Supporting information

A dual DNA-binding conjugate that selectively recognizes G-quadruplex structures.

Mitsuharu Ooga, [a] Vinodh J. Sahayasheela, [a] Yuki Hirose, [a] Daisuke Sasaki [a], Kaori Hashiya, [a] Toshikazu Bando, *[a] and Hiroshi Sugiyama*[b]

[a]*Department of Chemistry, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan.*
[b]*Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Yoshida-ushinomiyacho, Sakyo-ku, Kyoto 606-8501, Japan.*

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Material and methods

General procedures

Reagents and solvents were purchased from standard suppliers and used without further purification. ^{1}HNMR spectra were recorded on JEOL JNM ECA-600 spectrometer (600 MHz for ^{1}H), with chemical shifts reported in parts per million relative to residual solvent and coupling constants in hertz. The following abbreviations were applied to spin multiplicity: s (singlet), d (doublet), t (triplet), br (broad), and m (multiplet). Silica-gel preparative thin-layer chromatography (PTLC) was performed using plates from Silica gel 70 PF254 (Wako Pure Chemical Ind.Ltd.). Automated polyamide synthesis was performed on a PSSM-8 system (Shimadzu). All of the reactions were tracked with an analytical high-performance liquid chromatography (HPLC) on a PU-2089 plus series system (JASCO) using a Chemcobond 5-ODS-H 4.6 mm × 150 mm column (Chemco Plus Scientific) in 0.1% TFA in water with acetonitrile as the eluent at a flow rate of 1.0 mL/min and a linear gradient elution of 0–100% acetonitrile in 40 min with detection at 254 nm. Collected fractions were analyzed by MALDI-TOF-MS Microflex-KS II (Bruker). HPLC purification was carried out by PU-2080 plus series (JASCO) using a COSMOSIL 150 × 10 mm 5C_{18}-MS-II Packed Column (Nacalai Tesque Inc.) in 0.1% TFA in water with acetonitrile as the eluent at a flow rate of 3.0 mL/min with detection at 254 nm. PyPDS assumed to exist as the TFA salts were dissolved in the exact volume of DMSO (≥99.5% purity, Nacalai Tesque, Inc.) to yield 10 mM master solution and stored at –80 °C. The concentration of PIPs and conjugates solution in DMSO was prepared based on the following formula.

$\varepsilon = 9900 \times$ (sum number of Py and Im)

$Abs = \varepsilon cl$

ε, Abs, c, and l are molar extinction coefficients of PIPs in DMSO solution at around 310 nm, absorbance at around 310 nm measured by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific), molar concentration, and the path length, respectively.

Synthesis of PyPDS

4-(2-aminoethoxy)-N^2,N^6-bis(4-(2-(pyrrolidin-1-yl)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (PyPDS) was synthesized nearly according to the reported procedures.[1,2]

4-(2-((tert-butoxycarbonyl)amino)ethoxy)pyridine-2,6-dicarboxylate

Dimethyl-4-hydroxy-2,6-pyridinedicarboxylate monohydrate (202.0 mg, 0.881 mmol), *N*-Boc-ethanolamine (244.4 mg, 1.52 mmol) and triphenylphosphine (604.1 mg, 2.30 mmol) were dissolved in 4 ml dry THF and cooled to 0 °C. DIAD (298 µL, 1.52 mmol) was added dropwise under argon. The mixture was allowed to warm to rt and stirred overnight. The solvent was removed *in vacuo* and this compound was dissolved in MeOH (5 mL) and deprotected by slowly adding 1M NaOH in H_2O (2 mL). After stirring for 1 h at rt, the remaining solid dissolved in H_2O. The solution was washed with EtOAc. The aqueous layer was acidified with 7% HCl (aq.) and extracted with EtOAc. The organic layer was dried over Na_2SO_4, filtered, and the solvent removed *in vacuo* to obtain the title compound as a white powder (216.9 mg, 0.665 mmol, 75 %). ^{1}H NMR (594 MHz, DMSO-D6) δ 7.70 (s, 2H), 7.04 (br, 1H), 7.54 (t, J = 5.7 Hz, 2H), 1.36 (s, 9H), One peak might be overlapped with H_2O.

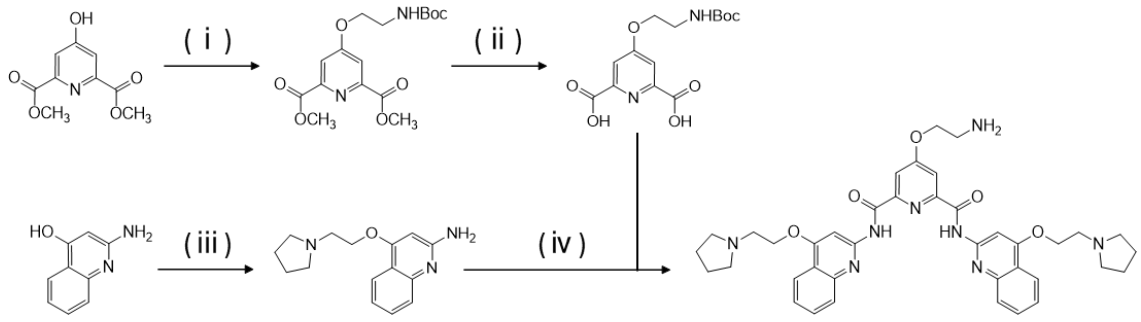
4-(2-(pyrrolidin-1-yl)ethoxy)quinolin-2-amine

2-Amino-4-hydroxyquinoline (502.8 mg, 3.14 mmol), *N*-(2-hydroxyethyl)-pyrrolidine (901.3 mg, 7.82 mmol), and triphenylphosphine (2.05 g, 7.80 mmol) were dissolved in 35 ml dry THF and cooled to 0 °C. DIAD (1.54 mL, 7.84 mmol) was added dropwise under argon. The mixture was allowed to warm to rt and stirred 3 d. The solvent was removed *in vacuo* and this compound was dissolved in 1% HCl (aq.). The solution was washed with DCM. The aqueous layer was neutralized with 1M NaOH (aq.) and extracted with DCM. The organic layer was dried over Na_2SO_4, filtered, and the solvent removed *in vacuo*. The product was semi-purified by silica gel column chromatography (81% EtOAc, 16% MeOH, 3% TEA), and further purification was performed by preparative TLC (81% EtOAc, 16% MeOH, 3% TEA) to obtain the title compound as a white powder (305.9 mg, 1.19 mmol, 38 %). ^{1}H NMR (594 MHz, CHLOROFORM-D) δ 7.99 (d, J = 8.2 Hz, 1H), 7.60 (d, J = 8.2 Hz, 1H), 7.54 (t, J = 7.5 Hz, 1H), 7.23 (t, J = 7.5 Hz, 1H), 6.04 (s, 1H), 4.74 (br, 2H), 4.27 (t, J = 5.8 Hz, 2H), 3.05 (t, J = 5.8 Hz, 2H), 2.72–2.69 (m, J = 6.1 Hz, 4H), 1.85–1.80 (m, 4H).

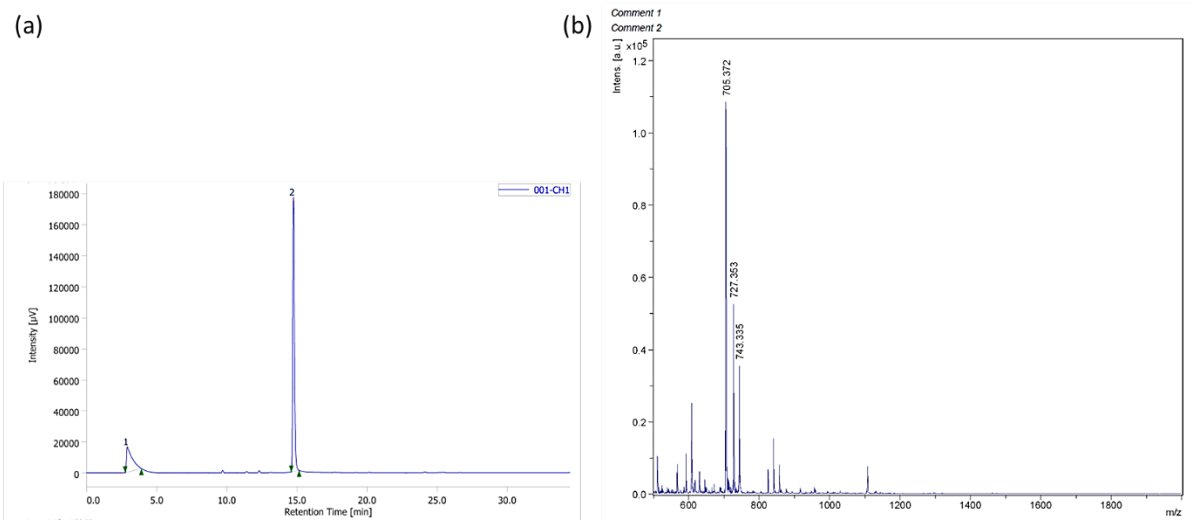
4-(2-aminoethoxy)-N^2,N^6-bis(4-(2-(pyrrolidin-1-yl)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (PyPDS)

4-(2-((tert-butoxycarbonyl)amino)ethoxy)pyridine-2,6-dicarboxylate (101.1 mg, 0.310 mmol) was dissolved in 3 ml DCM at 0 ºC and 1-chloro-*N*,*N*,2-trimethyl-1-propenyl-amine (95 µl, 0.718 mmol) was slowly added. The reaction was allowed to stir at rt for 4 h. The

solution was then cooled to 0 °C and triethylamine (108 µL, 0.775 mmol) was added dropwise. The reaction was then allowed to warm to rt and it was kept stirring for another 1.5 h. 4-(2-(pyrrolidin-1-yl)ethoxy)quinolin-2-amine (168.8 mg, 0.656 mmol) was added to the mixture as a DCM solution (2.5 mL) and stirred at rt overnight. The solvent was removed *in vacuo* to yield a yellow waxy solid. After crystallization from hot MeCN, filtered, washed with hexane, and dissolved in MeOH. The intermediate compound was purified by reverse-phase flash chromatography to afford the TFA salt of Boc-protected PyPDS as a white powder (38.1 mg, 47.3 µmol, 15%). Boc-protected PyPDS (16.49 mg, 16.0 µmol) was dissolved in TFA/DCM (1/2.5 mL) and the solution was stirred at rt for 1 h to deprotect the boc group. After the removal of the solvent, the resulting residue was dissolved in a minimum amount of DCM/MeOH and poured into diethylether (40 mL) to produce the TFA salt of the title compound as a white powder (16.64 mg, 15.9 µmol, 99%). ^{1}H NMR (594 MHz, DMSO-D6) δ 12.13 (s, 2H), 8.32 (d, J = 7.5 Hz, 2H), 8.14 (s, 2H), 8.06 (br, 2H), 7.97–7.96 (4H), 7.82 (t, J = 7.5 Hz, 2H), 7.58 (t, J = 7.8 Hz, 2H), 4.65 (t, J = 4.4 Hz, 4H), 4.51 (t, J = 4.8 Hz, 2H), 3.85–3.82 (m, 4H), 3.75–3.71 (m, 4H), 3.29–3.25 (m, 4H; partially overlapped with H_2O), 2.12–2.08 (m, 4H), 1.94–1.90 (m, 4H), One peak might be overlapped with H_2O. Analytical HPLC: t_R = 14.7 min. MALDI-TOF-MS m/z calcd for $C_{39}H_{45}N_8O_5^+$ $[M + H]^+$ 705.351 found 705.372; $[M + Na]^+$ 727.333 found 727.363; $[M + K]^+$ 743.307 found 743.335.



