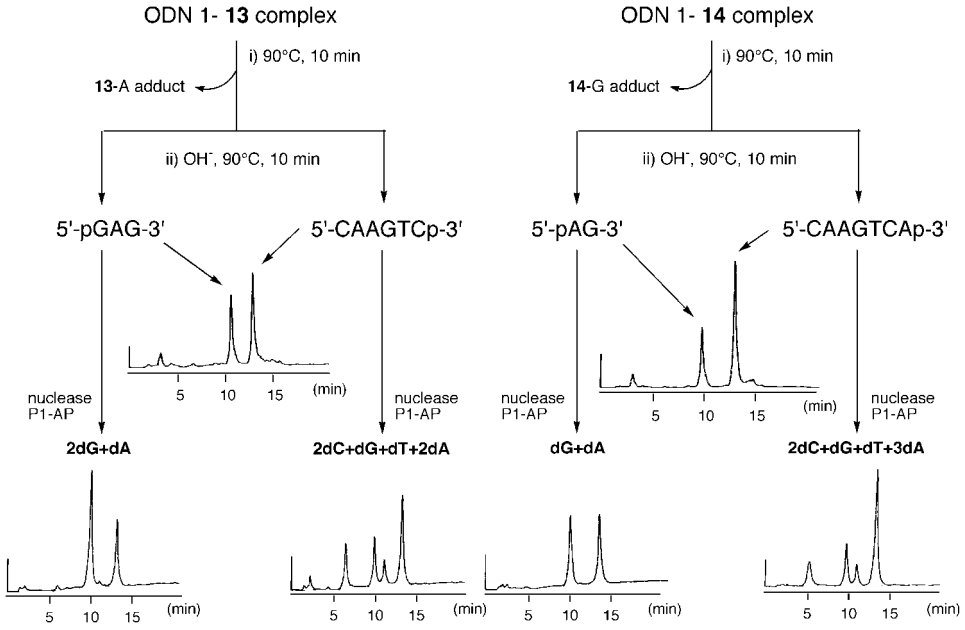


Figure 4. Schematic representation and HPLC analysis of ODN1-ODN2 with a) **12** b) **13**, and c) **14**, and d) ODN1(Z)-ODN2 with **13**. HPLC profiles correspond to a) 17 h, b) 5 min, c) 2 h, and d) 5 min incubations. e) Schematic representation of DNA alkylation by **13**.



Scheme 2. Characterization of alkyaltion sites in ODN1 – **13** and -**14**.

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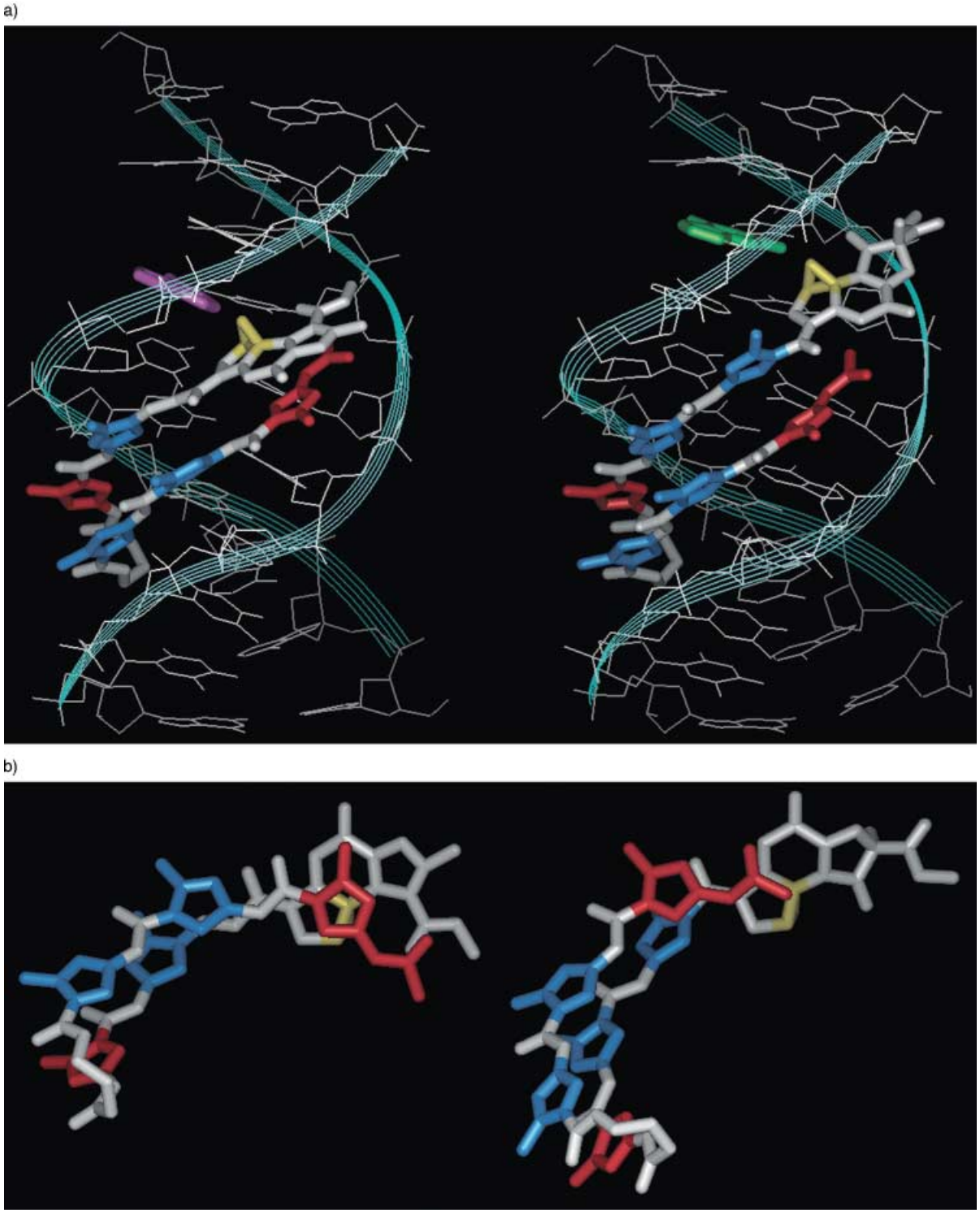

