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Synthesis of a Potent G-Quadruplex-Binding Macrocyclic Heptaoxazole

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Human telomeres are located at the ends of chromosomes and contain repeating $(TTAGGG)_n$ sequences, the 3′ ends of which exist as a single-stranded overhang.[1] This single G-rich strand forms a characteristic four-stranded helical conformation, called a G-quadruplex in the presence of high concentrations of monovalent cations such as potassium or sodium ions.[2] The G-quadruplex structure has recently been found not only in telomeres, but also in promoter regions of certain genes such as *c-kit*, *c-myc*, and *bcl-2*, and these G-quadruplexes have their own specific structures.[3–5] For example, telomere DNA sequences form parallel or antiparallel/parallel mixed-type G-quadruplex structures in the presence of potassium cations,[2a–e] whereas antiparallel-type structures are formed in the presence of sodium cations.[2f] In the case of the *c-myc* promoter region, the four-looped parallel-type structure is found.[3] In contrast, the G-quadruplex of the *c-kit* promoter region is composed of three stacked G-tetrads and four connecting loops.[4] The *bcl-2* promoter region forms an antiparallel/parallel mixed structure.[5] These DNA sequences and characteristic structures are involved in many important biological activities. For instance, telomeric G-quadruplex formation shortens the telomere drastically following the dissociation of TRF2 and/or Pot1, which bind at the end of the telomere, thereby inducing apoptosis in cancer cells.[6,7] Moreover, it was reported that the transcription of various oncogenes was directly suppressed by G-quadruplex formation in the gene promoter regions in vitro.[8] Because potent and sequence-selective G-quadruplex binders are candidate anticancer agents as well as useful biological tools, a number of studies have been aimed at the development of potent G-quadruplex binding compounds.[9]

Telomestatin (TMS; **1**), which has a macrocyclic structure containing five oxazoles, two methyloxazoles, and a thiazoline ring, is a natural product that was isolated from *Streptomyces anulatus* 3533-SV4 by screening with the telomeric repeat amplification protocol (TRAP) assay.[10] The macrocyclic polyoxazole structure of TMS (**1**) was reported to interact with telomeres, strongly stabilizing the G-quadruplex structure.[11a,o] Therefore, **1** is widely used as a standard G-quadruplex binder for exploring various functions.[6,7,11] However, TMS is not available in great quantity, and many synthetic approaches have been explored.[12] We recently synthesized 6OTDs **2**–**5** (Figure 1), which have a C_2-symmetrical macrocyclic hexaoxazole structure with various functional groups, as analogues of **1**.[13,14] Structure–activity relationship (SAR) studies with 6OTDs indicate that the functional groups play a critical role in the stabilization of G-quadruplex structure. L2H2-6OTD (**3**), with an aminopropyl group as R^1, strongly interacts with G-quadruplexes, and was observed by circular dichroism (CD) analysis to induce the randomly structured single stranded $d[TTAGGG]_4$ 24-mer (ss-telo24) into the antiparallel form efficiently, whereas the *N*-acetylaminopropyl derivative, L2A2-6OTD (**4**), does not.[13] With our focus on increasing the planarity of 6OTDs, we designed L1H1-7OTD (**6**), which is a macrocyclic heptaoxazole with an amino group side chain. We anticipated that the increased planarity of the macrocyclic structure relative to the 6OTDs would allow strong intercalation with G-quadruplexes through π–π interactions.[9] The amino group in **6** was also