Scheme S1. Synthesis of PyPDS. (i) *N*-Boc-ethanolamine, Ph_3P, DIAD, THF, 0 °C to rt; (ii) NaOH, H_2O, MeOH, rt; (iii) 1-(2-hydroxyethyl)pyrrolidine, Ph_3P, DIAD, THF, 0 °C to rt; (iv) 1-chloro-*N*,*N*,2-trimethylpropenyl-amine, TEA, DMF, rt; then TFA, DCM.



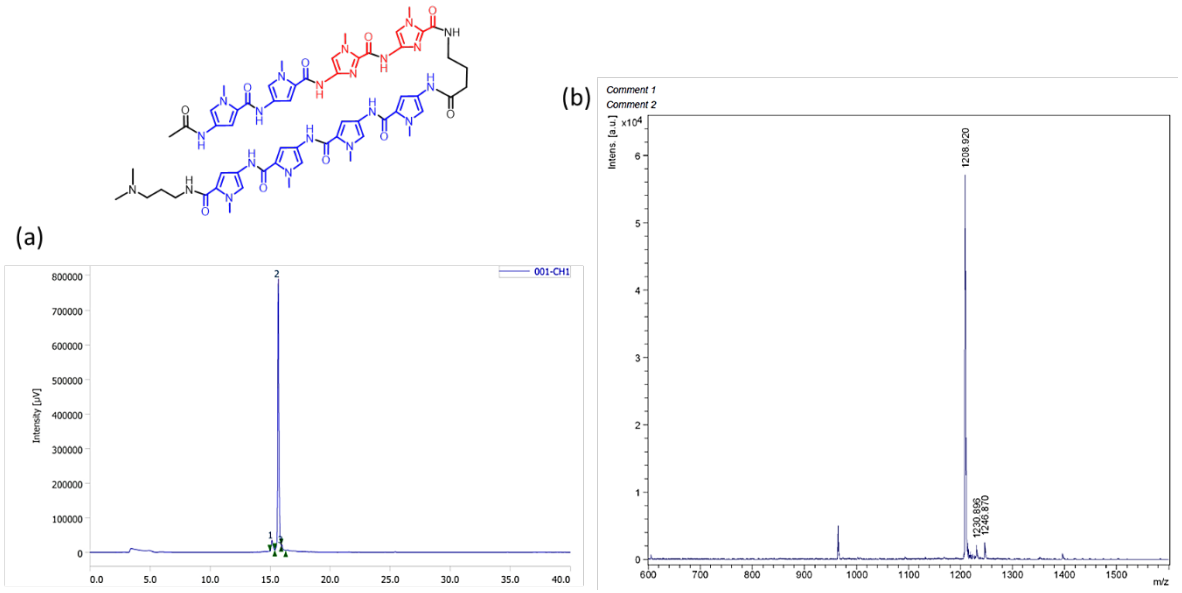
(a) HPLC spectrum of PyPDS. Conditions: 0.1% TFA containing 0−100% acetonitrile over a linear gradient for 40 min at a flow rate of 1.0 mL/min detected at 254 nm. The retention time was 14.7 min. (b) MALDI-TOF MS spectrum of PyPDS. *m/z* calcd for $C_{39}H_{45}N_8O_5^+$ $[M + H]^+$ 705.351, found; 705.372.

Synthesis of PIP 1, 2 and conjugates 1–3

The solid-phase synthesis of each PIP was performed as described previously[3]. The building blocks used in this study were FmocHN-Py-CO_2H, FmocHN-Im-CO_2H, FmocHN-PyIm-CO_2H, FmocHN-β-alanine-CO_2H, FmocHN-PEG-CO_2H (9 atoms), FmocHN-γ-aminobutyric acid, FmocHN-Boc-(*R*)-α-aminobutyric acid. Each of them was introduced sequentially to FmocHN-Py-oxime resin (for PIP **1**) or FmocHN-Im-CLEAR acid resin (for PIP **2**) or FmocHN-β-alanine-trityl resin (for conjugate **1** and **2**). The terminal Py-NH_2 was capped by an Ac-group.

Ac-Py-Py-Im-Im-γ-Py-Py-Py-Py-Dp (PIP 1)

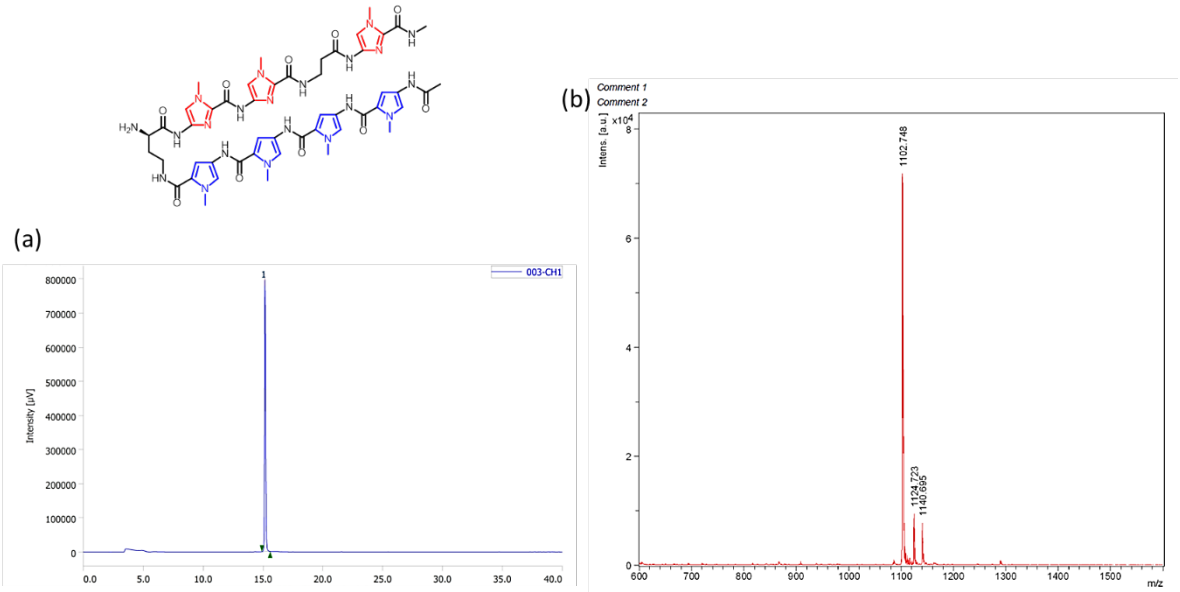
By using 80 mg of FmocHN-Py-oxime resin (0.443 mmol/g) and proper building blocks, Ac-Py-Py-Im-Im-γ-Py-Py-Py-Py-oxime resin was prepared by solid-phase synthesis. Following cleavage reaction with *N*, *N*-dimethyl propandiamine (750 μL) for 7.5 h at 55 °C, and then the residues removed from the resin were directly poured into diethylether (40 mL) to be precipitated. A portion of the precipitates (9.54 mg) was purified by reverse-phase HPLC to give PIP **1** (0.48 mg, 0.40 μmol, 8.4% yield from resin loading). Analytical HPLC: t_R = 16.0 min. MALDI-TOF-MS m/z calcd for $C_{57}H_{70}N_{21}O_{10}^+$ $[M + H]^+$ 1208.561 found 1208.920; $[M + Na]^+$ 1230.543 found 1230.896; $[M + K]^+$ 1246.517 found 1246.870.



(a) HPLC spectrum of PIP **1**. Conditions: 0.1% TFA containing 0−100% acetonitrile over a linear gradient for 40 min at a flow rate of 1.0 mL/min detected at 254 nm. The retention time was 16.0 min. (b) MALDI-TOF MS spectrum of PIP **1**. *m/z* calcd for $C_{57}H_{70}N_{21}O_{10}^+$ $[M + H]^+$ 1208.561, found 1208.920.

Ac-Py-Py-Py-Py-(*R*)$^{\alpha\text{-}NH_2}$γ-Im-Im-β-Im-NHMe (PIP 2)

By using 83 mg of FmocHN-Im-CLEAR acid resin (0.464 mmol/g) and proper building blocks, Ac-Py-Py-Py-Py-(*R*)$^{\alpha\text{-}NH_2}$γ-Im-Im-β-Im-CLEAR acid resin was prepared by solid-phase synthesis. Following a cleavage reaction with 2M $MeNH_2$ (1.5 mL) in THF for 4.5 h at 55 °C, then the residues removed from the resin were directly poured into diethylether (40 mL) to be precipitated. The dried precipitates were dissolved in TFA/DCM (1.3/3.5 mL) and the solution was stirred at rt for 20 min to deprotect the boc group. After concentration *in vacuo*, the residues were dissolved in a minimum amount of DCM/MeOH and poured into diethylether (40 mL). The dried precipitants were purified by reverse-phase flash chromatography to afford a semi-purified product (14.7 mg). Further purification was performed using a portion of the semi-purified product (2.0 mg) by reverse-phase HPLC to give pure PIP **2** (1.1 mg, 0.97 μmol, 18% yield from resin loading). Analytical HPLC: t_R = 15.2 min. MALDI-TOF-MS m/z calcd for $C_{49}H_{60}N_{21}O_{10}^+$ $[M + H]^+$ 1102.483 found 1102.748; $[M + Na]^+$ 1124.465 found 1124.723; $[M + K]^+$ 1140.438 found 1140.695.



