SAGE-Hindawi Access to Research
Journal of Nucleic Acids
Volume 2010, Article ID 217627, 9 pages
doi:10.4061/2010/217627

Research Article

Synthesis of Macrocyclic Hexaoxazole (6OTD) Dimers, Containing Guanidine and Amine Functionalized Side Chains, and an Evaluation of Their Telomeric G4 Stabilizing Properties

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Received 15 February 2010; Accepted 1 March 2010

Academic Editor: R. Eritja

Structure-activity relationship studies were carried out on macrocyclic hexaoxazole (6OTD) dimers, whose core structure stabilizes telomeric G-quadruplexes (G4). Two new 6OTD dimers having side chain amine and guanidine functional groups were synthesized and evaluated for their stabilizing ability against a telomeric G4 DNA sequence. The results show that the 6OTD dimers interact with the DNA to form 1:1 complexes and stabilize the antiparallel G4 structure of DNA in the presence of potassium cation. The guanidine functionalized dimer displays a potent stabilizing ability of the G4 structure, as determined by using a FRET melting assay ($\Delta T_m = 14\,^{\circ}\mathrm{C}$).

1. Introduction

G-quadruplexes (G4), secondary DNA structures consisting of G-quartet planers in G-rich regions, play significant biological roles for example, control of transcription and telomeric lengths [1–19]. One typical G4 forming DNA sequence is a telomere, which exists at the ends of chromosomes consisting of $(TTAGGG)_n$ repeating single-stranded sequences [1–12]. Telomeres protect chromosomes from end to end fusion events, which result in replication of the chromosome (the Hayflick limit) [20]. The telomere repeats are elongated by the reverse transcriptase telomerase, which is overexpressed in most tumor cells. In contrast, telomerase activity is not observed in normal somatic cells [21]. Since the activity of this enzyme is inhibited by the G4 structure of telomeres owing to steric hindrance, small molecules that selectively bind and stabilize the telomeric G4 should be potential anticancer agents. As a result, a number of G4 ligands, inspired by artificial DNA intercalators as well as natural products, have been developed during the past decade [22].

Telomestatin (TMS) is a natural product isolated from *Streptomyces anulatus* 3533-SV4, which displays one of the most potent telomeric G4 binding activity (Figure 1) [23–28]. Interaction analysis has shown that two molecules of TMS induce conversion of telomeric G4 into an antiparallel type by way of an end stacking mode [25–28]. We have recently developed macrocyclic hexaoxazole compounds 6OTD, containing a variety of side chain functional groups, that serve as a novel TMS derivative [29–32]. In addition, by considering the proposed binding mode of TMS with telomeric G4, we have carried out further structural development of dimeric 6OTD derivatives (Figure 1) [33]. The results of molecular dynamics calculations guided the selection of 6OTD dimer **1** that contains an appropriate length of a linker between the monomeric units of 6OTD. Studies showed that dimer **1** binds to telomeric G4 more tightly than do other 6OTD dimers with linkers of shorter or

longer lengths. One possible structural development strategy to enhance the stabilizing ability of **1** against the G4 would be to install cationic functional groups on the side chain [30]. Below, we describe synthesis of new 6OTD dimers **2** and **3** that derivatize **1** but possess cationic amine and guanidine functional groups on their side chains. In addition, the ability of these substances to stabilize telomeric G4 along with their interaction mode was investigated.

2. Materials and Methods

2.1. General. Flash chromatography was performed on Silica gel 60 (spherical, particle size 0.040 ~ 0.100 μm; Kanto). Optical rotations were measured on a JASCO P-2200 polarimeter, using the sodium D line. ^{1}H and ^{13}C NMR spectra were recorded on JEOL JNM-ECX 300, 400, and 500. The spectra are referenced internally according to residual solvent signals of $CDCl_3$ (^{1}H NMR; δ = 7.26 ppm, ^{13}C NMR δ = 77.0; ppm) and DMSO $d-6$ (^{1}H NMR; δ = 2.50 ppm, ^{13}C NMR; δ = 39.5 ppm). Data for ^{1}H NMR are recorded as follows: chemical shift (δ, ppm), multiplicity (s, singlet; d, doublet; m, multiplet; br, broad), integration, and coupling constant (Hz). Data for ^{13}C NMR are reported in terms of chemical shift (δ, ppm). Mass spectra were recorded on a JEOL JMS-T100X spectrometer with ESI-MASS mode using methanol as solvent. All oligonucleotides purified were obtained from Sigma Genosys and dissolved in double-distilled water to be used without further purification. Fluorescence resonance energy transfer (FRET) melting assay was made with an excitation wavelength of 470–505 nm and a detection wavelength of 523–543 nm using the DNA Engine Opticon 2 Real-Time Cycler PCR detection system (BioRad). CD spectra were recorded on a JASCO-810 spectropolarimeter (Jasco, Easton, MD) using a quartz cell of 1 mm optical path length and an instrument scanning speed of 500 nm/min with a response time of 1 s, and over a wavelength range of 220–320 nm. CD spectra are representative of ten averaged scans taken at 25°C.

