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Sequence-Specific Alkylation by Y-Shaped and Tandem Hairpin Pyrrole–Imidazole Polyamides

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Abstract: To extend the target DNA sequence length of the hairpin pyrrole–imidazole (Py–Im) polyamide **1**, we designed and synthesized Y-shaped and tandem hairpin Py–Im polyamides **2** and **3**, which possess 1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (*seco*-CBI) as DNA-alkylating moieties. High-resolution denaturing polyacrylamide gel electrophoresis by using 5′-Texas-Red-labeled 465 base pair (bp) DNA fragments revealed that conjugates **2** and **3** alkylated the adenine of the target DNA sequences at nanomolar concentrations. Conjugate **2** alkylated adenine N3 at the 3′ end of two 8 bp match sequences, 5′-AATAACCA-3′ (site A) and 5′-AAATTCCA-3′ (site C), while conjugate **3** recognized one 10 bp match sequence, 5′-AGAATAACCA-3′ (site A) in the 465 bp DNA fragments. These results demonstrate that *seco*-CBI conjugates of Y-shaped and tandem hairpin polyamides have extended their target alkylation sequences.

Keywords: antigens · DNA alkylation · DNA recognition · molecular recognition · nucleic acids

Introduction

Minor-groove-binding compounds that are composed of *N*-methylpyrrole (Py) and *N*-methylimidazole (Im) recognize each of the Watson–Crick base pairs. An antiparallel pairing of Im opposite Py (Im–Py pair) distinguishes a G–C from a C–G, whereas a Py–Py pair binds both A–T and T–A in preference to G–C/C–G base pairs.[1] We have developed a variety of sequence-specific alkylating agents by conjugation of Py–Im polyamides and alkylating moieties, including a Py–Im polyamide–cyclopropapyrroloindolone (CPI) conjugate with a vinyl linker.[2] We have introduced 1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (*seco*-CBI)[3] as an alkylating moiety and 2-carbonyl-5-aminoindole as a linker.[4] The introduction of *seco*-CBI and the indole moiety greatly facilitated the synthesis of sequence-specific alkylating Py–Im polyamides; this made it possible to supply sufficient quantities of polyamides for in vitro and in vivo experiments for drug development. We have demonstrated that indole-linked alkylating polyamides with 6 bp recognition ability sequence-specifically alkylate DNA fragments. The alkylation of the template strand of the coding regions by these polyamides causes sequence-specific gene silencing of the green fluorescent protein gene.[5] We have also developed 10-ringed hairpin polyamides that specifically alkylate the adenine of a targeted 9 bp matching sequence.[4] To develop an alkylating polyamide by causing selective gene silencing in an endogenous target sequence of a specific gene, a new type of hairpin motif for alkylating polyamides with sequence-specific binding-site sizes over 10 bp might be required. For this reason, we searched for alternative motifs to increase the targetable binding-site size of these hairpin motifs, which possess high alkylating activity and sequence-specificity. We selected Y-shaped and tandem hairpin Py–Im polyamides as the binding moiety for the alkylating agent. Dervan and co-workers have synthesized several tandem motifs and have investigated the DNA-binding affinities of these polyamides by quantitative DNase I footprinting.[6] They synthesized tandem polyamides that bind an 11 bp specific sequence through hydrogen bonds. Laemmli and co-workers reported that tandem hairpin Py–Im polyamides specifically bind vertebrate telomeres.[7]

Here, we report the syntheses and evaluation of hairpin conjugate **1**, Y-shaped conjugate **2**, and tandem hairpin Py–Im polyamide **3**, which alkylate target DNA sequences (Figure 1). Conjugates **2** and **3** were designed so that the

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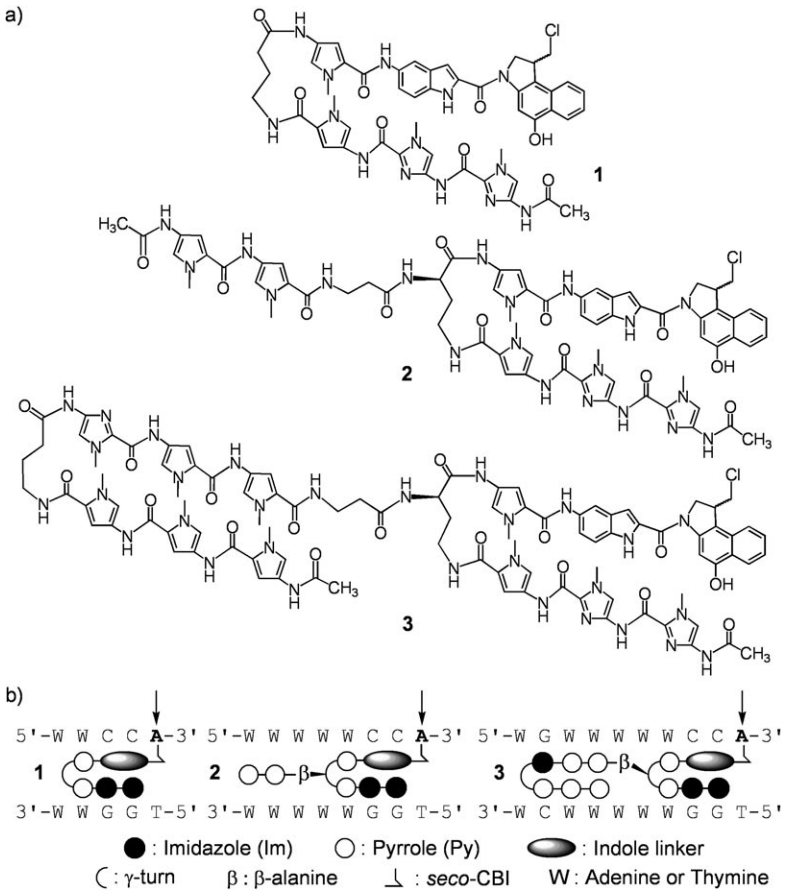