(a) HPLC spectrum of PIP **2**. Conditions: 0.1% TFA containing 0−100% acetonitrile over a linear gradient for 40 min at a flow rate of 1.0 mL/min detected at 254 nm. The retention time was 15.2 min. (b) MALDI-TOF MS spectrum of PIP **2**. *m/z* calcd for $C_{49}H_{60}N_{21}O_{10}^+$ $[M + H]^+$ 1102.483, found 1102.748.

conjugate 1, 2

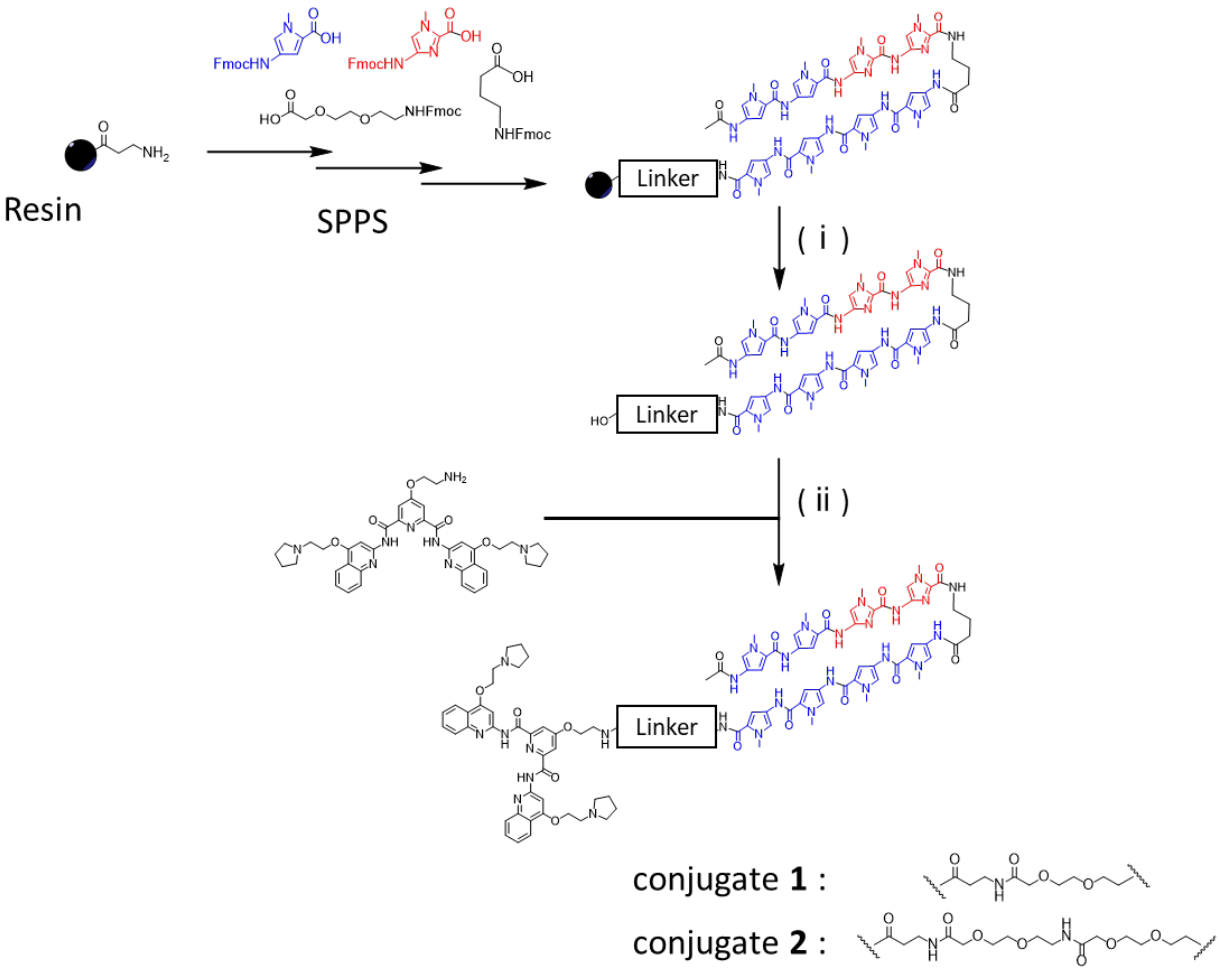
By using 80 mg of FmocHN-Py-oxime resin (0.426 mmol/g) and proper building blocks, Ac-Py-Py-Im-Im-γ-Py-Py-Py-Py-PEG-β-trityl resin was prepared by solid-phase synthesis. Following cleavage reaction with 30% hexafluoroisopropanol (HFIP) in DCM at rt for 3 h, and then the residues removed from the resin were directly poured into diethylether (40 mL) to be precipitated. The precipitates were purified by reverse-phase HPLC to give Ac-Py-Py-Im-Im-γ-Py-Py-Py-Py-PEG-β-CO_2H (9.8 mg, 7.34 μmol, 21% yield from resin loading). Analytical HPLC: t_R = 17.0 min. MALDI-TOF-MS m/z calcd for $C_{61}H_{74}N_{21}O_{15}^+$ $[M + H]^+$ 1340.567 found 1341.069; $[M + Na]^+$ 1362.549 found 1363.068; $[M + K]^+$ 1378.523 found 1379.043.

In the same way, Ac-Py-Py-Im-Im-γ-Py-Py-Py-Py-PEG-PEG-β-CO_2H was obtained after reverse-phase flash chromatography (32.1 mg, 21.58 μmol, 56% yield from resin loading). Analytical HPLC: t_R = 16.8 min. MALDI-TOF-MS m/z calcd for $C_{67}H_{85}N_{22}O_{18}^+$ $[M + H]^+$ 1485.641 found 1485.614; $[M + Na]^+$ 1507.623 found 1508.578; $[M + K]^+$ 1523.597 found 1523.697.

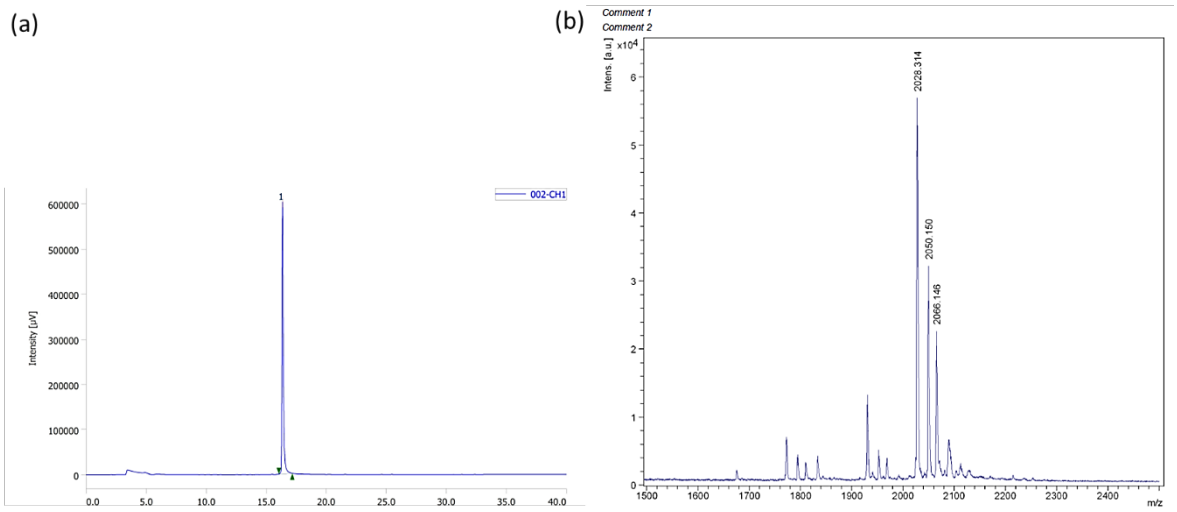
To a solution of PyPDS (5.0 mg, 7.14 μmol) in dry DMF (140 μL) was added a solution of Ac-Py-Py-Im-Im-γ-Py-Py-Py-Py-PEG-β-CO_2H (9.8 mg, 7.34 μmol) and PyBOP (7.5 mg, 14.4 μmol) in dry DMF (50 μL) and DIEA (4.9 μL, 28.0 μmol), which had been stirred at rt for 20 min. The mixture was stirred at rt for 3.5 h, and then the residues were directly poured into diethylether (40 mL) to be precipitated. The crude compound was purified by reverse-phase HPLC to afford the conjugate **1** as a yellow powder (4.8 mg, 2.34 μmol, 32%). Analytical

HPLC: t_R = 16.4 min. MALDI-TOF-MS m/z calcd for $C_{100}H_{116}N_{29}O_{19}^+$ $[M + H]^+$ 2026.900 found 2028.314; $[M + Na]^+$ 2048.882 found 2050.150; $[M + K]^+$ 2064.856 found 2066.146.

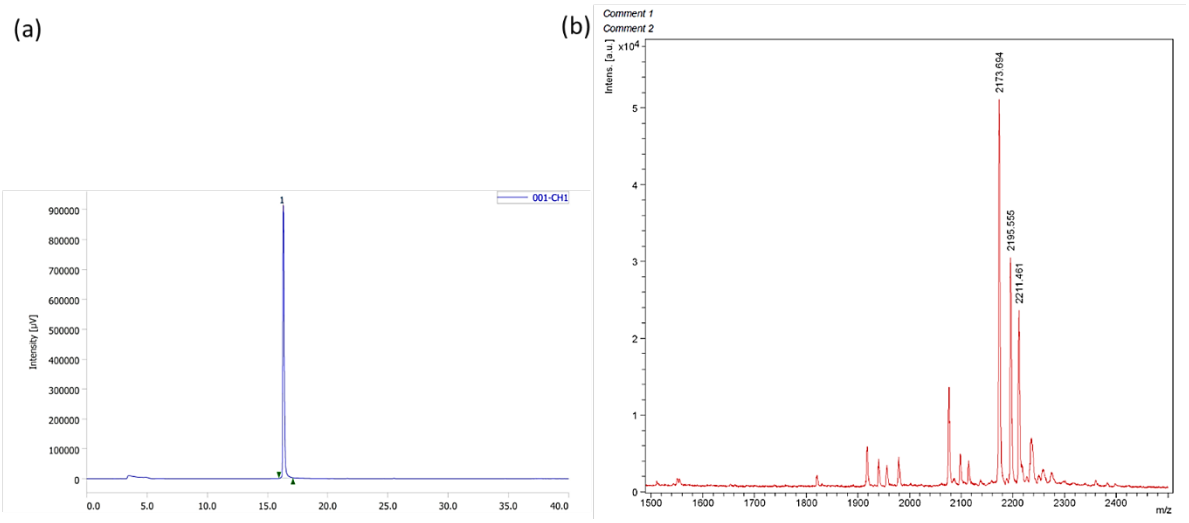
In the same way, conjugate **2** was obtained from Ac-Py-Py-Im-Im-γ-Py-Py-Py-Py-PEG-PEG-β-CO_2H (2.1 mg 0.94 μmol, 33% yield). Analytical HPLC: t_R = 16.3 min. MALDI-TOF-MS m/z calcd for $C_{106}H_{127}N_{30}O_{22}^+$ $[M + H]^+$ 2171.974 found 2173.694; $[M + Na]^+$ 2193.956 found 2195.555; $[M + K]^+$ 2209.930 found 2211.461.



Scheme S2. Synthesis of conjugate**1**, **2**. (ⅰ) HFIP, DCM, rt; (ⅱ) PyBOP, DIEA, DMF, rt.



