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Synthesis of Pyrrole–Imidazole Polyamide *seco*-1-Chloromethyl-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole Conjugates with a Vinyl Linker Recognizing a 7 bp DNA Sequence

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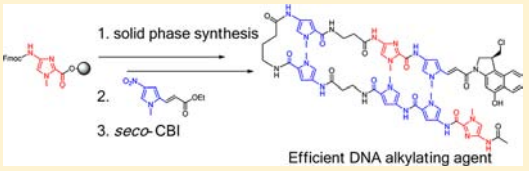
Supporting Information

ABSTRACT: Convergent synthetic routes for *N*-methylpyrrole (P) and *N*-methylimidazole (I) *seco*-1-chloromethyl-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (CBI) conjugates with a vinyl linker were developed. New hairpin polyamide–*seco*-CBI conjugates, compounds **16**–**19**, were synthesized, and their DNA sequence-specific alkylating activities were evaluated via high-resolution denaturing gel electrophoresis and high-performance liquid chromatography (HPLC) product analysis. The new synthetic route for PI conjugates with a vinyl linker consisted of the introduction of a vinylpyrrole unit (**8**–**11**) into the C terminal of a PI polyamide synthesized by (fluorenylmethoxy)carbonyl solid-phase peptide synthesis (SPPS), followed by liquid-phase coupling with *seco*-CBI. The yield of the conjugates was significantly improved compared with that of the method reported previously, which allows us to synthesize various substituted conjugates containing a vinyl linker. Conjugates **16**–**19** were designed to investigate the substituent effect of the vinyl linker, and conjugate **16*S*** was synthesized to evaluate the reactivity between racemic and *S* enantiomers of the *seco*-CBI derivative. The results of high-resolution denaturing gel electrophoresis using 208 bp DNA fragments indicated that alkylation by compounds **16** and **17**, in which the H of the vinyl linker of compound **16** was replaced with F, occurred predominantly at the A of the 5′-TTTGTCA-3′ sequence at nanomolar concentrations. In clear contrast, compounds **18** and **19**, which were methyl or Br derivatives of compound **16**, did not exhibit any DNA alkylating activity. Moreover, HPLC product analysis using synthetic oligonucleotides demonstrated that alkylation occurred between the N3 of the adenine of the oligomer and the cyclopropane ring of **16*S***. Density functional calculation of substituted vinylpyrrole *seco*-CBI units indicated that methyl and Br substituents led to a significantly distorted geometry of the vinyl group with the pyrrole ring compared with H and F derivatives. Molecular modeling studies offered the additional information that steric hindrance reduced the DNA alkylating activity of these derivatives.



INTRODUCTION

In recent years, the mechanisms of many diseases, including cancer, have been understood at the DNA sequence level. Biological characteristics, such as genetic polymorphisms and gene-expression profiles, are closely related to drug responses; hence, tailor-made therapies might be realized using a knowledge-based design.[1−3] For this reason, small DNA-binding molecules, including DNA alkylating agents,[4,5] are being widely studied as a new type of knowledge-based chemotherapy.[6−8] Dervan et al. demonstrated that *N*-methylpyrrole (P) and *N*-methylimidazole (I) polyamides, as minor-groove-binding molecules, recognize each of the four Watson–Crick base pairs uniquely.[9] Antiparallel pairing of I opposite P (I/P) recognizes a G-C base pair, whereas antiparallel pairing of P/P recognizes A-T or T-A base pairs.[10] For efficient binding, the introduction of a *β*-alanine acts as an aliphatic substitute for a P ring.[11] These PI polyamides have strong binding affinity and sequence selectivity for targeting DNA sequences, comparable to those of transcription factors. Using these polyamides as DNA-binding moieties, we and other groups have developed sequence-specific DNA alkylating agents via coupling with alkylating moieties.[12−14] We demonstrated that PI polyamide 1-chloromethyl-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (CBI)[15] conjugates act as highly sequence-specific alkylating agents via introduction of various motifs, such as hairpins, homodimers, and heterodimers, to extend the recognition sequence to 10 base pairs (bp). Initially, we found that insertion of the vinyl linker between the carboxylic acid of the PI moiety and the alkylating agents dramatically enhanced their DNA alkylating activity by adjusting the reacting position.[16] We demonstrated that conjugates such as **1** efficiently alkylated at the adenine (A) of the matching sequences, 5′-WGWCCA-3′, in DNA fragments (Figure 1a).[17] However, it was difficult to synthesize conjugates that process the vinyl linker because of its instability. For example, methods reported previously for conjugate **1** required time-consuming liquid-phase synthesis at all steps, to avoid unwanted side reactions. To overcome this problem, we introduced an indole linker between the

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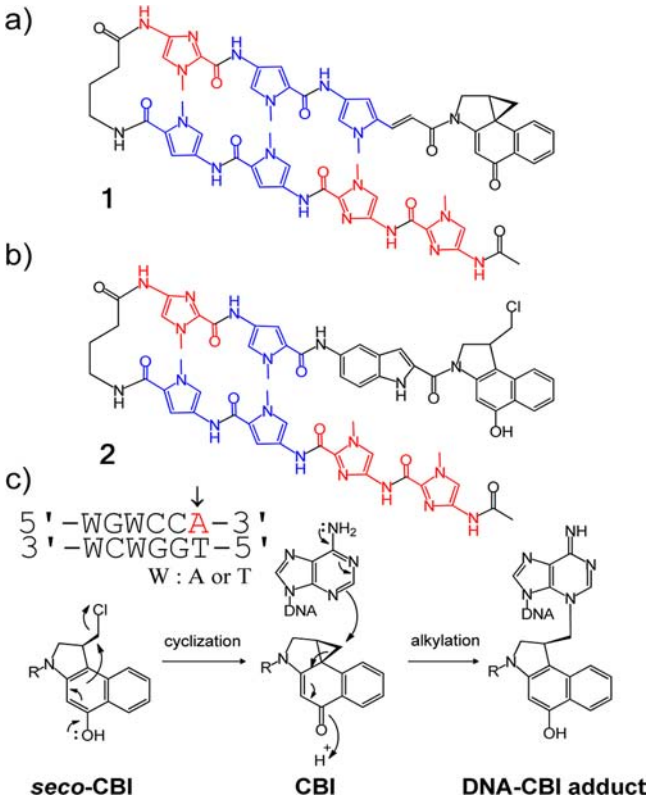

Figure 1. Chemical structures of PI polyamide *seco*-CBI conjugates of (a) **1**[17] and (b) **2**.[18] (c) Chemical mechanism of DNA N3 alkylation by *seco*-CBI via the formation of CBI. The arrow indicates the sequence-specific site of adenine N3 alkylation by **1** and **2**.

polyamide and CBI or *seco*-CBI. Conjugates such as **2** are stable and easy to synthesize from a PI polyamide prepared using a solid-phase synthesizer, which enabled us to synthesize various motifs of PI polyamides (Figure 1b).[18] However, their DNA alkylating activity was lower than that of conjugates with a vinyl linker; e.g., in the presence of ~10 nM DNA, a 2.5 nM concentration of the conjugates with a vinyl linker yielded a strong alkylating band after 1 h, whereas a concentration of 25 nM and a 12 h incubation period were necessary to obtain a similar extent of alkylation using the conjugates with an indole linker.

To overcome the difficulties associated with the synthesis of conjugates with a vinyl linker, we herein report a new synthetic route to PI conjugates with a vinyl linker in which a vinylpyrrole unit (**8**–**11**) is introduced into the C terminal of a PI polyamide synthesized via (fluorenylmethoxy)carbonyl (Fmoc) solid-phase peptide synthesis (SPPS), followed by liquid-phase coupling with *seco*-CBI. This new synthetic route is more efficient than the previously reported method[17] in terms of total yield of the conjugate, and it can be applied to various types of PI sequences and vinyl analogues. As a demonstration of the general utility of this method, we synthesized five conjugates (**16**–**19** and **16*S***). Conjugates **16**–**19** were synthesized to evaluate the substituent effect of the vinyl linker, and **16*S*** was synthesized to evaluate the reactivity between racemic and enantiopure *seco*-CBI derivatives. Each conjugate was examined for its DNA alkylating activity by polyacrylamide gel electrophoresis (PAGE), and some conjugates were also examined via high-performance liquid chromatography (HPLC) product analysis.