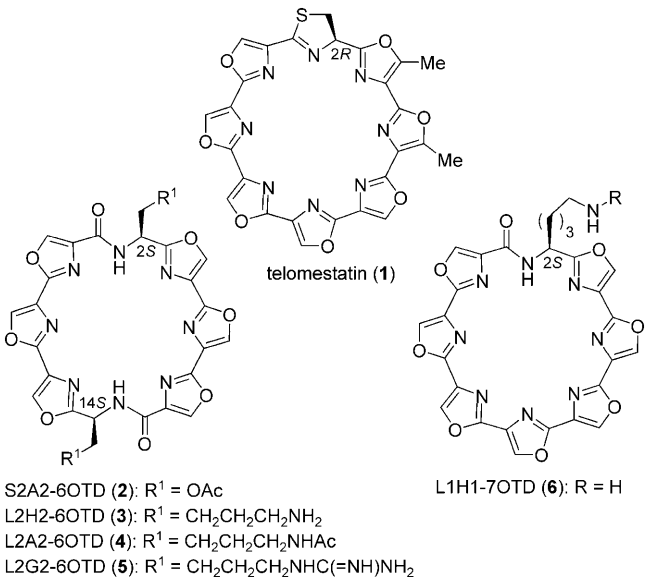

Figure 1. Structures of TMS (**1**), 6OTDs **2**–**5**, and L1H1-7OTD (**6**).

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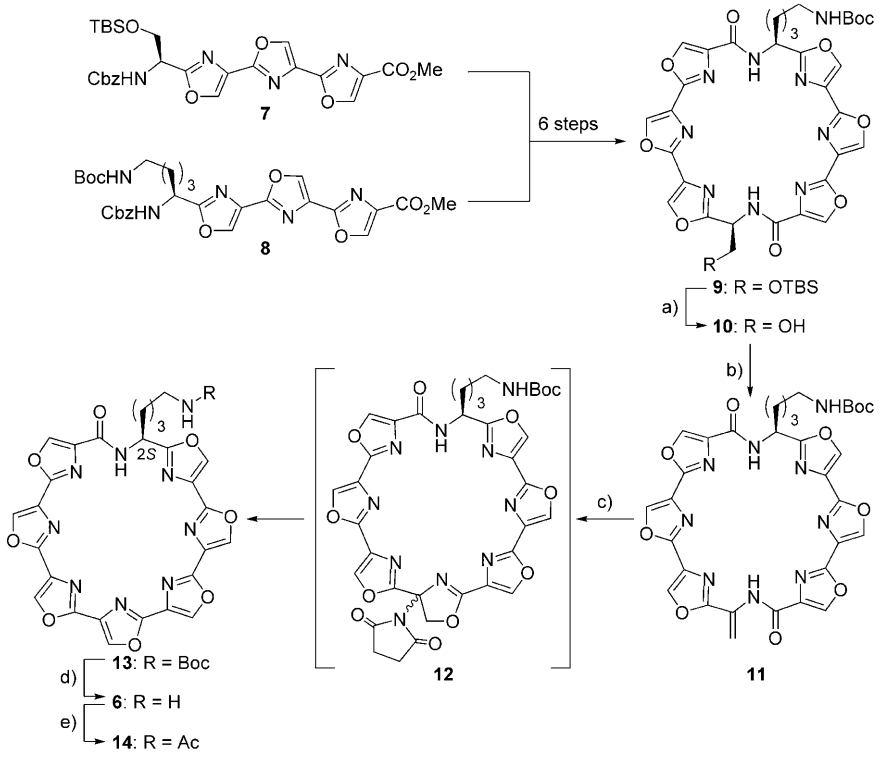

Scheme 1. Synthesis of L1H1-7OTD (**6**) and L1A1-7OTD (**14**): a) HF·pyridine, THF; b) MsCl, Et_3N, CH_2Cl_2 then DBU, 96% from **9**; c) NBS, Cs_2CO_3, MeCN, 65 °C, 31%; d) TFA, $CHCl_3$, 96%; e) Ac_2O, CH_2Cl_2, 64%. MsCl = methanesulfonyl chloride; DBU = 1,8-diazabicyclo[5,4,0]undec-7-ene; NBS = *N*-bromosuccinimide; DMAP = 4-dimethylaminopyridine; TFA = trifluoroacetic acid.

expected to stabilize the G-quadruplex structure efficiently through interaction with phosphate groups in the G-quadruplex.[9,13] Herein we describe the synthesis of L1H1-7OTD (**6**) and our evaluation of its biological activities.

