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Design of tetraplex specific ligand: cyclic naphthalene diimide

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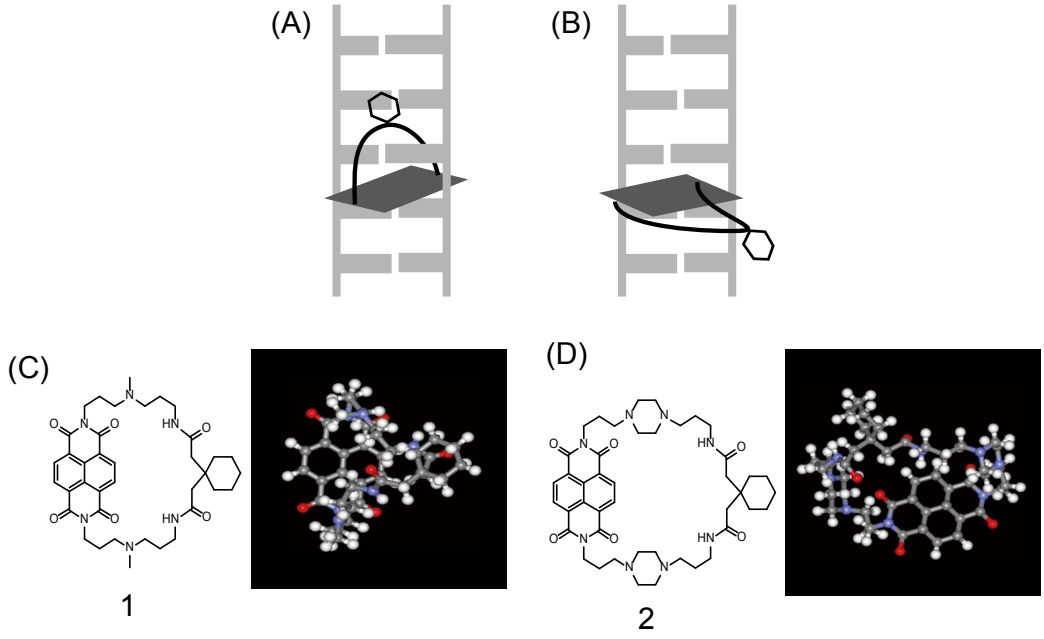

Figure S1. A cartoon for the complex of a cyclic naphthalene diimide with double stranded DNA. (A) The cyclohexyl part prevents the cyclic naphthalene diimide from threading intercalation into double stranded DNA. (B) The linker chains of the cyclic naphthalene diimide also prevent partial intercalation into double stranded DNA. According to the molecular modeling for (C) and (D), **1** is expected to prevent either of the binding modes, whereas **2** is expected to have a "bare" naphthalene diimide plane to allow partial interaction.

Synthesis of cyclic naphthalene diimide **1** and **2**

As precursors of **1** and **2**, N,N-bis[3-(3-Aminopropyl)methylaminopropyl]-naphthalene- 1,4,5,8-tetracarboxylic acid diimide (**4**) and N,N'-bis[[3-(3-Aminopropyl)piperazin-1-yl]- propyl]-naphthalene-1,4,5,8-tetracarboxylic acid diimide (**5**) were synthesized according to the procedure reported previously [1]. **3** was synthesized according to the procedure reported previously [2].

[1] Sato, S.; Takenaka, S. *J. Organomet. Chem*. **2009**, *693*, 1177-1185.

[2] Tanious, F. A., Yen, S.-F., Wilson, W. D. *Biochemistry*, **1991**, *30*, 1813-1819.

Synthesis of **1**

A suspension of **4** (0.305 g, 0.46 mmol), 1,1-cyclohexanediacetic acid (0.175 g, 0.46 mmol), 1-Hydroxybenzotriazole Monohydrate (0.268 g, 1.37 mmol) and (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (0.910 g, 1.37 mol) was dissolved in mixture of chloroform (1.0 l) and triethylamine (30 ml) and the mixture was stirred at room temperature for 120 h. The reaction mixture concentrated to around 100 ml was washed with 50 ml of saturated sodium hydrogen carbonate, and dried over $MgSO_4$. The solvent was removed and the residue was chromatographed on silica gel with chloroform/diethylamine = 1/0.02 as eluent. The fraction showing Rf of 0.31 on TLC with the same solvent was collected and the solvent was removed under reduced pressure. 1 was purified as a collection with a peak at retention time of 16 min by reversed phase HPLC on Inertsil ODS-3 (inner diameter 5mm, size 4.6mm×250mm, GL Science Inc., Tokyo, Japan) in a gradient mode at a flow rate of 1.0 $mLmin^{-1}$, where the concentration of acetonitrile was changed linearly to 90% from 10% in water containing 0.1% trifluoroacetic acid over 30 min at 40 ˚C. Elution was monitored by absorption at 250–400 nm. **1** was utilized in all of the experiment after reversed phase HPLC. Yield: 0.06 g (14 %), MALDI-TOFMS (positive mode, α-CHCA) m/z = 687.9 (theory for $C_{38}H_{50}N_6O_6$ + H^+ = 687.4). ^{1}H-NMR (500 MHz, $CDCl_3$): δ= 1.21 (4H, m), 1.32 (2H, t), 1.55 (4H, m), 1.80 (8H, m), 1.94 (10H, m), 2.19 (4H, t), 2.44 (4H, t), 3.15 (4H, m), 4.34 (4H, m), 7.88 (2H, bs), 8.68 (4H, s) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 21.05, 25.08, 25.49, 25.0, 35.56, 36.29, 37.84, 39.10, 41.04, 45.81, 45.85, 55.73, 56.21, 126.00, 130.41, 162.50, and 170.74 ppm. HRMS Calcd. for $C_{38}H_{50}N_6O_6$+H^+: M, 687.3864. Found: m/z 687.3864.

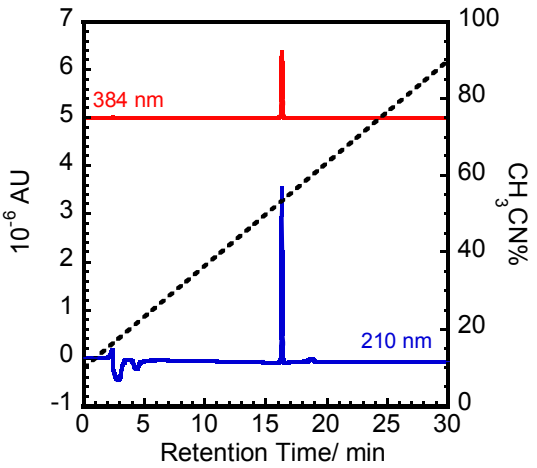

Figure S2. Reversed phase HPLC of **1**. The concentration of acetonitrile was changed linearly to 90% from 10% in water containing 0.1% trifluoroacetic acid over 30 min at 40 ˚C

