Supplementary information

Structural regulation by a G-quadruplex ligand increases binding abilities of G-quadruplex-forming aptamers.

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Materials and Methods

Chemicals, oligonucleotides, and proteins

L1H1-7OTD (**Fig. S1A**) was synthesized as previously described[1] and dissolved in dimethyl sulfoxide (DMSO). Finally, a 1 mM stock solution of L1H1-7OTD was prepared by diluting it with DMSO. L2H2-6OTD (**Fig. S1B**) was also synthesized as previously described.[2] Phen-DC3 (**Fig. S1C**) and TmPyP4 (**Fig. S1D**) were purchased from Polysciences, Inc. and DOJINDO, respectively.

All oligonucleotides employed in this study were synthesized elsewhere (FASMAC Co., Ltd., Eurofins Genomics K.K.). The DNA sequences of the aptamers are shown in Supplementary material (**Table S1**). The aptamers were labeled with fluorescein isothiocyanate (FITC) at the 5′-terminus. A recombinant α-synuclein (α-syn) monomer

and α-syn oligomer were prepared as previously described.[3, 4] Human VEGF was purchased from R&D Systems, Inc.

Preparation of DNA aptamers

Aliquots of the α-syn oligomer-binding aptamers were diluted with TBS buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM KCl, pH 7.4) or TK buffer (10 mM Tris-HCl, 100 mM KCl, pH 7.4). For the VEGF-binding aptamer, TBS buffer containing either 10 mM Tris-HCl, 100 mM NaCl, and 5 mM KCl or 10 mM Tris-HCl and 100 mM NaCl was used. An aptamer solution was mixed with G4 ligands before heat treatment for folding. Folding of the DNA aptamers was performed at 95°C for 10 min, and then the solutions were gradually cooled to 25°C.

Circular dichroism spectrum measurement and gel electrophoresis

The circular dichroism (CD) spectra of the aptamers were measured at 20°C in a wavelength range of 200–320 nm using a J-720 or J-820 spectropolarimeter (JASCO, Tokyo, Japan) equipped with a 0.1-cm path cuvette. Aliquots of the DNA aptamers were diluted in TBS buffer to a final concentration of 10 μM and folded by thermal process as described above. DNA–polyacrylamide gel electrophoresis (PAGE) was performed in a 15% polyacrylamide gel. The FITC-modified aptamers were heat-treated

as described above and separated in Tris–borate–ethylenediaminetetraacetic acid (TBE) buffer.

Dimethyl sulfate (DMS) footprinting

FITC-labeled oligonucleotides (500 pmol) were prepared in a total volume of 100 μL as described above, and 10 μL of DMS was added to obtain a final concentration of 1% (v/v).The solution was incubated for 3 min at room temperature. After incubation, we added 10 μL of the stop solution containing 45% (v/v) β-mercaptoethanol, 1.3 M sodium acetate (pH 7.0), and 1.8 μg/mL tRNA (Roche Diagnostics K.K.). After ethanol precipitation, we performed the piperidine cleavage reaction by heating the samples at 95ºC for 30 min in 100 μL of 10% (v/v) piperidine. The DNA solution was lyophilized, and the lyophilized powder was dissolved with loading buffer (formamide:0.5M EDTA, pH 8.0:MQ; 197:10:43, vol/vol). The samples were separated by 20% polyacrylamide gel containing 7 M urea in TBE buffer. To determine the folding of T-SO530 and 5′-side of bivalent aptamers, electrophoresis was performed at 1000 V for 10 min and at 2500 V for 95 min. To determine the folding of 3′-side of bivalent aptamers, electrophoresis was performed at 1000 V for 10 min and at 2500 V for 210 min. The gel image was obtained by Typhoon8600 (GE healthcare). Band intensity was quantified by ImageJ.