L1H1-7OTD (**6**) was synthesized as shown in Scheme 1. The trioxazoles **7**[14] and **8**[13] were prepared as previously reported, and the corresponding macrocyclic bisamide **9** was synthesized in six steps (see the Supporting Information). The TBS group of **9** was deprotected with HF·pyridine to give the alcohol **10**. Insertion of the seventh oxazole ring in proceeding from **10** to **13** was problematic because of the strained structure of the β-hydroxyamide moiety.[12] After many attempts, it was achieved by modifying the protocol described by Pattenden and colleagues.[15,16a] Thus, alcohol **10** was converted into enamide **11** by mesylation, followed by treatment with DBU. The resulting enamide **11** was converted into heptaoxazole **13** via oxazoline **12**[16b] by reaction with NBS and Cs_2CO_3 in acetonitrile. Finally, the Boc group was deprotected with TFA to give L1H1-7OTD (**6**) in 30% yield from **11**. To evaluate the role of the amino group in the biological activities of **6**, the *N*-acetyl derivative of L1A1-7OTD (**14**) was synthesized for comparison, in 64% yield from **6** (Scheme 1).

With the macrocyclic heptaoxazole derivative L1H1-7OTD (**6**) in hand, we examined the interaction of **6** with telomeric DNA by CD analysis with ss-telo24. The ss-telo24 oligonucleotide has a random structure (Figure 2 ▲), which is known to adopt a G-quadruplex conformation in the presence of monovalent cations or G-quadruplex binders.[2,11n] Telomestatin (**1**) is known to induce an intramolecular antiparallel G-quadruplex structure in ss-telo24; this has a characteristic CD spectrum including a positive signal at 292 nm and a negative signal at 262 nm.[11n] Upon treatment of ss-telo24 with **6**, the CD spectrum changed, and a positive peak at 292 nm and a negative peak at 262 nm were observed. These characteristic spectral changes clearly showed that L1H1-7OTD (**6**) induced a change in the conformation of ss-telo24 to an antiparallel G-quadruplex (Figure 2 A, ○).[17] Interestingly, L1H1-7OTD (**6**) was found to convert the parallel/antiparallel mixed-type structure of ss-telo24, induced by potassium cations (Figure 2 A ■), into an antiparallel G-quadruplex structure (Figure 2 A □).[11n] Therefore, L1H1-7OTD (**6**) was revealed to strongly induce an antiparallel G-quadruplex structure in ss-telo24. These conformational changes were also observed with L1A1-7OTD (**14**), although the efficacy was weaker than that of **6** (Figure 2 B).[17] These results show that the planar heptaoxazole skeleton of 7OTD is more favorable than 6OTD[13] for stabilizing the G-quadruplex structure of ss-telo24. To quantify the binding affinity of 7OTDs **6** and **14** toward ss-telo24, K_a values were examined by CD titration experiments (Figures S1 and S2; Supporting Information), and respective binding constants for **6** and **14** of 5.9×10^4 M and 3.3×10^4 M were obtained.

To verify that this antiparallel G-quadruplex structural change is "intramolecular", an electrophoresis mobility shift assay (EMSA) was carried out between ss-telo24 and compounds **6** and **14**. At the higher concentrations, the new band with high mobility which corresponds to the "intramolecular" G-quadruplex structure was significantly increased with both **6** and **14** (Figure 3). EC_{50} values of **6** and **14** were determined, and were found to be 151 ± 16 μM and 459 ± 22 μM, respectively, with an ss-telo24 concentration of 50 μM (Figure S4, Supporting Information).