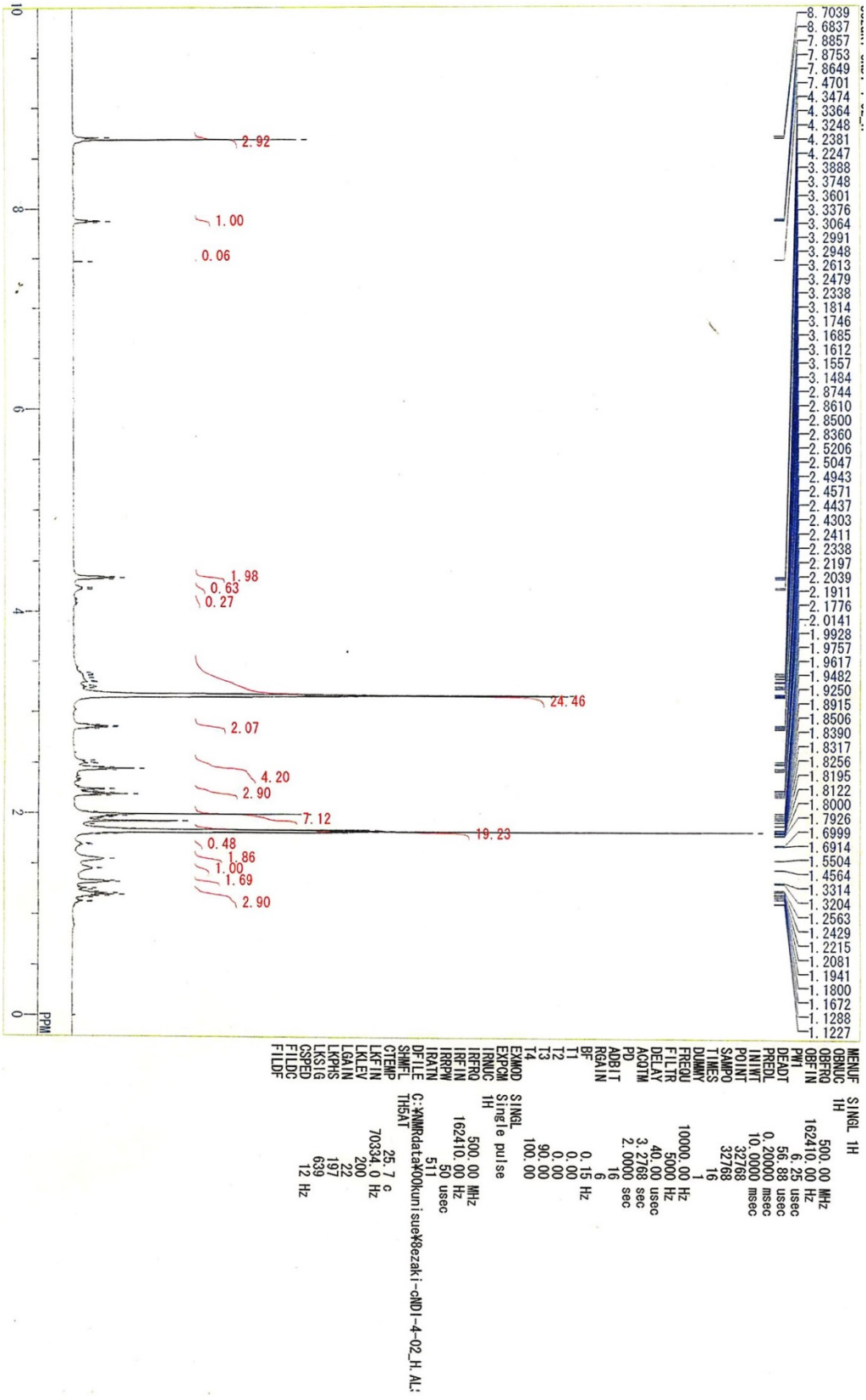

Figure S3. ^{1}H-NMR chart of **1** in $CDCl_3$ using TMS as internal standard.

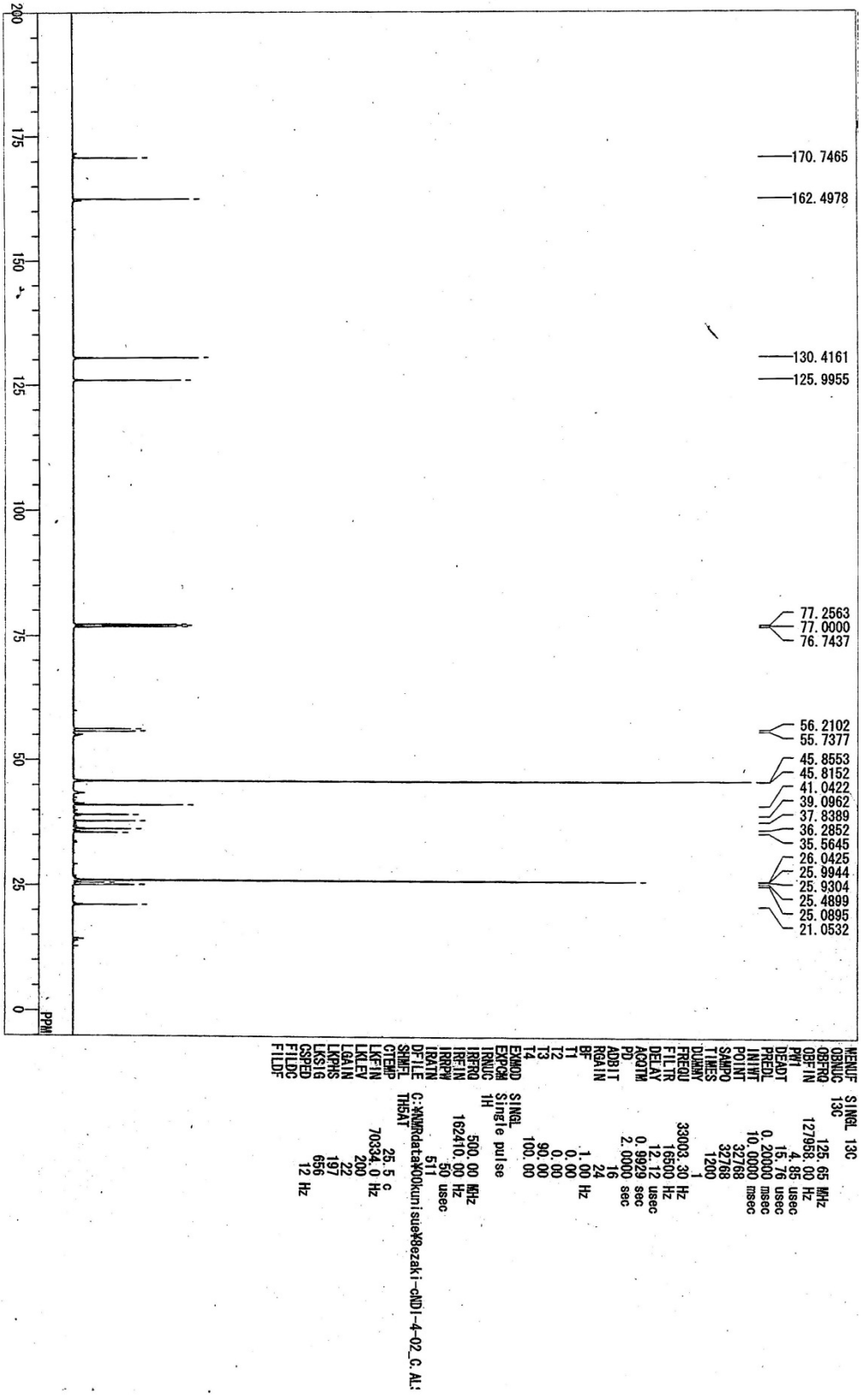

Figure S4. ^{13}C-NMR chart of **1** in $CDCl_3$ using TMS as internal standard.