Investigation of melting temperature (T_m)

We measured the ellipticity of a DNA solution containing the α-syn oligomer-binding aptamers with or without L1H1-7OTD by using a J-820 spectropolarimeter equipped with a 0.1-cm path cuvette. Aliquots of the DNA aptamers were diluted in TBS buffer to a final concentration of 10 μM and folded by the thermal process, as described above. In the spectrum obtained at 20ºC, we detected a positive peak around 280 nm, which is the characteristic wavelength for anti-parallel structure of T-SO530 and the bivalents. Melting curve was recorded at the identified wavelength around 280 nm during cuvette heating from 20ºC to 95ºC. We determined maximum ellipticity ($[\theta_{max}]$) and minimum ellipticity ($[\theta_{min}]$) from the melting curve (**Fig. S4A**). The Gibbs free energy change, ΔG, was calculated using the following equations.

$$\Delta G=-RT\ln[G4]/[ssDNA]$$

$$[G4]=([\theta]-[\theta_{min}])/([\theta_{max}]-[\theta_{min}])$$

$$[ssDNA]=([\theta_{max}]-[\theta])/([\theta_{max}]-[\theta_{min}])$$

R; the gas constant. T; the absolute temperature.

We plotted the ΔG and temperature to confirm the linear decrease (**Fig. S4B-G**), which can be approximated by the primary expression. Melting temperature, T_m, was obtained from the linear expression using ΔG=0 (**Fig. S4H**).

Evaluation of binding abilities to targeted proteins

Enzyme-linked oligonucleotide assay (ELONA)

We evaluated the binding ability of aptamers for α-syn oligomer by an enzyme-linked oligonucleotide assay (ELONA).[5] We immobilized 1 μg of the α-syn oligomer prepared in 100 μL of PBS buffer, pH 7.4 in a 96-well polystyrene plate at 37°C for 90 min. After washing the wells with TBS buffer containing 0.05% Tween-20 (TBS-T buffer), their surface was blocked by using 4% (w/v) skim milk in TBS-T buffer. The FITC-modified DNA aptamers were diluted to 100 nM in TBS-T buffer, with or without L1H1-7OTD (final concentration 1 μM), then added to each well, and the plate was incubated for 1 h. After washing the wells by TBS-T buffer, diluted horseradish peroxidase (HRP)-conjugated anti-FITC antibodies were added to each well and incubated for 1 h. Excess antibodies were removed by washing five times. To measure the HRP activity, BM chemiluminescence HRP substrate (Roche Diagnostics K.K.) was added, and chemiluminescence was detected by a multi label plate counter (Perkin Elmer). All steps of the ELONA, except immobilization of the α-syn oligomer, were performed at room temperature.

Aptamer blotting

The FITC-labeled VEGF-binding aptamer bivalent 3R02 was prepared at 100 nM in the presence or absence of 500 nM L1H1-7OTD, L2H2-6OTD, Phen-DC3, and

TmPyP4. Aliquots of VEGF were immobilized on a nitrocellulose membrane. The VEGF-spotted membrane was blocked with 2% (w/v) bovine serum albumin and then incubated with the prepared FITC-labeled bivalent 3R02. After washing with TBS-T buffer, the membrane was incubated with the HRP-conjugated anti-FITC antibody and developed using the Immobilon™ western chemiluminescent HRP substrate (Millipore). All steps of aptamer blotting were performed at room temperature.