Next, selective interactions between L1H1-7OTD (**6**) and the telomere DNA sequence were examined by a polymerase chain reaction (PCR) stop assay with ss-telo24 and its mutant sequence, ss-telo24 mut.[11o,13] In this protocol, the selectivity of **6** was evaluated by PCR inhibitory activity, and **6** showed strong inhibition of the extension of ss-telo24, with an IC_{50} value of 0.67 ± 0.01 μM. In contrast, weak inhibition of ss-telo24 mut extension by **6** was observed ($IC_{50}=5.2\pm0.8$ μM),

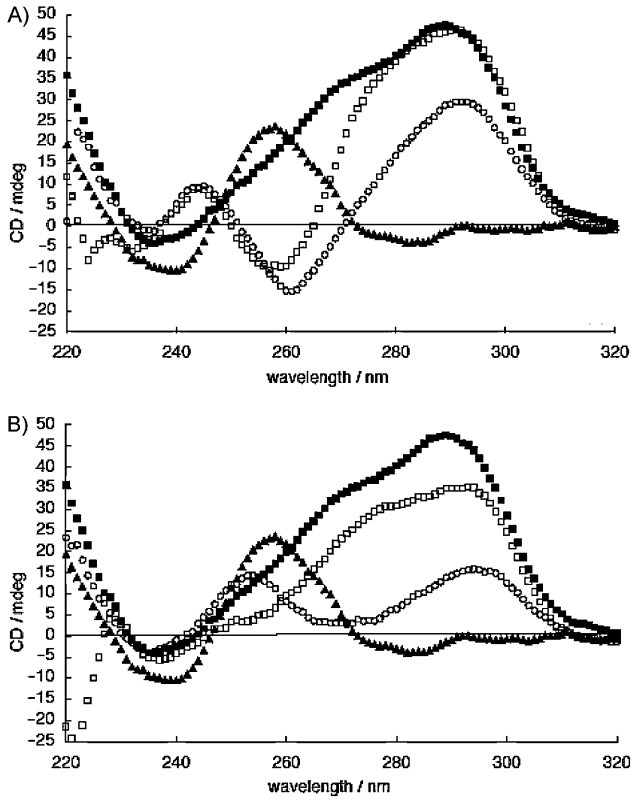

Figure 2. CD spectra of ss-telo24 (10 μM) in Tris-HCl buffer (50 mM pH 7.6) with 50 μM 7OTDs and/or 100 mM K^+. A) ▲: ss-telo24 (no salt added); ○: ss-telo24 + L1H1-7OTD (**6**) (no salt added); ■: ss-telo24 + KCl; □: ss-telo24 + KCl + L1H1-7OTD (**6**). B) ▲: ss-telo24 (no salt added); ○: ss-telo24 + L1A1-7OTD (**14**) (no salt added); ■: ss-telo24 + KCl; □: ss-telo24 + KCl + L1A1-7OTD (**14**).[17]

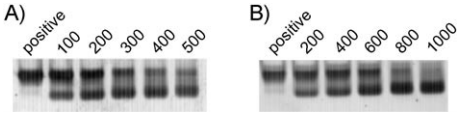

Figure 3. Effects of L1H1-7OTD (**6**) and L1A1-7OTD (**14**) on the formation of intramolecular G-quadruplexes. A) ss-telo24 (50 μM) was incubated for 60 min with various concentrations (indicated in μM) of L1H1-7OTD (**6**) in 50 mM Tris-HCl buffer (no salt added). After incubation, samples were mixed with Ficoll 400 and separated by 12% native PAGE in 1×TBE buffer at 4 °C. All oligonucleotides were stained by Stains-All. B) L1A1-7OTD (**14**) was used as G-quadruplex ligand (concentrations indicated in μM). The oligonucleotides were quantified with ImageQuant 5.1 (Molecular Dynamics).

indicating that compound **6** selectively interacts with ss-telo24 (Figure 4). Supporting the results of the CD experiments discussed above, L1A1-7OTD (**14**) showed weaker activity in the PCR protocol, with IC_{50} values of 2.2 ± 0.1 μM for ss-telo24 and > 80 μM toward ss-telo24 mut (Table 1).