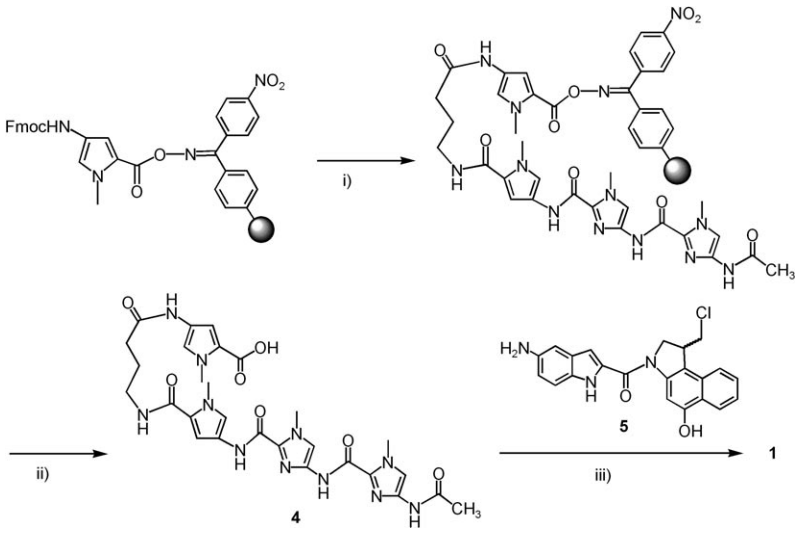
Figure 1. a) Chemical structures of Py–Im conjugates **1**, **2** and **3**. b) Schematic representation of the sequence specific recognition by conjugates **1**, **2** and **3**. Arrows indicate sites of adenine N3 alkylation.

C terminus of two-ring linear polyamides or six-ringhairpin polyamides, respectively, is covalently tethered to the α-amino group of the γ-turn of the alkylating polyamide **1** through β-alanine. High-resolution denaturing polyacrylamide gel electrophoresis using a 5′-Texas-Red-labeled 465 bp DNA fragment revealed that conjugates **2** and **3** alkylate the adenine of target DNA sequences at nanomolar concentrations with 8 and 10 bp recognition, respectively.

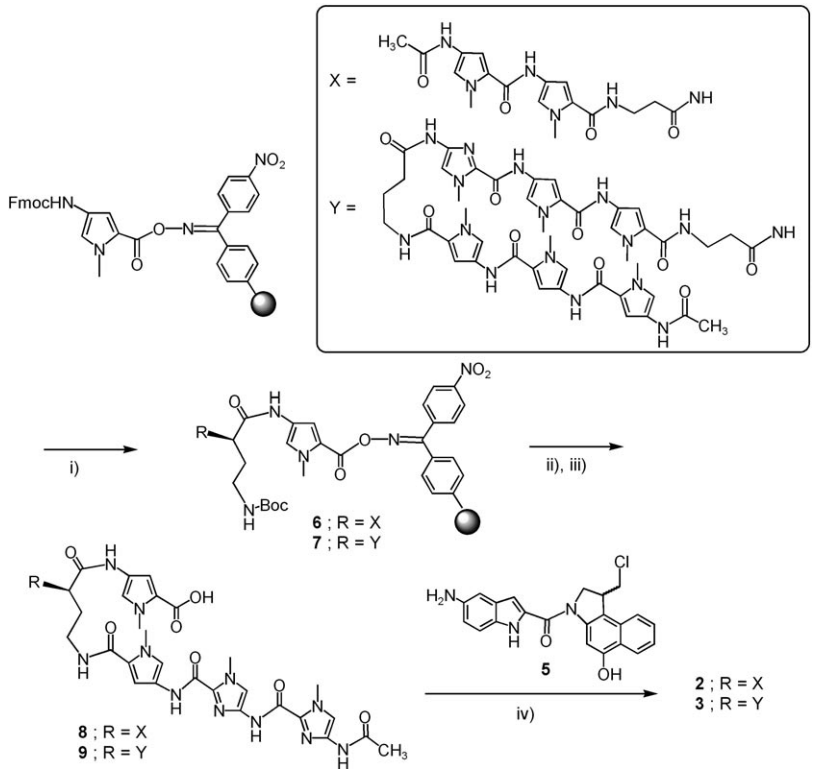
Results and Discussion

Synthesis of conjugates 1–3: Conjugate **1** was synthesized as shown in Scheme 1. The four-ring Py–Im hairpin polyamide **4**, which has a terminal carboxylic acid group, was prepared by fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis by using a Py-coupled oxime resin, followed by treatment with NaOH. The carboxylic acid of **4** was converted to an activating ester by using *O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HCTU), *i*Pr$_2$NEt, followed by coupling with Indole–CBI (**5**), which was synthesized by coupling *seco*-CBI with 5-*tert*-butoxyamino-1*H*-indole-2-carboxylic acid followed by deprotection with trifluoroacetic acid (TFA), to produce **1**.