(a) HPLC spectrum of conjugate **1**. Conditions: 0.1% TFA containing 0−100% acetonitrile over a linear gradient for 40 min at a flow rate of 1.0 mL/min detected at 254 nm. The retention time was 16.4 min. (b) MALDI-TOF MS spectrum of conjugate **1**. *m/z* calcd for $C_{100}H_{116}N_{29}O_{19}^{+}$ [M + H]$^{+}$ 2026.900, found 2028.314.



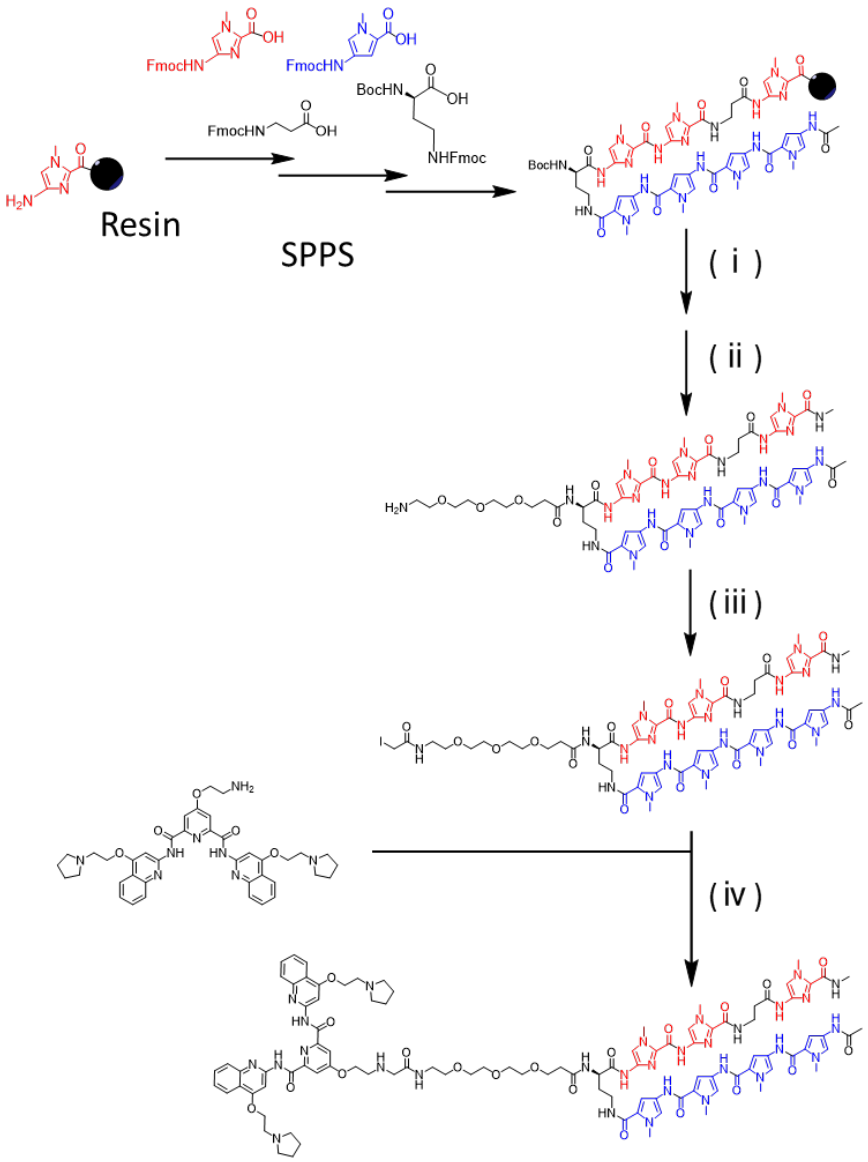
(a) HPLC spectrum of conjugate **2**. Conditions: 0.1% TFA containing 0−100% acetonitrile over a linear gradient for 40 min at a flow rate of 1.0 mL/min detected at 254 nm. The retention time was 16.3 min. (b) MALDI-TOF MS spectrum of conjugate **2**. *m/z* calcd for $C_{106}H_{127}N_{30}O_{22}^{+}$ [M + H]$^{+}$ 2171.974 found 2173.694.

conjugate 3

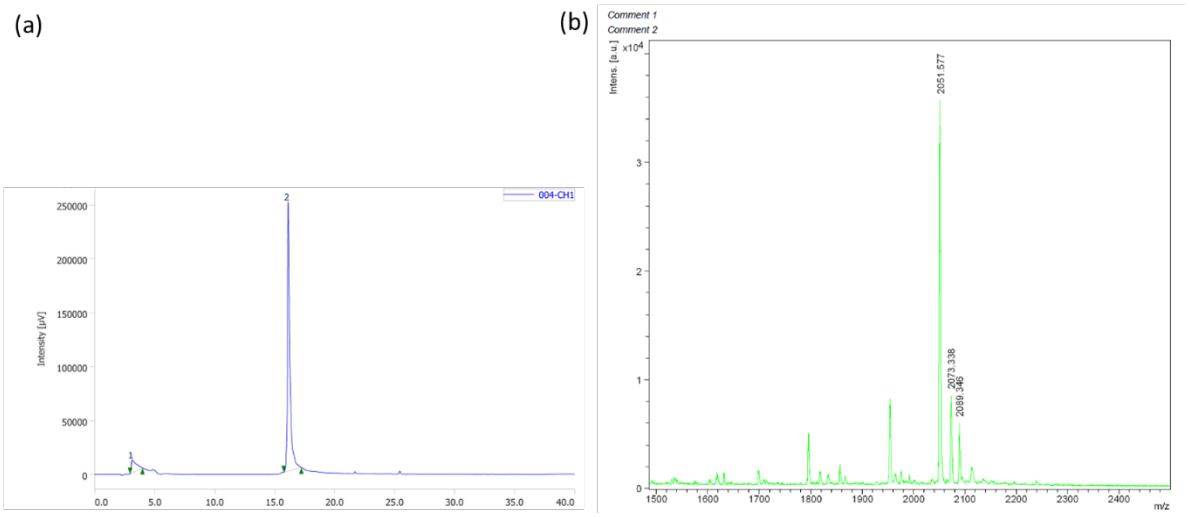
By using 91 mg of FmocHN-Im-CLEAR acid resin (0.356 mmol/g) and proper building blocks, Ac-Py-Py-Py-Py-(*R*)$^{\alpha\text{-}NH_2}$γ-Im-Im-β-Im-CLEAR acid resin was prepared by solid-phase synthesis. Following a cleavage reaction with 2M $MeNH_2$ (1000 µL) in THF for 3.5 h at 55 °C, the residues removed from the resin were directly poured into diethylether (40 mL) to be precipitated. The dried precipitates were dissolved in TFA/DCM (1.3/3.5 mL) and the solution was stirred at rt for 1.5 h to deprotect the boc group. After concentration *in vacuo*, the residues were dissolved in a minimum amount of DCM/MeOH and poured into diethylether (40 mL). The dried precipitates of PIP **2** (31.6 mg) were used for the next steps without further purification.

To a solution of PIP **2** (31.6 mg) in dry DMF (250 µL) was added a solution of *t*-Boc-*N*-amido-PEG3-acid (17.6 mg, 54.9 µmol) and PyBOP (30.4 mg, 58.4 µmol) in dry DMF (100 µL) and DIEA (18.9 µL, 108.5 µmol), which had been stirred at rt for 10 min. The mixture was stirred at rt for 3.5 h, and then the residues were directly poured into AcOEt (40 mL) to produce the precipitates, which were used for the next steps without further purification. The dried precipitates were dissolved in TFA/DCM (1.3/3.5 mL) and the solution was stirred at rt for 1 h to deprotect the boc group. After evaporation and the diethylether precipitation described above, the crude amine-containing polyamide (22.7 mg) was dissolved in dry DMF (200 µL) and DIEA (12.1 µL, 69.5 µmol). To the mixture was added *N*-succinimidyl iodoacetate (15.1 mg, 53.3 µmol), then stirred at rt for 2 h. The residues were directly poured into diethylether (40 mL) to be precipitated. The crude iodoacetate-containing polyamide was purified by reverse-phase HPLC (2.5 mg, 1.72 µmol, 5% yield over four steps). Analytical HPLC: t_R = 16.8 min. MALDI-TOF-MS m/z calcd for $C_{60}H_{78}IN_{22}O_{15}^+$ $[M + H]^+$ 1473.506 found 1474.083; $[M + Na]^+$ 1495.488 found 1496.135; $[M + K]^+$ 1511.462 found 1512.112.

PyPDS (2.5 mg, 3.55 µmol) was dissolved in dry DMF (150 µL) and DIEA (3.0 µL, 17.2 µmol). After stirring for 10 min, to the mixture was added the iodoacetate-containing polyamide (2.5 mg, 1.72 µmol), then stirred at 40 °C for 5 h. The evaporation afforded the crude title compound, which was purified by reverse-phase HPLC to give conjugate **3** (2.6 mg, 1.26 µmol, 73% yield). Analytical HPLC: t_R = 16.1 min. MALDI-TOF-MS m/z calcd for $C_{99}H_{121}N_{30}O_{20}^+$ $[M + H]^+$ 2049.937 found 2051.577; $[M + Na]^+$ 2071.919 found 2073.338; $[M + K]^+$ 2087.893 found 2089.346.



Scheme S3. Synthesis of conjugate **3**. (ⅰ) $MeNH_2$, THF, 55 °C; then TFA, DCM, rt; (ⅱ) Boc-*N*-amido-PEG3-acid, PyBOP, DIEA, DMF, rt; then TFA, DCM, rt; (ⅲ) *N*-succinimidyl iodoacetate, DIEA, DMF, rt; (ⅳ) DIEA, DMF, rt.



(a) HPLC spectrum of compound **3**. Conditions: 0.1% TFA containing 0−100% acetonitrile over a linear gradient for 40 min at a flow rate of 1.0 mL/min detected at 254 nm. The retention time was 16.1 min. (b) MALDI-TOF MS spectrum of compound **3**. *m/z* calcd for $C_{99}H_{121}N_{30}O_{20}^{+}$ $[M + H]^{+}$ 2049.937 found 2051.577.

Circular Dichroism (CD) spectroscopy experiments.

DNA samples (5 µM, 100 µL) for CD were prepared in 10 mM Tris-HCl (pH 7.5) and 4 mM KCl buffer. Before analysis, samples were heated to 95 °C and cooled down to 25 °C on a 0.8 °C/min rate. CD spectra were recorded at 25 °C over the range of 220−380nm using a J-805LST spectrometer (JASCO) in a 1-cm path-length quartz cuvette.

CD melting assays.