2.2. Synthesis

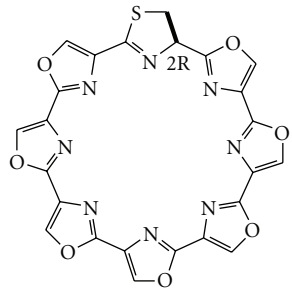
*Synthesis of **5**.* To a solution of trioxazole **4** (2.1 g, 3.6 mmol) in MeOH-THF (1 : 1, 60 mL) was added $Pd(OH)_2/C$ (420 mg), and the reaction mixture was stirred at room temperature under hydrogen gas (balloon). After 3 h, the catalyst was removed by filtration through a pad of Celite, and the filtrates were concentrated in vacuo to give amine **5**, which was used without further purification.

*Synthesis of **7**.* To a solution of trioxazole **6** (2.1 g, 3.6 mmol) in THF-H_2O (3 : 1, 80 mL) was added LiOH (230 mg, 5.4 mmol) at 0°C. After stirring at room temperature for 1 h,to the resulting mixture was added 1 N HCl, to give carboxylic acid **7**, which was used without further purification.

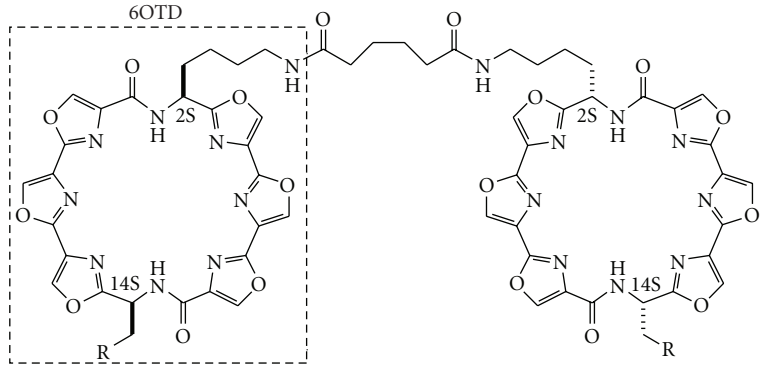
*Synthesis of **8**.* To a solution of amine **5** (abovementioned) in THF-H_2O (1 : 1) was added the carboxylic acid **7** (above-mentioned), *N*-methylmorpholine (1.2 mL, 11 mmol), and 4-(4, 6-dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) (3.0 g, 11 mmol), and the mixture was stirred at room temperature for 15 h. To the reaction mixture was added H_2O and precipitate was formed. This precipitate was collected with filtration using filter paper, to give **8** as a white solid (3.3 g, 3.2 mmol 89% 2 steps, mp = 200–203°C). Spectral data for **8**: $[\alpha]^{25}_D = -2.7$ (c 1.1, $CHCl_3$–MeOH (1 : 1)); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (s, 1H), 8.35–8.27 (m, 5H), 7.53 (d, J = 8.9 Hz, 1H), 7.34 (m, 5H), 5.98–5.81 (m, 1H), 5.57–5.44 (m, 2H), 5.30 (d, J = 18 Hz, 1H), 5.21 (d, J = 11 Hz, 1H), 5.15–4.97 (m, 2H), 4.82 (br, 1H), 4.58 (d, J = 5.5 Hz, 3H), 3.95 (s, 3H), 3.30–3.01 (m, 4H), 2.25–1.80 (m, 4H), 1.70–1.30 (m, 17H); ^{13}C NMR (125 MHz, DMSO $d-6$) δ 165.7, 165.2, 160.9, 159.8, 156.1, 155.8, 155.7, 155.6, 155.0, 154.4, 145.7, 142.8, 141.0, 140.9, 140.7, 137.3, 136.6, 133.4, 133.3, 130.1, 130.0, 128.9, 128.8, 128.3, 127.7, 117.3, 77.3, 65.1, 64.9, 64.7, 52.0, 48.9, 46.8, 31.8, 31.3, 29.2, 28.9, 28.2, 28.1, 22.8, 22.6; HRMS (ESI, M + Na) calcd for $C_{48}H_{52}N_{10}O_{15}Na$ 1031.3511, found 1031.3479.

*Synthesis of **9**.* To a solution of bis-trioxazole **8** (510 mg, 0.50 mmol) in DMF-THF (1 : 5, 30 mL) was added morpholine (440 μL, 5.0 mmol) and $Pd(PPh_3)_4$ (29 mg, 0.025 mmol), and the mixture was stirred at room temperature for 1 h. To the reaction mixture was added ether and precipitate was formed. This precipitate was collected with filtration using filter paper. The solid was purified by column chromatography on silica gel ($CHCl_3$-MeOH = 9 : 1) to give **9** (460 mg, 0.50 mmol 99%). Spectral data for **9**: $[\alpha]^{25}_D = 28$ (c 1.0, $CHCl_3$-MeOH (1 : 1)); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (s, 1H), 8.35–8.25 (m, 5H), 7.53 (d, J = 8.9 Hz, 1H), 7.33 (m, 5H), 5.56–5.43 (m, 1H), 5.08 (br, 2H), 4.80 (br, 1H), 4.56 (br, 1H), 4.14–4.05 (m, 1H), 3.95 (s, 3H), 3.26–3.04 (m, 4H), 2.25–1.75 (m, 4H), 1.73–1.30 (m, 17H); ^{13}C NMR (125 MHz, DMSO $d-6$) δ 169.3, 165.2, 160.9, 159.8, 156.1, 155.9, 155.7, 155.6, 154.9, 154.4, 145.7, 142.7, 141.0, 140.9, 140.6, 140.5, 137.3, 136.6, 133.3, 130.0, 129.9, 128.8, 128.5, 128.3, 127.7, 77.3, 65.1, 52.0, 49.4, 46.7, 35.3, 31.3, 29.2, 28.2, 28.1, 22.8, 22.6; HRMS (ESI, M + Na) calcd for $C_{44}H_{48}N_{10}O_{13}Na$ 947.3300, found 947.3308.