Figure 5. Energy-minimized structure of the d(CAAGTCAGAG) ODN1/d(CTCTGACTTG) ODN2–**13** (top left) and -**1** (top right) complexes. Minimization was performed in the presence of 18 sodium cations and a 10 Å layer of H_2O by CFF force-field. For simplicity, sodium ions and H_2O are not represented. Each strand of DNA is drawn in white and ribbon representation is drawn in light blue. Py and Im are drawn in blue and red, respectively. The reacting G and A bases are drawn in green and purple, respectively. The cyclopropane units in the Du moiety of the hybrids are drawn in yellow. Top views of hairpin polyamides, **13** (bottom left) and **1** (bottom right).

Experimental Section

General: 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; iPr$_2$NEt: *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

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abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), qu (quintet), m (multiplet), br (broad). Electrospray ionization mass spectra (ESMS) were produced on a PE SCIEX API 150 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, the thermo sequenase core sequencing kit and loading dye (dimethylformamide with fuschin red) from Amersham, 5′-Texas-Red-modified DNA oligomer (18 mer) from Kurabo, decanucleotides including 5′-CAAGTCZGAG-3′ from JBioS, and 50% Long Ranger™ gel solution from FMC Bioproducts. P1 nuclease and calf intestine alkaline phosphatase (AP, 1000 units mL^{-1}) were purchased from Roche Diagnostics. The following precursors were prepared by the reported procedures.[9–11] **$NO_2Py\gamma CO_2CH_3$ (2)**: ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 8.39$ (br t, $J = 6.0$ Hz, 1H; NH), 8.10 (d, $J = 2.0$ Hz, 1H; CH), 7.41 (d, $J = 2.0$ Hz, 1H; CH), 3.88 (s, 3H; NCH_3), 3.57 (s, 3H; OCH_3), 3.19 (dt, $J = 6.0$, 7.5 Hz, 2H; CH_2), 2.35 (t, $J = 7.5$ Hz, 2H; CH_2), 1.74 (qu, $J = 7.0$ Hz, 2H; CH_2); ESMS: m/z calcd for $C_{11}H_{16}N_3O_5$ [M^++H]: 270.1; found: 270.1.

AcImPyCO$_2$H (3): ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 12.22$ (br s, 1H; OH), 10.22 (s, 1H; NH), 10.01 (s, 1H; NH), 7.50 (d, $J = 2.0$ Hz, 1H; CH), 7.41 (s, 1H; NH), 6.92 (d, $J = 2.0$ Hz, 1H; CH), 3.92 (s, 3H; NCH_3), 3.81 (s, 3H; NCH_3), 2.01 (s, 3H; $COCH_3$); ESMS: m/z calcd for $C_{13}H_{16}N_5O_4$ [M^++H]: 306.1; found: 306.1.

NO_2ImPyCO$_2$H (7): ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 12.28$ (br s, 1H; OH), 10.84 (s, 1H; NH), 8.60 (s, 1H; CH), 7.48 (d, $J = 2.0$ Hz, 1H; CH), 7.02 (d, $J = 2.0$ Hz, 1H; CH), 4.03 (s, 3H; NCH_3), 3.82 (s, 3H; NCH_3); ESMS: m/z calcd for $C_{11}H_{12}N_5O_5$ [M^++H]: 294.1; found: 294.0.

NO_2ImPyPyCO$_2$CH$_3$ (8a): ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 10.87$ (s, 1H; NH), 9.98 (s, 1H; NH), 8.60 (s, 1H; CH), 7.46 (s, 1H; CH), 7.31 (s, 1H; CH), 7.21 (s, 1H; CH), 6.91 (s, 1H; CH), 4.05 (s, 3H; NCH_3), 3.84 (s, 3H; NCH_3), 3.83 (s, 3H; NCH_3), 3.73 (s, 3H; OCH_3); ESMS: m/z calcd for $C_{18}H_{20}N_7O_6$ [M^++H]: 430.1; found: 430.1.