DNA samples (5 µM, 100 µL) for CD melting measurements were prepared in 10 mM Tris-HCl (pH 7.5) and 4 mM KCl buffer. Before analysis, samples were heated to 95 °C and cooled down to 25 °C on a 0.8 °C/min rate. Temperature scans were performed in the presence or absence of compounds (5 µM, 0.5% DMSO) by monitoring continuously from 20 to 95 °C at 267 nm on a 0.5 °C /min rate in a 1-cm quartz cuvette using a J-805LST spectrometer (JASCO) and a refrigerated and heating circulator F25-ME (Julabo). The T_m values were determined as the minimum of the first derivative of the sigmoidal approximate curve.

CD titration assays.

The target DNA sample (5 μM, 500 μL) for CD titration was prepared in 10 mM Tris-HCl (pH 7.5) and 4 mM KCl. Before analysis, samples were heated to 95 °C and cooled down to 25 °C on a 0.8 °C/min rate. Aliquots of the master solution of compounds (2 mM in DMSO) were added continuously and incubated at least 3 min to reach the equilibrium. CD spectra were recorded at 25 °C over the range of 220−380 nm using JASCO J-805LST spectrometer in a 1-cm path length quartz cuvette.

UV melting assays.

UV melting assays of duplex were carried out on a spectrophotometer V-650 (JASCO) equipped with a thermocontrolled PAC-743R cell changer (JASCO) and a refrigerated and heating circulator F25-ED (Julabo). DNA samples (2.5 μM, 100 μL) were prepared in 10 mM Tris-HCl (pH 7.5) and 4 mM KCl buffer. Before analysis, samples were heated to 95 °C and cooled down to 10 °C on a 1 °C/min rate. Temperature scans were performed in the presence or absence of compounds (7.5 μM, 0.75% DMSO) by monitoring continuously from 10 to 75 °C at 260 nm on a 1 °C/min rate. The ΔT_m values shown in Table 2 are the average of all data (n=2). The representative denaturing curves of each compound are shown in Figure S4. The T_m values were determined as the maximum of the first derivative of the denaturing profile.

Fluorescence resonance energy transfer (FRET) melting assays.

DNA samples (1 μM, 25 μL) were prepared in 10 mM Tris-HCl (pH 7.5) and 4 mM KCl buffer on a 96-well plate. The shorter strand and the longer strand of the target sequence (Figure S9a) were labeled at the 5'-end with a fluorescein (FAM) and the 3'-end with a tetramethylrhodamine (TAMRA), respectively. Before analysis, samples were heated to 95 °C and cooled down to 10 °C on a 1 °C/min rate. Temperature scans were performed in the presence or absence of compounds (3 μM) by monitoring continuously from 20 to 70 °C with FAM fluorescence monitored on a 1 °C /min rate using a LightCycler 480 System II (Roche). The fluorescence was detected by the FAM filter (excitation: 465 nm, detection: 510 nm). The ΔT_m values shown in Table S1 are the average of all data (n=2). The T_m values were determined as the temperature at half of the maximum signal increased.

Native gel electrophoretic mobility shift assays.

DNA samples (0.2 μM, 10 μL) were prepared in 10 mM Tris-HCl buffer (pH 7.5) and 50 mM KCl buffer. The DNA samples were heated at 95 °C for 5min, then gradually cooled to 25 °C on a 0.8 °C/min rate. After annealing, compounds were added to the resulting DNA solution (1 μM, 0.1% DMSO) and incubated for 60 min at 25 °C. The samples mixed with 6 × loading dye (BioLabs Inc.) were loaded on a 15% polyacrylamide gel containing 50 mM KCl and electrophoresed at 4 °C, 100 V for 110 min in 1 × TBE buffer containing 50 mM KCl. The gel was stained with SYBR gold for 15 min and then imaged on a GelDoc Go Gel Imaging System (Bio-Rad laboratories).

Fluorescent indicator (ThT or EtBr) displacement assays.

DNA samples (0.5 μM, 100 μL) were prepared in 10 mM Tris-HCl (pH 7.5) and 50 mM KCl buffer. The samples were heated at 95 °C for 5 min and cooled down to 25 °C a 1 °C/min rate for annealing, then incubated with the fluorescent indicator (ThT 0.5 μM or EtBr 1 μM) and compounds (0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4, 5 μM, 0.5% DMSO) for 60 min at 25 °C. The samples were excited at 425 or 525 nm and the fluorescence emission intensity of ThT or EtBr at 490 or 596 nm were measured respectively with a SpectraMax M2e microplate reader (Molecular Devices). All the measurements were performed in technical duplicate. The percentage of displacement was calculated using the following formula: $(1 - F/F_0) \times 100$. F and F_0 indicate the fluorescence intensity in the presence or absence of compounds and the background fluorescence was subtracted from all measurements. The DC_{50} values (the concentration required for 50 % displacement of fluorescent indicator) of each compound were determined with the fitting curve calculated by SoftMax Pro 6.5.

Molecular modeling studies.

Molecular modeling studies were performed with Discovery Studio (BIOVIA) using a CHARMm force field. The initial structures were constructed based on the previous crystal and NMR structures (PBD ID: 3I5L, 1VAV, and 7X2Z). PyPDS was manually inserted between the G4 and duplex and connected with PIP located in a minor groove of the duplex through the linker using the builder module. After the pre-minimization of initial structures, the complex was solvated in cubic water with 50 mM KCl. The entire structure was finally minimized to the stage where the root-mean-square was less than 0.001 kcal/mol·Å using the conjugate gradient algorithm with fixed restraints of the terminal two base pairs of DNA.

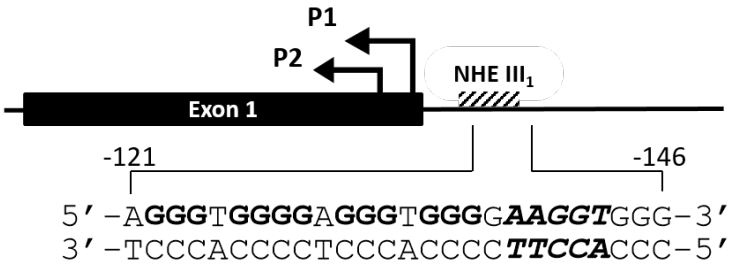


Fig. S1 The natural *c-MYC* G4 sequence and its proximal duplex in the nuclease hypersensitive element (NHE) III_1 upstream of the P1 promoter. Nucleotides in bold represent G4-forming sequences, in bold and italics represent PIP recognition sites.

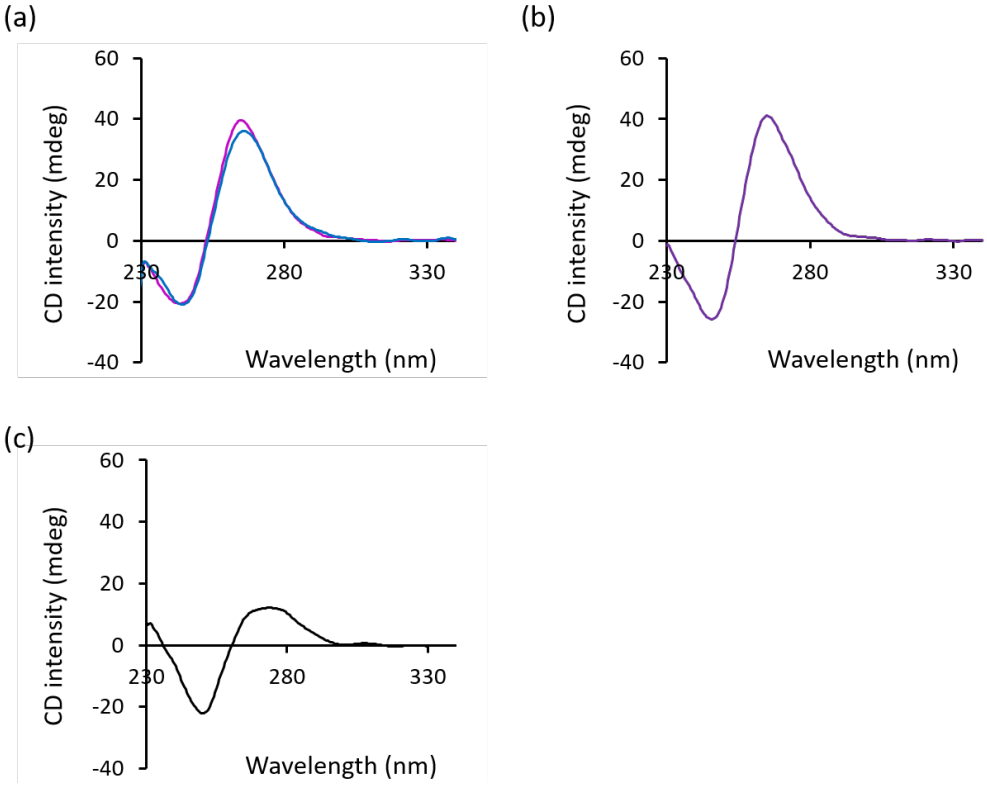


Fig. S2 CD spectrum of (a) the target sequence (purple), the sum of CD spectra of the G4 and ds sequences (blue), (b) the non-target sequence (dark purple), and (c) the non-G4 sequence (black).

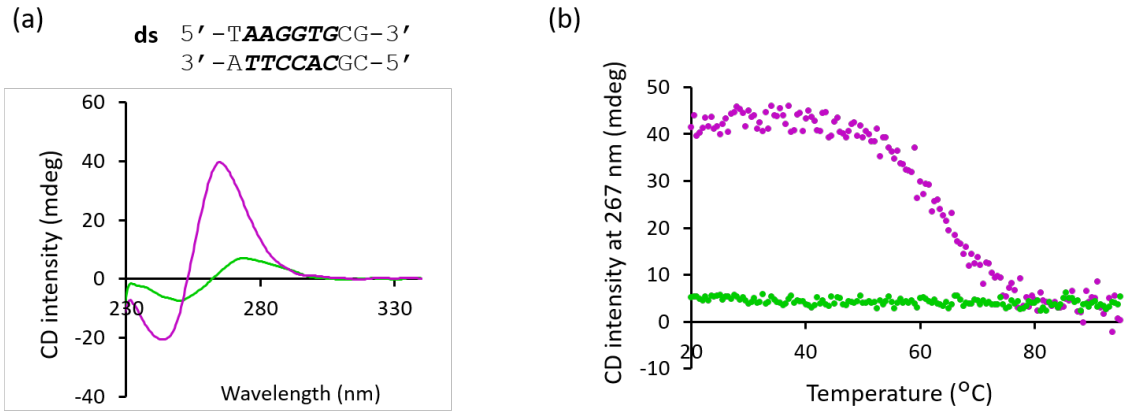


Fig. S3 (a) CD spectra of the target sequence (purple) and the ds sequence (green). (b) CD melting curves for the target sequence (purple) and the ds sequence (green) detected at 267 nm.