*Synthesis of **10**.* To a solution of bis-trioxazole **9** (2.2 g, 2.4 mmol) in THF-H_2O (3 : 1, 200 mL) was added lithium hydroxide (300 mg, 7.2 mmol), and the mixture was stirred at room temperature for 2 h. To the reaction mixture was added 1N HCl, and the resulting mixture was concentrated in vacuo. To the residual solution in DMF-CH_2Cl_2 (1 : 2, 800 mL) was added DMAP (1.5 g, 12 mmol), diisopropylethylamine (2.0 mL, 12 mmol), and DPPA (2.6 mL, 12 mmol), and the resulting mixture was stirred for 22 h at 90°C. To the reaction mixture was added H_2O and the organic layer was extracted with ethyl acetate. The extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (ethyl acetate 100%) to give **10** as a white solid (1.7 g, 1.9 mmol 79%, mp = 220–225°C



Telomestatin (TMS)



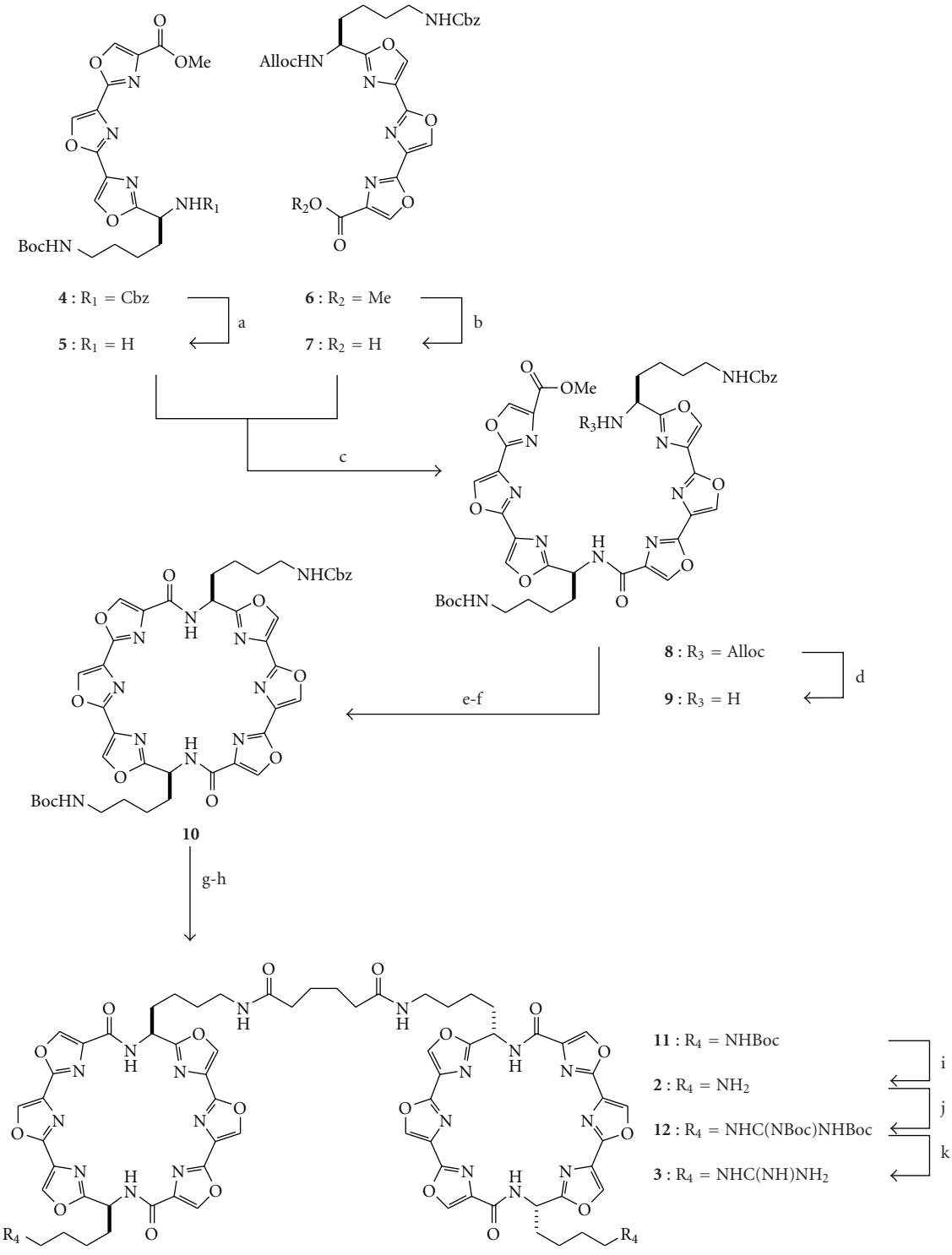
6OTD dimers (**1**) : R = OAc
(**2**) : R = $(CH_2)_3NH_2$
(**3**) : R = $(CH_2)_3NHC(NH)NH_2$