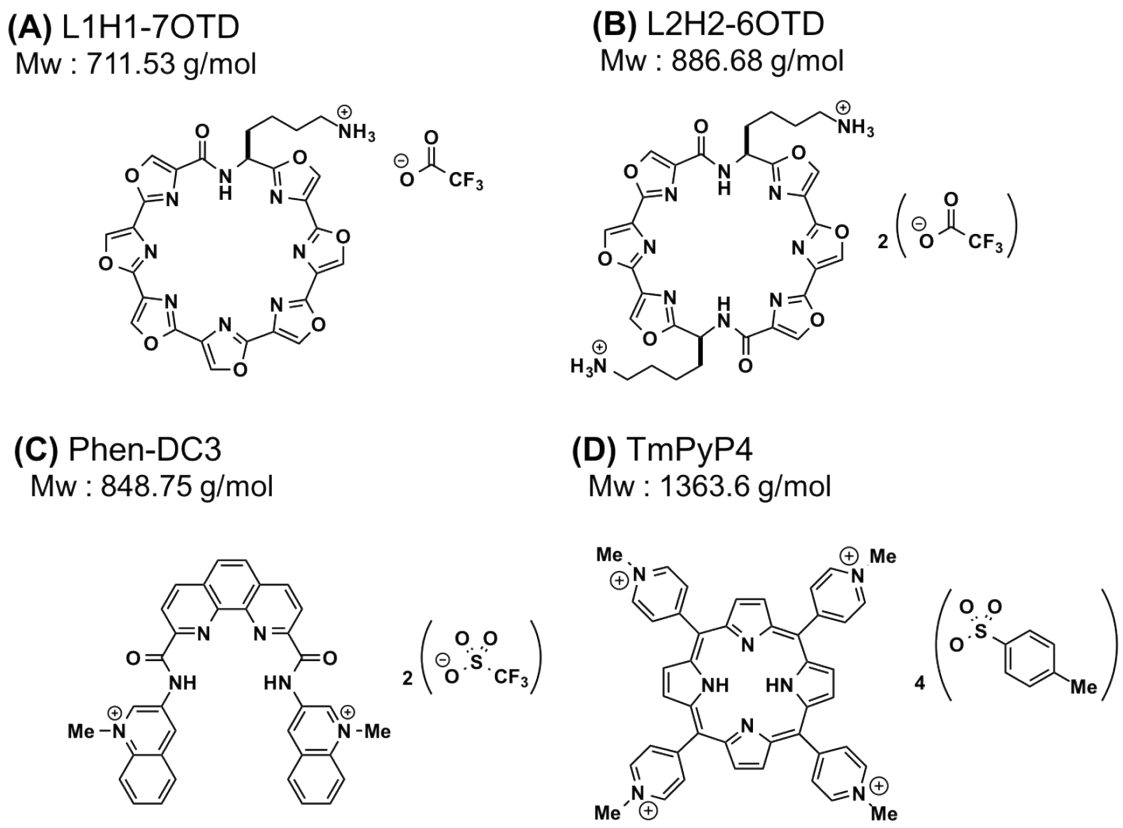


Fig. S1. The molecular structural formula of the G4 ligands used in this study.

Table S1. Sequences of DNA aptamers used in this study.

Name	Sequence (5′→3′)
T-SO530	ttttGGTGCGGCGGGACTAGTGGGTGTG
Bivalent T-SO530-1	ttttGGTGCGGCGGGACTAGTGGGTGTG-ttttGGTGCGGCGGGACTAGTGGGTGTG
Bivalent T-SO530-2	ttttGGTGCGGCGGGACTAGTGGGTGTG-ttttttttttGGTGCGGCGGGACTAGTGGGTGTG
Bivalent 3R02	TGTGGGGGTGGACTGGGTGGGTACC-ttttttttttTGTGGGGGTGGACTGGGTGGGTACC-