NO_2ImPyLCO$_2$CH$_2$CH$_3$ (8b): ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 10.79$ (s, 1H; NH), 8.59 (s, 1H; CH), 7.50 (d, $J = 16.0$ Hz, 1H; C*H*=CHCO), 7.45 (d, $J = 2.0$ Hz, 1H; CH), 6.92 (d, $J = 2.0$ Hz, 1H; CH), 6.07 (d, $J = 16.0$ Hz, 1H; CH=C*H*CO), 4.14 (q, $J = 7.0$ Hz, 2H; CH_2), 4.03 (s, 3H; NCH_3), 3.69 (s, 3H; NCH_3), 1.23 (t, $J = 7.0$ Hz, 3H; CH_3); ESMS: m/z calcd for $C_{15}H_{18}N_5O_5$ [M^++H]: 348.1; found: 348.1.

Molecular modeling studies: Minimizations were performed with the Discover (MSI, San Diego, CA) program by using CFF force-field parameters. The starting structure was built on the basis of the NMR structure of the ImPyPyγPyPyPy-d(CGCTAACAGGC)/d(GCCTGTTAGCG) complex[15] and the duocarmycin A/distamycin A/octamer complex.[16] The connecting parts between them were built by using standard bond lengths and angles. The Du moiety of the assembled initial structure was energy minimized with a distance-dependent dielectric constant of $\varepsilon = 4r$ (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^{-1} $Å^{-1}$. 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^{-1} $Å^{-1}$.

AcImPyPyγCO$_2$CH$_3$ (4): 10% Pd/C (220 mg) was added to a solution of compound **2** (1.0 g, 3.72 mmol) in MeOH/AcOEt (1:1, 30 mL), and the reaction mixture was stirred for 3 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 (859 mg), which was used in the next step without further purification. Compound **3** (820 mg, 2.69 mmol) and FDPP (1.70 g, 4.42 mmol), followed by iPr$_2$NEt (1.54 mL, 8.84 mmol) was added to the solution of crude amine (859 mg, 3.59 mmol) in DMF (15 mL). The solution was stirred for 24 h and concentrated to a residue, which was subjected to column chromatography (silica gel, 0–10% MeOH in $CHCl_3$, gradient elution) to produce compound **4** (1.33 g, 94% yield) as a yellow powder. ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 10.23$ (s, 1H; NH), 9.94 (s, 1H; NH), 9.88 (s, 1H; NH), 8.02 (br t, 1H; NH), 7.42 (s, 1H; CH), 7.27 (d, $J = 1.5$ Hz, 1H; CH), 7.17 (d, $J = 1.5$ Hz, 1H; CH), 7.12 (d, $J = 1.5$ Hz, 1H; CH), 6.87 (d, $J = 1.5$ Hz, 1H; CH), 3.95 (s, 3H; NCH_3), 3.85 (s, 3H; NCH_3), 3.80 (s, 3H; NCH_3), 3.59 (s, 3H; OCH_3), 3.16 (dt, $J = 6.0$, 7.0 Hz, 2H; CH_2), 2.34 (t, $J = 7.0$ Hz, 2H; CH_2), 2.03 (s, 3H; $COCH_3$), 1.74 (qu, $J = 7.0$ Hz, 2H; CH_2); ESMS: m/z calcd for $C_{24}H_{31}N_8O_6$ [M^++H]: 527.2; found: 527.1.

AcImPyPyγCO$_2$H (5): NaOH (800 mg, 20 mmol) was added to a suspension of compound **4** (1.33g, 2.52 mmol) in H_2O (50 mL). The solution was stirred for 24 h at room temperature and was acidified by addition of HCl (aq) to pH 2 at 0 °C. The precipitate was collected by filtration, washed with water, and dried to produce compound **5** (1.10 g, 85% yield) as a white powder. ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 10.24$ (s, 1H; NH), 9.95 (s, 1H; NH), 9.89 (s, 1H; NH), 8.02 (br t, 1H; NH), 7.42 (s, 1H; CH), 7.26 (d, $J = 1.5$ Hz, 1H; CH), 7.17 (d, $J = 1.5$ Hz, 1H; CH), 7.11 (d, $J = 1.5$ Hz, 1H; CH), 6.86 (d, $J = 1.5$ Hz, 1H; CH), 3.94 (s, 3H; NCH_3), 3.84 (s, 3H; NCH_3), 3.78 (s, 3H; NCH_3), 3.17 (dt, $J = 6.0$, 7.0 Hz, 2H; CH_2), 2.24 (t, $J = 7.0$ Hz, 2H; CH_2), 2.01 (s, 3H; $COCH_3$), 1.70 (qu, $J = 7.0$ Hz, 2H; CH_2); ESMS: m/z calcd for $C_{23}H_{29}N_8O_6$ [M^++H]: 513.2; found: 513.3.