Figure 1: Structures of telomestatin and 6OTD dimers.

dec). Spectral data for **10**: $[\alpha]^{25}{}_D = -12$ (*c* 0.4, $CHCl_3$-MeOH (1 : 1)); ^{H}H NMR (400 MHz, $CDCl_3$) δ 8.54 (d, J = 7.3 Hz, 2H), 8.25–8.16 (m, 6H), 7.36–7.27 (m, 5H), 5.47–5.37 (m, 2H), 5.05 (br, 2H), 4.89 (br, 1H), 4.59 (br, 1H), 3.22–2.98 (m, 4H), 2.30–1.89 (m, 4H), 1.62–1.18 (m, 17H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.8, 164.7, 161.2, 159.9, 159.8, 156.3, 156.0, 155.9, 154.6, 141.0, 140.9, 139.1, 138.4, 136.9, 136.8, 136.6, 130.9, 129.6, 128.4, 128.1, 128.0, 79.0, 66.5, 47.8, 47.7, 40.7, 40.3, 34.6, 29.5, 29.2, 28.4, 21.9, 21.8; HRMS (ESI, M + Na) calcd for $C_{43}H_{44}N_{10}O_{12}Na$ 915.3038, found 915.2999.

*Synthesis of **11***. To a solution of macrocyclic bis-amide **10** (200 mg, 220 μmol) in MeOH (50 mL) was added $Pd(OH)_2$/C (80 mg) and the reaction mixture was stirred at room temperature under hydrogen (balloon). After 5 h, the reaction mixture was filtered through a pad of Celite, and the filtrates were concentrated in vacuo. To the residual solution in DMF-MeCN (1 : 1, 4.0 mL) was added diisopropylethylamine (190 μL, 1.1 mmol) and adipoyl chloride (16 μL, 110 μmol), and the mixture was stirred at room temperature for 11 h. The reaction mixture was concentrated in vacuo, and the residue was acidified with 0.1 N HCl and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$, filtrated, and concentrated in vacuo. The residue was purified by column chromatography on silica gel ($CHCl_3$–AcOEt–MeOH = 3 : 2 : 1) to give **11** (51 mg, 31 μmol, 28%). Spectral data for **11**: $[\alpha]^{25}{}_D = -11$ (*c* 0.95, $CHCl_3$-MeOH (1 : 1)); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67–8.48 (m, 4H), 8.27–8.15 (m, 12H), 6.38 (br, 2H), 5.47–5.38 (m, 4H), 4.84 (br, 2H), 3.30–2.98 (m, 8H), 2.15–1.90 (m, 12H), 1.65–1.10 (m, 38H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.0, 164.9, 164.8, 159.8, 159.7, 156.2, 156.1, 156.0, 154.7, 154.6, 141.0, 140.9, 139.5, 139.3, 138.7, 138.6, 136.9, 136.8, 130.8, 129.5, 129.3, 78.9, 47.8, 47.7, 40.3, 38.9, 36.0, 34.5, 34.3, 29.7, 29.5, 28.8, 28.4, 25.1, 21.9, 21.7; HRMS (ESI, M + Na) calcd for $C_{76}H_{82}N_{20}O_{22}Na$ 1649.5810, found 1649.5811.

*Synthesis of **2***. A solution of **11** (50 mg, 31 μmol) in CH_2Cl_2-TFA (95 : 5, 25 mL) was stirred at room temperature for 2 h. The reaction mixture was concentrated in vacuo to give **2** as a white solid (50 mg, 30 μmol, 98%, mp = 225–230°C dec). Spectral data for **2**: $[\alpha]^{25}{}_D = 72$ (*c* 0.3, $CHCl_3$-MeOH (1 : 1)); ^{1}H NMR (500 MHz, DMSO $d-6$) δ 9.15–9.08 (m, 8H), 8.95–8.89 (m, 4H), 8.38 (d, J = 6.9 Hz, 2H), 8.30 (d, J = 6.9 Hz, 1H), 7.80–7.53 (m, 6H), 5.50–5.35 (m, 4H), 2.98–2.89 (m, 4H), 2.78–2.89 (m, 4H), 2.15–1.85 (m, 12H), 1.55–1.00 (m, 20H); ^{13}C NMR (125 MHz, DMSO d – 6) δ 171.7, 164.5, 164.3, 158.8, 158.7, 155.6, 154.5, 142.5, 141.9, 141.8, 141.1, 136.0, 129.8, 129.7, 128.5, 128.4, 47.4, 47.3, 38.6, 38.1, 35.1,