The synthetic route of conjugates **2** and **3** is shown in Scheme 2. Conjugates **2** and **3** contain (*R*)-2,4-diaminobutyric acid as the γ-turn element that connects the two parts of the recognition Py–Im polyamide. Three-ring polyamide-coupled resin **6** and seven-ring polyamide-coupled resin **7** were prepared by Fmoc solid-phase synthesis. Subsequently, the Boc protecting group of the γ-turn was removed by treatment with TFA, and the Fmoc solid-phase synthesis was continued. Following cleavage of the sample from the resin with NaOH, the six-ring Py–Im Y-shaped



Scheme 1. Synthetic scheme for the preparation of *seco*-CBI conjugate **1** with an indole linker. i) Stepwise reaction by Fmoc solid-phase synthesis; ii) treatment with 1 N NaOH and DMF; iii) HCTU, *i*Pr$_2$NEt, DMF then **5**, *i*Pr$_2$NEt, DMF.



Scheme 2. Synthetic scheme for the preparation of *seco*-CBI conjugates **2** and **3** with an indole linker. i) Stepwise reaction by Fmoc solid-phase synthesis; ii) 20% TFA/DCM; iii) stepwise reaction by Fmoc solid-phase synthesis was followed by processing with 1 N NaOH and DMF; iv) HCTU, iPr$_2$NEt, DMF then **5**, iPr$_2$NEt, DMF.

polyamide **8** and the ten-ring Py–Im tandem polyamide **9** were isolated. Carboxylic acids **8** and **9** were converted to activated esters by using HCTU and iPr$_2$NEt, followed by coupling with Indole–CBI to produce **2** and **3**. The structure of conjugates **4**, **8**, and **9** were identified by using ^{1}H NMR spectroscopy and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). The structures of conjugates **1–3** were confirmed by ESI-TOF-MS after purification by using reverse-phase HPLC. Purified conjugates **1–3** were used for the evaluation of DNA alkylation.

DNA alkylating activities of conjugates 1–3: Sequence-specific DNA alkylation by compounds **1–3** was examined by using 5′-Texas-Red-labeled 465 bp DNA fragments. Sequence specificities were analyzed by high-resolution denaturing polyacrylamide gel electrophoresis by using an automated DNA sequencer, as described previously.[8] Alkylation was carried out at 37 °C for 4.5 h, followed by quenching with calf thymus DNA. The samples were heated at 95 °C under neutral conditions for 20 min. The sites of the N3 alkylation were visualized by thermal cleavage of the DNA strand at the alkylated sites.[9] Under these heating conditions, all alkylation sites are cleaved quantitatively. The cleaved DNA products were observed as bands after electrophoresis. The results of analyses of the alkylated DNA fragments after heat treatment are shown in Figure 2. The 465 bp DNA fragment has three target sites (sites A–C) for conjugate **1**, two target sites (sites A and C) for conjugate **2**, and one target site (site A) for conjugate **3**. Denaturing polyacrylamide gel analysis demonstrated that conjugates **1–3** presented distinct cleavage patterns at nanomolar concentrations. As expected, DNA alkylation by conjugate **1** occurred at three match sites with sequences 5′-AACC*A*-3′ (site A), 5′-AACC*A*-3′ (site B), and 5′-TTCC*A*-3′ (site C) with two minor mismatch alkylation sites at 5′-AGAT*A*-3′ (site 2′) and 5′-CTTT*A*-3′ (site 3′). DNA alkylation by conjugate **2** occurred at two match sites with sequences of 5′-AATAACC*A*-3′ (site A) and 5′-AAATTCC*A*-3′ (site C). DNA alkylation by conjugate **3** occurred mainly at one match site with a sequence of 5′-AGAATAACC*A*-3′ (site A). From the densitometric analysis of the gel electrophoresis, the sequence specificities of conjugates **1–3** at the match sites are at least threefold higher than those at the mismatch sites (Figure 2).

In particular, conjugate **2** showed relatively high alkylating activity and sequence specificity in lanes 7–11. In contrast, we investigated the alkylation of the complementary strand of the 465 bp DNA fragment, the sequence of which does not contain the target sites for conjugates **1–3**, and it was not significantly alkylated by **1–3** (Supporting Information). These results demonstrate that the Y-shaped and tandem hairpin alkylating polyamides **2** and **3** extend the recognition site size of hairpin conjugates **1** from 5 bp to 8 and 10 bp without losing the high alkylating activity and sequence specificity of **1**.