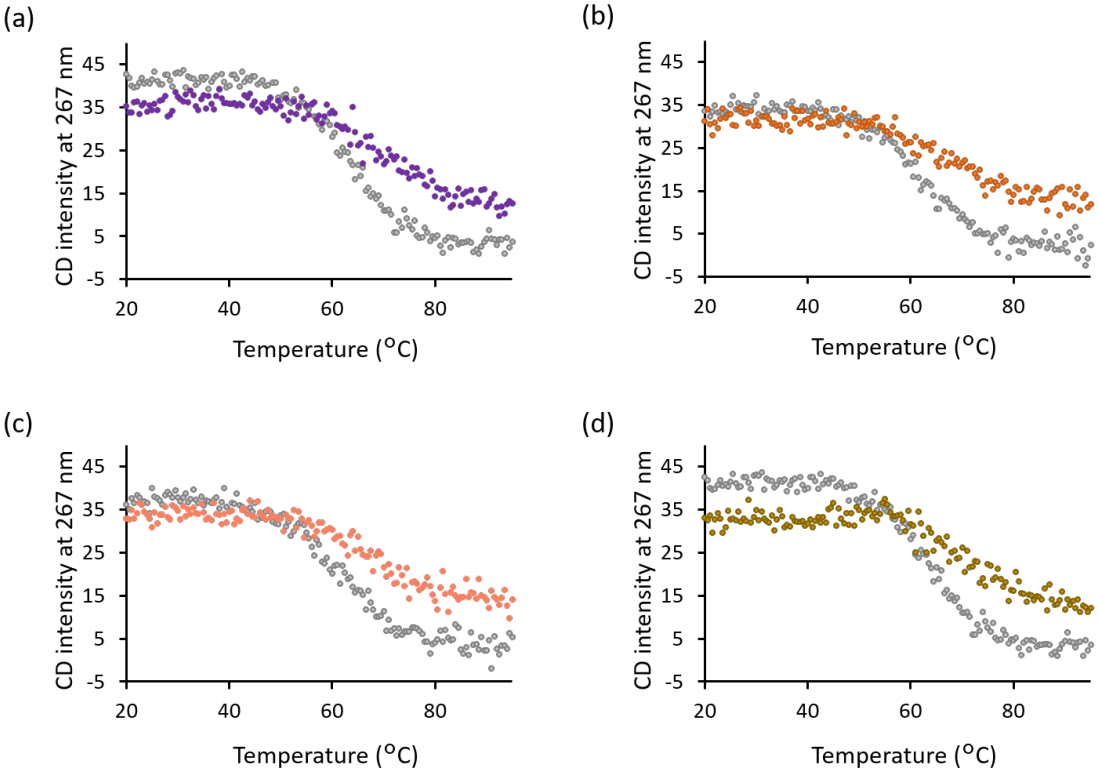


Fig. S4 The raw data of CD melting curves for the target (G4–duplex chimera) sequence in the absence (gray) or presence of 1 molar equivalent of compounds (colored): (a) PyPDS; (b) conjugate **1**; (c) conjugate **2**; (d) conjugate **3**.

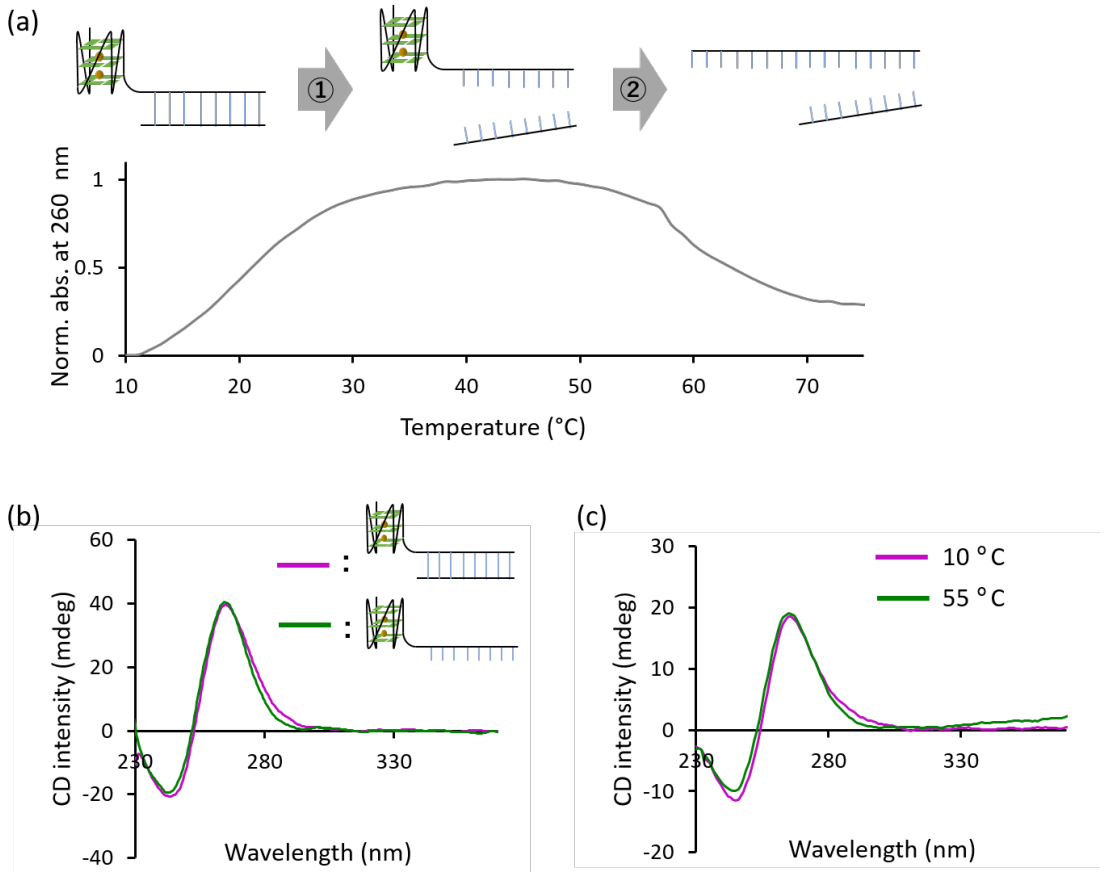


Fig. S5 (a) Schematic representation of the DNA melting for thermal denaturing assays and UV melting curves at 260 nm for the target sequence. (b) CD spectra of the target DNA sequence (purple) and the DNA sequence from which the complementary strand forming the duplex region was excluded (green). (c) CD spectra of the target sequence used in the UV melting assay detected at 10 °C (purple) and 55 °C (green).

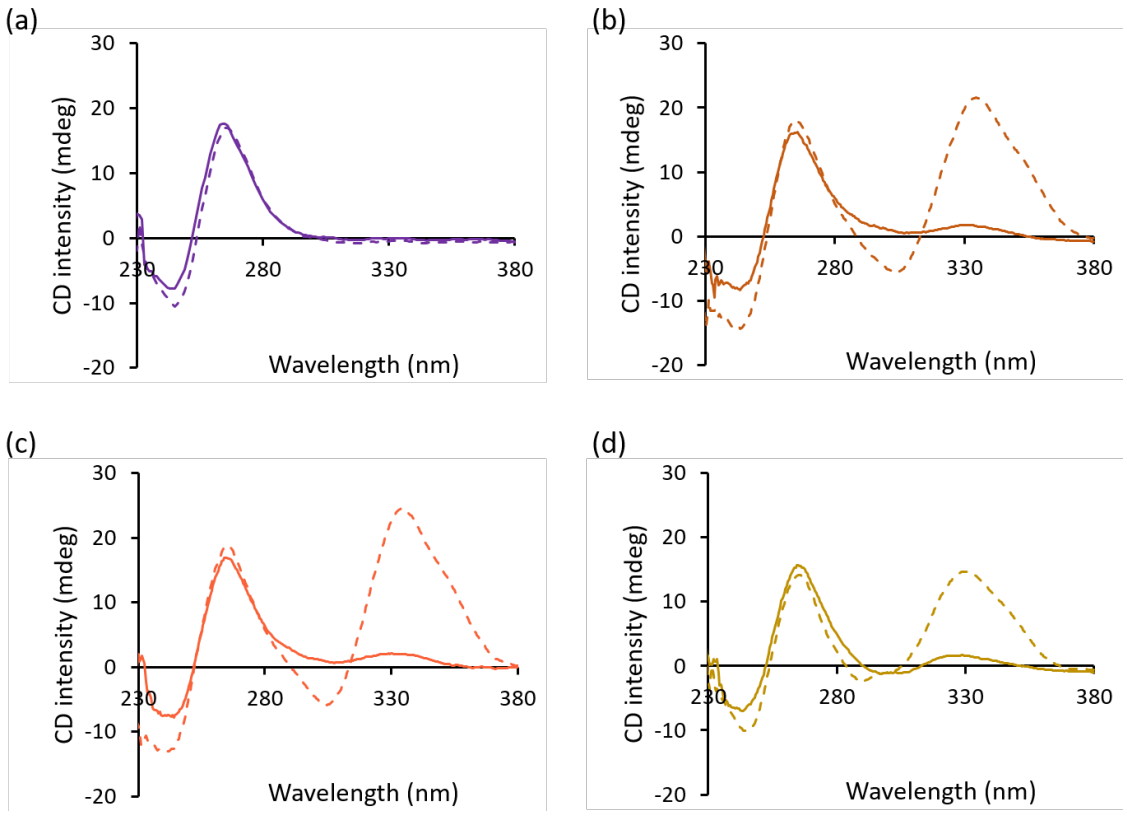


Fig. S6 CD spectra of the target sequence in the presence of (a) PyPDS, (b) conjugate **1**, (c) conjugate **2** and (d) conjugate **3** detected at 10 °C (dashed lines) and 55 °C (solid lines). The samples were prepared in the same condition as the UV melting assay detected at 260 nm.

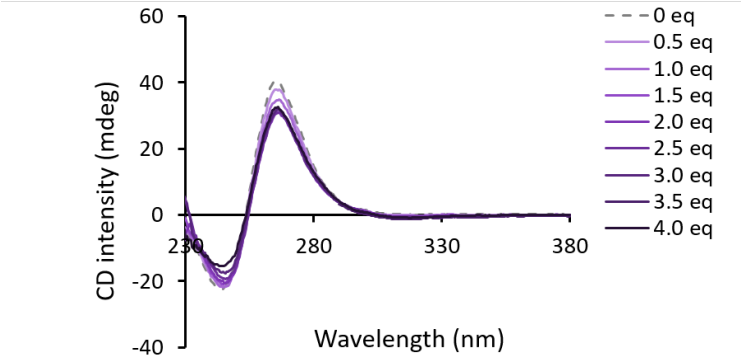


Fig. S7 CD spectra of the target sequence titrated with PyPDS.