RESULTS AND DISCUSSION

Synthesis of Conjugates 16–19 and 16*S*. We synthesized the hairpin polyamide *seco*-CBI conjugates **16**–**19** and **16*S*** as shown in Scheme 1. Polyamide **3** was synthesized via Fmoc SPPS using CLEAR-Acid resin and cleavage using trifluoroacetic acid (TFA). The detached crude **3** was used in the next step without further purification. Reduction of compounds **4**–**7** was achieved using Pd–C and sodium borohydride ($NaBH_4$), to generate compounds **8**–**11**, which were directly coupled with the polyamide **3** using benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) and *N*,*N*-diisopropylethylamine (DIEA), to produce the ethyl esters of polyamides **12**–**15**. The crude polyamides **12**–**15** were triturated by addition of diethyl ether and then washed with ethyl acetate. The ethyl esters were hydrolyzed to obtain the key carboxylic acids **12**–**15** using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and H_2O. Finally, *seco*-CBI was coupled with polyamides **12**–**15** using PyBOP and DIEA, to synthesize conjugates **16**–**19** and **16*S***. After purification via HPLC, these conjugates were used in DNA alkylation studies. Using this new synthetic scheme, we improved the total yield of **16**–**19** compared with that obtained in the methods reported previously, which used liquid-phase synthesis.[16,17] For example, conjugate **16** was synthesized from **4** with an overall yield of 11.1%, which is significantly higher than the previous yield for the syntheses of conjugates **1** (0.1%)[17] and **2** (6.4%).[18]

Evaluation of DNA Alkylating Activity Using DNA Fragments of 208 bp. We investigated the DNA alkylating ability and sequence specificity of conjugates **16**–**19** and **16*S*** on a 5′ Texas Red-labeled 208 bp DNA fragment using an automated DNA sequencer. DNA alkylation was carried out at 23 °C for 1 h, followed by quenching with calf thymus DNA. The samples were heated at 95 °C for 5 min. Under these heating conditions, all purine N3 alkylated sites in DNA molecules yielded cleavage bands on the gel. Figure 2a shows DNA alkylation by conjugates **16*S*** and **16**–**19**. A 2.5 nM concentration of **16** and **17** yielded a strong alkylating band after 1 h, with no DNA alkylation of any mismatch sites within this fragment. Compared with conjugates with an indole linker, which require a concentration of 25 nM and 12 h incubation period to achieve a similar extent of alkylation, these conjugates exhibited very strong DNA alkylating activities. In clear contrast, conjugates **18** and **19** did not show any DNA alkylating activity. These results suggest that the steric hindrance of a vinyl moiety as the substituent group reduces the DNA alkylating activity (**16** and **17** vs **18** and **19**). A detailed discussion of these findings based on molecular modeling studies will be provided later. Moreover, the enantiomerically pure **16*S*** alkylated the target sequences more efficiently. In particular, at 2.5 nM, its efficiency of alkylation reached 66%, measuring the amount of DNA cleavage divided by the amount of agent used. Densitometric analysis revealed that the alkylation yield of **16*S*** was approximately twice that of **16**. These results suggest that *S*-*seco*-CBI alkylates DNA much more efficiently than the *R*-*seco*-CBI isomer, which apparently shows minimal cleavage. These results are consistent with previous findings.[17]

HPLC Analysis of DNA Alkylation by Conjugates 16 and 16*S*. To obtain further information on the chemistry of alkylation, the reaction of an oligomer duplex with conjugate **16*S*** was examined via HPLC product analysis. We investigated

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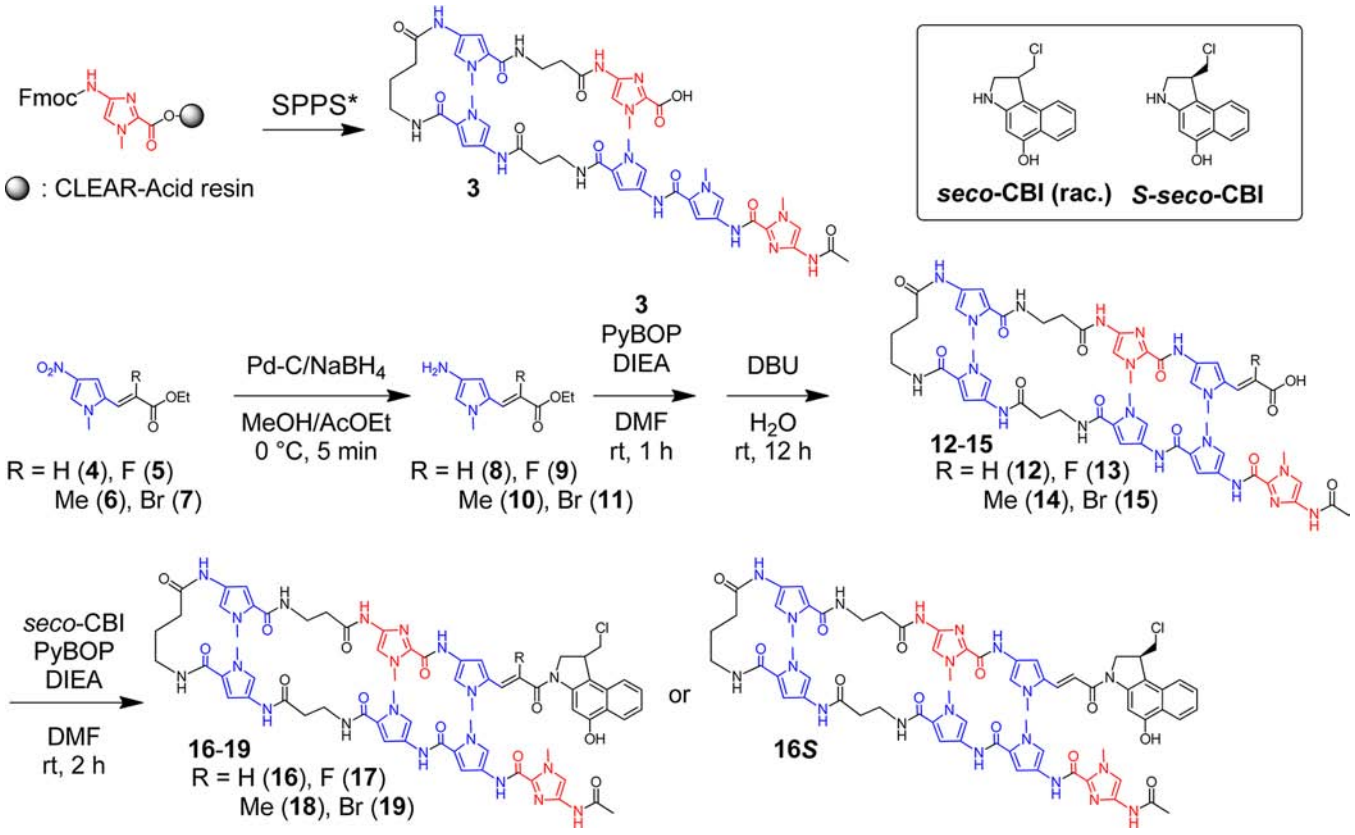
Scheme 1. Synthetic Route to PI Hairpin *seco*-CBI Conjugates 16−19 and 16*S*



*SPPS = solid-phase peptide synthesis.

the alkylation of the duplex oligonucleotide 5′-CGCTTTGTCACGC-3′ (ODN1)/5′-GCGTGACAAAGCG-3′ (ODN2), which was designed according to the results of the PAGE analysis described above. HPLC analysis of the reaction mixtures containing ODN1/ODN2 and **16*S*** after 5 min revealed that conjugate **16*S*** and ODN1 containing the target sequence were consumed, with the formation of a new peak (retention time, 22.3 min; Figure 3b−d). The new peak was determined to be that of the ODN1−**16*S*** adduct by electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) (Figure 3e).