Antitumor activities of conjugates 1–3: To evaluate the cytotoxic potencies of *seco*-CBI conjugates **1–3**, we investigated the 50% cell growth inhibition (GI_{50}) values of conjugates **1–3** by using seven mammalian cell lines as shown in Figure 3.[10] The mammalian cell lines were composed of six human cancer cell lines (Jurkat, Molt-4, Raji, HCT116, 293, WI38) and one mouse fibroblast cell line (M5S). The mean $-\log GI_{50}$ for conjugates **1–3** were 7.66 (21.9 nM), 6.83 (148 nM), and 7.16 (69.2 nM), respectively. These results indicated that conjugates **1–3** have strong cytotoxicities against human cancer cell lines. Further, all of the conjugates **1–3** have particularly strong cytotoxiciy against Jurkat (acute T cell leukemia) and Molt-4 (acute lymphoblastic leukemia)

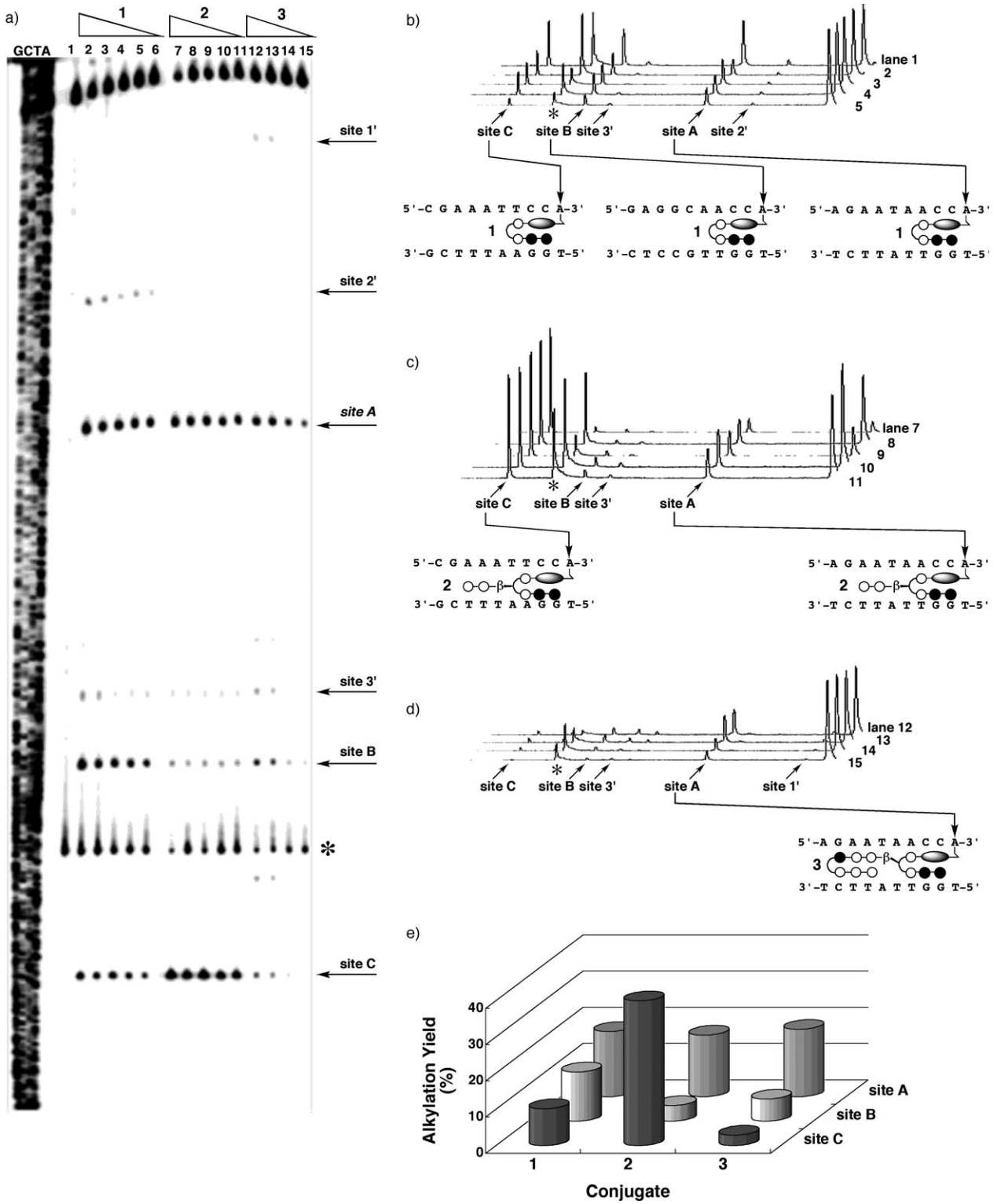


Figure 2. Thermally induced strand cleavage by conjugates **1**, **2** and **3** of 465 bp DNA fragments 5-end-labeled with Texas Red. a) lane 1: DNA control; lanes 2–6: 150, 125, 100, 75, 50 nM of conjugate **1**; lanes 7–11: 150, 125, 100, 75, 50 nM of conjugate **2**; lanes 12–15: 100, 75, 50, 25 nM of conjugate **3**. Arrows indicate sites of DNA alkylation. *The smeared DNA bands are presumably due not to alkylation, but rather to higher order structures of substrate DNA; these bands appeared in the control lane and their appearance depended on the concentration of substrate DNA. Densitometric analyses of lanes 1–6 (b), lanes 7–11 (c), and lanes 12–15 (d). e) The alkylation yield was estimated from the averaged yield of DNA alkylation at match sites, A, B and C by conjugates **1**–**3** at 50 nM, 75 nM and 100 nM. Yields were based on the amount of starting substrate DNA.