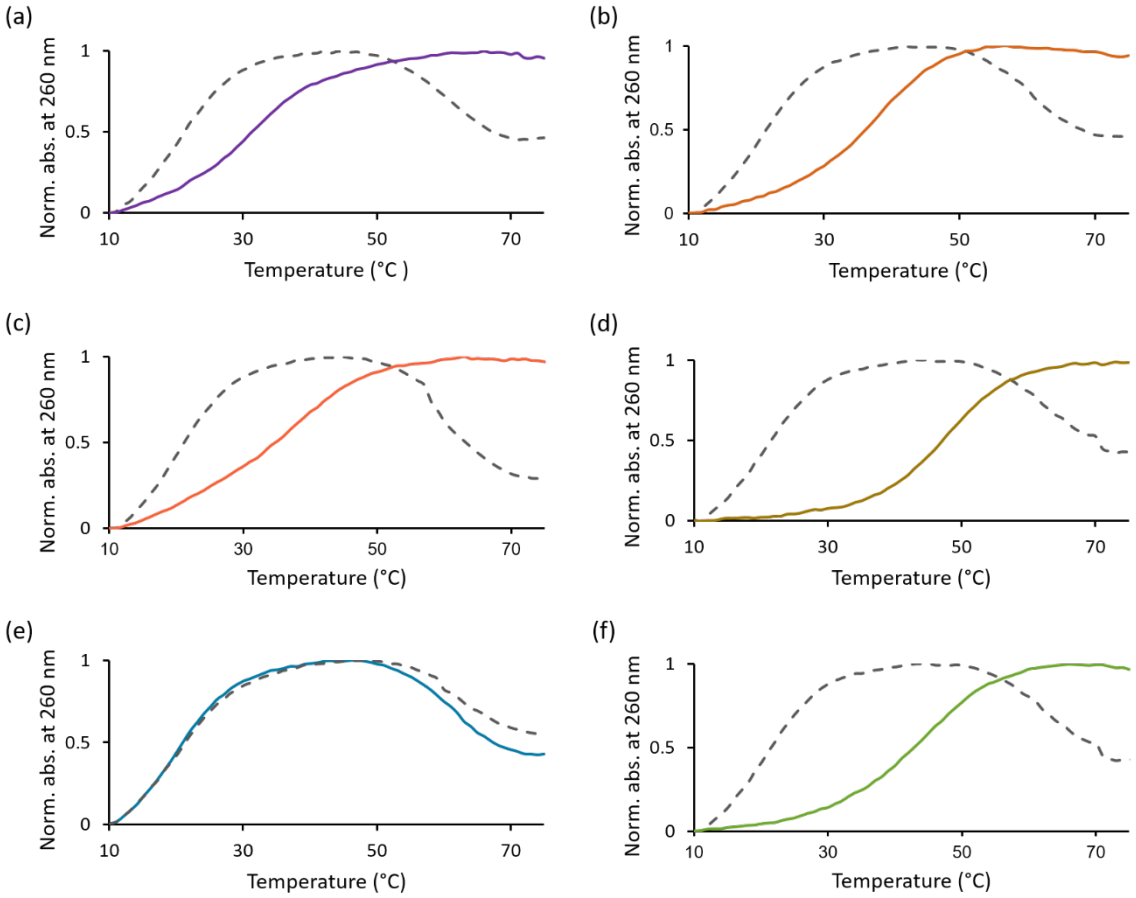


Fig. S8 Normalized absorbance-base melting curves for the target sequence in the absence (gray-dashed lines) and presence of 3 molar equivalents of compounds (colored lines) detected at 260 nm: (a) PyPDS; (b) conjugate **1**; (c) conjugate **2**; (d) conjugate **3**; (e) PIP **1**; (f) PIP **2**.

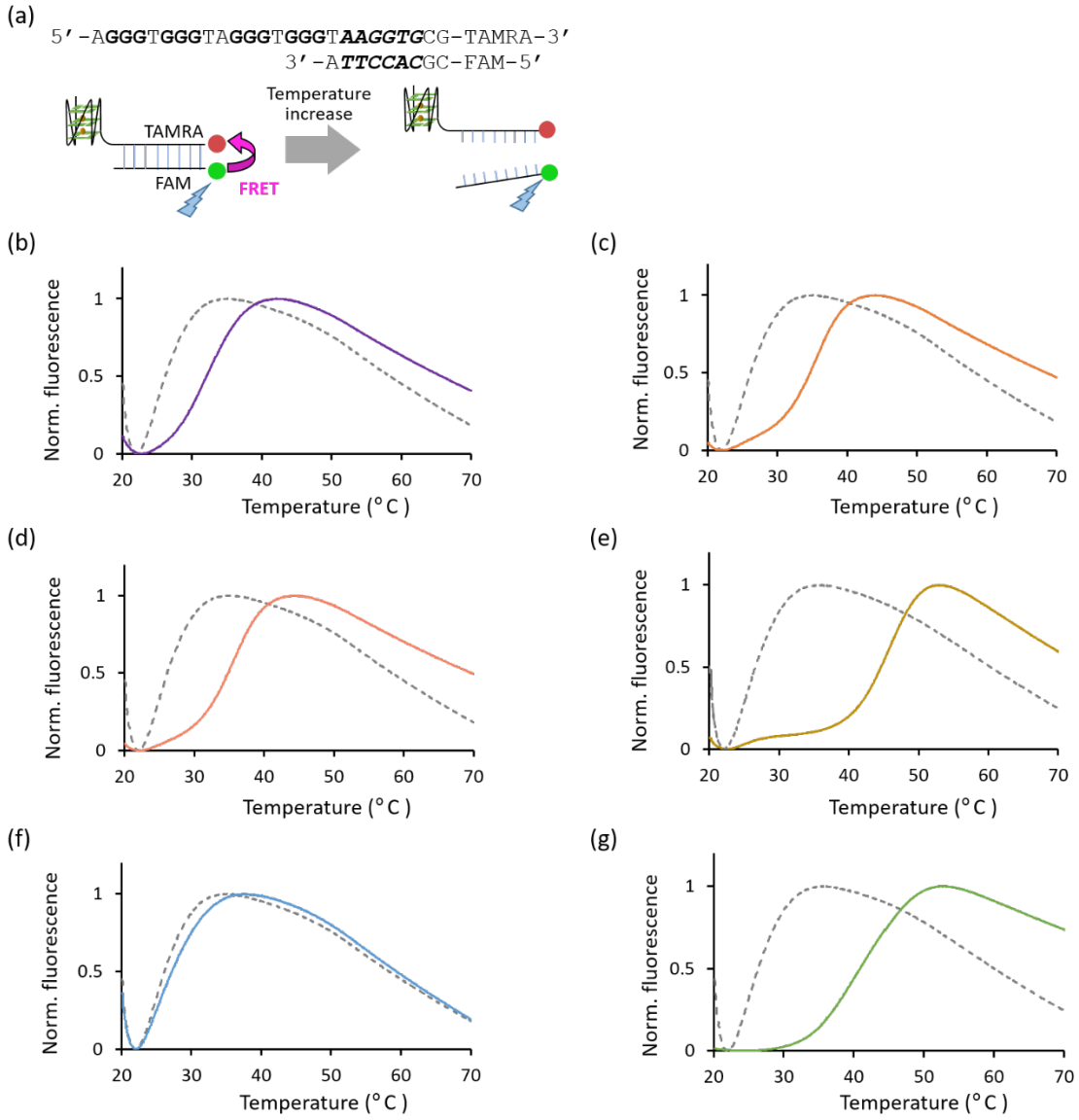


Fig. S9 (a) The fluorescent labeled DNA sequence and the schematic representation of the FRET melting system; Normalized melting curves monitoring the FAM fluorescence for the target sequence in the absence (gray-dashed lines) and presence of 3 molar equivalents of compounds (colored lines): (b) PyPDS; (c) conjugate **1**; (d) conjugate **2**; (e) conjugate **3**; (f) PIP **1**; (g) PIP **2**.

Table S1 Changes in duplex melting temperature values (ΔT_m) of the target sequence (1 μM) in the presence of compounds (3 molar equivalents) by FRET melting assay.

	PyPDS	PIP **1**	conjugate **1**	conjugate **2**	PIP **2**	conjugate **3**
ΔT_m [°C]	6.2±0.2	0.5±0.7	8.8±0.2	9.1±0.4	14.1±0.6	17.9±0.1

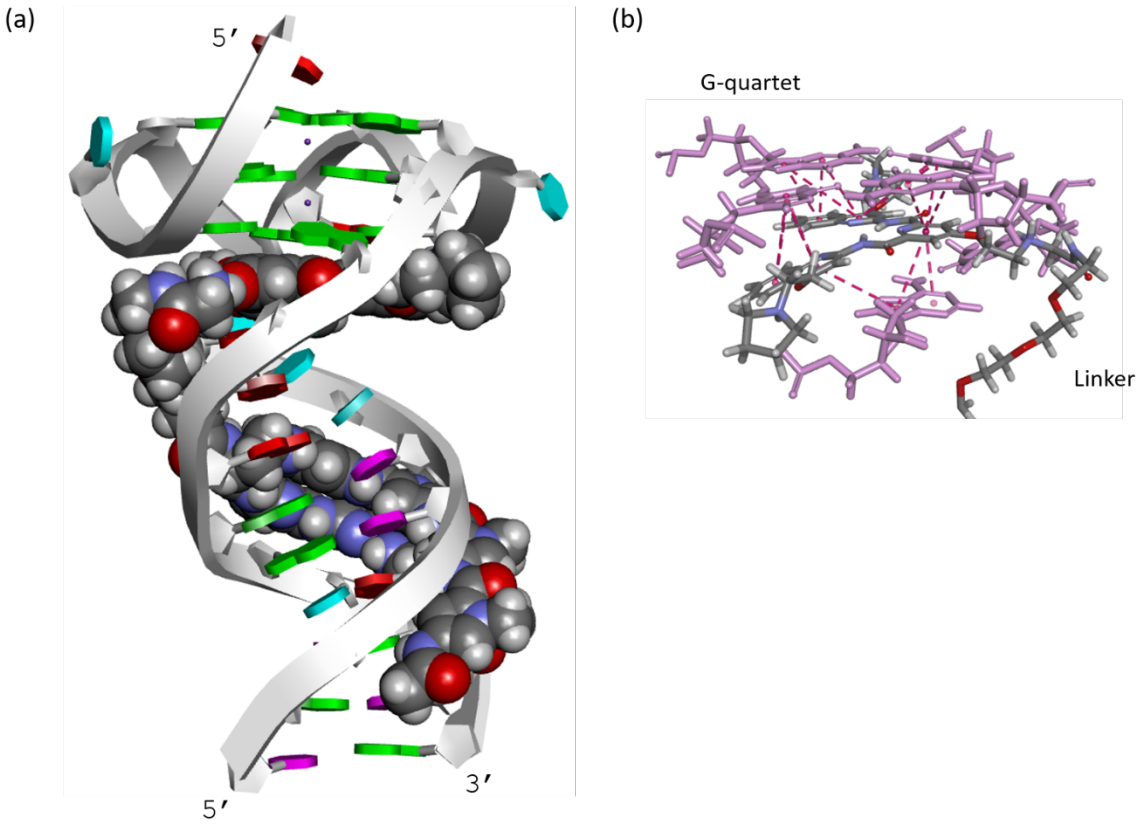


Fig. S10 The optimized binding model of conjugate **3** towards the target DNA sequence; (a) overview (b) The π–π interactions of PyPDS in the 3′ G4–duplex interface.