The ODN1−**16*S*** adduct obtained was determined using a procedure reported previously.[19] The adduct was heated at 90 °C for 10 min, to yield an abasic-site-containing ODN1 and a **16*S***−A adduct. These products were analyzed by HPLC (Figure S1). Quantitative HPLC analysis indicated that ODN1 was consumed to 33, 57, and 81% after 5 min, 1 h, and 16 h, respectively. Based on these results, we calculated that the efficiency of alkylation by **16*S*** reached 20, 36, and 76% after 5 min, 1 h, and 16 h, respectively. HPLC analysis indicated that the complementary ODN2 was not consumed during the alkylation reaction. The results therefore demonstrated that **16*S*** selectively alkylated N3 of adenine in the recognition sequence of the ODN1/ODN2 duplex. We also observed another new peak with a retention time of 39.7 min (Figure 3b−d). ESI-TOFMS analysis suggested that this was the peak for the cyclized CBI product of the unreacted conjugate (**16*S*′**, Figure S2). These results are consistent with the similar result that *seco*-CBI derivatives react with the target adenine after the cyclization.[20] The racemic *seco*-CBI conjugate **16** gave the same adduct with a yield of 16, 19, and 25% after 5 min, 1 h, and 16 h, respectively, which represented a lower efficiency than that of **16*S*** (Figure S3). These results are consistent with those of the PAGE analysis and the previous results.

Conformational Analysis of the CBI-Vinyl Moiety. To gain insight into the origin of the varying reactivity of conjugates **16**−**19**, we carried out a conformational analysis of the CBI-vinyl moiety. Since we showed that DNA alkylation by *seco*-CBI occurred through formation of CBI with cyclopropane, we constructed a binding model of cyclized CBI-vinyl motifs. The optimized structures obtained by using B3LYP density functional theory calculation at the 6-31G(d,p) level (Gaussian 09W)[21] are shown in Figure 4b (for a stereo image of each model, see Figure S4 in the Supporting Information). From these structures, the dihedral angle (χ_1) of the analogues of **16** and **17** is −179.38° and −171.20°, respectively (Figure 4a). The results clearly indicate that these analogues retain a planar conformation of the CBI-vinyl moiety (Figure 4b). In contrast, the analogues of **18** and **19** exhibited a distorted conformation at the position of the C2−C3 bond due to the steric hindrance resulting from the bulky substituent; as a result, the cyclopropane subunit was positioned far from the adenine N3, thus reducing the DNA alkylating activity of these analogues. These results fit well with those of the PAGE analysis of **16**−**19**.

Molecular Modeling Studies of Conjugate 16*S*′. To gain insight into the varying reactivity of conjugates **16**−**19**, we carried out molecular modeling studies of d(CGCTTTGTCACGC) ODN1/d(GCGTGACAAAGCG) ODN2−**16*S*′** complex, as shown in Figure 5 (for the stereo

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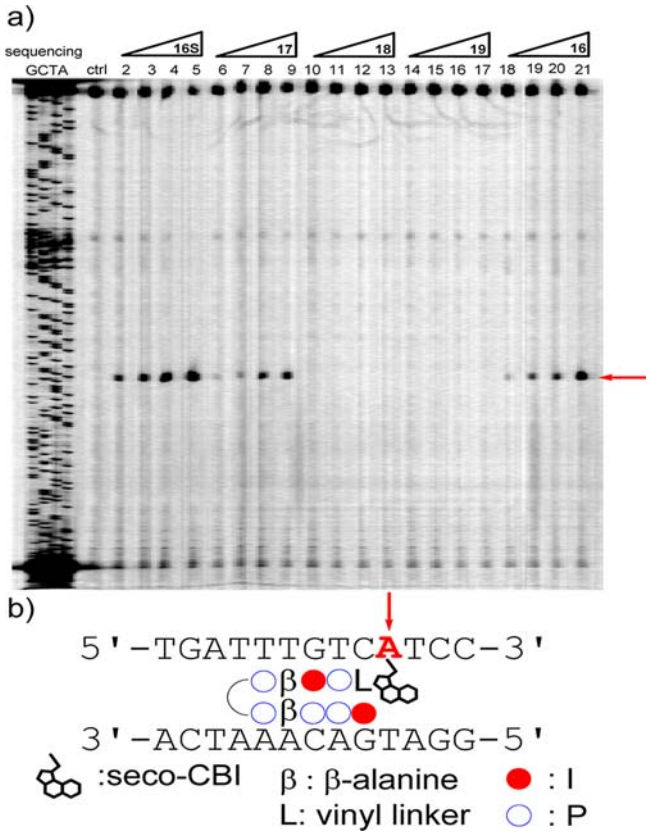

Figure 2. (a) Thermally induced strand cleavage of the 5′ Texas Red-labeled 208 bp DNA fragment (6 nM) by conjugates **16*S*** and **16**−**19** via incubation for 1 h at 23 °C: lane 1, DNA control; lanes 2−5, 2.5, 5, 10, and 20 nM **16*S***; lanes 6−9, 2.5, 5, 10, and 20 nM **17**; lanes 10−13, 2.5, 5, 10, and 20 nM **18**; lanes 14−17, 2.5, 5, 10, and 20 nM **19**; and lanes 18−21, 2.5, 5, 10, and 20 nM **16**. Alkylation site is shown with a red arrow. (b) Schematic representation of sequence-specific alkylation by conjugates **16**−**19**.

image, see Figure S5 in the Supporting Information). The overall energy-minimized structure (Figure 5) shows that the **16*S*′** conjugate bound snugly to the minor groove of the helix. We found that conjugate **16*S*′** retained a planar conformation, which kept it close to the nucleophilic nitrogen of the adenine. The structure of the ODN1/ODN2−**16*S*′** complex indicated that the distance between the cyclopropane subunit of CBI and the nucleophilic adenine N3 was only 2.88 Å, and that the dihedral angle (χ_1, cf. Figure 4) was −178.43°, which implies a better orientation for alkylation. In contrast, the corresponding distance and dihedral angle of conjugate **18** were 3.08 Å and −145.77°, because of the distortion of the C2−C3 bond (Figure 4c). The distance and dihedral angle of other conjugates are 2.95 Å/−171.23° for **17** and 3.27 Å/−152.55° for **19**. These differences might explain the low reactivity of **18** and **19**.

CONCLUSIONS

In this study, we developed a synthetic scheme for PI polyamide *seco*-CBI conjugates with a vinyl analogue. Compared with the method reported previously, this scheme dramatically improved the total yield, by ∼100 times. Additionally, we succeeded in inserting a substituent group into the vinyl moiety and expanding the PI sequences (**16**−**19**). Conjugates **16** and **17** exhibited high DNA alkylating activity, even at a concentration of 2.5 nM. Conversely, conjugates **18** and **19**, which were modified by the addition of a bulky substituent group, exhibited a dramatic reduction in DNA alkylating activity because of steric hindrance. Moreover, the efficiency of the enantiopure *S-seco*-CBI coupled to conjugate **16*S*** was displayed in HPLC and PAGE analyses. This novel synthetic method will simplify the synthesis of various types of conjugates with potent and sequence-selective DNA alkylating ability, including one with a longer DNA sequence recognition. We are currently investigating more potent DNA alkylation methods, such as cross-link alkylation.