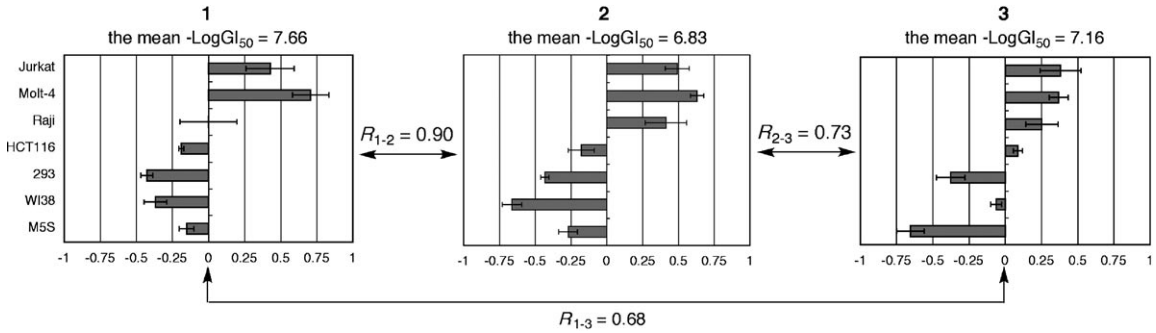


Figure 3. $-\mathrm{Log}\,GI_{50}$ (50% growth inhibition) concentrations of conjugates **1**–**3** for 7 cell lines, respectively, are shown. Columns extending to the right indicate cell lines that were sensitive to the agents and those extending to the left indicate cell lines that were less sensitive to the agents. *R* indicates the correlation coefficients for the mean GI_{50} values of compounds **1**–**3**.

cell lines compared to the other five cell lines. We compared the cytotoxic patterns of conjugates **1**–**3** against seven cell lines. Interestingly, a correlation coefficient value (R_{1-3}= 0.68) between conjugates **1** and **3**, and one other correlation coefficient value (R_{2-3}=0.73) between conjugates **2** and **3**, are lower than the value (R_{1-2}=0.90) between conjugates **1** and **2**. These results suggest that differences in sequence specificity might affect the pattern of cytotoxicities.[8c,10]

To gain an insight into the binding orientation of the tandem motif **3** on the DNA minor groove, we constructed a molecular model of the d(GCAGAATAACCA$_{12}$TG)/d(CATGGTTATTCTGC)-**3** complex (Figure 4). The energy-minimized structures of the binding of **3** to the minor groove of d(GCAGAATAACCA$_{12}$TG)/d(CATGGTTATTCTGC) suggest that the C9 position of the CBI is in close proximity to the nucleophilic N3 of the A_{12} residues: 2.95 Å (Figure 4). The binding orientation of the tandem conjugate **3** implies that the induction of alkylation at a specific 10 bp sequence occurs.

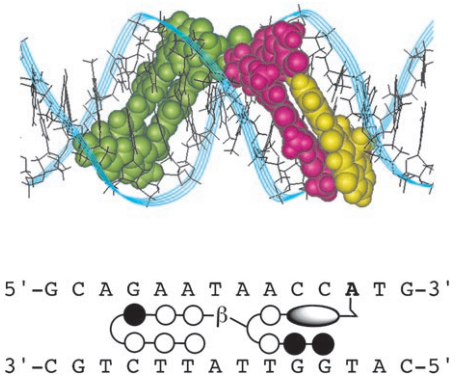


Figure 4. The energy-minimized structure of d(GCAGAATAACCA$_{12}$TG)/d(CATGGTTATTCTGC)-cyclized CBI form of **3**. Minimization was carried out in the presence of 26 sodium cations and a 10 Å layer of H_2O by using a consistent force field. Each strand of the DNA is drawn as a line; the cyclized form of conjugate **3** is drawn as a CPK model. The indole–CBI unit is drawn in yellow, the first hairpin polyamide of conjugate **3** is drawn in purple, and the second hairpin polyamide, which is covalently tethered to the first hairpin polyamide, is drawn in green.

Conclusion

We designed and synthesized Y-shaped and tandem hairpin conjugates **2** and **3**. High-resolution-sequencing gel analysis demonstrated that conjugates **2** and **3** selectively recognize 8 and 10 bp target DNA sequences, respectively, and alkylate the adenine in these sequences at nanomolar concentrations. These results suggest that Y-shaped and tandem motifs of alkylating polyamides could be used to expand target sequences. Furthermore, cytotoxicity assay revealed that conjugates **1**–**3** have strong cell toxicities against human cancer cell lines.

Experimental Section

General methods: Reagents and solvents were purchased from standard suppliers and were used without further purification. Oxime resin (200–400 mesh) was purchased from Novabiochem, California, USA. 1-(Chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole was synthesized and purified by reported methods.[3] ^{1}H NMR spectra were recorded in parts per million (ppm) downfield relative to tetramethylsilane. Electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) was carried out on a BioTOF II (Bruker Daltonics) mass spectrometer. Polyacrylamide gel electrophoresis was performed on a HITACHI 5500-S DNA sequencer. λ-DNA plasmid and Ex Taq DNA polymerase were purchased from Takara Bio, Shiga, Japan; the Thermo Sequenase core sequencing kit and loading dye (*N*,*N*′-dimethylformamide with fuschin red) were from GE Healthcare Bio-Sciences, Buckinghamshire, England; 5′-Texas-Red-modified DNA oligonucleotides (21-mer) was purchased from Sigma-Aldrich, St. Louis, USA; and 50% Long Ranger™ gel solution was from FMC Bioproducts, Philadelphia, USA.