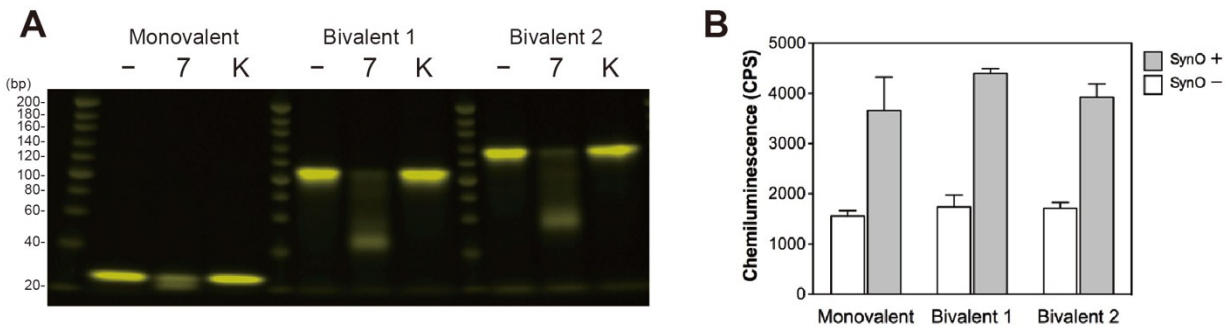
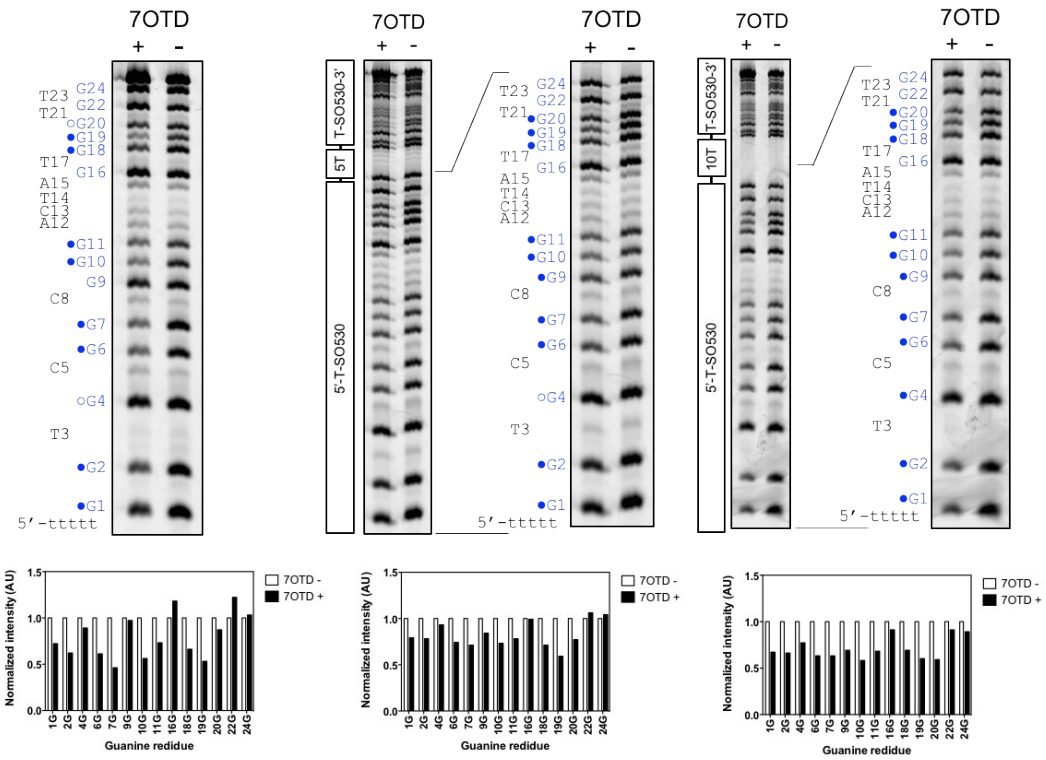


Fig. S2. Structural and binding properties of bivalent T-SO530 aptamers in the presence of 100 mM KCl. (**A**) DNA-PAGE data (–, TBS buffer; 7, TBS buffer with L1H1-7OTD; K, TBS buffer containing 100 mM KCl). (**B**) Results of ELONA.


(A) T-SO530
(B) Bivalent 1, 5'-side
(C) Bivalent 2, 5'-side
7OTD
+ -
T-SO530-3'
5T
10T
5'-T-SO530
5'-ttttt
Normalized intensity (AU)
7OTD -
7OTD +
Guanine redidue