MATERIALS AND METHODS

Materials. Reagents and solvents were purchased from standard suppliers and used without further purification. Silica gel thin-layer chromatography was performed using Silica Gel 70 PF_{254} plates from Wako; plates were visualized by UV illumination. ^{1}H NMR spectra were recorded with a JEOL JNM ECA-600 spectrometer operating at 600 MHz for ^{1}H NMR; the spectra were recorded in parts per million (ppm) downfield relative to tetramethylsilane used as an internal standard. The following abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). HPLC analysis was performed with a Shimadzu LC-10AD liquid chromatograph pump, an SPD-10A UV/vis detector, and a Chemcobond 5-ODS-H reversed-phase column (4.6 × 150 mm) in 0.1% TFA in water with CH_3CN as eluent at a flow rate of 1.0 mL/min, and a linear gradient elution of 0−100% CH_3CN over 20 min with detection at 254 nm. HPLC purification was performed with a JASCO CCPS HPLC pump, a JASCO UV8020 HPLC UV/vis detector, and a Chemcobond 5-ODS-H reversed-phase column (10 × 150 mm) in 0.1% TFA in water with CH_3CN as eluent at a flow rate of 3.0 mL/min, and a linear gradient elution of 40−60% CH_3CN over 30 min with detection at 254 nm. ESI-TOFMS was produced on a BioTOF II (Bruker Daltonics) mass spectrometer using a positive ionization mode. UV spectra were measured on a Nanodrop ND-1000 spectrophotometer. All DNA fragments, 5′-Texas Red-labeled primers, and cold primers were purchased from Sigma Aldrich. A Thermo Sequence core sequencing kit was purchased from GE Healthcare. Polymerase chain reaction (PCR) was performed on an iCyclear (Bio-Rad). Loading dye was composed of 10 mL of formamide, 200 μL of H_2O, 300 μL of a 0.5 M aqueous solution of disodium dihydrogen ethylenediaminetetraacetate dehydrate (Nacalai Tesque Inc.), and 2.5 mg of new fuchsin (Merck). A 50% Long Ranger gel solution was purchased from Lonza Rockland, Inc. PAGE was performed on a Hitachi 5500-S DNA sequencer, and data were analyzed by FLAGLYS version 2 software (Hitachi). *seco*-CBI was synthesized by reported procedures.[15]

Syntheses and Analytical Data of Compounds 3−19. *AcIPP-β-P-γ-P-β-I-COOH (**3**).* Compound **3** was prepared in a stepwise reaction by manual Fmoc SPPS according to reported procedures. Polyamide supported by CLEAR-Acid resin was cleaved with acidic conditions (TFA, H_2O, triisopropylsilane, rt, 0.5 h). Synthesized compound **3** was used in the next step without HPLC purification (total 74% yield, from 100 μmol of I-CLEAR-Acid resin). Analytical HPLC: t_R = 13.3 min. ESI-TOFMS: m/z calcd for $C_{46}H_{55}N_{17}O_{11}$ $[M+H]^+$ 1022.4345, found 1022.4349. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.55 (s, 1H; NH), 10.23 (s, 1H; NH), 9.95 (s, 1H; NH), 9.89 (s, 1H; NH), 9.84 (s, 1H; NH), 9.75 (s, 1H; NH), 8.06−7.98 (m, 3H; NH×3), 7.48 (s, 1H; CH) 7.42 (s, 1H; CH), 7.26 (d, 1H, J = 2.0 Hz; CH), 7.19 (d, 1H, J = 2.0 Hz; CH), 7.12 (s, 1H; CH), 7.10 (d, 1H, J = 1.4 Hz; CH), 6.84 (d, 1H, J = 2.0 Hz; CH), 6.68 (d, 1H, J = 1.3 Hz; CH), 6.63 (d, 1H, J = 1.4 Hz; CH), 3.92 (s, 3H; NMe), 3.95 (s, 3H; NMe), 3.89 (s, 3H; NMe), 3.84 (s, 3H; NMe), 3.776 (s, 3H; NMe), 3.767 (s, 3H; NMe), 3.46−3.36 (m, 4H; CH_2×2), 3.17 (m, 2H; CH_2), 2.56 (t, 2H, J = 7.6 Hz; CH_2), 2.52 (m, 2H; CH_2), 2.22 (t, 2H, J = 7.6 Hz; CH_2), 2.02 (s, 3H; Ac) 1.75 (m, 2H; CH_2).

*General Procedure for the Synthesis of NO_2PLCO_2Et (**4**−**7**).* Compounds **4**−**7** were synthesized from 1-methyl-2-pyrrolecarbalde-

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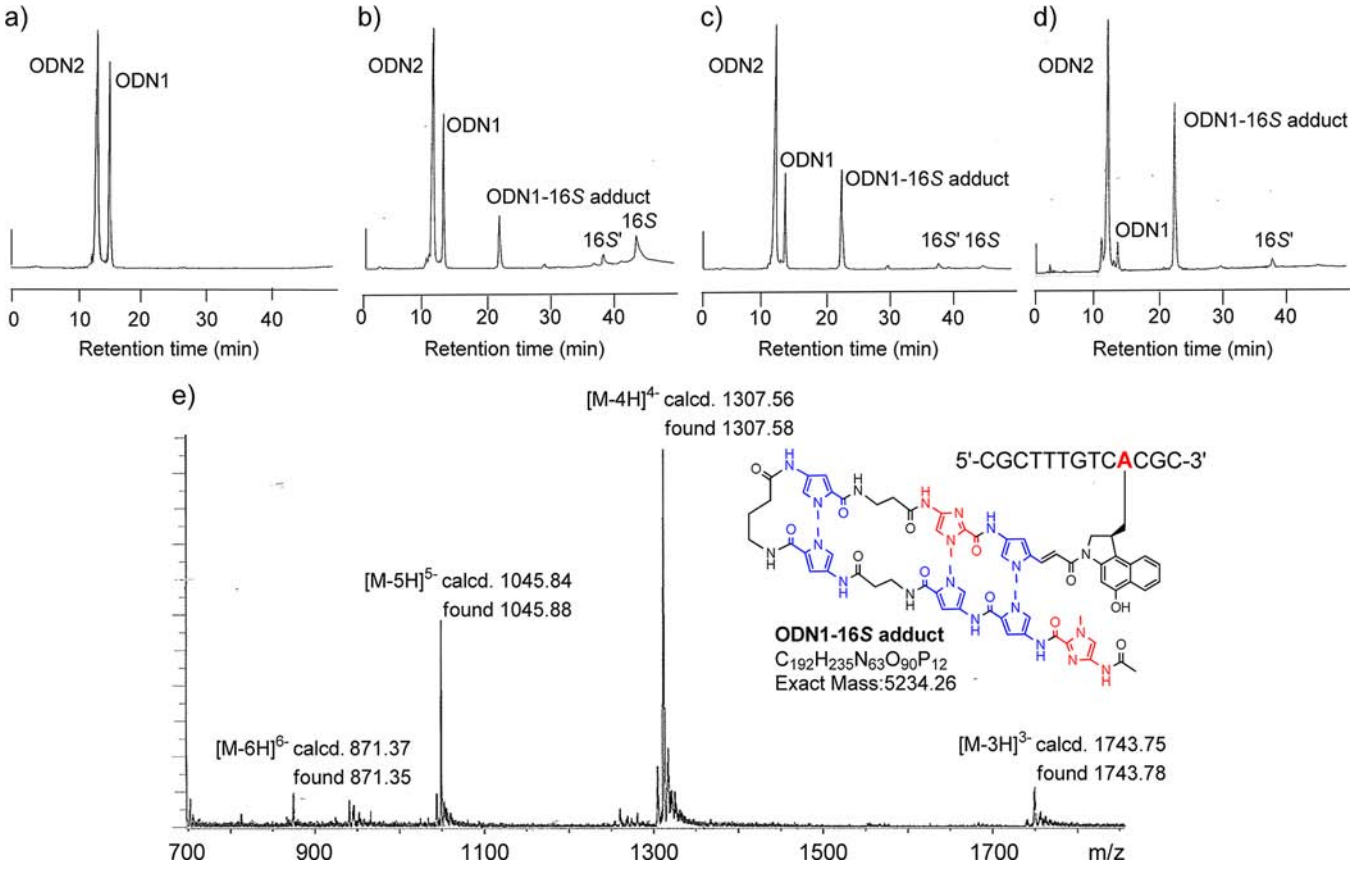

Figure 3. HPLC analysis of the alkylation of ODN1/ODN2 with **16*S***: (a) DNA control, (b) 5 min, (c) 1 h, and (d) 16 h. (e) Chemical structure and ESI-TOFMS spectrum of the ODN1−**16*S*** adduct.