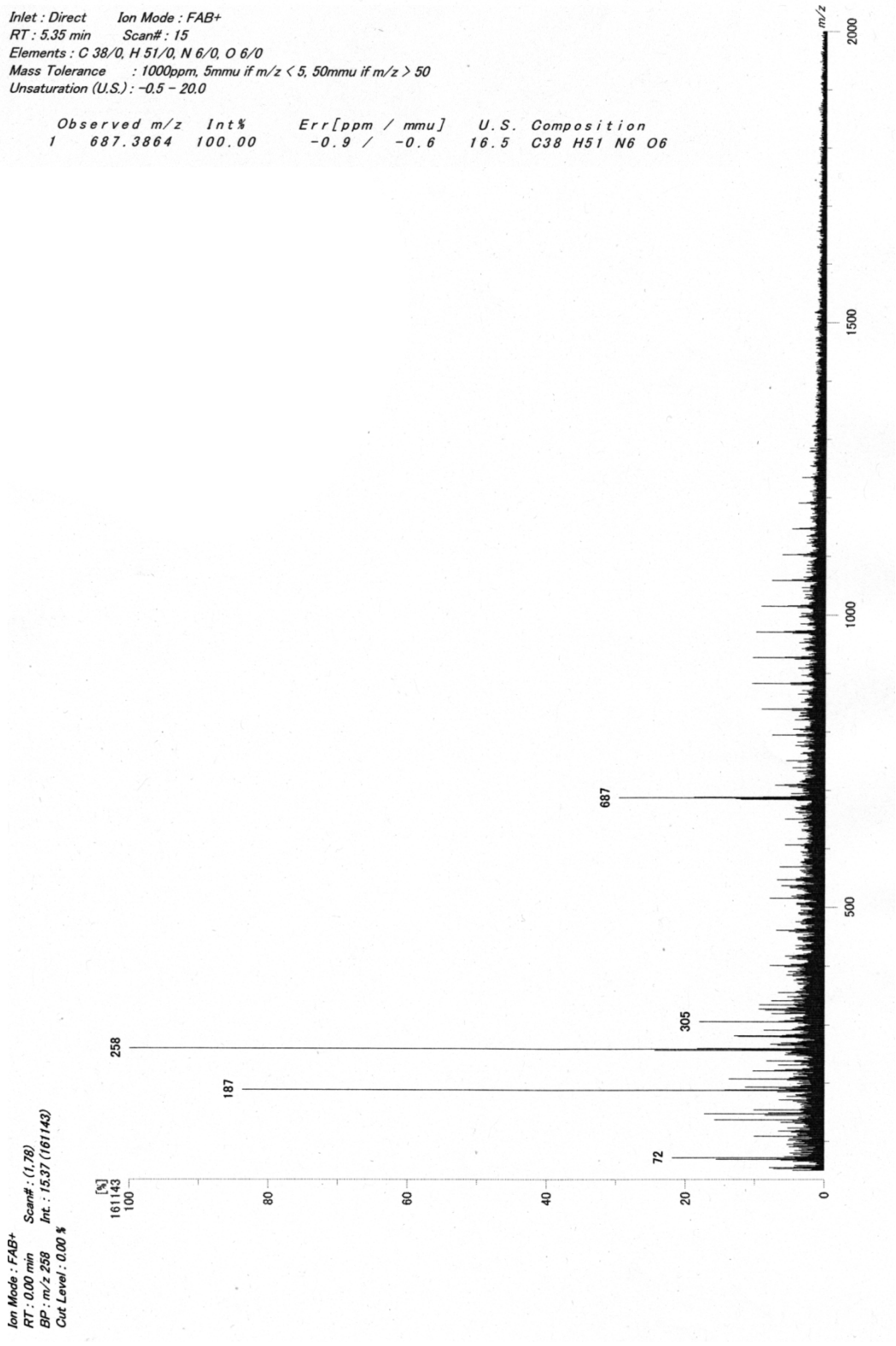

Figure S5. High-resolution mass spectra (HRMS-FAB) of **1.**

Synthesis of **2**

A suspension of **5** (0.396 g, 0.47 mmol), 1,1-cyclohexanediacetic acid (0.0945 g, 0.47 mmol), 1-Hydroxybenzotriazole Monohydrate (0.217 g, 1.42 mmol) and (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (0.737 g, 1.42 mmol) was dissolved in mixture of chloroform (500 ml) and triethylamine (15 ml) and the mixture was stirred at room temperature for 42 h. The reaction mixture concentrated to around 50 ml was washed with saturated sodium hydrogen carbonate (50 ml each), and dried over $MgSO_4$. The solvent was removed and the residue was chromatographed on silica gel with chloroform/diethylamine = 1/0.02 as eluent. The fraction showing Rf of 0.21 on TLC with the same solvent was collected and the solvent was removed under reduced pressure. The residue was taken up in a small amount of methanol, sonicated, and then the solvent evaporated. This process was repeated several times until the orange product (6) solidified. **2** was utilized in all of the experiment after reversed phase HPLC. Yield: 0.054 g (14%), MALDI-TOFMS (positive mode, α-CHCA) m/z = 798.70 (theory for $C_{44}H_{60}N_8O_6 + H^+$ = 798.0). ^{1}H-NMR (500 MHz, $CDCl_3$): δ= 1.32 (4H, m), 1.45 (10H, m), 1.66 (4H, m), 1.93 (4H, t), 1.98 (4H, t), 2.16-2.25 (16H, m), 2.48 (4H, m), 3.13 (4H, m), 4.38 (4H, t), 6.90 (2H, bs), 8.76 (4H, s) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ= 21.57, 23.62, 25.89, 26.01, 36.20, 36.77, 37.38, 39.66, 44.05, 52.94, 52.94, 55.63, 56.56, 126.73, 126.78, 130.84, 163.17, and 171.68 ppm. HRMS Calcd. for $C_{44}H_{60}N_8O_6$+H^+: M, 797.4709. Found: m/z 797.4725.

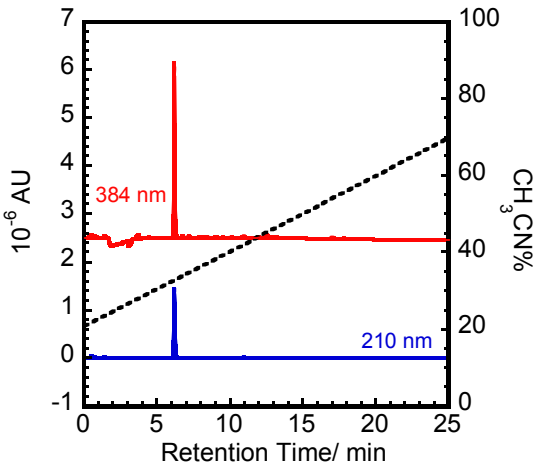

Figure S6. Reversed phase HPLC of **1**. The concentration of acetonitrile was changed linearly to 70% from 21% in water containing 0.1% trifluoroacetic acid over 25 min at 40 ˚C.

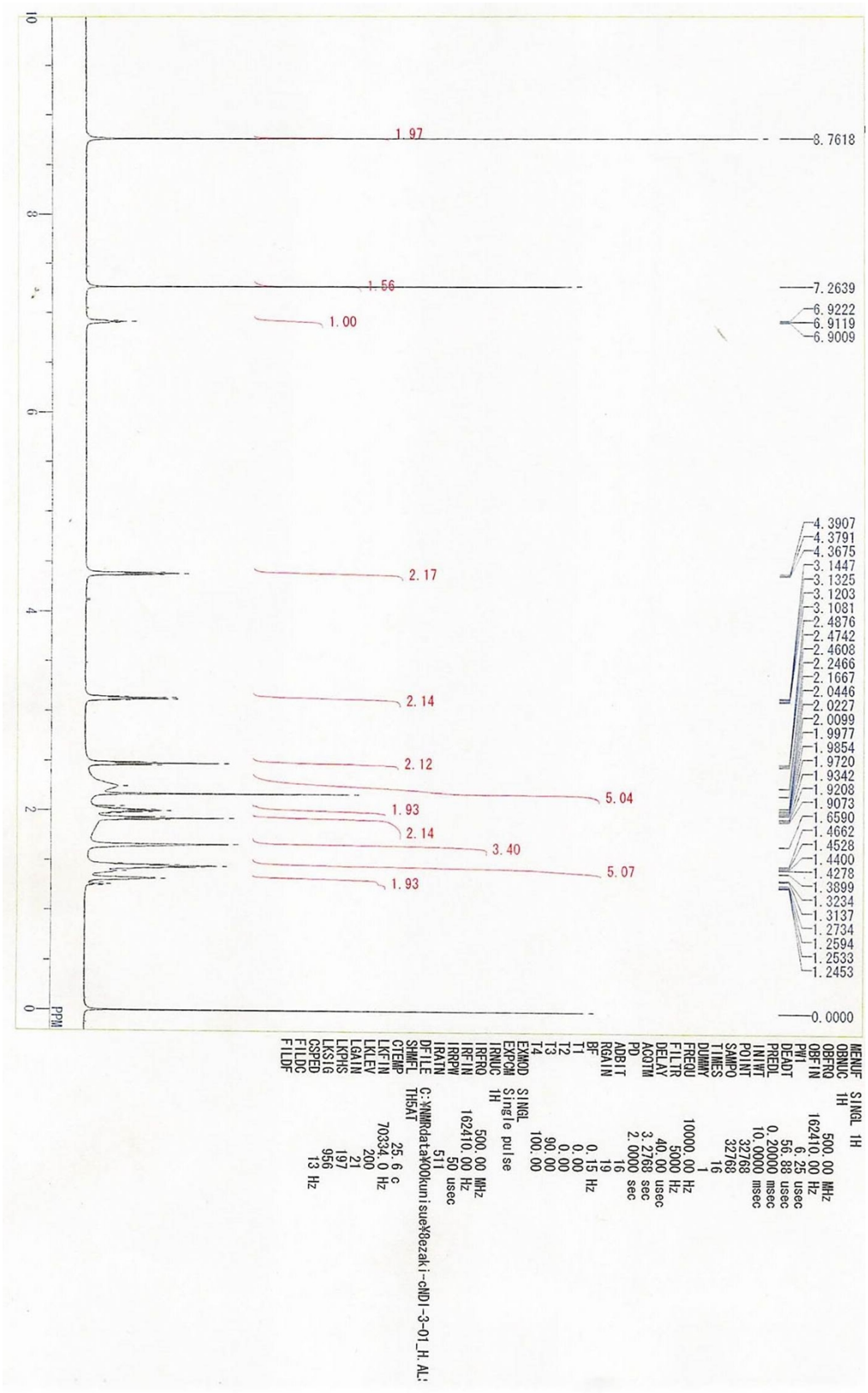

Figure S7. ^{1}H-NMR chart of **2** in $CDCl_3$ using TMS as internal standard.