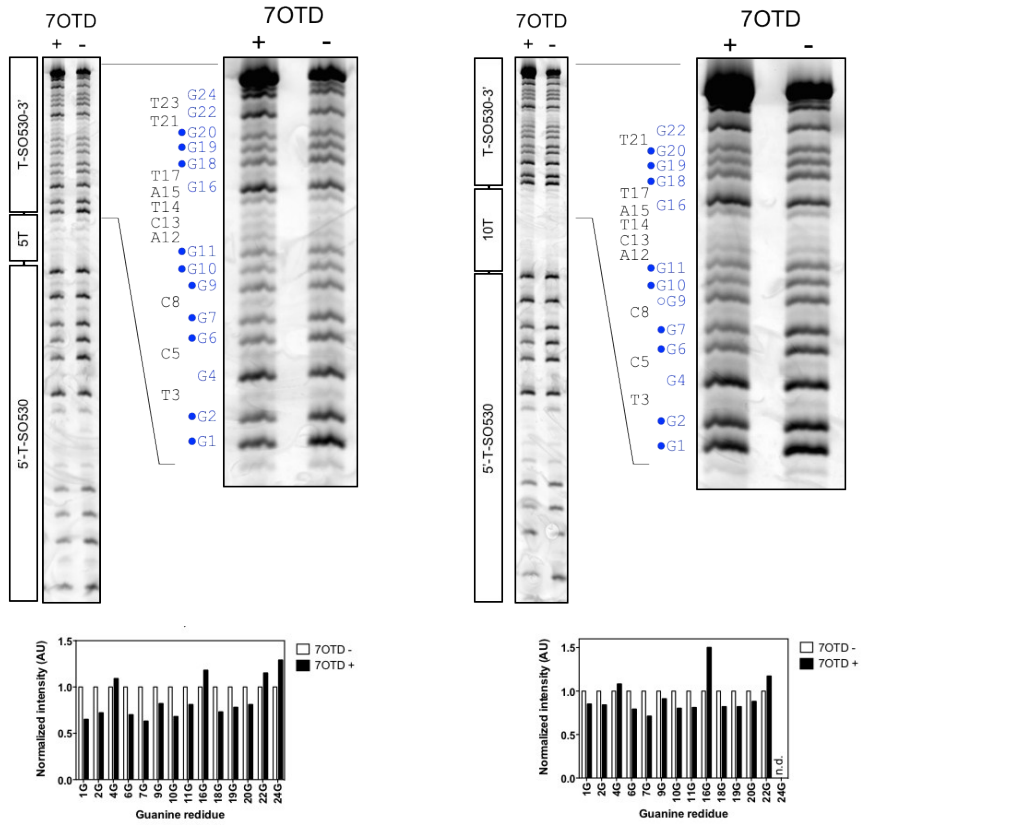
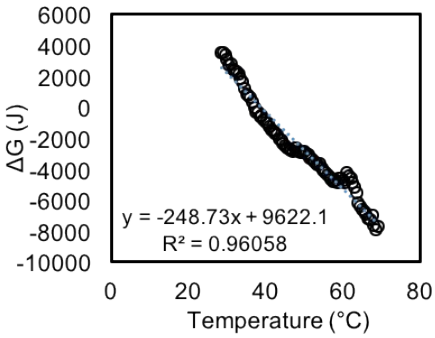

(D) Bivalent 1, 3'-side
(E) Bivalent 2, 3'-side
7OTD
+ -
T-SO530-3'
5T
10T
5'-T-SO530
Normalized intensity (AU)
7OTD -
7OTD +
Guanine redidue
n.d.

Fig. S3. DMS footprinting of intramolecular G4 structures of T-SO530 (**A**) and bivalents (**B**-**E**). Close circles indicate the protected guanines, and open circles indicate the partially protected guanines. The graphs indicate quantified band intensities normalized by signals obtained from the 7OTD-negative lane (n.d.; no data).

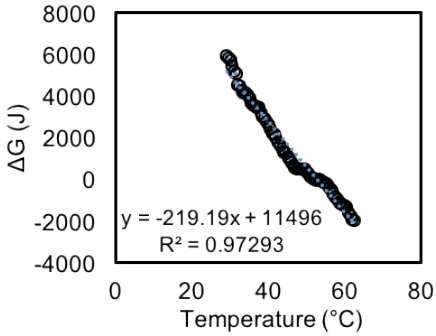
(**A**)

Aptamer	without 7OTD		with 5 e.q. 7OTD	
	$[\theta_{max}]$	$[\theta_{min}]$	$[\theta_{max}]$	$[\theta_{min}]$
T-SO530	3.5445	2.5434	4.3408	2.7136
Bivalent 1	4.9335	3.8152	6.0496	4.1987
Bivalent 2	5.8999	4.5223	7.2429	4.5959