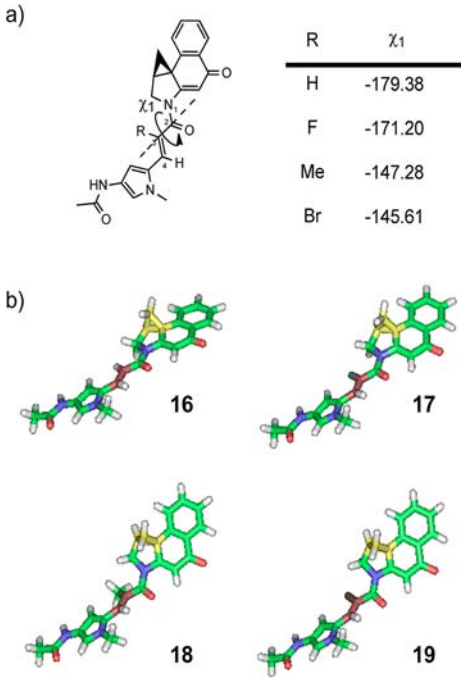

Figure 4. (a) Chemical structure of the AcPL(R)CBI moiety and dihedral angle (χ_1) of each compound. (b) Geometry-optimized structures of the AcPL(R)CBI model obtained by density functional calculation at the B3LYP/6-31G(d,p) level.

hyde and the corresponding phosphonoacetate. The general procedure for the synthesis of compounds **4**−**7** is as follows. To a solution of the corresponding phosphonoacetate (3.9 mmol) in THF (4 mL) was added sodium hydride (60% assay, 3.9 mmol, 156 mg) at 0 °C. After the solution was stirred for 5 min, a solution of 1-methyl-2-pyrrolecarbaldehyde (3.0 mmol, 462 mg) in 4 mL of THF was dropwise added at 0 °C. After this solution was stirred for 2 h at rt, the reaction solution was evaporated and then quenched by H_2O. The mixture was extracted with ethyl acetate. Combined organic layers were dried over $MgSO_4$ and concentrated. The resulting residue was washed by hexane, giving target compounds. Each phosphonoacetate was purchased from TCI (for **3**−**5**) or synthesized by a reported procedure (for **6**).[22]

*NO_2PL(H)CO_2Et (**4**).* Compound **4** was obtained as a yellow powder (445 mg, 66% yield). ESI-TOFMS: *m*/*z* calcd for $C_{10}H_{12}N_2O_4$ $[M+H]^+$ 225.0875, found 225.0892. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.56 (d, *J* = 1.4 Hz, 1H; CH), 7.49 (d, 1H, *J* = 16.4 Hz; CH), 7.65 (d, 1H, *J* = 2.0 Hz; CH), 6.30 (d, 1H, *J* = 15.8 Hz; CH), 4.26 (q, 2H, *J* = 11.0 Hz; CH_2), 3.77 (s, 3H; NMe), 1.33 (t, 3H, *J* = 7.5 Hz; CH_3).

*NO_2PL(F)CO_2Et (**5**).* Compound **5** was obtained as a brown powder (388 mg, 53% yield; mixture of *E* and *Z* forms, *E*:*Z* = 2:5). ESI-TOFMS: *m*/*z* calcd for $C_{10}H_{11}FN_2O_4$ $[M+H]^+$ 243.0781, found 243.0827. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.58−7.56 (m, 2H; 2CH), 7.30[a] and 7.26[b] (s, 1H; CH), 6.82[a] and 6.60[b] (d, 1H, *J* = 31.6[a] and 21.3[b] Hz; CH), 4.40−4.30 (m, 2H; CH_2), 3.75[a] and 3.64[b] (s, 3H; NMe), 1.38[a] and 1.36[b] (t, 3H, *J* = 6.8[a] and 7.6[b] Hz; CH_3)[[a]= *E* form; [b]= *Z* form].

*NO_2PL(Me)CO_2Et (**6**).* Compound **6** was obtained as a yellow powder (356 mg, 62% yield). ESI-TOFMS: *m*/*z* calcd for $C_{11}H_{14}N_2O_4$ $[M+H]^+$ 239.1032, found 239.1053. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.58 (d, *J* = 2.0 Hz, 1H; CH), 7.44 (s, 1H; CH), 6.98 (d, 1H, *J* = 1.4 Hz; CH), 4.27 (q, 2H, *J* = 10.6 Hz; CH_2), 3.73 (s, 3H; NMe), 2.15 (s, 3H; CH_3), 1.33 (t, 3H; *J* = 6.9 Hz; CH_3).

*NO_2PL(Br)CO_2Et (**7**).* Compound **7** was obtained as a brown powder (612 mg, 67% yield). ESI-TOFMS: *m*/*z* calcd for $C_{10}H_{11}BrN_2O_4$ [M

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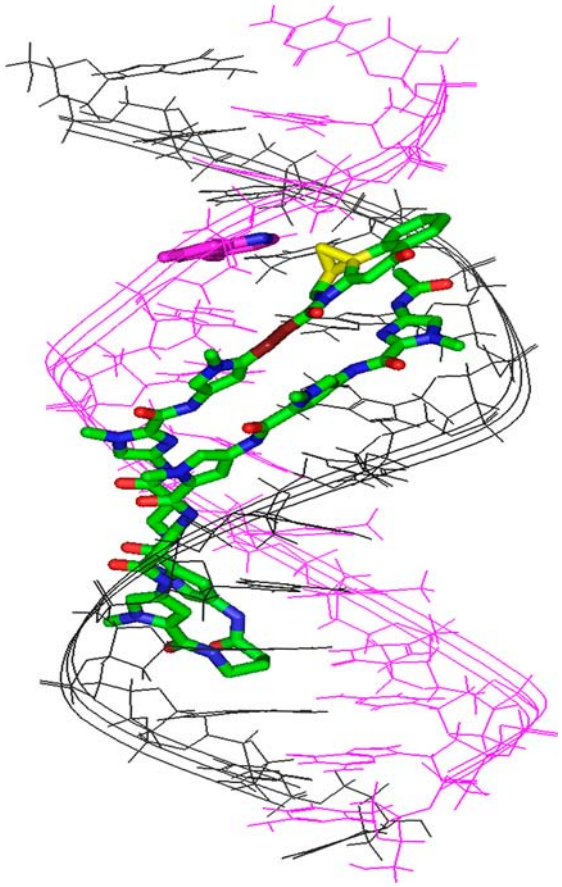

Figure 5. Energy-minimized structure of the d-(CGCTTTGTCACGC) ODN1/d(GCGTGACAAAGCG) ODN2–**16*S′*** complex. The vinyl linker is drawn in brown, the cyclopropane unit of CBI in yellow, ODN1 in purple, and the reacting adenine in bold purple.