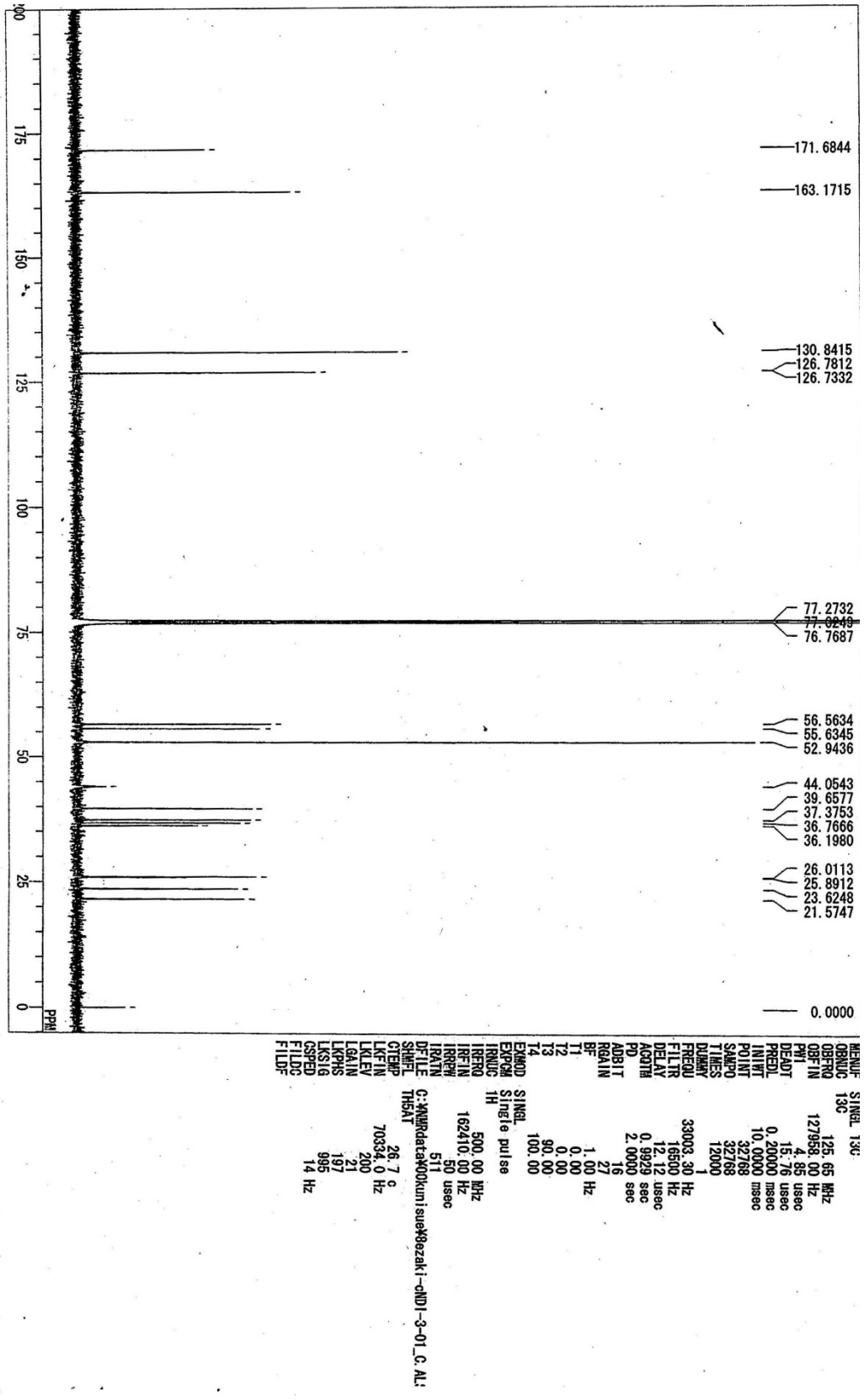

Figure S8. ^{13}C-NMR chart of **2** in $CDCl_3$ using TMS as internal standard.

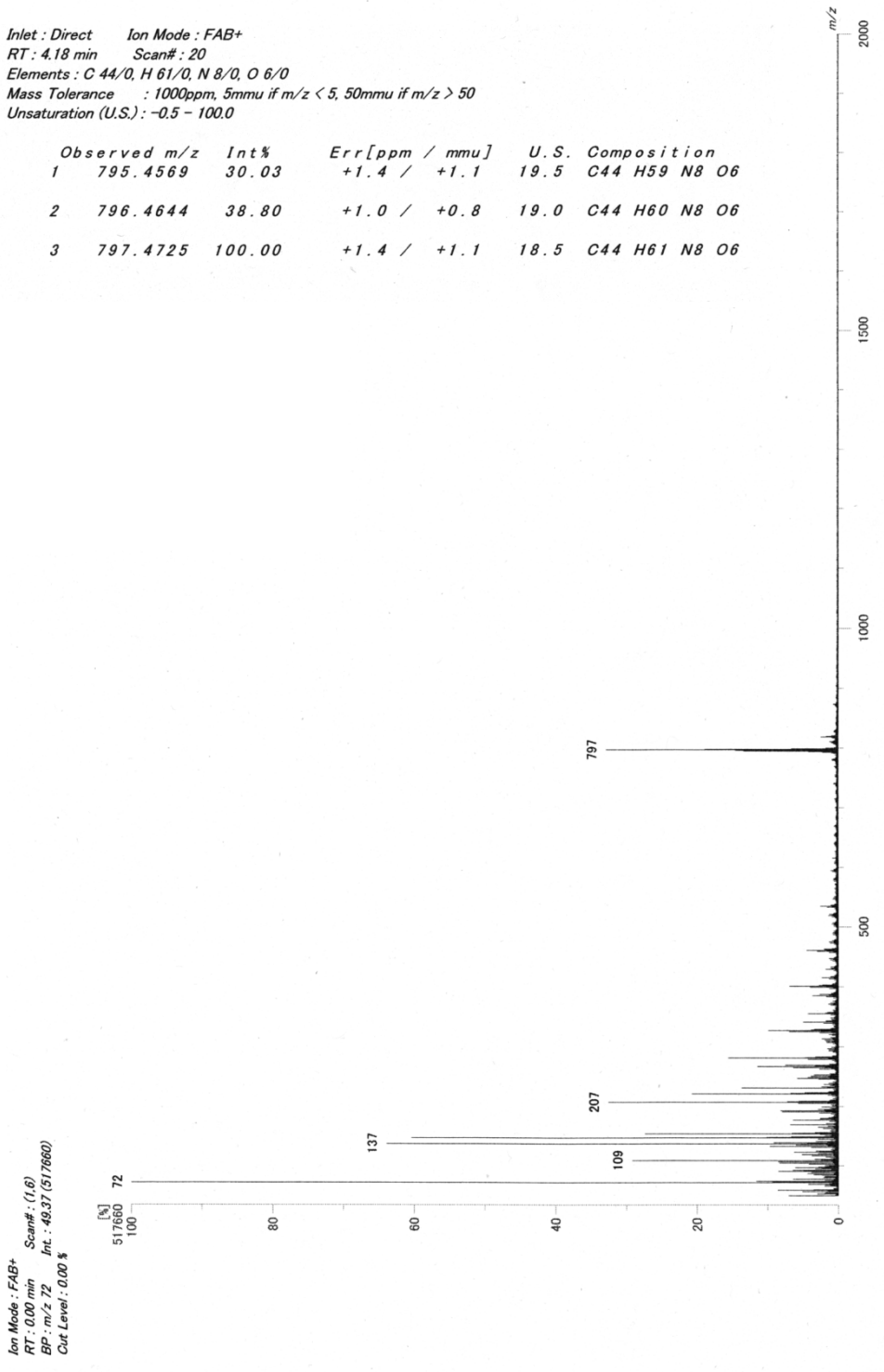

Figure S9. High-resolution mass spectra (HRMS-FAB) of **2.**

Experimental procedures

Topoisomerase I assay

Topoisomerase I assay was carried out according to the method previously reported [3]. Briefly, 0.25 μg of pUC19 were incubated with 5 U of topoisomerase I in 0.1% bovine serum albumin (BSA) and 1 × reaction buffer, composed of 35 mM Tris-HCl (pH 8.0), 72 mM potassium chloride, 5 mM magnesium chloride, 5 mM dithiothreitol (DTT) and 5 mM spermidine at 37 ˚C for 5 min. A various concentration of **1-3** was then added, and the mixture was incubated at 37 ˚C for 1 h. The reaction was terminated by adding 2 μl of 10% sodium dodecylsulfate (SDS) and 0.5 μl of 20 mg/ml proteinase K, and the solution was incubated at 37 ˚C for 15 min. It was then extracted with phenol containing chloroform and isoamyl alcohol, and then with chloroform containing isoamyl alcohol. After ethanol precipitation and dissolution, it was analyzed by gel electrophoresis on 1% agarose in 1 × TAE at 18 V for 3.5 h. The gel was stained with Gelstar (Takara Bio, Shiga, Japan) for 30 min in 1 × TAE.