Scheme 1: Synthesis of 6OTD dimers. (a) $Pd(OH)_2/C$, H_2, THF-MeOH; (b) $LiOH \cdot H_2O$, THF-H_2O; (c) DMT-MM, *N*-methylmorpholine, THF-H_2O, 89% over 2 steps from **4** and **6**; (d) $Pd(PPh_3)_4$, morpholine, DMF-THF 99%; (e) $LiOH \cdot H_2O$, THF-H_2O; (f) *N*,*N*-diisopropylethylamine, DMAP, DPPA, DMF-CH_2Cl_2, 78% over 2 steps from **9**; (g) $Pd(OH)_2/C$, H_2, MeOH; h) *N*,*N*-diisopropylethylamine, adipoyl chloride, 28% over 2 steps from **10**; (i) TFA, CH_2Cl_2 98%; (j) Et_3N, $HgCl_2$, 1,3-bis(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea; (k) TFA, CH_2Cl_2, 42%; Boc = tert-butoxycarbonyl, Cbz = benzyloxycarbonyl, DMT-MM = 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, DMAP = 4-dimethylaminopyridine, DPPA = diphenylphosphoryl azide, TFA = trifluoroacetic acid.

33.1, 28.7, 26.7, 24.9, 21.3, 20.8; HRMS (ESI, M + H) calcd for $C_{66}H_{67}N_{20}O_{18}$ 1427.4942, found 1427.4961.

*Synthesis of **12**.* To a solution of **2** (50 mg, 30 μmol) in MeOH (5.0 mL) was added Amberlyst A-26(OH) ion-exchange

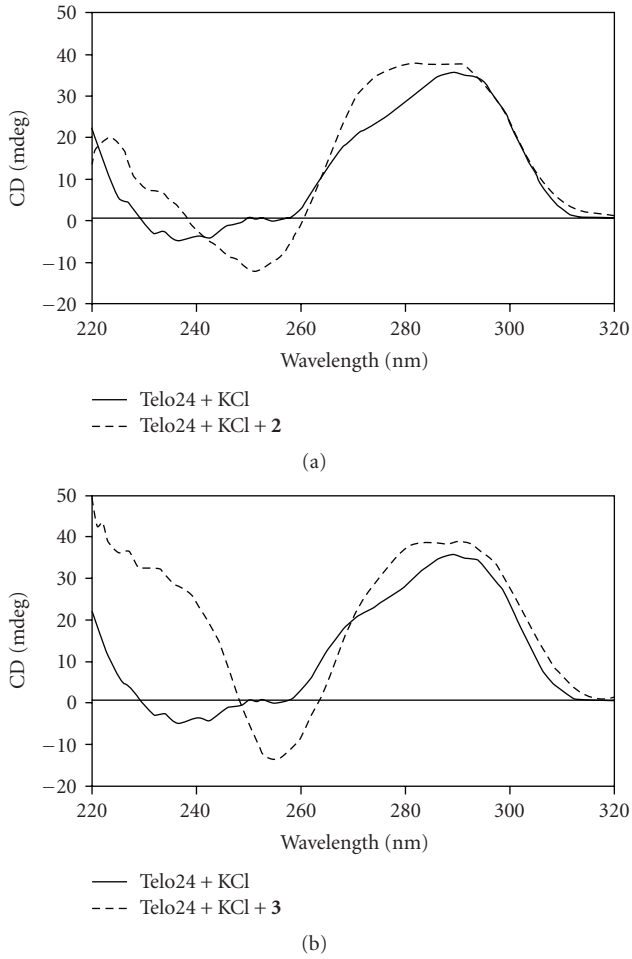


FIGURE 2: CD spectra of telo24 (10 $\mu\Delta$M) in Tris-HCl buffer (50 mM, pH 7.0) with KCl (100 mM) and/or ligands (50 μM). (a) solid line: telo24 + KCl; dashed line: telo24 + KCl + **2**. (b) solid line: telo24 + KCl; dashed line: telo24 + KCl + **3**.

resin, and the mixture was stirred for 30 minutes. The resulting mixture was filtered through a cotton with MeOH, and the filtrates were concentrated in vacuo. To a residual solution of **2** in DMF (5.0 mL) was added diisopropylethylamine (52 μL, 0.31 mmol), $HgCl_2$ (50 mg, 0.18 mmol), and 1,3-Bis(*tert*butoxycarbonyl)-2-methyl-2-thiopseudourea (66 mg, 0.18 mmol), and the mixture was stirred for 1 h at room temperature. To the reaction mixture was added H_2O, and the organic layer was extracted with ethyl acetate. The extracts were dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was chromatographed on silica gel ($CHCl_3$-ethyl acetate-MeOH = 3 : 2 : 1) to give **12** as a white solid (30 mg, 16 μmol, 52%). Spectral data for **12**: $[\alpha]^{25}{}_D$ = 3.8 (*c* 1.4, $CHCl_3$-MeOH (1 : 1)); ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.5 (br, 2H), 8.57–8.48 (m, 4H), 8.30–8.17 (m, 14H), 6.17 (br, 2H), 5.45–5.36 (m, 4H), 3.41–3.32 (m 4H), 3.28–3.10 (m, 4H), 2.20–1.88 (m, 12H), 1.65–1.20 (m, 56H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.9, 164.8, 164.7, 163.5, 159.9, 159.7, 156.1, 156.0, 154.7, 154.6, 153.2, 141.0, 140.9, 139.3, 139.2, 138.6, 138.5, 136.9, 136.8, 130.8, 129.6, 129.5, 82.9, 79.1, 47.7, 47.6, 40.5, 39.1, 36.0, 34.7, 28.7, 28.6, 28.2, 28.0, 25.0, 22.1, 22.0; HRMS (ESI, M + Na) calcd for $C_{88}H_{102}N_{24}O_{26}Na$ 1933.7295, found 1933.7332.