AcImImPy-γ-Py-indole-*seco*-CBI (1): The four-ring Py–Im polyamide-coupled oxime resin (AcImImPy-γ-Py-CO_2-oxime resin) was synthesized in a stepwise reaction by Fmoc solid-phase methods on the Py-coupled oxime resin with Fmoc-Py-CO_2H and Fmoc-Im-CO_2H monomers. A sample resin was cleaved from the oxime resin to yield primary carboxylic acid under alkali conditions (1 N NaOH 2 mL and DMF 2 mL, 1 h, 55 °C) leading to **4** (53 mg, 83%) as a yellow powder. Compound **4** was rinsed with 1% aq HCl and used in the next coupling step without further purification. *i*Pr$_2$NEt (11 μL, 63 μmol) and HCTU (26 mg, 63 μmol) were added to a solution of **4** (20 mg, 31 μmol) in DMF (200 μL), and the reaction mixture was stirred for 4.5 h at room temperature. After the conversion from **4** to the activated ester was confirmed by HPLC and ESI-TOF-MS analysis, NH_2-Indole-*seco*-CBI (**5**; 18 mg, 46 μmol) and *i*Pr$_2$NEt (11 μL, 63 μmol) were added to the reaction vessel. The reaction

mixture was stirred overnight at room temperature under a N_2 atmosphere. Evaporation of the solvent gave a yellow residue, which was washed with Et_2O (2×2 mL) to produce conjugate **1** as a yellow powder, which was further purified by HPLC, by using a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN, 0–50% linear gradient, 0–40 min, 254 nm). Yield: 3.7 mg (12%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ= 11.66 (s, 1H; NH), 10.43 (s, 1H; OH), 10.30 (s, 1H; NH), 10.27 (s, 1H; NH), 9.86 (s, 1H; NH), 9.77 (s, 1H; NH), 9.36 (s, 1H; NH), 8.13 (s, 1H; CH), 8.10 (s, 1H; CH), 8.08 (s, 1H; NH), 8.03 (d, J=8.0 Hz, 1H; CH), 7.96 (brs, 1H; CH), 7.85 (d, J=8.4 Hz, 1H; CH), 7.56 (s, 1H; CH), 7.52 (t, J=8.0 Hz, 1H; CH), 7.50 (s, 1H; CH), 7.39 (d, J=8.0 Hz, 1H; CH), 7.35 (t, J=7.5 Hz, 1H; CH), 7.21 (s, 2H; CH), 7.16 (s, 1H; CH), 7.00 (d, J=1.6 Hz, 1H; CH), 6.93 (d, J=1.6 Hz, 1H; CH), 4.80 (t, J=10.0 Hz, 1H; CH), 4.55 (d, J=11.0 Hz, 1H; CH), 4.22 (brs, 1H; CH), 3.99 (s, 3H; CH_3), 3.97 (s, 3H; CH_3), 3.84 (s, 3H; CH_3), 3.77 (s, 3H; CH_3), 3.76 (m, 2H; CH_2), 3.28 (m, 2H; CH_2), 2.29 (m, 2H; CH_2), 2.03 (s, 3H; CH_3), 1.79 ppm (m, 2H; CH_2); ESI-TOF-MS: m/z calcd for $C_{50}H_{50}ClN_{14}O_9$: 1009.35; found: 1009.43 $[M+H]^+$.

AcImImPy-(*R*)[PyPy-β]^{HN}γ-Py-indole-*seco*-CBI (2): Compound **2** was prepared by using a synthetic procedure similar to that used for the preparation of compound **1** from **4**, by starting from Py–Im polyamide carboxylic acid **8**. iPr$_2$NEt (3.0 μL, 17 μmol) and HCTU (4.0 mg, 9.7 μmol) were added to a solution of conjugate **8** (8.4 mg, 8.6 μmol) in DMF (150 μL), and the reaction mixture was stirred for 5 h at room temperature. After the conversion from conjugate **8** to the activated ester was confirmed by HPLC and ESI-MS analysis, NH_2-Indole-*seco*-CBI (**5**) (5.1 mg, 13 μmol) and iPr$_2$NEt (3.0 mL, 17 μmol) were added to the reaction vessel. The reaction mixture was stirred overnight at room temperature under a N_2 atmosphere. Evaporation of the solvent gave a yellow residue, which was washed with Et_2O (2×2 mL) to give conjugate **2** as a yellow powder, which was further purified by HPLC by using a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 25–75% linear gradient, 0–30 min, 254 nm). Yield: 2.1 mg (18%); ESI-TOF-MS: m/z calcd for $C_{67}H_{70}ClN_{20}O_{12}$: 691.26; found: 691.36 $[M+2H]^{2+}$.

AcImImPy-(*R*)-[PyPyPy-γ-ImPyPy-β]^{HN}γ-Py-indole-*seco*-CBI (3): A synthetic procedure similar to that used for the preparation of compound **2** was followed to prepare compound **3** (6.9 mg, 4.0%). ESI-TOF-MS: m/z calcd for $C_{94}H_{100}ClN_{30}O_{17}$: 978.37; found: 978.54 $[M+2H]^{2+}$.