[3] Pommier, Y.; Covey, J. M.; Kerrigan, D.; Markovits, J.; Pham, R. *Nucleic Acids Res.* **1987**, 15, 6713-6731.

Evaluation of the binding abilities of **1-3** with a-core or dsOligo

Binding of **1** to A-core or dsOligo was studied spectroscopically on a Hitachi U-3310 spectrophotometer (Tokyo, Japan). Aliquots of 200 μM a-core or 50 μM dsOligo were added to 4-5 μM **1-3** in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl or KCl and spectra taken at 25 ˚C. The absorbance at various DNA concentrations were analyzed by Scatchard formulation: $r/L=K(n-r)$, where r is the stoichiometry (the moles of ligand bound per DNA molecule), L is the free ligand concentration, K is the observed binding constant, and n is the number of bound ligand per DNA or Benesi-Hilidebrand formulation: $1/\Delta Abs = 1/(l\Delta\varepsilon\,[ligand]) + 1/(nKl\Delta\varepsilon\,[ligand])\times(1/DNA)$, where $\Delta\varepsilon$ is molar absorptivity change of ligand and l is 1 cm.

Circular dichroism (CD) spectral measurements

Various concentrations of **1-3** were added to 1.5 μM a-core or dsOligo in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl or KCl at 25 ˚C, and CD spectra taken at a scan rate 50 nm/min on a Jasco J-820 spectropolarimeter (Tokyo, Japan). Other conditions were: response 2 s, data interval 0.1 nm, sensitivity 100 mdeg, bandwidth 2 nm, and scan number 4 times.

Melting curve measurements

Melting curves of A-core or dsOligo were measured on a Hitachi 3300 spectrophotometer (heating

rate of 0.5°C/min to 90°C) or Jasco J-820 spectrophotometer (response, 100 mdeg; temperature gradient, 30°C/h; response, 8 s; data collecting interval, 0.5 ˚C; bandwidth, 2 nm) equipped with a temperature controller, respectively. The melting curves based on circular dichroism (CD) at 292.6 nm of A-core or absorption change at 260 nm of dsOligo were measured in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl or KCl. A mixture of 1.5 μM A-core or dsOligo and 1.5 μM of **1**-**3** was placed in a cell of 1 cm in light path length (total 3 ml).

Isothermal Titration Calorimetry (ITC) measurements

Before measurement, A-core DNA and HP-27 (5'- GCG ATT CTC GGC TTT GCC GAG AAT CGC - 3') were annealed by heating to 95˚C for 10 min, cooled to 25˚C at 0.5˚C/min. Binding studies were performed using a low volume nano ITC (TA instruments, USA) with a cell volume of 190 μL at 25 °C. Samples were degassed for 5 min prior to use. The sample cell was filled with the 10 μM A-core DNA or HP-27 in 50 mM sodium or potassium phosphate buffer. One μL of 250 μM **1**, **2** or **3** in 50 mM sodium or potassium phosphate buffer was added into the thermostated cell by means of a syringe.

Atomic Force Microscopy (AFM) of pUC19

The mica surface was exfoliated with adhesive tape, 40 μl of 10 ppm poly-L-lysine placed on it and allowed to stand for 5 min. They were washed twice with Milli-Q water (100 μl), dried with an N_2 gas for 30 min. Two concentrations (2.0 and 16 μM) of **1**, **2** or **3** were added to pUC19 (1.3 ng/μl, 2.0 μM-bp) in MilliQ. They were placed on the substrate, and washed twice with Milli-Q water (100 μl), dried overnight in a desiccator and finally with an N_2 gas for 30 min. AFM images were taken on an Nanonavi IIs station and nanocute in the dynamic force mode with a cantilever (PRC-DF40P, SII Nanotechnology, Tokyo, Japan).

Electrochemical Telomerase assay by using chronocoulometry (CC) [4]

Disposable chips carrying a gold electrode (Hano Manufacturing, Fukuoka, Japan) were prepared as described previously [5]. Three hundred microliters of 1.0 mM 6-mercaptohexanol were added on the gold electrode and kept at 45˚C for 1 h, and washed with 300 μL of Milli-Q water. Oligonucleotide TS-primer 5'-HO$(CH_2)_6$-SS-$(CH_2)_6$-TTT TTT TTA ATC CGT CGA GCA GAG TTA GGG-3' was custom-synthesized by Genenet (Fukuoka, Japan). Ten microliters of 25 nM TS primer in 50 mM NaCl were added on the 6-mercaptohexanol immobilized gold electrode and kept at 37˚C for 30 min. After the addition of 300 μL of 1 mM 6-mercaptohexanol, the chip was incubated at 37˚C for 1 h and washed with 300 μL of Milli-Q water twice to yield TS primer-immobilized electrodes.

The electrochemical measurement was made with a three-electrode configuration in a portable apparatus described previously [5], where a platinum electrode acted as the counter electrode, Ag/AgCl as the reference electrode, and the TS primer-immobilized electrode as the working electrode. CC was measured in 10 mM Tris-HCl (pH 7.4) containing 50 μM RuHex and the electrode was washed with 300 μL of Milli-Q water twice and subsequently washed with 300 μL of Milli-Q water containing 40 units/mL RNase Inhibitor. For the telomerase reaction, 20 μL of a reaction solution prepared above was placed on the electrode and incubated at 37˚C for 30 min. After washing the electrode with 300 μL of Milli-Q water twice, multipotentical step and CC were taken again under the same conditions.

In the Cottrell equation (1) for chronocoulograms (plot of Q vs. $t_{1/2}$) in the absence and presence of RuHex, the y-intercept at time zero represents Q_{dl} or Q_{before} and Γ_{DNA} is derived from equations (2) and (3) using the obtained Q_{dl} and Q_{before}. After telomerase reaction of this electrode, the y-intercept at time zero in the chronocoulogram in the presence of RuHex gives Q_{after}. Accordingly, the elongation time x in $(TTAGGG)_x$ is given by equations (4) and (5) using the obtained Q_{before} and Q_{after}.

$$Q = (2nFAD_0^{1/2}C_0^*/\pi^{1/2})t^{1/2} + Q_{dl} + nFA\Gamma_0 \quad (1)$$

$$\Gamma_0 = (Q_{before} - Q_{dl})/nFA \quad (2)$$

$$\Gamma_{DNA} = \Gamma_0(z/m)N_A \quad (3)$$

$$\Delta\Gamma_0 = (Q_{after} - Q_{before})/nFA \quad (4)$$

$$(TTAGGG)x = \Delta\Gamma_0(z/6)N_A/\Gamma_{DNA} \quad (5)$$

The parameters used are as follows: n, number of electrons per molecule for reduction (n = 3); F, Faraday constant (C/equiv); A, electrode area (cm^2); Q_{dl}, capacitive charge (C); Γ_0, amount of redox marker, RuHex (mol/cm^2); Γ_{DNA}, probe surface density ($molecules/cm^2$); m, the number of bases in the probe DNA (m = 30); $\Delta\Gamma$, elongated products per electrode area (mol/cm^2); z, charge of the redox molecule (z = 3); N_A, Avogadro's number (molecules/mol); Q_{before}, charge before elongation reaction; Q_{after}, charge after elongation reaction, and $(TTAGGG)_x$, average elongation time per hexanucleotide per primer molecule.