+H]$^+$ 302.9980, found 303.0022. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.08 (s, 1H; CH), 7.95 (d, 1H, J = 1.3 Hz; CH), 7.65 (d, 1H, J = 2.0 Hz; CH), 4.35 (d, 2H, J = 10.0 Hz; CH_2), 3.78 (s, 3H; NMe), 1.58 (s, 3H; CH_3), 1.38 (t, 3H, J = 7.6 Hz; CH_3).

*AcIPP-β-P-γ-P-β-IP-L(H)-COOH (**12**).* To a solution of compound **4** (44.8 mg, 0.20 mmol) in CH_3OH–AcOEt (2:1, total 1.5 mL) was added 10% Pd–C (30 mg) and then $NaBH_4$ (11.3 mg, 0.30 mmol) in H_2O (0.1 mL). The reaction mixture was stirred at 0 °C for 5 min under an Ar atmosphere. Pd–C was removed by filtration through a short pad of silica gel. The filtrate was concentrated to produce compound **8**, which was used in the next step without further purification. To the solution of crude **8** in 0.7 mL of *N*,*N*-dimethylformamide (DMF) was added polyamide **3** (30.6 mg, 0.03 mmol) and PyBOP (46.8 mg, 0.09 mmol), followed by DIEA (31.4 μL, 0.18 mmol). The solution was stirred at rt for 1 h and then concentrated to a residue. The residue was washed and triturated from Et_2O and then AcOEt to produce the ethyl ester of compound **12** (23.8 mg, quant) as a brown powder. The resulting powder was dissolved in DBU (0.6 mL) and H_2O (0.6 mL). After being stirred at room temperature for 12 h, the solution was concentrated in vacuo and then washed with Et_2O, AcOEt, and 1 N HCl. The resulting residue was dried to produce polyamide **12** (20.6 mg, 75% yield) as a brown powder. Analytical HPLC: t_R = 14.3 min. ESI-TOFMS: m/z calcd for $C_{54}H_{63}N_{19}O_{12}$ $[M+H]^+$ 1170.4982, found 1170.4988. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.29 (s, 1H; NH), 10.22 (s, 1H; NH), 9.94 (s, 1H; NH), 9.89 (s, 1H; NH), 9.88 (s, 1H; NH), 9.83 (s, 1H; NH), 9.75 (s, 1H; NH), 8.05–7.99 (m, 3H; NH×3), 7.45 (d, 1H, J = 15.8 Hz; CH) 7.44 (s, 1H; CH), 7.42 (s, 1H; CH), 7.40 (d, 1H, J = 1.4 Hz; CH), 7.26 (d, 1H, J = 1.4 Hz; CH), 7.19 (d, 1H, J = 1.4 Hz; CH), 7.11–7.10 (m, 2H; CH×2), 7.09 (s, 1H; CH) 6.83 (d, 1H, J = 1.4 Hz; CH), 6.80 (d, 1H, J = 1.4 Hz; CH), 6.68 (d, 1H, J = 1.4 Hz; CH), 6.63 (d, 1H, J = 1.4 Hz; CH), 6.02 (d, 1H, J = 15.8 Hz; CH), 3.941 (s, 3H; NMe), 3.939 (s, 3H; NMe), 3.84 (s, 3H; NMe), 3.81 (s, 3H; NMe), 3.77 (s, 6H; NMe×2), 3.67(s, 3H; NMe), 3.46–3.36 (m, 4H; CH_2×2), 3.17 (m, 2H; CH_2), 2.56 (t, 2H, J = 7.6 Hz; CH_2), 2.52 (m, 2H; CH_2), 2.22 (t, 2H, J = 7.6 Hz; CH_2), 2.02 (s, 3H; Ac) 1.75 (m, 2H; CH_2).

*AcIPP-β-P-γ-P-β-IP-L(F)-COOH (**13**).* A synthetic procedure similar to that used for the preparation of polyamide **12** provided polyamide **13** (74% yield in two steps). Analytical HPLC: t_R = 15.2 min. ESI-TOFMS: m/z calcd for $C_{54}H_{62}FN_{19}O_{12}$ $[M+H]^+$ 1188.4888, found 1188.4864. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.29 (s, 1H; NH), 10.23 (s, 1H; NH), 10.05 (s, 1H; NH), 9.95 (s, 1H; NH), 9.89 (s, 1H; NH), 9.83 (s, 1H; NH), 9.76 (s, 1H; NH), 8.05–7.98 (m, 3H; NH×3), 7.42–7.39 (m, 3H; CH×3), 7.26 (d, 1H, J = 2.1 Hz; CH), 7.19 (d, 1H, J = 1.4 Hz; CH), 7.13–7.08 (m, 3H; CH×3), 7.07 (s, 1H; CH), 6.99 (s, 1H; CH), 6.84 (d, 1H, J = 2.0 Hz; CH), 6.68 (d, 1H, J = 2.0 Hz; CH), 6.64 (d, 1H, J = 2.0 Hz; CH), 3.94 (s, 3H; NMe), 3.93 (s, 3H; NMe), 3.84 (s, 3H; NMe), 3.81 (s, 3H; NMe), 3.77 (s, 6H; NMe×2), 3.63 (s, 3H; NMe), 3.46–3.40 (m, 4H; CH_2×2), 3.17 (q, 2H, J = 6.8 Hz; CH_2), 2.57 (t, 2H, J = 7.6 Hz; CH_2), 2.52 (m, 2H; CH_2), 2.24 (t, 2H, J = 7.6 Hz; CH_2), 2.02 (s, 3H; Ac), 1.75 (m, 2H; CH_2).

*AcIPP-β-P-γ-P-β-IP-L(Me)-COOH (**14**).* A synthetic procedure similar to that used for the preparation of polyamide **12** provided polyamide **14** (66% yield in two steps). Analytical HPLC: t_R = 14.9 min. ESI-TOFMS: m/z calcd for $C_{55}H_{65}N_{19}O_{12}$ $[M+H]^+$ 1184.5138, found 1184.5182. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.26 (s, 1H; NH), 10.23 (s, 1H; NH), 9.97 (s, 1H; NH) 9.94 (s, 1H; NH), 9.89 (s, 1H; NH), 9.83 (s, 1H; NH), 9.75 (s, 1H; NH), 8.05–7.98 (m, 3H; NH×3), 7.52 (s, 1H; CH), 7.44 (s, 1H; CH), 7.42–7.39 (m, 2H; CH×2), 7.26 (d, 1H, J = 2.1 Hz; CH), 7.19 (d, 1H, J = 1.4 Hz; CH), 7.14–7.09 (m, 3H; CH×3), 6.83 (d, 1H, J = 2.0 Hz; CH), 6.76 (d, 1H, J = 1.4 Hz; CH), 6.68 (d, 1H, J = 2.0 Hz; CH), 6.63 (d, 1H, J = 2.0 Hz; CH), 3.95 (s, 6H; NMe×2), 3.84 (s, 3H; NMe), 3.81 (s, 3H; NMe), 3.775 (s, 3H; NMe), 3.773 (s, 3H; NMe), 3.65 (s, 3H; NMe) 3.46–3.40 (m, 4H; CH_2×2), 3.17 (q, 2H, J = 6.8 Hz; CH_2), 2.57 (t, 2H, J = 7.6 Hz; CH_2), 2.52 (m, 2H; CH_2), 2.24 (t, 2H, J = 7.6 Hz; CH_2), 2.02 (s, 3H; Ac), 2.00 (s, 3H; CH_3), 1.75 (m, 2H; CH_2).