*Synthesis of **3**.* A solution of **12** (29 mg, 31 μmol) in CH_2Cl_2-TFA (3 : 1, 2.0 mL) was stirred at room temperature for 2 h. To the reaction mixture was added ether and precipitate was formed. This precipitate was collected with filtration using filter paper, to give **3** as a white solid (20 mg, 12 μmol, 80%, mp = 220–225°C dec). Spectral data for **3**: $[\alpha]^{25}{}_D$ = −18 (*c* 0.75, $CHCl_3$-MeOH (1 : 1)); ^{1}H NMR (400 MHz, DMSO *d* − 6) δ 9.14–9.08 (m, 8H), 8.94–8.90 (m, 4H), 8.37 (d, *J* = 7.3 Hz, 1H), 8.32 (d, *J* = 7.3 Hz, 1H), 7.75–7.69 (m, 2H), 7.51–7.45 (m, 2H), 5.48–5.37 (m, 4H), 3.08–2.90 (m, 8H), 2.15–1.84 (m, 12H), 1.50–1.00 (m, 20H); ^{13}C NMR (125 MHz, DMSO *d* − 6) δ171.7, 164.5, 164.4, 158.8, 158.7, 156.7, 155.7, 155.6, 154.5, 142.5, 141.8, 141.1, 136.0, 129.8, 129.7, 128.5, 128.4, 47.4, 40.5, 38.1, 35.1, 33.4, 33.3, 28.7, 28.2, 24.9, 21.3, 21.0; HRMS (ESI, M + H) calcd for $C_{68}H_{71}N_{24}O_{18}$ 1511.5378, found 1511.5368.

2.3. FRET Melting Assay. FRET melting assays were performed as reported methods [34, 35]. The dual fluorescently labeled oligonucleotides Flu-telo21 5′-FAM-[GGG(TTAGGG)3]-TAMRA-3′ and Flu-ds26 5′-FAM-[(TA)2GC(TA)2T6(TA)2GC(TA)2]-TAMRA-3′ were used in this protocol. The donor fluorophore was 6-carboxy-fluorescein, FAM, and the acceptor fluorophore was 6-carboxytetramethylrhodamine, TAMRA. The oligonucleotides were initially dissolved as a 100 μM stock solution in MilliQ water; further dilutions were carried out in 60 mM potassium cacodylate buffer (pH 7.4). Dual-labeled DNA was annealed at a concentration of 400 nM by heating at 94°C for 5 minutes followed by cooling to room temperature. We added the different concentrations of ligands into different samples, using a total reaction volume of 40 μL, with 200 nM of labelled oligonucleotide. Then we lay them at 25°C. Following experiments should keep the temperature procedure in real-time PCR and procedure was finished as following: 25°C for 5 minutes, then a stepwise increase of 1°C every minute from 25°C to reach 99°C. During the procedures, we measured the FAM after each stepwise.

2.4. CD Spectroscopy. The 10 μM oligonucleotide of telo24: ([TTAGGG]$_4$) was dissolved in Tris-HCl buffer (50 mM, pH 7.0) and the solution was heated to 90°C for 5 minutes, then slowly cooled to 25°C. G4 ligands were diluted from 10 mM stock solutions to give a concentration of 1 mM with water and added into the oligonucleotide samples at 50 μM (the 10 mM stock solutions of **2** and **3** were made in DMSO).

2.5. ESI-MASS Spectrometry. ESI-MASS spectra were recorded in a negative-ion mode with JEOL JMS-T100X spectrometer. The direct-infusion flow rate was 5.0 μL min^{-1}. All experiments were performed in 20 mM

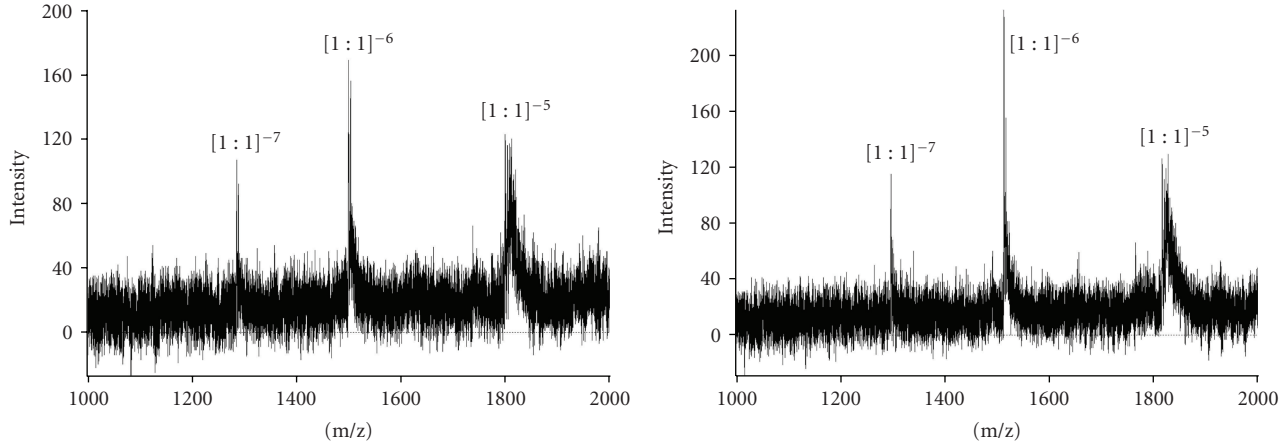


Figure 3: ESI-MASS spectra of 10 μM telo24 with a 40 μM ligands **2** (a) and **3** (b).