The condition of multi potential steps was as follows: potential 1 = -0.16 V, time 1 = 1.0 s, potential 2 = -0.55 V, time 2 = 1.0 s, potential 3 = -0.16 V, time 3 = 1.0 s, sample interval = 2.0 ms, quiet time = 2.0 s, sensitivity = 5e-4 A/V. The condition of CC measurements was as follows: potential step from 0.1 to -0.4 V, step = 1, pulse width = 0.25 s, sample interval = 5.0 ms, quiet time = 2.0 s, sensitivity = 1e-5 A/V. The telomerase reaction solution (20 μL) consisted of 20 mM Tris-HCl (pH 8.5), 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 20 μM dNTP mixture, 25 HeLa cell extracts, and 2.5 μM **1**, **2** or **3**.

[4] Sato, S.; Takenaka, S. *Anal. Chem*. **2012**, 84, 1772-1775.

[5] Ohtsuka, K.; Endo, H.; Morimoto, K.; Vuong, B. N.; Ogawa, H.; Imai, K.; Takenaka, S. *Anal. Sci*. **2008**, 24, 1619-1622.

Computer modeling of the complex of hybrid type tetraplex structure of a-core with **1** or **2**

Molecular modeling of these complexes was constructed by MOE [6]. Data of NMR in the aqueous solution of K^+ (PDBID: 2GKU) [7] was utilized in the structural construction of a-core. Structure of a-core was fixed except for the bases of T1T2T18T19T20 located upper site of three G-tetraplex planes which is expected as the binding site of **1** or **2**. Ligand, **1** or **2** was placed on the binding site of a-core and energy minimization of these complexes was carried out. Molecular dynamics calculation of these mineralized complexes was further carried out until **1** or **2** was located in the binding site as stable condition. Finally, energy minimization of the complexes was obtained as shown in Figure 3. These calculations were used the force field of MMFF94x.

[6] Molecular Operating Environment (MOE).2011.10., Chemical Computing Group (CCG) http://www.chemcomp.com/

[7] Luu, K.N.; Phan, A.T.; Kuryavyi, V.; Lacroix, L.; Patel, D.J. *J. Am. Chem. Soc*., **2006**, *128* (30), 9963-9970.

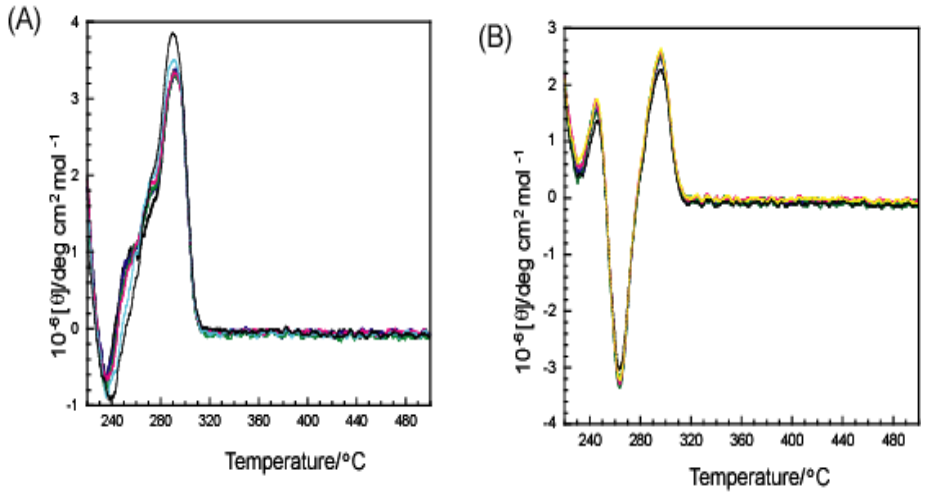

Figure S10. CD spectra of 1.5 μM a-core in 50 mM Tris-HCl (pH 7.4),100 mM KCl (A) or NaCl (B) in addition of **1** (0, 0.38, 0.75, 0.80, 2.25, and 3.00 μM from bottom to top) at 25˚C.

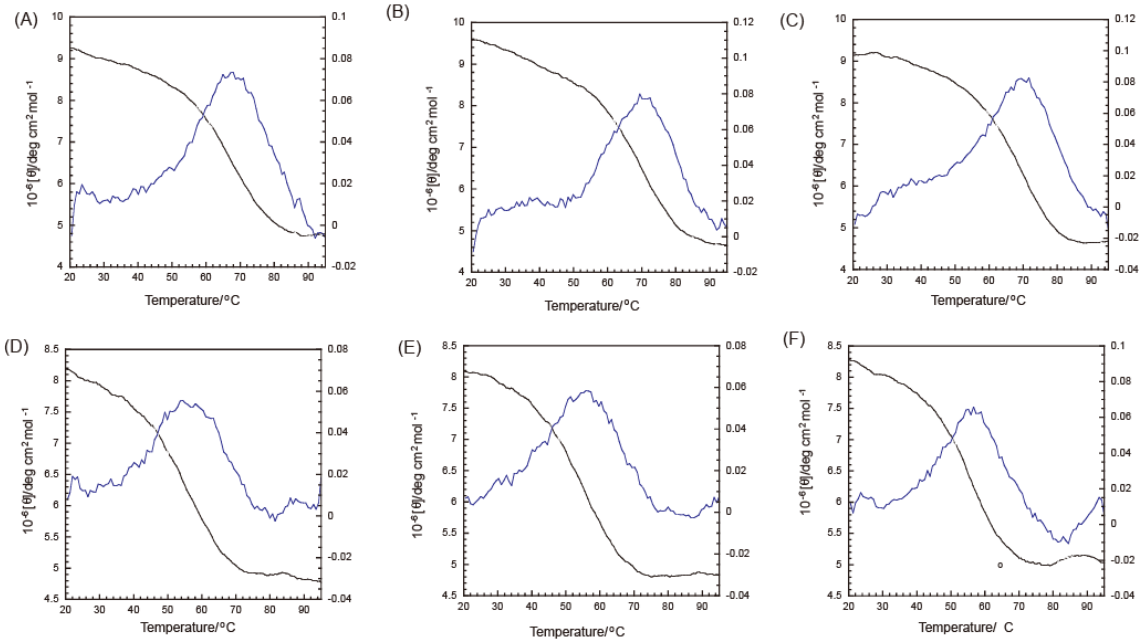

Figure S11. Temperature dependence of 1.5 μM a-core in the absence (A, D) or presence of 1.5 μM **1** (B, E) and **2** (C, F) under 50 mM Tris-HCl (pH7.4) and 100 mM KCl (A-C) or NaCl (D-F).

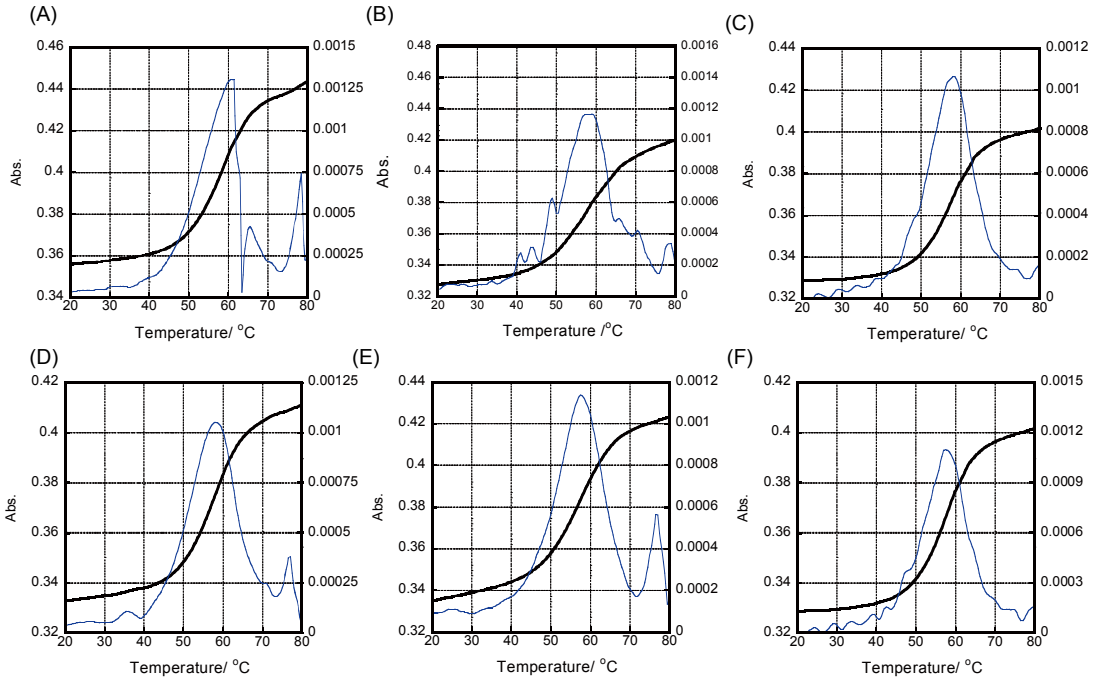

Figure S12. Temperature dependence of 1.5 μM dsOligo in the absence (A, B) or presence of 1.5 μM **1** (C, D) and **2** (E, F) under 50 mM Tris-HCl (pH7.4) and 100 mM KCl (A, C, E) or NaCl (B, D, F).