Next, the selectivity of the interaction between 7OTDs and single- and double-stranded DNA was examined using ds-telo24 (the double-stranded telomeric DNA 24-mer) under the same EMSA conditions. An interaction between **6** and ds-telo24 was not observed even under the higher concentrations (Figure 5). These results clearly suggest that L1H1-7OTD (**6**) selectively interacts with "single"-stranded telomeric DNA sequences.[18]

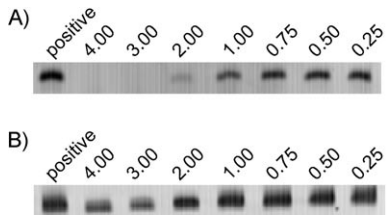

Figure 4. Effects of L1H1-7OTD (**6**) in the PCR stop assay at the concentrations indicated (μM) with G-quadruplex-forming A) ss-telo24 and B) control mutated ss-telo24 mut. The corresponding PCR products were mixed with Ficoll 400 and separated by 12% native PAGE in 0.5×TBE buffer. All oligonucleotides were stained by ethidium bromide. The oligonucleotides were quantified with ImageQuant 5.1 (Molecular Dynamics).

Table 1. PCR stop assays for L1H1-7OTD and L1A1-7OTD.

7OTD	IC_{50} [μM][a]	
	ss-telo24	ss-telo24 mut
L1H1-7OTD (**6**)	0.67 ± 0.01	5.2 ± 0.8
L1A1-7OTD (**14**)	2.2 ± 0.1	> 80

[a] Values represent the means ± SD of triplicate assays.

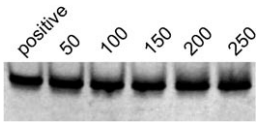

Figure 5. Evaluation of the interaction between ds-telo24 and L1H1-7OTD (**6**). EMSA of ds-telo24 (25 μM) in the presence of 7OTDs by native PAGE was performed; ds-telo24 was incubated in the presence of various concentrations (indicated in μM) of L1H1-7OTD (**6**) for 60 min in 50 mM Tris-HCl buffer (no salt added). After incubation, samples were mixed with Ficoll 400 and separated by 12% native PAGE in 1×TBE buffer at 4 °C. All oligonucleotides were stained by ethidium bromide.

Because L1H1-7OTD (**6**) potently interacts with telomeric DNA and induces intramolecular antiparallel G-quadruplex formation, we next examined the cytotoxicity of **6** toward HeLa cells, a telomerase-positive cell line, and Saos-2 cells, a telomerase-negative cell line.[19] Compound **6** exhibited cytotoxicity with an IC_{50} value of 2.2 ± 0.5 μM in HeLa cells. In contrast, no cytotoxicity was observed with Saos-2 cells up to concentrations of 30 μM and incubation for six days. On the other hand, doxorubicin, used as a telomerase-independent cytotoxic agent, showed potent cytotoxicity in both HeLa and Saos-2 cells, with IC_{50} values of 0.03 ± 0.001 and 0.07 ± 0.005 μM, respectively. These results suggest that the cytotoxicity of L1H1-7OTD (**6**) is related to its telomerase inhibitory activity.[20]

In conclusion, we have developed macrocyclic heptaoxazoles as G-quadruplex binders, with L1H1-7OTD being especially potent. This planar G-quadruplex binder strongly and selectively interacts with ss-telo24 and induces a conformational change to an intramolecular antiparallel G-quadruplex struc-

ture. The macrocyclic heptaoxazole appears to be a powerful scaffold for stabilizing telomeric antiparallel G-quadruplexes. The amino group in the planar 7OTD scaffold also plays a significant role in stabilizing G-quadruplex structure. Results of the cell-based assays suggest that the cytotoxicity of L1H1-7OTD (**6**) can be attributed to its interaction with telomeres.