*AcIPP-β-P-γ-P-β-IP-L(Br)-COOH (**15**).* A synthetic procedure similar to that used for the preparation of polyamide **12** provided polyamide **15** (71% yield in two steps). Analytical HPLC: t_R = 14.8 min. ESI-TOFMS: m/z calcd for $C_{54}H_{62}BrN_{19}O_{12}$ $[M+H]^+$ 1248.4087, found 1248.4071. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.25 (s, 1H; NH), 10.22 (s, 1H; NH), 10.13 (s, 1H; NH), 9.94 (s, 1H; NH), 9.88 (s, 1H; NH), 9.83 (s, 1H; NH), 9.75 (s, 1H; NH), 8.14 (s, 1H; CH), 8.05–7.98 (m, 3H; NH×3), 7.62 (d, 1H, J = 1.4 Hz; CH), 7.55 (d, 1H, J = 1.4 Hz; CH), 7.44 (s, 1H; CH), 7.41 (s, 1H; CH), 7.26 (d, 1H, J = 1.4 Hz; CH), 7.18 (d, 1H, J = 1.4 Hz; CH), 7.12–7.08 (m, 3H; CH×3), 6.83 (d, 1H, J = 1.4 Hz; CH), 6.68 (d, 1H, J = 1.4 Hz; CH), 6.64 (d, 1H, J = 1.4 Hz; CH), 3.94 (s, 6H; NMe×2), 3.84 (s, 3H; NMe), 3.80 (s, 3H; NMe), 3.77 (s, 6H; NMe×2), 3.70 (s, 3H; NMe) 3.46–3.36 (m, 4H; CH_2×2), 3.17 (m, 2H; CH_2), 2.57 (t, 2H, J = 6.9 Hz; CH_2), 2.52 (m, 2H; CH_2), 2.23 (t, 2H, J = 7.9 Hz; CH_2), 2.02 (s, 3H; Ac) 1.75 (m, 2H; CH_2).

*AcIPP-β-P-γ-P-β-IP-L(H)-seco-CBI (rac-**16**).* A solution of polyamide **12** (11.7 mg, 0.010 mmol), *seco*-CBI (4.7 mg, 0.020 mmol), and PyBOP (10.4 mg, 0.020 mmol) in DMF (100 μL) and DIEA (10.5 μL, 0.06 mmol) was stirred at room temperature for 2 h. Et_2O was added to the mixture, and the resultant was collected by centrifugation and washed by Et_2O and CH_2Cl_2. After further purification by HPLC, **16** was obtained as a yellow powder (2.8 mg, 20% yield). Analytical HPLC: t_R = 16.0 min. ESI-TOFMS: m/z calcd for $C_{67}H_{73}ClN_{20}O_{12}$ $[M+H]^+$ 1385.5484, found 1385.5477. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.38 (s, 1H; OH), 10.29 (s, 1H; NH), 10.22 (s, 1H; NH), 9.94 (s, 1H; NH) 9.89 (s, 1H; NH), 9.83 (s, 1H; NH), 9.79 (s, 1H; NH), 9.76 (s, 1H; NH), 8.13–7.96 (m, 4H; NH×3,CH), 7.79 (d, 1H, J = 12.0 Hz; CH), 7.58 (d, 1H, J = 15.0 Hz; CH), 7.51–7.47 (m, 1H; CH), 7.46 (s, 1H; CH), 7.42 (s, 1H; CH), 7.38 (s, 1H; CH), 7.33–

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7.31 (m, 1H; CH), 7.26 (d, 1H, J = 2.0 Hz; CH), 7.19 (d, 1H, J = 2.0 Hz; CH), 7.17 (d, 1H, J = 2.0 Hz; CH), 7.14−7.07 (m, 3H; CH×3), 6.85 (d, 1H, J = 1.4 Hz; CH), 6.83 (d, 1H, J = 1.4 Hz; CH), 6.82 (d, 1H, J = 1.4 Hz; CH), 6.68 (s, 1H; CH), 6.64 (d, 1H, J = 2.0 Hz; CH), 4.50−4.40 (m, 1H; CH), 3.96 (s, 3H; NMe), 3.94 (s, 3H; NMe), 3.84 (s, 3H; NMe), 3.81 (s, 3H; NMe), 3.78 (s, 3H; NMe), 3.77 (s, 3H; NMe), 3.73 (s, 3H; NMe), 3.47−3.33 (m, 4H; CH_2×2), 3.18 (m, 2H; CH_2), 2.61 (m, 2H; CH_2), 2.54−2.49 (m, 2H; CH_2), 2.47−2.45 (m, 2H; CH_2), 2.38 (m, 2H; CH_2), 2.24 (t, 2H, J = 7.6 Hz; CH_2), 2.02 (s, 3H; CH_3), 1.75 (m, 2H; CH_2). Conjugate **16*S*** was synthesized by the same procedure as conjugate **16**.

*AcIPP-β-P-γ-P-β-IP-L(F)-seco-CBI (rac-**17**).* A synthetic procedure similar to that used for the preparation of conjugate **16** provided conjugate **17** in 21% yield. Analytical HPLC: t_R = 16.8 min. ESI-TOFMS: m/z calcd for $C_{67}H_{72}ClFN_{20}O_{12}$ $[M+H]^+$ 1403.5389, found 1403.5393. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.46 (s, 1H; OH), 10.29 (s, 1H; NH), 10.24 (s, 1H; NH), 9.96 (s, 1H; NH) 9.90 (s, 1H; NH), 9.84 (s, 1H; NH), 9.76 (s, 1H; NH), 9.75 (s, 1H; NH), 8.14−8.10 (m, 1H; CH), 8.07−8.00 (m, 3H; NH×3), 7.84 (d, 1H, J = 12.0 Hz; CH), 7.80 (d, 1H, J = 12.0 Hz; CH), 7.54−7.48 (m, 2H; CH×2), 7.45 (s, 1H; CH), 7.44 (d, 1H, J = 2.0 Hz; CH), 7.42 (s, 1H; CH), 7.26 (d, 1H, J = 2.0 Hz; CH), 7.19 (s, 1H; CH), 7.14−7.07 (m, 3H; CH×3), 6.99 (d, 1H, J = 1.4 Hz; CH), 6.84 (s, 1H; CH), 6.80 (s, 1H; CH) 6.69 (s, 1H; CH), 6.64 (d, 1H, J = 2.0 Hz; CH), 4.50−4.40 (m, 1H; CH), 3.95 (s, 3H; NMe), 3.94 (s, 3H; NMe), 3.84 (s, 3H; NMe), 3.81 (s, 3H; NMe), 3.78 (s, 3H; NMe), 3.77 (s, 3H; NMe), 3.75 (s, 3H; NMe), 3.47−3.33 (m, 4H; CH_2×2), 3.18 (m, 2H; CH_2), 2.61 (m, 2H; CH_2), 2.54−2.49 (m, 2H; CH_2), 2.47−2.45 (m, 2H; CH_2), 2.38 (m, 2H; CH_2), 2.24 (t, 2H, J = 7.6 Hz; CH_2), 2.02 (s, 3H; CH_3), 1.75 (m, 2H; CH_2).