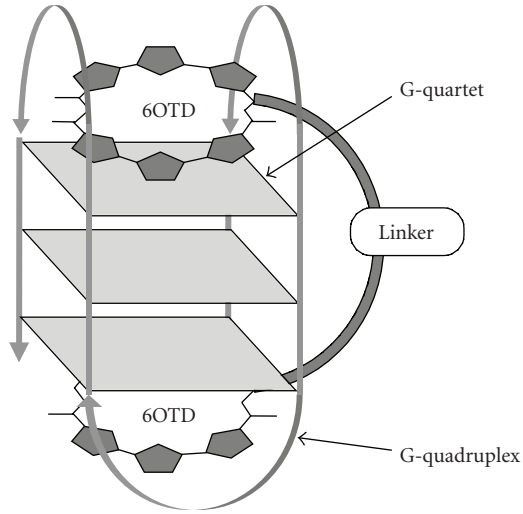


Figure 4: Plausible binding mode of the 6OTD dimers with telo24 [33].

NH_4OAc containing 10 μM of telo24 and 40 μM of **2** and **3**. Methanol (15%) was added just before injection.

3. Results and Discussion

3.1. Synthesis of 6OTD Dimers 2 and 3. The 6OTD dimers **2** and **3** were synthesized by using the sequences as shown in Scheme 1. Trioxazoles **4** and **6** were synthesized starting with L-serine and L-lysine, respectively by using the previously reported procedure [29, 30, 36–38]. The Cbz group of **4** was removed by treatment with hydrogen in the presence of $Pd(OH)_2/C$ to give amine **5**. Hydrolysis of the ester group in **6** with lithium hydroxide followed by coupling of the resulting acid with amine **5** using DMT-MM [39] gave the bis-trioxazole amide **8**. Cleavage of the allyloxycarbonyl group in **8** and hydrolysis of the ester group produced an amino acid, which was subjected to macrocyclization under high dilution conditions (5 mM) to give 6OTD **10**. The Cbz group in **10** was removed with hydrogen in the presence of $Pd(OH)_2/C$ to give corresponding amine. The procedure for synthesis of dimer **11** involved coupling of the amine with adipoyl chloride. Bis-amine **2** was obtained by removal of the Boc group of **11** with TFA. Preparation of the guanidine derivative **3** was carried out by guanidination of the amine moiety in **2** by using 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea followed by deprotection of Boc group with TFA.

3.2. Binding Properties of 2 and 3 toward Telomeric G4. With the desired 6OTD dimers **2** and **3** in hand, their mode of interaction with the telomeric DNA (telo24) was investigated. Firstly, conformational changes of telo24, induced by these substances were evaluated using circular dichroism (CD) spectroscopy. Upon treatment of telo24 with 6OTD dimers **2** and **3** (50 μM) in the presence of potassium chloride (100 mM), the mixed-type structure induced by potassium cation (solid line in Figure 2) is transformed to a typical antiparallel-type G4 structure (dashed line in Figure 2) [28, 40][1]. The binding stoichiometries of the complexes formed between the telo24 and ligands and **3** (molar ratio = 1 : 4) were determined by using ESI-MASS spectrometric analysis [41, 42]. In both cases, only mass peaks that correspond to 1 : 1 complexes of both **2** and **3** with telo24 were observed (at the 7-, 6- or in the 5-charge states). Since these interaction modes are the same as that of 6OTD dimer **1**, the newly synthesized 6OTD dimers **2** and **3**