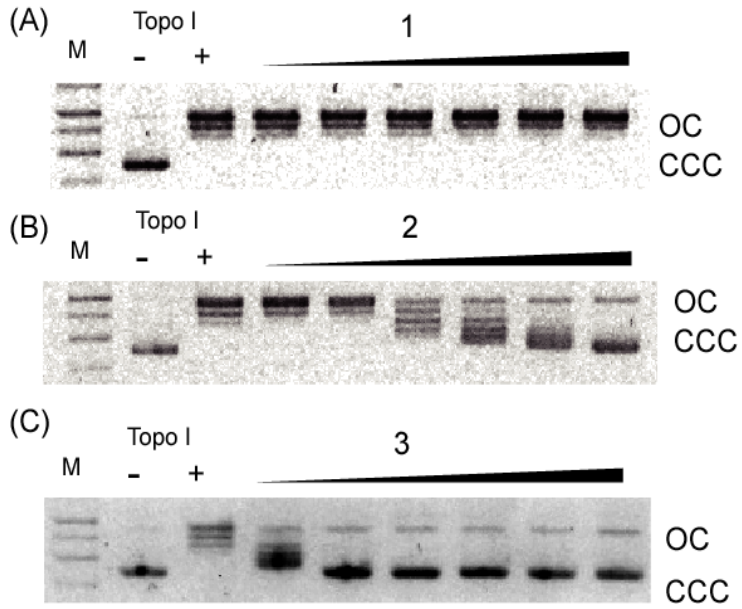

Figure S13. Topoisomerase I assay for pUC19 treated with 5.0 U of enzyme. Following incubation a various concentration of **1** (A), **2** (B), and **3** (C) (2.0, 5.0, 10, 20, 30, 40, 50 μM from left to right) was added and the mixture incubated further. After work-up, DNA was electrophoresed Lanes M and Topo I - represent 1 kb size markers and pUC19, respectively. OC and CCC refer to open circler and covalent closed circular, respectively. Ligand **1** seems not to inhibit Topoisomerase I activity because of the obtaining pattern from forth to ninth gel bands same as third gel band from the left side in Figure S13(A) and this result also shows that **1** doesn't unwind the superhelicity of pUC19.

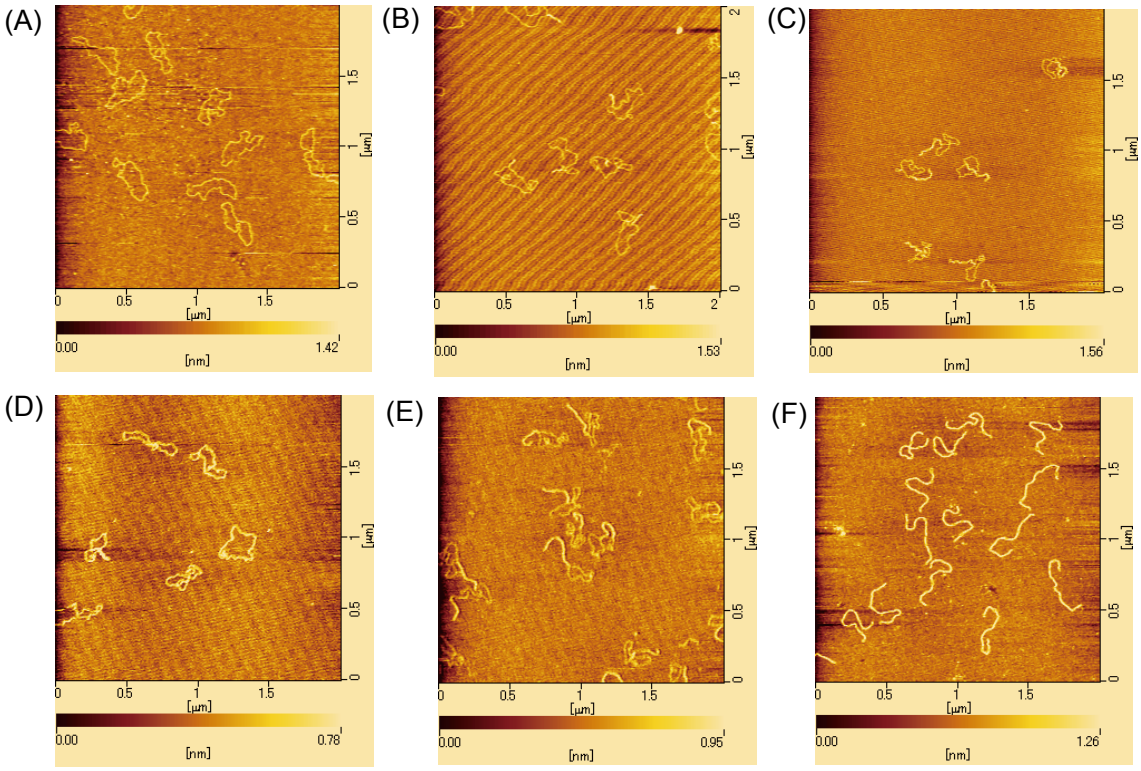

Figure S14. Atomic force microscopic images for 2 µM-bp pUC19 in the absence (A) and presence of 2 µM of **1** (B), 16 µM of **1** (C), 16 µM of **2** (D), 2 µM of **3** (E), and 16 µM of **3** (F). **3** as threading intercalator led to solely plectonemic supercoiling (E and F) of pUC19 as described previously [8], whereas almost no structural changes were observed in the case of **1** and **2**.

[8] Pope, L. H.; Davies, M. C.; Laughton, C. A.; Roberts, C. J.; Tendler, S. J. B.; Williams, P. M. *J. Microscopy*, **2000**,*199*, 68-78.