NO_2ImPyPyLCO$_2$CH$_2$CH$_3$ (8c): 10% Pd/C (200 mg) was added to a solution of compound **6** (500 mg, 2.23 mmol) in MeOH/AcOEt (1:1, 20 mL). After $NaBH_4$ (170 mg, 4.47 mmol) in H_2O (1 mL) was added dropwise at 0 °C, the reaction mixture was stirred for 20 min at room temperature. The catalyst was removed by filtration through silica gel. The filtrate was concentrated in vacuo to produce crude amine (418 mg), which was used in the next step without further purification. Ccompound **7** (330 mg, 1.13 mmol) and FDPP (1.3 g, 3.39 mmol), followed by iPr$_2$NEt (1.18 mL, 6.78 mmol) was added to a solution of crude amine (418 mg, 2.15 mmol) in DMF (14 mL). The solution was stirred for 20 h and concentrated to a residue, which was subjected to column chromatography (silica gel, 0–10% MeOH in $CHCl_3$, gradient elution) to produce compound **8c** (359 mg, 83% yield) as a yellow powder. ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 10.86$ (s, 1H; NH), 9.98 (s, 1H; NH), 8.61 (s, 1H; CH), 7.51 (d, $J = 16.0$ Hz, 1H; C*H*=CHCO), 7.41 (s, 1H; CH), 7.30 (s, 1H; CH), 7.22 (s, 1H; CH), 6.74 (s, 1H; CH), 6.07 (d, $J = 16.0$ Hz, 1H; CH=C*H*CO), 4.14 (q, $J = 7.0$ Hz, 2H; CH_2), 4.05 (s, 3H; NCH_3), 3.85 (s, 3H; NCH_3), 3.68 (s, 3H; NCH_3), 1.23 (t, $J = 7.0$ Hz, 3H; CH_3); ESMS: m/z calcd for $C_{21}H_{24}N_7O_6$ [M^++H]: 470.2; found: 470.1.

AcImPyPyγImPyPyCO$_2$CH$_3$ (9a): 10% Pd/C (100 mg) was added to a solution of compound **8a** (200 mg, 0.466 mmol) in MeOH/AcOEt (1:1, 8 mL); the reaction mixture was stirred for 5 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 (178 mg), which was used in the next step without further purification. compound **5** (190 mg, 0.373 mmol) and FDPP (268 mg, 0.699 mmol), followed by iPr$_2$NEt (0.243 mL, 1.39 mmol) was added to a solution of crude amine (178 mg, 0.446 mmol) in DMF (2 mL). 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 **9a** (154.5 mg, 49% yield) as a brown powder. ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 10.26$ (s, 1H; NH), 10.23 (s, 1H; NH), 9.99 (s, 1H; NH), 9.96 (s, 1H; NH), 9.94 (s, 1H; NH), 9.90 (s, 1H; NH), 8.02 (br t, 1H; NH), 7.45 (s, 1H; CH), 7.42 (s, 2H; CH), 7.26 (s, 2H; CH), 7.17 (s, 1H; CH), 7.14 (s, 1H; CH), 7.12 (s, 1H; CH), 6.89 (s, 1H; CH), 6.88 (s, 1H; CH), 3.94 (s, 6H; NCH_3), 3.84 (s, 3H; NCH_3), 3.83 (s, 3H; NCH_3), 3.82 (s, 3H; NCH_3), 3.81 (s, 3H; NCH_3), 3.79 (s, 3H; OCH_3), 3.20 (m, 2H; CH_2), 2.35 (m, 2H; CH_2), 2.01 (s, 3H; $COCH_3$), 1.78 (m, 2H; CH_2); ESMS: m/z calcd for $C_{41}H_{48}N_{15}O_9$ [M^++H]: 894.4; found: 894.3.

AcImPyPyγImPyLCO$_2$CH$_2$CH$_3$ (9b): 10% Pd/C (30 mg) was added to a solution of compound **8b** (68 mg, 0.196 mmol) in MeOH/AcOEt (1:1, 4 mL). After $NaBH_4$ (20 mg, 0.528 mmol) in H_2O (0.2 mL) was added dropwise at 0 °C, the reaction mixture was stirred for 20 min at room temperature. The catalyst was removed by filtration through silica gel. The filtrate was concentrated in vacuo to produce crude amine (60 mg), which was used in the next step without further purification. Compound **5** (97 mg, 0.189 mmol) and FDPP (109 mg, 0.284 mmol), followed by iPr$_2$NEt (99 μL, 0.568 mmol) was added to a solution of crude amine (60 mg, 0.189 mmol) in DMF (0.6 mL). The solution was stirred for 18 h and concentrated to a residue, which was subjected to column chromatography (silica gel, 0–10% MeOH in $CHCl_3$, gradient elution) to produce **9b** (107 mg, 69% yield) as a brown powder. ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 10.27$ (s, 1H; NH), 10.23 (s, 1H; NH), 9.95 (s, 1H; NH), 9.89 (s, 2H; NH), 8.01 (br t, 1H; NH), 7.51 (d, $J = 16.0$ Hz, 1H; C*H*=CHCO), 7.45 (s, 1H; CH), 7.43 (d, $J = 1.0$ Hz, 1H; CH), 7.42 (s, 1H; CH), 7.27 (d, $J = 1.0$ Hz, 1H; CH), 7.17 (d, $J = 2.0$ Hz, 1H; CH), 7.12 (d, $J = 2.0$ Hz, 1H; CH), 6.89 (d, $J = 2.0$ Hz, 1H; CH), 6.83 (d, $J = 2.0$ Hz, 1H; CH), 6.11 (d, $J = 16.0$ Hz, 1H; CH=C*H*CO),

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4.15 (q, $J = 7.0$ Hz, 2H; CH_2), 3.95 (s, 3H; NCH_3), 3.94 (s, 3H; NCH_3), 3.85 (s, 3H; NCH_3), 3.80 (s, 3H; NCH_3), 3.69 (s, 3H; NCH_3), 3.20 (dt, $J = 5.5$, 7.0 Hz, 2H; CH_2), 2.36 (t, $J = 7.0$ Hz, 2H; CH_2), 2.02 (s, 3H; $COCH_3$), 1.79 (qu, $J = 7.0$ Hz, 2H; CH_2), 1.24 (t, $J = 7.0$ Hz, 3H; CH_3); ESMS: *m/z* calcd for $C_{38}H_{46}N_{13}O_8$ [M^++H]: 812.4; found: 812.3.