Experimental Section

PCR stop assays: PCR stop assays were performed as previously reported.[13] Oligonucleotides ss-telo24 and ss-telo24 mut d[TTA GAG TTA GAG TTA GAG TTA GGG], the complementary sequence of telo24 d[TCT CGT CTT CCC TAA] (telo24 rev), were used. The chain-extension reaction was performed in 1×PCR buffer containing 0.2 mM dNTP, 5 U Taq polymerase, 7.5 pmol oligonucleotides and various concentrations of **6** and **14**. The mixtures were incubated in a thermocycler under the following conditions: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 47 °C for 30 s, and 72 °C for 30 s. Amplified PCR products were resolved on 12% native polyacrylamide gels in 0.5×TBE buffer and stained with ethidium bromide. The IC_{50} values were calculated based on the fluorescence intensity scanned with a phosphorimager (Typhoon 8600, Molecular Dynamics).

CD experiments: CD was carried out according to published procedures.[11n,13] The ss-telo24 oligonucleotide was dissolved in Tris buffer (50 mM, pH 7.6), and the solution was heated at 90 °C for 5 min, then slowly cooled to 25 °C. L1H1-7OTD (**6**) and L1A1-7OTD (**14**) was diluted from a 10 mM stock solution to a concentration of 1 mM with H_2O and added to the oligonucleotide samples at 50 μM. The final DNA concentration was 10 μM, and the CD spectra are representative of three averaged scans taken at 25 °C.

EMSA: EMSAs were performed by using a modified protocol of the reported procedure.[11n] The ss-telo24 oligonucleotide was dissolved in Tris buffer (50 mM, pH 7.6), and the solution was heated at 95 °C for 3 min, then slowly cooled to 25 °C. Various concentrations of L1H1-7OTD (**6**) and L1A1-7OTD (**14**) with 10% DMSO were prepared from 10 mM stock solutions and added to the ss-telo24 (50 μM) sample. After incubation, samples were mixed with Ficoll 400 and resolved on 12% native polyacrylamide gels in 1× TBE buffer at 4 °C and stained with Stains-All (3,3′-diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine bromide). The EC_{50} values were calculated based on the fluorescence intensity scanned with a phosphorimager (Typhoon 8600, Molecular Dynamics). The EMSA of ds-telo24 was also performed according to the above procedure.

Cell culture: HeLa and Saos-2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 μg mL^{-1} streptomycin, and 5 U mL^{-1} penicillin. Cells (2×10^3 per well) were seeded 96-well plates and then treated with various concentrations of compounds (**6** and doxorubicin) for six days. Cell viability was examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. IC_{50} values are defined as the concentration resulting in 50% cell viability after incubation for six days (Figure S7, Supporting Information).

Keywords: G-quadruplexes · macrocycles · polyoxazoles · telomerase inhibitors · telomeres

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[16] a) According to the original procedure reported by Pattenden and coworkers,[15] vinyl bromide was generated from enamide **12** by using NBS and Et_3N in dioxane. The cyclization reaction of the resulting vinyl bromide with Cs_2CO_3 gave a complex mixture, and the desired heptaoxazole **14** was obtained in only 12% yield; b) The intermediate oxazoline **12**, which was generated by the nucleophilic addition of succinimide to innamide, could be isolated, and the structure was determined by ^1H NMR and MS analysis (see the Supporting Information).

[17] The intensity (CD/mdeg) of the positive 292 nm and negative 262 nm signals reflects the ratio of the antiparallel G-quadruplexes.[11n]

[18] a) Interaction of L1A1-7OTD (**14**) with ds-telo24 was not observed (Figures S3 and S4); b) The difference in ΔT_m values of ds-telo24 (10 μM) in the absence or presence of L1H1-7OTD (**6**; 50 μM) was not observed (see the Supporting Information and Ref. [21]).

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[20] It has been reported that telomeric G-quadruplex binders induce collapse of the telomeric structure in addition to inhibition of telomerase activity. Therefore, we think that L1H1-7OTD (**6**) induces both biological effects in HeLa cells, resulting in potent cytotoxicity, and might induce collapse of telomeric structure in Saos-2 cells at high concentration.

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