*AcIPP-β-P-γ-P-β-IP-L(Me)-seco-CBI (rac-**18**).* A synthetic procedure similar to that used for the preparation of conjugate **16** provided conjugate **18** in 17% yield. Analytical HPLC: t_R = 16.8 min. ESI-TOFMS: m/z calcd for $C_{68}H_{75}ClN_{20}O_{12}$ $[M+H]^+$ 1399.5640, found 1399.5598. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.40 (s, 1H; OH), 10.27 (s, 1H; NH), 10.22 (s, 1H; NH), 9.94 (s, 1H; NH) 9.92 (s, 1H; NH), 9.89 (s, 1H; NH), 9.83 (s, 1H; NH), 9.75 (s, 1H; NH), 8.08 (d, 1H, J = 12.0 Hz; CH), 8.06−7.99 (m, 3H; NH×3), 7.79 (d, 1H J = 12.0 Hz; CH), 7.51−7.47 (m, 1H; CH), 7.44 (s, 1H; CH), 7.42 (s, 1H; CH), 7.33 (s, 1H; CH), 7.25 (d, 1H, J = 2.0 Hz; CH), 7.18 (d, 1H, J = 2.0 Hz; CH), 7.15 (s, 1H; CH), 7.14−7.07 (m, 3H; CH×3), 7.06 (s, 1H; CH), 6.98 (s, 1H; CH), 6.83 (s, 1H; CH), 6.82 (s, 1H; CH), 6.68 (d, 1H, J = 2.0 Hz; CH), 6.64 (d, 1H, J = 2.0 Hz; CH), 4.40−4.36 (m, 1H; CH), 3.95 (s, 3H; NMe), 3.94 (s, 3H; NMe), 3.84 (s, 3H; NMe), 3.81 (s, 3H; NMe), 3.78 (s, 3H; NMe), 3.77 (s, 3H; NMe), 3.57 (s, 3H; NMe) 3.47−3.37 (m, 4H; CH_2×2), 3.18 (q, 2H, J = 6.8 Hz; CH_2), 2.61 (m, 2H; CH_2), 2.54−2.48 (m, 4H; CH_2×2), 2.38 (m, 2H; CH_2), 2.24 (t, 2H, J = 7.6 Hz; CH_2), 2.02 (s, 3H; CH_3), 1.75 (m, 2H; CH_2).

*AcIPP-β-P-γ-P-β-IP-L(Br)-seco-CBI (rac-**19**).* A synthetic procedure similar to that used for the preparation of conjugate **16** provided conjugate **19** in 16% yield. Analytical HPLC: t_R = 17.3 min. ESI-TOFMS: m/z calcd for $C_{67}H_{72}BrClN_{20}O_{12}$ $[M+H]^+$ 1463.4589, found 1463.4583. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.51 (s, 1H; OH), 10.27 (s, 1H; NH), 10.23 (s, 1H; NH), 10.09 (s, 1H; NH), 9.95 (s, 1H; NH), 9.89 (s, 1H; NH), 9.84 (s, 1H; NH), 9.76 (s, 1H; NH), 8.46 (s, 1H; CH), 8.08−7.98 (m, 3H; NH×3), 7.83 (d, 1H, J = 1.4 Hz; CH), 7.73 (d, 1H, J = 12.0 Hz; CH), 7.68 (s, 1H; CH), 7.48−7.40 (m, 3H; CH×3), 7.26 (s, 1H; CH), 7.19 (d, 1H, J = 2.0 Hz; CH), 7.17 (s, 1H; CH), 7.14−7.08 (m, 3H; CH×3), 7.08 (s, 1H; CH), 6.99 (s, 1H; CH), 6.90 (s, 1H; CH), 6.69 (s, 1H; CH), 6.65 (s, 1H; CH), 4.38−4.32 (m, 1H; CH), 3.97 (s, 3H; NMe), 3.95 (s, 3H; NMe), 3.84 (s, 3H; NMe), 3.81 (s, 3H; NMe), 3.79 (s, 3H; NMe), 3.78 (s, 3H; NMe), 3.77 (s, 3H; NMe) 3.67−3.62 (m, 2H; CH_2), 3.46−3.40 (m, 2H; CH_2), 3.20−3.16 (m, 2H; CH_2), 2.63−2.56 (m, 2H; CH_2), 2.54−2.50 (m, 2H; CH_2), 2.49−2.46 (m, 2H; CH_2), 2.40−2.37 (m, 2H; CH_2), 2.25 (m, 2H; CH_2), 2.02 (s, 3H; CH_3), 1.76 (m, 2H; CH_2).

Cloning of 208 bp DNA Fragments. DNA fragments were annealed in a final volume of 50 μL, containing 10 μM of each strand (5′-TGTCATCCAGTGCCATTCAGTTCCATCCAGA3′ and 5′-CTGGATGGAACTGAATGGCACTGGATGACAA3′) and ligated into pGEM-T Easy vector (Promega). *Escherichia coli* DH5a competent cells (TOYOBO) were transformed and cultured on an LB plate with 100 μg/mL ampicillin and 32 μg of X-gal (20 mg/mL), 25 μL/IPTG 100 mM, 25 μL overnight at 37 °C. White colonies were identified by colony PCR in 20 μL of the reaction mixtures containing 250 nM of each primer (T7 primer, 5′-TAATACGACTCACTA-TAGGG-3′; SP6 primer, 5′-TATTTAGGTGACACTATAG-3′), 200 μM dNTPs (Sigma Aldrich), 2 units of Taq polymerase, and 1× ThermoPol reaction buffer (New England Bio Labs). Amplification cycles were carried out with an iCycler (Bio-Rad). The reaction mix was incubated at 95 °C for 5 min, followed by 35 cycles of 95 °C for 35 s, 50 °C for 35 s, 72 °C for 30 s, with a final extension step of 72 °C for 7 min. The appropriate colony was selected for transfer to 5 mL of LB medium with 100 μg/mL ampicillin and cultured overnight at 37 °C. The plasmids were extracted using GenElute Plasmid Miniprep Kit (Sigma Aldrich) and identified by PCR (program and reaction mixtures were the same as above). The sequence of this PCR product (208 bp) is 5′-TATTTAGGTGACACTATAGAATACTCAAGC-TATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTC-GACCTGCAGGCGGCCGCGAATTCACTAGTGATTTGTCATC-C A G T G C C A T T C A G T T C C A T C C A G A A T C - GAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGC-GACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTA-3′.

High-Resolution Gel Electrophoresis. The 5′-Texas Red-labeled 208 bp DNA fragments (6 nM) were alkylated by various concentration of conjugates **16*S*** and **16**−**19** in 10 μL of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 20 °C. After incubation for 1 h, the reaction mixture was quenched by calf thymus DNA and heating for 5 min at 95 °C. The solution was concentrated by vacuum centrifugation. The pellet was dissolved with 6 μL of loading dye (formamide with fuschin red), heated at 95 °C for 20 min, and then immediately cooled to 0 °C. A 1.2 μL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a Hitachi DNA sequencer. Densitometric analyses were carried out by Multi Gauge ver3.1.

Alkylation of Oligonucleotides by Conjugates 16 and 16*S* As Monitored by HPLC. Oligonucleotides were purchased from Sigma Aldrich. A reaction mixture (50 μL) containing conjugates **16** and **16*S*** (100 μM) and the duplex oligonucleotide (90 μM duplex concentration) in 5 mM sodium phosphate buffer (pH 7.0) was incubated at 23 °C for the indicated periods. The progress of the reaction was monitored by HPLC using a Chemcobond 5-ODS-H column (4.6 × 150 mm). Elution was performed with 50 mM ammonium formate and a 0−50% acetonitrile linear gradient (0−50 min) at a flow rate of 1.0 mL/min at 260 nm.

Molecular Modeling Studies on Conjugate 16*S*′−ODN1 Complex. Minimizations were performed with the Discover (MSI, San Diego, CA) program using CVFF force-field parameters. The starting structure was constructed using builder module of program Insight II using standard bond lengths and angles. Where the every three upper and lower sides of Watson−Crick base pairs were fixed, **16*S*′** was inserted in ODN1/ODN2. Twenty-four Na cations were placed at the bifurcating position of the O−P−O angle at a distance of 2.21 Å from the phosphorus atom. The resulting complex was soaked in a 10 Å layer of water. Layer of water was minimized without constraints to the stage where the rms was less than 0.001 kcal/mol·Å by steepest then conjugate algorithm; successively the whole complex was also minimized in the same way.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Characterization of product analysis for ODN1−**16*S*** adduct; chemical structure and TOF mass spectra of the conjugate **16*S*′**; HPLC analysis of ODN1/ODN2 with conjugate **16**; stereo images of structures of the AcPL(R)CBI model and the d(CGCTTTGTCACGC) ODN1/d(GCGTGACAAAGCG) ODN2−**16*S*′** complex; and ESI-TOFMS spectra of conjugates

16−19. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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