AcImPyPyγImPyPyLCO$_2$CH$_2$CH$_3$ (9c): A similar synthetic procedure as that described for **9b** was followed in the preparation of **9c**, with a yield of 28%. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 10.26$ (s, 1H; NH), 10.23 (s, 1H; NH), 9.97 (s, 1H; NH), 9.95 (s, 1H; NH), 9.94 (s, 1H; NH), 9.89 (s, 1H; NH), 8.02 (br t, 1H; NH), 7.52 (d, $J = 16.0$ Hz, 1H; C*H*=CHCO), 7.46 (s, 1H; CH), 7.42 (s, 1H; CH), 7.41 (s, 1H; CH), 7.27 (s, 2H; CH), 7.17 (s, 1H; CH), 7.15 (s, 1H; CH), 7.13 (s, 1H; CH), 6.90 (s, 1H; CH), 6.75 (s, 1H; CH), 6.08 (d, $J = 16.0$ Hz, 1H; CH=C*H*CO), 4.15 (q, $J = 7.0$ Hz, 2H; CH_2), 3.95 (s, 3H; NCH_3), 3.94 (s, 3H; NCH_3), 3.85 (s, 3H; NCH_3), 3.84 (s, 3H; NCH_3), 3.80 (s, 3H; NCH_3), 3.69 (s, 3H; NCH_3), 3.21 (m, 2H; CH_2), 2.36 (m, 2H; CH_2), 2.02 (s, 3H; $COCH_3$), 1.79 (m, 2H; CH_2), 1.24 (t, $J = 7.0$ Hz, 3H; CH_3); ESMS: *m/z* calcd for $C_{44}H_{52}N_{15}O_9$ [M^++H]: 934.4; found: 934.4.

AcImPyPyγImPyPyCO$_2$H (10a): DBU (0.2 mL, 1.34 mmol) was added to a suspension of compound **9a** (154.5 mg, 0.181 mmol) in H_2O (0.6 mL). The solution was stirred for 2 h and the was acidified with HCl (aq) to pH 2 at 0 °C. The precipitate was collected by filtration, washed with water, and dried to produce **10a** (131.5 mg, 86% yield) as a brown powder. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 10.25$ (s, 1H; NH), 10.23 (s, 1H; NH), 9.97 (s, 1H; NH), 9.95 (s, 1H; NH), 9.90 (s, 1H; NH), 9.89 (s, 1H; NH), 8.02 (br t, 1H; NH), 7.45 (s, 1H; CH), 7.41 (s, 2H; CH), 7.26 (s, 2H; CH), 7.17 (s, 1H; CH), 7.12 (s, 2H; CH), 6.89 (s, 1H; CH), 6.84 (s, 1H; CH), 3.94 (s, 6H; NCH_3), 3.84 (s, 3H; NCH_3), 3.83 (s, 3H; NCH_3), 3.81 (s, 3H; NCH_3), 3.79 (s, 3H; NCH_3), 3.18 (m, 2H; CH_2), 2.35 (m, 2H; CH_2), 2.01 (s, 3H; $COCH_3$), 1.78 (m, 2H; CH_2); ESMS: *m/z* calcd for $C_{40}H_{46}N_{15}O_9$ [M^++H]: 880.4; found: 880.4.

AcImPyPyγImPyLCO$_2$H (10b): DBU (0.6 mL, 4.01 mmol) was added to a suspension of compound **9b** (143 mg, 0.176 mmol) in H_2O (0.6 mL). The solution was stirred for 6 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% AcOH. The precipitate was collected by filtration, washed with water, and dried to produce **10b** (70 mg, 51% yield) as a brown powder. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 10.27$ (s, 1H; NH), 10.23 (s, 1H; NH), 9.95 (s, 1H; NH), 9.89 (s, 2H; NH), 8.02 (br t, 1H; NH), 7.46 (d, $J = 16.0$ Hz, 1H; C*H*=CHCO), 7.45 (s, 1H; CH), 7.42 (s, 1H; CH), 7.41 (s, 1H; CH), 7.27 (d, $J = 2.0$ Hz, 1H; CH), 7.17 (s, 1H; CH), 7.13 (d, $J = 2.0$ Hz, 1H; CH), 6.89 (d, $J = 2.0$ Hz, 1H; CH), 6.80 (d, $J = 2.0$ Hz, 1H; CH), 6.03 (d, $J = 16.0$ Hz, 1H; CH=C*H*CO), 3.95 (s, 3H; NCH_3), 3.94 (s, 3H; NCH_3), 3.85 (s, 3H; NCH_3), 3.80 (s, 3H; NCH_3), 3.68 (s, 3H; NCH_3), 3.21 (m, 2H; CH_2), 2.36 (m, 2H; CH_2), 2.03 (s, 3H; $COCH_3$), 1.79 (m, 2H; CH_2); ESMS: *m/z* calcd for $C_{36}H_{42}N_{13}O_8$ [M^++H]: 784.3; found: 784.3.