AcImImPy-γ-Py-CO$_2$H (4): Compound **4** was synthesized in a stepwise reaction by Fmoc solid-phase methods on the Py-coupled resin (51 mg, 80% in 4 couplings). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.32 (s, 1H; NH), 10.26 (s, 1H; NH), 9.84 (s, 1H; NH), 9.76 (s, 1H; NH), 8.07 (s, 1H; NH), 7.56 (s, 1H; CH), 7.49 (s, 1H; CH), 7.28 (d, J=2.5 Hz, 1H; CH), 7.21 (d, J=2.0 Hz, 1H; CH), 6.99 (d, J=2.5 Hz, 1H; CH), 6.64 (d, J=2.0 Hz, 1H; CH), 3.99 (s, 3H; CH_3), 3.97 (s, 3H; CH_3), 3.79 (s, 3H; CH_3), 3.78 (s, 3H; CH_3), 3.19 (m, 2H; CH_2), 2.25 (m, 2H; CH_2), 2.03 (s, 3H; CH_3), 1.76 ppm (m, 2H; CH_2); ESI-TOF-MS: m/z calcd for $C_{28}H_{34}N_{11}O_7$: 636.26; found: 636.40 $[M+H]^+$.

NH$_2$-Indole-*seco*-CBI (5): Compound **5** was prepared by the reported procedure.[11]

AcImImPy-(*R*)-[PyPy-β]^{HN}γ-Py-CO$_2$H (8): The three-ring Py–Im polyamide-coupled oxime resin **6** was prepared by Fmoc solid-phase synthesis using oxime resin (170 mg, 0.1 mmol). A solution of TFA in CH_2Cl_2 (20% TFA (1 mL) in CH_2Cl_2 (4 mL)) was added to polyamide-coupled resin **6**, and the reaction mixture was stirred for 30 min at room temperature. After 30 min, deprotected conjugate **6** was filtrated by using a disposal tube and washed with CH_2Cl_2 (2×2 mL) and DMF (2 mL×2). Subsequently, **6** was extended from the deprotected amine by Fmoc solid-phase synthesis again. A sample resin was cleaved under alkali conditions (1N NaOH (2 mL) in DMF (2 mL), 1 h, 55°C) to yield primary carboxylic acid **8**. The solution of **8** was concentrated and washed by 1% aq HCl (100 mg, 99% in 7 couplings), and used in the next coupling step without further purification. ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.32 (s, 1H; NH), 10.26 (s, 1H; NH), 9.84 (s, 1H; NH), 9.81 (s, 1H; NH), 9.33 (s, 1H; NH), 8.27 (d, 1H; NH), 7.99 (m, 1H; NH), 7.94 (m, 1H; NH), 7.55 (s, 1H; CH), 7.50 (s, 1H; CH), 7.29 (s, 1H; CH), 7.22 (s, 1H; CH), 7.18 (s, 1H; CH), 7.13 (s, 1H; CH), 6.97 (s, 1H; CH), 6.82 (m, 2H; CH), 6.73 (s, 1H; CH), 4.39 (m, 1H; CH), 3.99 (s, 3H; CH_3), 3.97 (s, 3H; CH_3), 3.79 (s, 12H; CH_3), 3.21 (m, 2H; CH_2), 3.00 (m, 2H; CH_2), 2.32 (m, 2H; CH_2), 2.03 (s, 3H; CH_3), 2.01 (s, 3H; CH_3), 1.78 ppm (m, 2H; CH_2); ESI-TOF-MS: m/z calcd for $C_{45}H_{54}N_{17}O_{11}$: 1008.41; found: 1008.47 $[M+H]^+$.

AcImImPy-(*R*)-[PyPyPy-γ-ImPyPy-β]^{HN}γ-Py-CO$_2$H (9): A synthetic procedure similar to that used for the preparation of compound **8** was followed to prepare compound **9** (146 mg, 92% in 12 couplings). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.30 (s, 1H; NH), 10.27 (s, 1H; NH), 10.25 (s, 1H; NH), 9.99 (s, 1H; NH), 9.95 (s, 1H; NH), 9.88 (s, 3H; NH), 9.80 (s, 1H; NH), 9.35 (s, 1H; NH), 8.26 (d, J=7.6 Hz, 1H; NH), 8.03 (t, J=5.6 Hz, 2H; NH), 7.96 (t, J=5.2 Hz, 1H; NH), 7.55 (s, 1H; CH), 7.49 (s, 1H; CH), 7.44 (s, 1H; CH), 7.29 (d, J=2.0 Hz, 1H; CH), 7.25 (d, J=1.2 Hz, 1H; CH), 7.22 (s, 2H; CH), 7.18 (d, J=1.2 Hz, 1H; CH), 7.16 (d, J=1.2 Hz, 1H; CH), 7.13 (d, J=1.6 Hz, 1H; CH), 7.11 (s, 1H; CH), 7.03 (d, J=1.6 Hz, 1H; CH), 6.97 (d, J=1.6 Hz, 1H; CH), 6.89 (d, J=1.6 Hz, 1H; CH), 6.85 (s, 2H; CH), 6.72 (d, J=2.0 Hz, 1H; CH), 4.40 (m, 1H; CH), 3.98 (s, 3H; NCH_3), 3.97 (s, 3H; NCH_3), 3.94 (s, 3H; NCH_3), 3.83 (s, 6H; NCH_3), 3.82 (s, 3H; NCH_3), 3.79 (s, 12H; NCH_3), 3.39 (m, 2H; CH_2), 3.20 (m, 2H; CH_2), 2.45 (m, 2H; CH_2), 2.35 (m, 4H; CH_2), 2.03 (s, 3H; CH_3) 1.90 (s, 3H; CH_3) 1.78 ppm (m, 4H; CH_2). ESI-TOF-MS: m/z calcd for $C_{77}H_{90}N_{31}O_{17}$: 791.83; found: 791.97 $[M+2H]^{2+}$.