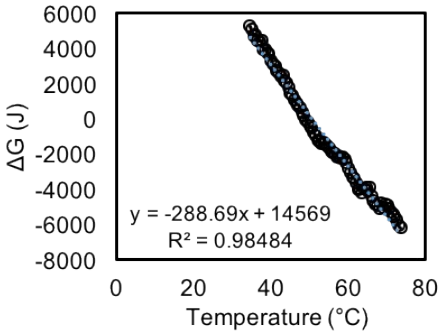
(**B**) T-SO530



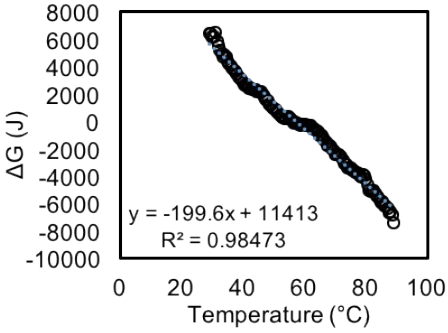
(**C**) T-SO530 with 5 e.q. 7OTD



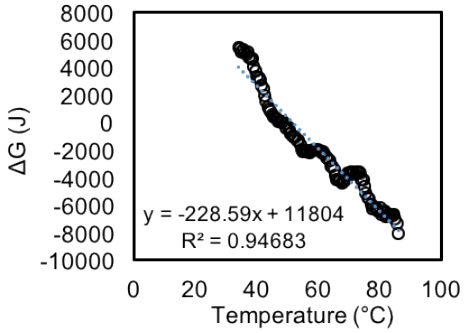
(**D**) Bivalent 1



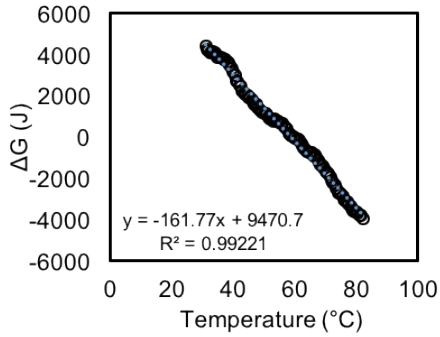
(**E**) Bivalent 1 with 5 e.q. 7OTD



(**F**) Bivalent 2



(**G**) Bivalent 2 with 5 e.q. 7OTD



(**H**)

Aptamer	T_m (°C)	
	without 7OTD	with 5 e.q. 7OTD
T-SO530	38.7	52.4
Bivalent 1	50.5	57.2
Bivalent 2	47.0	58.5

Fig. S4. Melting temperature (T_m) of T-SO530 and bivalent aptamers. (**A**) Maximum ellipticity ([θ_{max}]) and minimum ellipticity ([θ_{min}]) were determined from the melting curve at 20°C and 95°C, respectively. (**B-G**) Graphs indicating linear approximate equations to determine T_m from the linear expression using $\Delta G=0$. (**H**) Summary of T_m values of the aptamers with or without L1H1-7OTD.

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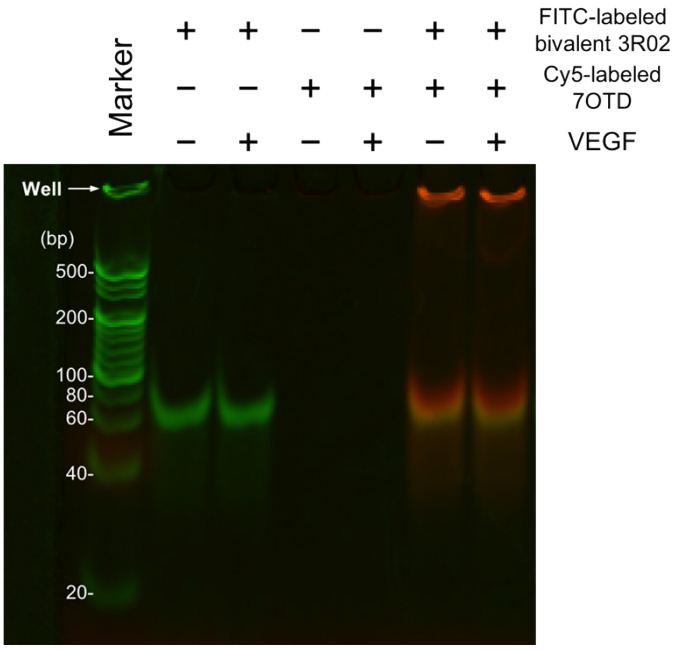