AcImPyPyγImPyPyLCO$_2$H (10c): A synthetic procedure similar to that described for compound **10b** was followed in the preparation of **10c**, with a yield of 50%. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 10.24$ (s, 1H; NH), 10.21 (s, 1H; NH), 9.95 (s, 1H; NH), 9.93 (s, 1H; NH), 9.91 (s, 1H; NH), 9.88 (s, 1H; NH), 8.01 (br t, 1H; NH), 7.45 (d, $J = 16.0$ Hz, 1H; C*H*=CHCO), 7.44 (s, 1H; CH), 7.41 (s, 1H; CH), 7.37 (s, 1H; CH), 7.26 (s, 2H; CH), 7.16 (s, 1H; CH), 7.12 (s, 1H; CH), 7.11 (s, 1H; CH), 6.88 (s, 1H; CH), 6.70 (s, 1H; CH), 5.99 (d, $J = 16.0$ Hz, 1H; CH=C*H*CO), 3.94 (s, 6H; NCH_3), 3.84 (s, 6H; NCH_3), 3.79 (s, 3H; NCH_3), 3.66 (s, 3H; NCH_3), 3.20 (m, 2H; CH_2), 2.35 (m, 2H; CH_2), 2.01 (s, 3H; $COCH_3$), 1.78 (m, 2H; CH_2); ESMS: *m/z* calcd for $C_{42}H_{48}N_{15}O_9$ [M^++H]: 906.4; found: 906.3.

AcImPyPyγImPyPyCOIm (11a): 1,1′-Carbonyldiimidazole (3.5 mg, 22.0 μmol) was added to a solution of compound **10a** (10 mg, 11.3 μmol) in DMF (0.2 mL) was added. The mixture was stirred for 5 h at room temperature. Evaporation of the solvent gave a yellow residue, which was triturated with ethyl ether (5 mL × 3) to produce **11a** (10 mg, 95%) as a yellow powder. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 10.25$ (s, 1H; NH), 10.23 (s, 1H; NH), 10.06 (s, 1H; NH), 10.03 (s, 1H; NH), 9.96 (s, 1H; NH), 9.90 (s, 1H; NH), 8.26 (s, 1H; CH), 8.01 (br t, 1H; NH), 7.77 (s, 1H; CH), 7.69 (s, 1H; CH), 7.45 (s, 1H; CH), 7.41 (s, 1H; CH), 7.26 (s, 2H; CH), 7.20 (s, 1H; CH), 7.17 (s, 1H; CH), 7.13 (s, 2H; CH), 6.95 (s, 1H; CH), 6.89 (s, 1H; CH), 3.94 (s, 6H; NCH_3), 3.90 (s, 3H; NCH_3), 3.85 (s, 3H; NCH_3), 3.84 (s, 3H; NCH_3), 3.79 (s, 3H; NCH_3), 3.20 (m, 2H; CH_2), 2.35 (m, 2H; CH_2), 2.01 (s, 3H; $COCH_3$), 1.78 (m, 2H; CH_2); ESMS: *m/z* calcd for $C_{43}H_{48}N_{17}O_8$ [M^++H]: 930.4; found: 930.3.

AcImPyPyγImPyLCOIm (11b): A synthetic procedure similar to that used to prepare compound **11a** was followed in the preparation of **11b**, with a yield of 94%. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 10.26$ (s, 1H; NH), 10.23 (s, 1H; NH), 10.08 (s, 1H; NH), 9.95 (s, 1H; NH), 9.89 (s, 1H; NH), 8.68 (s, 1H; CH), 8.02 (br t, 1H; NH), 7.90 (s, 1H; CH), 7.87 (d, $J = 15.0$ Hz, 1H; C*H*=CHCO), 7.63 (s, 1H; CH), 7.49 (s, 1H; CH), 7.47 (s, 1H; CH), 7.42 (s, 1H; CH), 7.31 (s, 1H; CH), 7.27 (s, 1H; CH), 7.18 (s, 1H; CH), 7.14 (d, $J = 15.0$ Hz, 1H; CH=C*H*CO), 7.13 (s, 1H; CH), 6.90 (s, 1H; CH), 3.96 (s, 3H; NCH_3), 3.95 (s, 3H; NCH_3), 3.86 (s, 3H; NCH_3), 3.81 (s, 3H; NCH_3), 3.78 (s, 3H; NCH_3), 3.21 (m, 2H; CH_2), 2.37 (m, 2H; CH_2), 2.02 (s, 3H; $COCH_3$), 1.80 (m, 2H; CH_2); ESMS: *m/z* calcd for $C_{39}H_{44}N_{15}O_7$ [M^++H]: 834.4; found: 834.3.