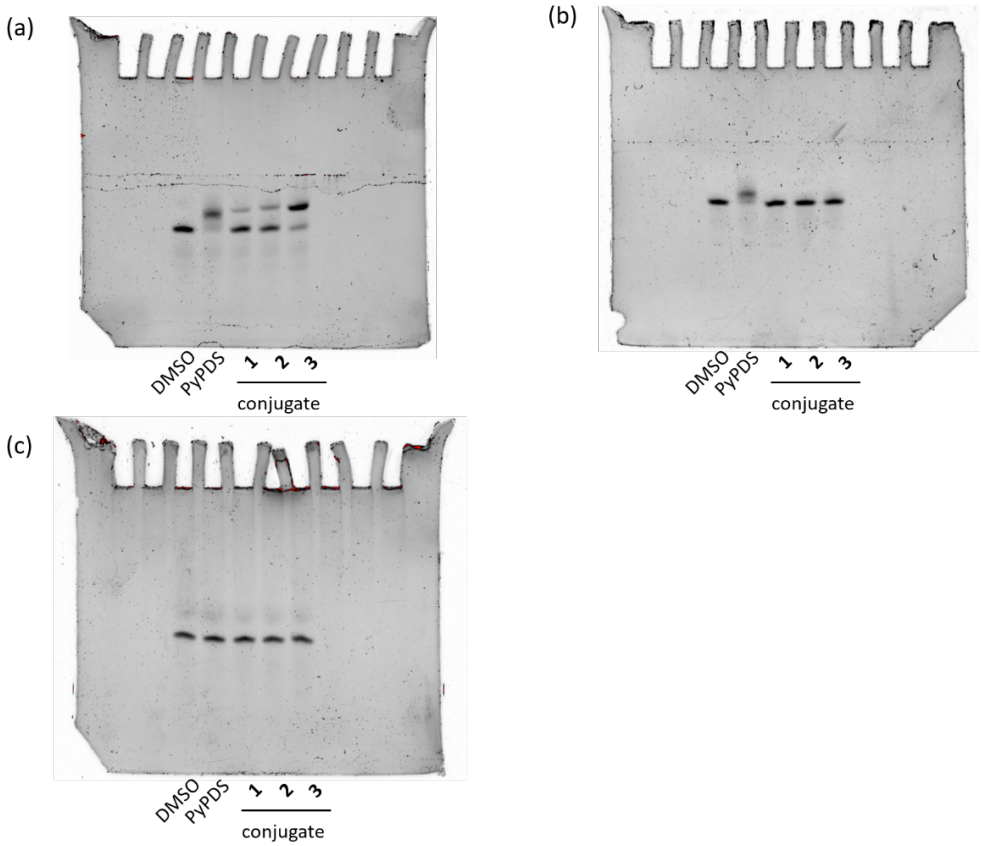


Fig. S11 The full gel images for comparing the binding preference of compounds (5 molar equivalents) towards (a) the target sequence; (b) the non-target sequence; (c) the non-G4 sequence.

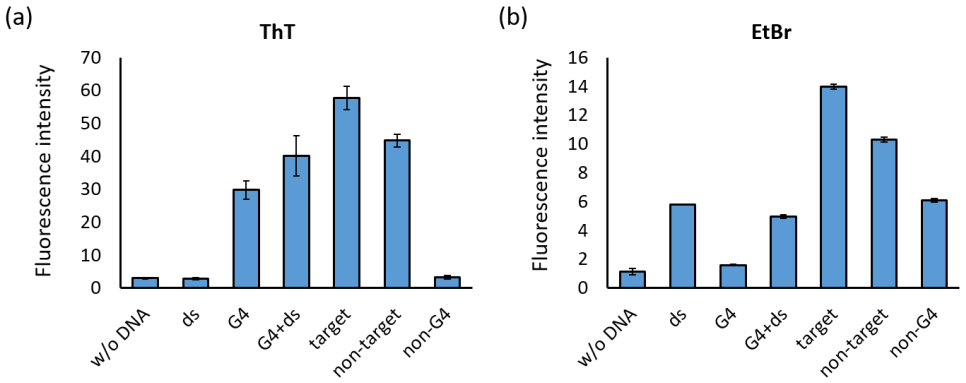


Fig. S12 The fluorescence intensity of (a) ThT at 490 nm and (b) EtBr at 596 nm in the absence or presence of various DNA sequences. G4+ds represents a mixture of equal amounts of G4 DNA sequence and ds DNA sequence, which are components of the target sequence. The error bars represent the standard deviation of technical duplicate measurements.

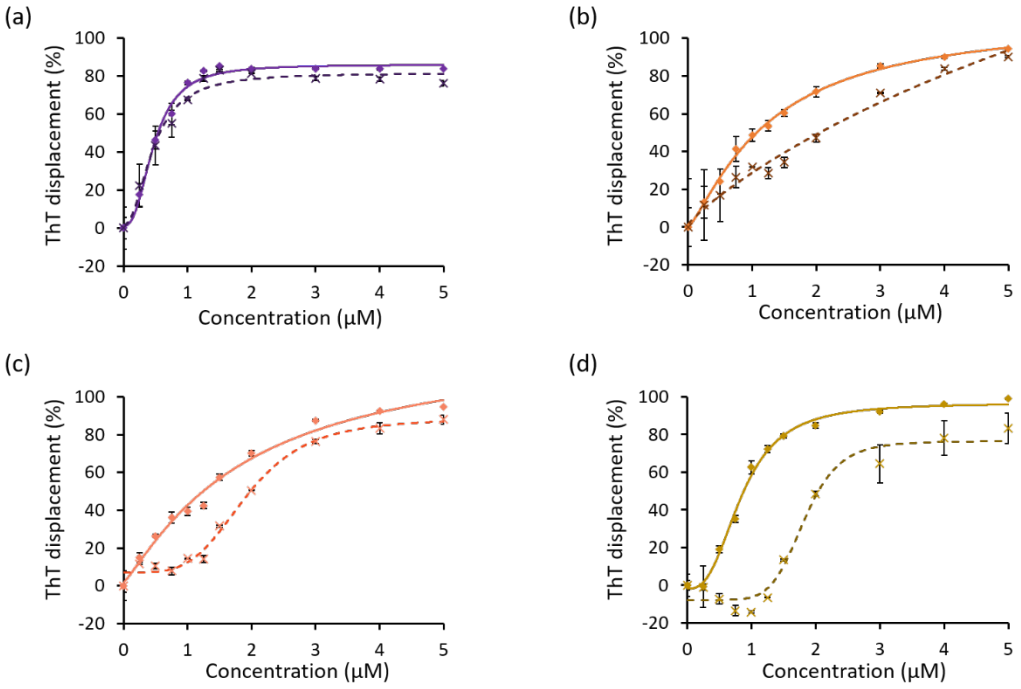


Fig. S13 ThT displacement curves from the target sequence (solid lines) and non-target sequence (dashed lines) by (a) PyPDS, (b) conjugate **1**, (c) conjugate **2** and (d) conjugate **3**. The error bars represent the standard deviation of technical duplicate measurements.

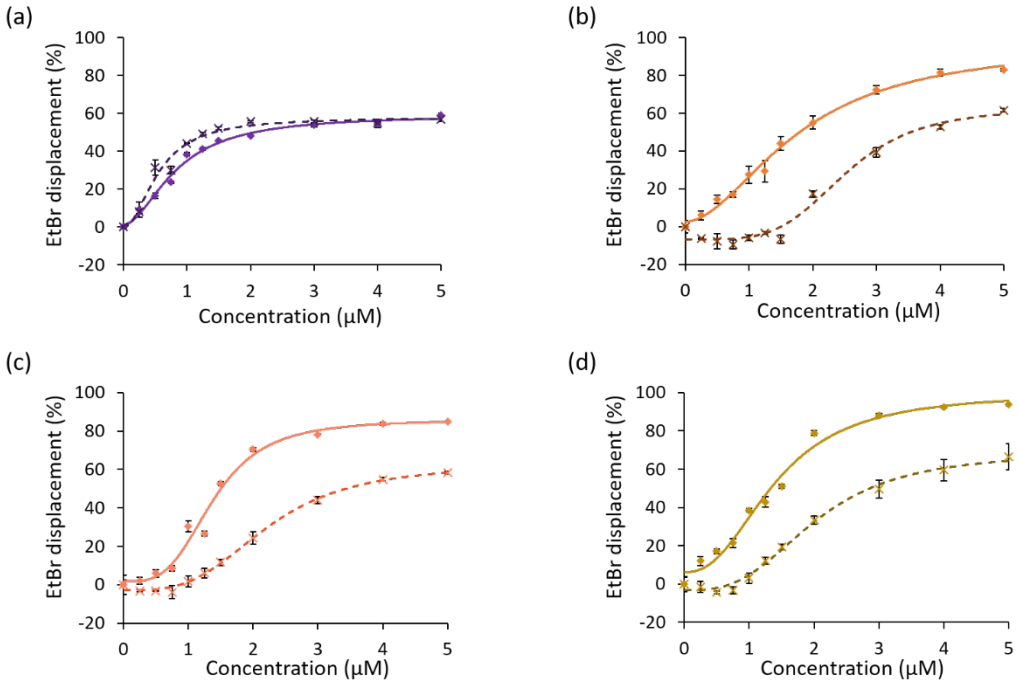


Fig. S14 EtBr displacement curves from the target sequence (solid lines) and non-target sequence (dashed lines) by (a) PyPDS, (b) conjugate **1**, (c) conjugate **2** and (d) conjugate **3**. The error bars represent the standard deviation of technical duplicate measurements.

References

1 S. Müller, D. A. Sanders, M. Di Antonio, S. Matsis, J.-F. Riou, R. Rodriguez and S. Balasubramanian, *Org. Biomol. Chem.*, 2012, **10**, 6537.

2 M. Di Antonio, A. Ponjavic, A. Radzevičius, R. T. Ranasinghe, M. Catalano, X. Zhang, J. Shen, L.-M. Needham, S. F. Lee, D. Klenerman and S. Balasubramanian, *Nat. Chem.*, 2020, **12**, 832–837.

3 S. Asamitsu, S. Obata, A. T. Phan, K. Hashiya, T. Bando and H. Sugiyama, *Chemistry*, 2018, **24**, 4428–4435.