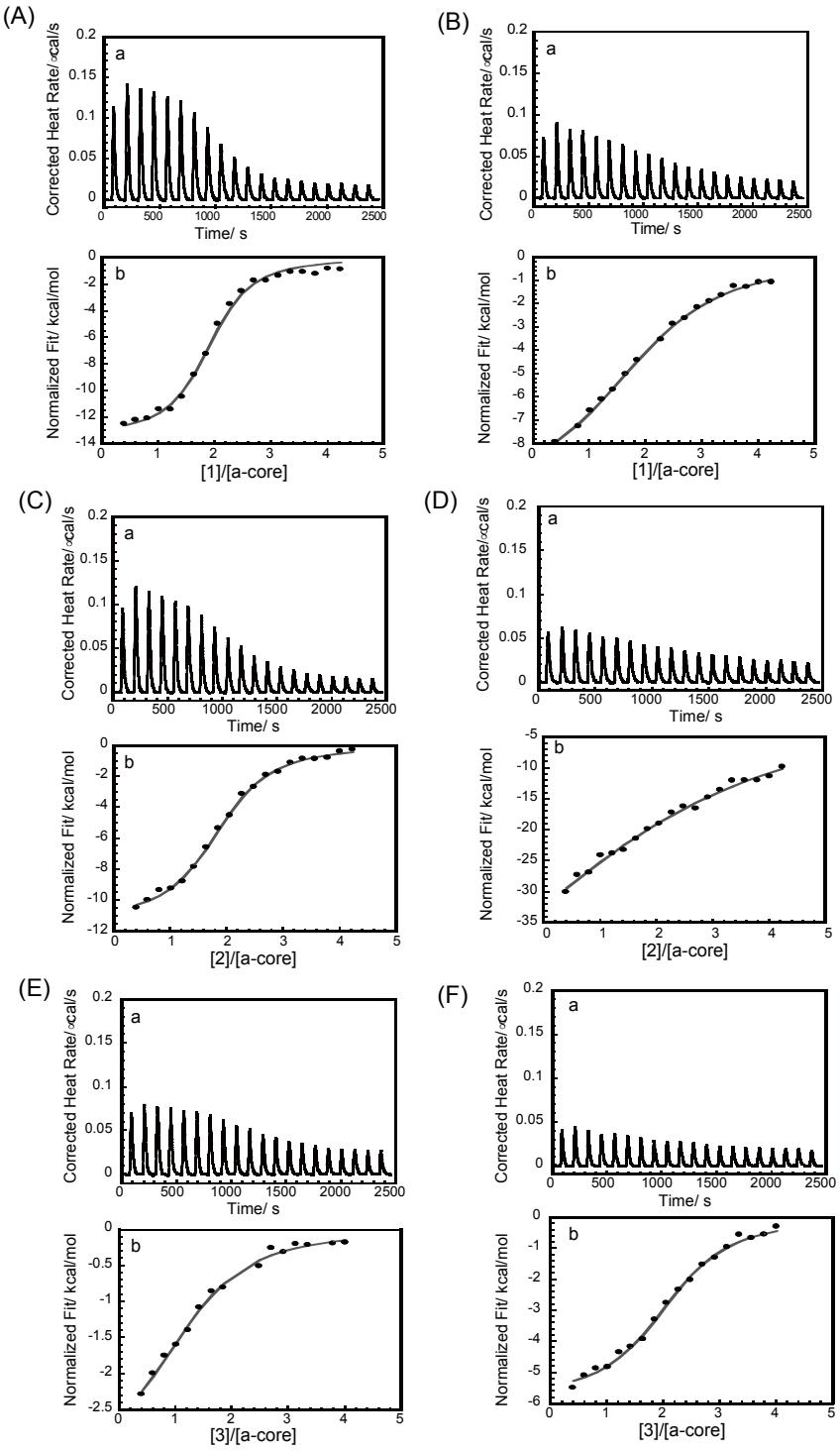

Figure S15. Calorimetric data for **1**-**3** binding to a-core. In each cases, 250 μM of **1**-**3** was titrated into 10 μM A-core at 25 °C. Data are shown for Raw calorimetric data (a) and binding isotherm (a, heat change vs ligand/a-core molar ratio) in 50 mM potassium (A, C, E) or sodium (B, D, F) phosphate buffer (pH7.4) with serial injections of **1** (A, B), **2** (C, D) or **3** (E, F).

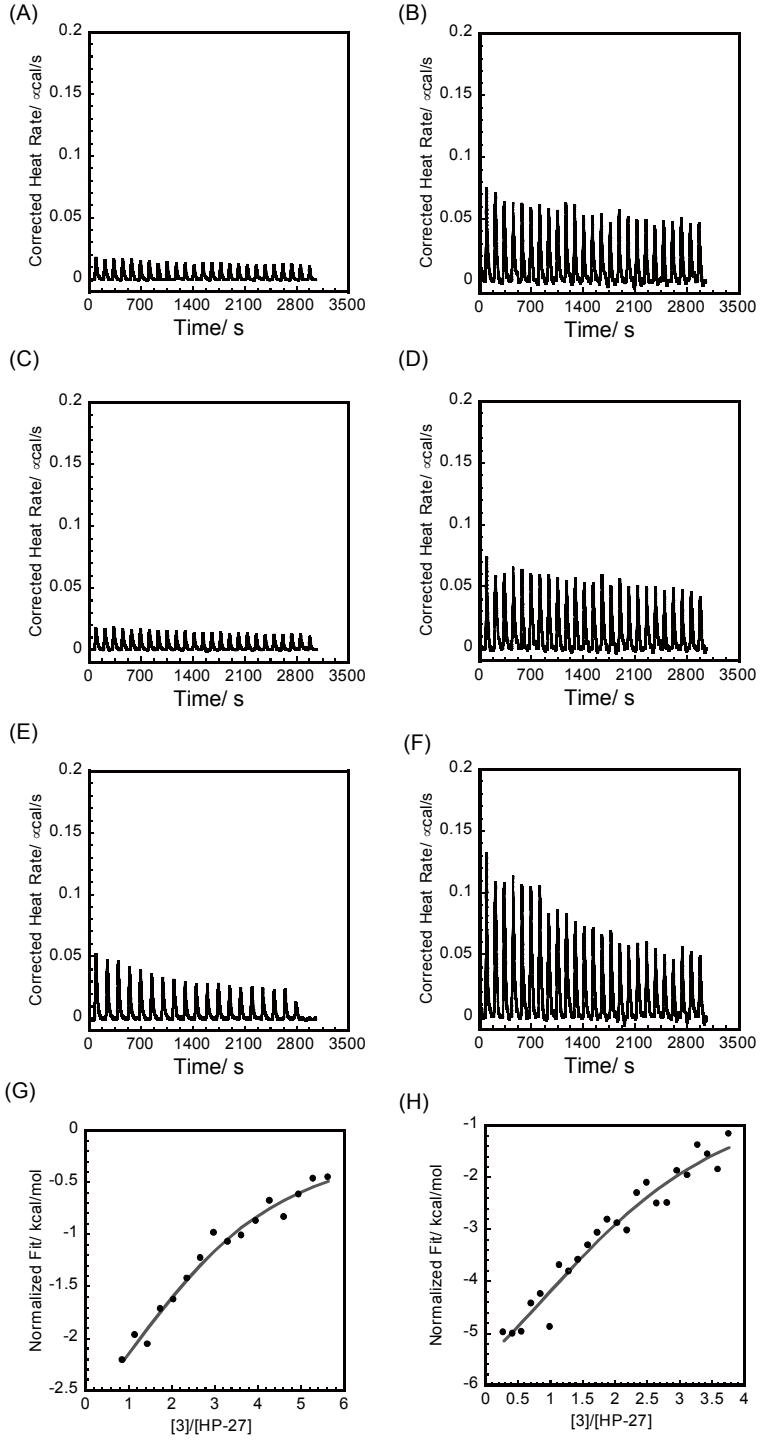

Figure S16. Calorimetric data for **1**-**3** binding to HP27. In each cases, 250 μM of **1**-**3** was titrated into 10 μM HP-27 at 25 °C. Data are shown for Raw calorimetric data (A-F) and binding isotherm (G, H, heat change vs ligand/HP27 molar ratio) in 50 mM potassium (A, C, E, G) or sodium (B, D, F, H) phosphate buffer (pH7.4) with serial injections of **1** (A, B), **2** (C, D) or **3** (E, F, G, H).

Table S1 Thermodynamic data for the binding of **3** to HP27 in the presence of K^+ or Na^+

	K_+	Na^+
$10^{-5}K/M^{-1}$	0.71	0.87
n	2.7	2.3
ΔH/kcal mol^{-1}	-3.95	-8.18
ΔS/cal mol^{-1}	8.94	-4.84

50 mM potassium or sodium phosphate buffer (pH7.4).

HP27: 5'- GCG ATT CTC GGC TTT GCC GAG AAT CGC-3'

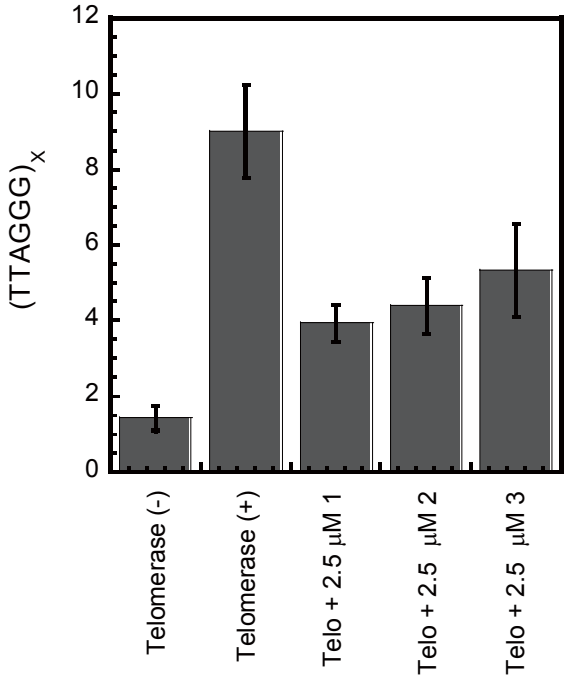

Figure S17. Telomerase inhibition assay by electrochemical method with 2.5 μM **1** – **3** in the presence of 25-cell extracts at 37 ˚C for 1 hr. Immobilization density of the TS-primer was 1.0×10^{11} molecules/cm^2 reported previously [4].