AcImPyPyγImPyPyLCOIm (11c): A synthetic procedure similar to that used in the preparation of compound **11a** was followed to prepare compound **11c**, with a yield of 94%. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta =$ 10.24 (s, 1H; NH), 10.21 (s, 1H; NH), 10.04 (s, 1H; NH), 9.99 (s, 1H; NH), 9.94 (s, 1H; NH), 9.88 (s, 1H; NH), 8.66 (s, 1H; CH), 8.01 (br t, 1H; NH), 7.89 (s, 1H; CH), 7.87 (d, $J = 15.0$ Hz, 1H; C*H*=CHCO), 7.63 (s, 1H; CH), 7.47 (s, 1H; CH), 7.45 (s, 1H; CH), 7.41 (s, 1H; CH), 7.28 (s, 1H; CH), 7.25 (s, 1H; CH), 7.22 (s, 1H; CH), 7.16 (s, 1H; CH), 7.13 (d, $J = 15.0$ Hz, 1H; CH=C*H*CO), 7.11 (s, 1H; CH), 7.09 (s, 1H; CH), 6.89 (s, 1H; CH), 3.95 (s, 3H; NCH_3), 3.94 (s, 3H; NCH_3), 3.86 (s, 3H; NCH_3), 3.84 (s, 3H; NCH_3), 3.79 (s, 3H; NCH_3), 3.77 (s, 3H; NCH_3), 3.20 (m, 2H; CH_2), 2.35 (m, 2H; CH_2), 2.01 (s, 3H; $COCH_3$), 1.79 (m, 2H; CH_2); ESMS: *m/z* calcd for $C_{45}H_{50}N_{17}O_8$ [M^++H]: 956.4; found: 956.5.

AcImPyPyγImPyPyCPI (12): Segment A of DU-86 (3.7 mg, 14.5 μmol) in DMF (0.1 mL) was added to a solution of sodium hydride (3.0 mg, 75 μmol, 60% oil suspension) in DMF (0.1 mL). Compound **11a** (10 mg, 10.7 μmol) in DMF (0.1 mL) was added at 0 °C, and the reaction mixture was then stirred for 1 h 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 **12** (5.7 mg, 49% yield) as a yellow powder. After further purification by HPLC with a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 0–50% linear gradient, 35.1 min/40 min, 254 nm), **12** was used in the DNA alkylation reaction. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 12.37$ (br s, 1H; NH), 10.25 (s, 1H; NH), 10.22 (s, 1H; NH), 9.96 (s, 1H; NH), 9.94 (s, 1H; NH), 9.93 (s, 1H; NH), 9.88 (s, 1H; NH), 8.01 (br t, 1H; NH), 7.44 (s, 2H; CH), 7.41 (s, 1H; CH), 7.25 (s, 2H; CH), 7.16 (s, 1H; CH), 7.14 (s, 1H; CH), 7.12 (s, 1H; CH), 6.88 (s, 1H; CH), 6.70 (s, 1H; CH), 6.14 (s, 1H; CH), 4.22 (m, 1H; NCH*H*), 4.08 (m, 1H; NC*H*H), 3.94 (s, 6H; NCH_3), 3.84 (s, 6H; NCH_3), 3.79 (s, 3H; NCH_3), 3.73 (s, 3H; NCH_3), 3.72 (s, 3H; OCH_3), 3.42 (m, 1H; CH), 3.21 (m, 2H; CH_2), 2.41 (s, 3H; CH_3), 2.34 (m, 2H; CH_2), 2.17 (m, 1H; CH*H*), 2.01 (s, 3H; $COCH_3$), 1.78 (m, 2H; CH_2), 1.41 (m, 1H; C*H*H); ESMS: *m/z* calcd for $C_{54}H_{58}N_{17}O_{11}$ [M^++H]: 1120.4; found: 1120.5.

AcImPyPyγImPyLCPI (13): A synthetic procedure similar to that used to prepare compound **12** was followed in the preparation of compound **13**, with a yield of 50%. After further purification by HPLC with a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 0–50% linear gradient, 32.9 min/40 min, 254 nm), **13** was used in the DNA alkylation reaction. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 12.18$ (br s, 1H; NH), 10.26 (s, 1H; NH), 10.22 (s, 1H; NH), 10.19 (s, 1H; NH), 9.94 (s, 1H; NH), 9.89 (s, 1H; NH), 8.02 (br t, 1H; NH), 7.57 (d, $J = 15.0$ Hz, 1H; C*H*=CHCO), 7.46 (s, 1H; CH), 7.42 (s, 1H; CH), 7.34 (s, 1H; CH), 7.27 (s, 1H; CH), 7.18 (s, 1H; CH), 7.13 (s, 1H; CH), 6.98 (s, 1H; CH), 6.89 (s, 1H; CH), 6.58 (d, $J =$ 15.0 Hz, 1H; CH=C*H*CO), 5.96 (s, 1H; CH), 4.28 (m, 1H; NCH*H*), 4.15 (m, 1H; NC*H*H), 3.95 (s, 6H; NCH_3), 3.85 (s, 3H; NCH_3), 3.80 (s, 3H; NCH_3), 3.73 (s, 3H; NCH_3), 3.72 (s, 3H; OCH_3), 3.54 (m, 1H; CH), 3.20 (m, 2H; CH_2), 2.46 (s, 3H; CH_3), 2.36 (m, 2H; CH_2), 2.09 (m, 1H; CH*H*), 2.02 (s, 3H; $COCH_3$), 1.79 (m, 2H; CH_2), 1.29 (m, 1H; C*H*H); ESMS: *m/z* calcd for $C_{50}H_{54}N_{15}O_{10}$ [M^++H]: 1024.4; found: 1024.4.