Fig. S5. DNA-PAGE analysis against bivalent 3R02. A FITC-labeled bivalent 3R02 was mixed with Cy5-labeled 7OTD and folded by heat treatment. Cy5 fluorescence was detected in the presence of bivalent 3R02, indicating 7OTD bound to bivalent 3R02. A small portion of the aptamer formed a polymerized G4 structure by addition of Cy5-labeled 7OTD. A DNA marker was stained by Midori Green DIRECT DNA stain (NIPPON Genetics Co, Ltd).

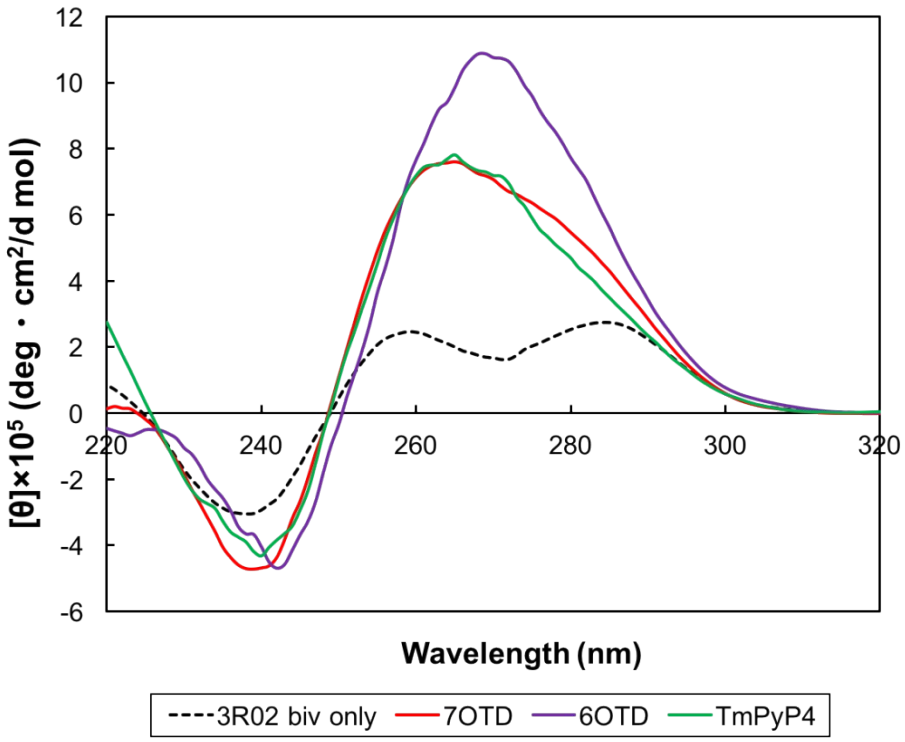

Fig. S6. CD spectrum of bivalent 3R02 with L1H1-7OTD (red solid line), L2H2-6OTD (purple solid line), and TmPyP4 (green solid line). Black dashed line indicates bivalent 3R02 with no ligand. In the presence of Phen-DC3, CD spectrum was not observed due to high level of applied voltage from photomultiplier tube.

Reference

1. M. Tera, K. Iida, H. Ishizuka, M. Takagi, M. Suganuma, T. Doi, K. Shin-ya and K. Nagasawa, *Chembiochem*, 2009, **10**, 431-435.
2. M. Tera, H. Ishizuka, M. Takagi, M. Suganuma, K. Shin-ya and K. Nagasawa, *Angew Chem Int Ed Engl*, 2008, **47**, 5557-5560.
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5. D. W. Drolet, L. Moon-McDermott and T. S. Romig, *Nat Biotechnol*, 1996, **14**, 1021-1025.