Preparation of 5′-Texas-Red-modified DNA fragment: The 5′-Texas-Red-modified 465 bp DNA fragments,λ-DNA F12612*-13076, was prepared by polymerase chain reaction (PCR) with 5′-Texas-Red-modified-TTCTGCTTAAGCAGGCAATGG-3′ (λ-DNA forward, 12612–12632) and 5′-CACCATCACGCATCTGTGTCT-3′ (λ-DNA reverse, 35426–35446). Fragments were purified by GenElute™ PCR clean-up kit (Sigma Aldrich), diluted to 64.5 ng μL^{-1}, stored at −20°C, and used within one week. The complementary strand of 465 bp DNA was prepared by similar methods that were used for the preparation of 465 bp DNA fragments; it was diluted to 62.3 ng μL^{-1}, stored at −20°C, and used within one week. The asterisk indicates the Texas Red modification and the nucleotide numbering starts with the replication site.

High-resolution gel electrophoresis: The 5′-Texas-Red-labelled DNA fragments were alkylated by various concentrations of **1**, **2**, and **3** in sodium phosphate buffer (5 mM, 10 μL, pH 7.0) that contained 10% DMF at 37°C for 4.5 h. The reaction was quenched by the addition of calf thymus DNA (1 mM, 1 μL), and was heated for 5 min at 95°C. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in loading dye (5 μL; formamide with fuschsin red), heated at 95°C for 20 min, and then immediately placed on ice. The 2 μL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a HITACHI SQ5500-E DNA Sequencer.

Analysis of growth inhibition against seven mammalian cell lines: Detailed assay procedures have been already reported.[10] The mammalian cell lines used were Jurkat (human T cell leukaemia), Molt-4 (human acute lymphoblastic leukemia), Raji (human Burkitt lymphoma), HCT116 (human colon carcinoma), 293 (human kidney epithelial), WI38 (human embryonic lung fibroblast), and M5S (mouse near-diploid fibroblast). The cell lines Jurkat, Molt-4, Raji, and HCT116 cell lines were cultured in RPMI-1640 medium that was supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 IU mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cell lines 293, WI38, and M5S were cultured in Dulbecco's modified Eagle's medium that was supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}). The cells were exposed to drugs for 48 h. Colorimetric assays that used WST-8 (Dojindo, Kumamoto, Japan) were carried out in 96-well plates. The cells were plated in each well at approximately 15% confluence in of culture medium (50 μL). One day later, when the cells were in the logarithmic growth phase, the medium was changed to fresh medium (100 μL) that contained various concentrations of the compounds **1**–**3** and 0.1% DMF (50 μL). After treatment with the compounds for 48 h, WST-8 reagent (10 μL) was added to each well, and the cells were incubated for 2 h at 37°C. Absorbance was then measured at 450 and 600 nm by using an MPR-A4I microplate reader (Tosoh, Tokyo, Japan). The absorbance of the control (C), the treated wells (T) and treated wells at time 0 (t_0) were measured. The GI_{50} was calculated as $100\times[(T\text{-}t_0)/(C-T_0)]=50$. The mean graphs, which show the differential

growth inhibition of the drugs in the cell line panel, were drawn based on a calculation by using a set of GI_{50}.[12] Pearson correlation coefficients (R) were calculated by using the following formula: $R=(\Sigma(x_i-x_m)-(y_i-y_m))/(\Sigma(x_i-x_m)^2\Sigma(y_i-y_m)^2)^{1/2}$, in which x_i and y_i are log GI_{50} of drug A and drug B, respectively, against each cell line, and x_m and y_m are the mean values of x_i and y_i, respectively.

Molecular modeling studies: Molecular modeling was performed with the Insight II (2005) Discover (Accelrys, San Diego, CA) program, by using CFF force field parameters. The B-form DNA, which consisted of d(GCAGAATAACCA$_{12}$TG)/d(CATGGTTATTCTGC), was constructed by using the builder module of Insight II with standard B-form helical parameters (pitch, 3.38 Å; twist, 36; tilt, 1°). Conjugate **3** was built by using the NMR spectroscopy structure of ImPyPy-γ-PyPyPy-d(CGCTAACAGGC)/d(GCCTGTTAGCG) complex[13] and the Duo-Dist-octamer complex, by using standard bond lengths and angles.[14] Docking was performed virtually by adjusting the DNA structure to permit minor-groove binding according to the NMR spectroscopy structure of the ImPyPy-γ-PyPyPy-5′-d(CGCTAACAGGC)-3′/5′-d(GCCTGTTAGCG)-3′ complex.[13] The assembled initial structure was energy minimized with distance constraints of the putative hydrogen bonds, such as for the amido H and O2 of a pyrimidine, or the N3 of a purine base, the N3 of imidazole and the $2NH_2$ of guanine and the fourteen Watson–Crick base pairs. The minimization was carried out by using a distance-dependent dielectric constant of $\varepsilon=4r$ (r stands for the distance between two atoms) and with convergence criteria having an RMS gradient of less than 0.001 kcal mol^{-1} Å. Twenty-six 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 water was minimized first to the stage where the RMS was less than 0.001 kcal mol^{-1} Å, and then the whole system was minimized without any constraint under the same conditions.

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