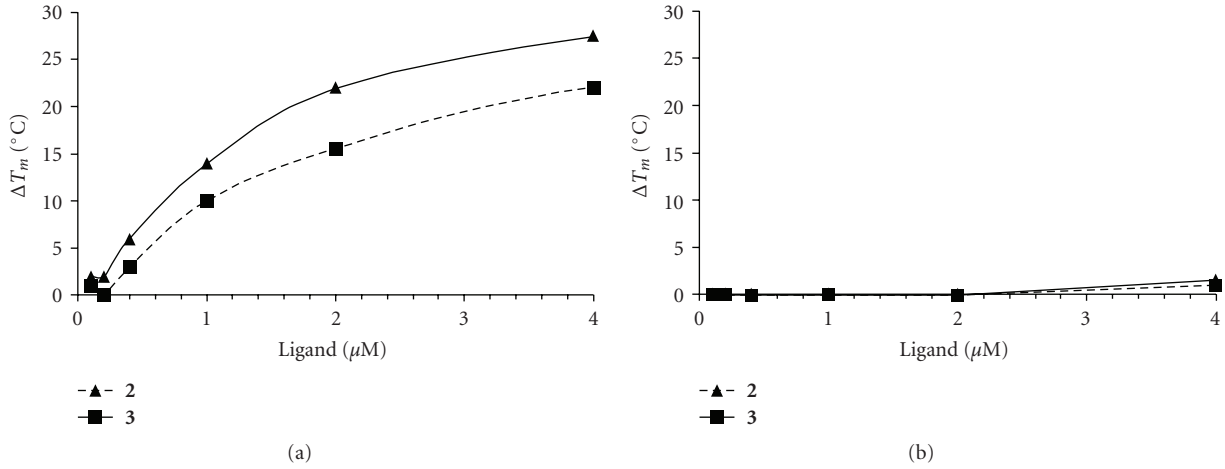


Figure 5: ΔT_m values of 0.2 μM Flu-telo21 (a) and Flu-ds26 (b) in the presence of ligands **2** (solid line) and **3** (dashed line).

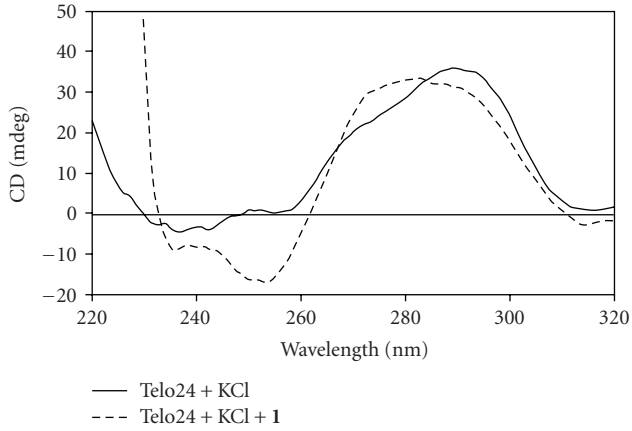


Figure 6

Table 1: ΔT_m values by FRET melting assay.

G4 ligands	**2**	**3**
	ΔT_m at 1 μM G4 ligands (ΔT_m/ °C)	
Flu-telo21	10	14
Flu-ds26	0	0

appear to interact with telo24 through an end stacking mode using two 6OTD moieties in a manner similar to that of TMS and/or 6OTD dimer **1** (Figures 4 and 6) [33][2,3].

The ability to stabilize the G4 structure of telo24 by 6OTD dimers **2** and **3** was evaluated by employing a fluorescence resonance energy transfer -(FRET-) based assay [34, 35]. The ΔT_m values of labeled oligonucleotide flu-telo21 with **2** and **3** at a concentration of 1 μM, which corresponds to 5 equivalents, are 10 and 14°C, respectively (Figure 5(a) and Table 1). Since under the same conditions the ΔT_m value for **1** was 12°C, among the substances explored to date guanidine **3** is a potent stabilizer of the G4 structure[4]. This stabilization effect is likely caused by the attractive interaction between positively charged guanidinium residue and anionic phosphates backbone of the telomeric G4. Interactions of the ligands with the duplex form of flu-ds26 were also investigated by using the same protocol (Figure 5(b) and Table 1). The observation that no differences in the ΔT_m values exist in the presence or absence of dimers **2** and suggests that these ligands are selective for the telomere DNA sequence.

4. Conclusions

In summary, the efforts described above have led to the design and syntheses of **2** and **3**, two novel macrocyclic hexaoxazole dimeric derivatives of 6OTD that have amine and guanidine groups in their respective side chains. These compounds, together with 6OTD dimer acetate **1**, were found to induce a change of the telomeric DNA sequence of telo24 into an antiparallel structure through the formation of 1 : 1 complexes with the DNA. The guanidine functionalized 6OTD dimer **3** was determined to have the greatest ability to stabilize the telomere DNA sequence. Also, both dimers selectively bind to the telomeric DNA sequence and not double-stranded DNA. Further studies, aimed at the structural development of 6OTD dimers with different linkers, are currently underway.

Acknowledgments

This work was supported in part by the Novartis Foundation (Japan) for the Promotion of Science, a Grant-in-Aid for Exploratory Research (21655060), and a Grant under the Industrial Technology Research Grant Program (01A04006b) from the New Energy and Industrial Technology Development Organization (NEDO) of Japan. Keisuke Iida and Masayuki Tera are grateful for a JSPS Research Fellowship for Young Scientists and a grant under the education program "Human Resource Development Program for Scientific Powerhouse" provided through Tokyo University of Agriculture & Technology.

Endnotes

1. Telomeric antiparallel intramolecular G-quadruplexes have characteristic CD spectra consisting of a positive peak at 290 nm and a negative peak at 260 nm [28, 40].

2. A similar conformational change was observed for 6OTD dimer **1** by using CD spectroscopic analysis of a solution of 10 μM of telo24 in the presence of potassium chloride (100 mM) and Tris-HCl buffer (50 mM, pH 7.0) as shown in Figure 6 (solid line: telo24 + KCl, dashed line: telo24 + KCl + dimer **1**).

3. The 6OTD dimer **1** also interacts with telo24 by forming 1 : 1 complex based on ESI-MASS analysis [33].

4. Under the same measurement conditions, the ΔT_m values in the presence of 1 μM **1** are 12°C (Flu-telo21) and 0°C (Flu-ds26), respectively.

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