AcImPyPyγImPyPyLCPI (14): A synthetic procedure similar to that used for the preparation of compound **12** was followed to prepare compound **14**, with a yield of 51%. After further purification by HPLC with a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 0–50% linear gradient, 36.8 min/40 min, 254 nm), **14** was used in the DNA alkylation

reaction. ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 12.37$ (br s, 1H; NH), 10.26 (s, 1H; NH), 10.22 (s, 1H; NH), 10.19 (s, 1H; NH), 9.97 (s, 1H; NH), 9.94 (s, 1H; NH), 9.89 (s, 1H; NH), 8.02 (br t, 1H; NH), 7.58 (d, $J = 15.0$ Hz, 1H; C*H*=CHCO), 7.46 (s, 1H; CH), 7.42 (s, 1H; CH), 7.39 (s, 1H; CH), 7.28 (s, 1H; CH), 7.27 (s, 1H; CH), 7.17 (s, 1H; CH), 7.15 (s, 1H; CH), 7.13 (s, 1H; CH), 6.90 (s, 2H; CH), 6.58 (d, $J = 15.0$ Hz, 1H; CH=C*H*CO), 6.00 (s, 1H; CH), 4.29 (m, 1H; NCH*H*), 4.15 (m, 1H; NC*H*H), 3.96 (s, 3H; NCH$_3$), 3.95 (s, 3H; NCH$_3$), 3.86 (s, 3H; NCH$_3$), 3.85 (s, 3H; NCH$_3$), 3.80 (s, 3H; NCH$_3$), 3.73 (s, 3H; NCH$_3$), 3.72 (s, 3H; OCH$_3$), 3.54 (m, 1H; CH), 3.19 (m, 2H; CH$_2$), 2.47 (s, 3H; CH$_3$), 2.38 (m, 2H; CH$_2$), 2.09 (m, 1H; CH*H*), 2.02 (s, 3H; COCH$_3$), 1.80 (m, 2H; CH$_2$), 1.29 (m, 1H; C*H*H); ESMS: *m*/*z* calcd for $C_{56}H_{60}N_{17}O_{11}$ [M^++H]: 1146.5; found: 1146.5.

Preparation of 5′-Texas-Red-modified 450-bp DNA fragments: The 5′-Texas-Red-modified 450-bp DNA fragments pUC18 F780*-1229 and pUC18 R1459*-1908 (these two DNA fragments are complementary) were prepared by polymerase chain reaction (PCR) with 5′-Texas-Red-modified 20-mer primers: 5′-AGAATCAGGGGATAACGCAG-3′ (pUC18 forward, 780–799) and 5′-TTACCAGTGGCTGCTGCCAG-3′ (pUC18 reverse, 1459–1478). Fragments were purified by filtration with Suprec-02 and their concentrations were determined by UV absorption. The asterisk indicates Texas Red modification and the nucleotide numbering starts with the replication site.

High-resolution gel electrophoresis: The 5′-Texas-Red-labeled DNA fragments (9 nM) were alkylated by various concentrations of **1**, **12**, **13**, and **14** in sodium phosphate buffer (5 mM, 10 μL, pH 7.0) containing 10% DMF at 23 °C. The reaction was quenched by the addition of calf thymus DNA (1 mM, 1 μL) and heating for 5 min at 90 °C. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in loading dye (formamide with fuschin red, 8 μL), heated at 94 °C for 20 min, and then immediately cooled to 0 °C. A 2 μL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a Hitachi 5500-S DNA Sequencer.

Alkylation of oligonucleotides by conjugates 1, 12, 13, and 14, as monitored by HPLC: A reaction mixture (50 μL) containing conjugates **1**, **12**, **13**, or **14** (150 μM) and the duplex oligonucleotide (100 μM duplex concentration) in sodium cacodylate buffer (50 mM, pH 7.0) was incubated at 23 °C for the indicated periods. The progress of the reaction was monitored by HPLC with a Chemcobond 5-ODS-H column (4.6 × 150 mm). Elution was performed with ammonium formate (50 mM) and a 0–50% acetonitrile linear gradient (0–40 min) at a flow rate of 1.0 mL min^{-1}. Products were detected at 254 nm.

Characterization of ODN–conjugate 1, 12, 13, and 14 alkylation complexes: The ODN1 alkylation products shown in Figure 4 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^{-1}). The collected fractions were evaporated, then heated at 90 °C for 10 min and heated in the presence of 0.1N NaOH at 90 °C for 10 min. After neutralization, the solution 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^{-1}). The composition of the cleaved oligonucleotides was confirmed by enzymatic digestion. Oligonucleotides were digested with nuclease P1 (10 units mL^{-1})/AP (5 units mL^{-1}) in Na cacodylate buffer (5 mM, pH 7.0) at 37 °C for 2 h, and 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^{-1}).

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