DNA Recognition

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Macrocyclic Hexaoxazoles as Sequence- and Mode-Selective G-Quadruplex Binders**

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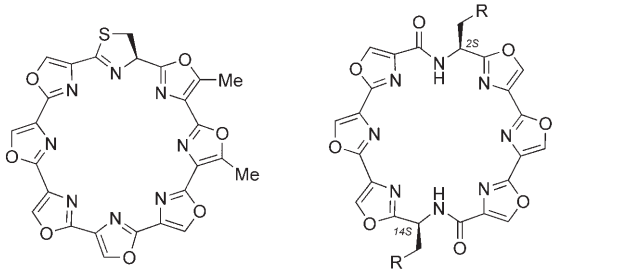
Human telomeric DNA, which is located at the end of chromosomes, contains repeating single-strand sequences of (TTAGGG)*n*.[1] This G-rich single strand forms a characteristic four-stranded helical conformation, called a G-quadruplex, in the presence of monovalent cations such as potassium or sodium ions.[2] The formation of G-quadruplexes in vitro has been shown to inhibit the activity of telomerase, an enzyme related to the elongation of telomeres in tumor cells; thus, inhibiting telomerase activity by stabilizing the telomeric G-quadruplex is considered to have potential for the treatment of cancer.[1] The G-quadruplex structure exists not only in the telomeric DNA, but also in the promoter regions of some oncogenes, such as c-myc, c-kit, and bcl-2.[3–5] These G-quadruplexes have different sequences from telomeric DNA, and have their own inherent conformational mode, namely, the telomere G-quadruplex has an antiparallel mode,[2] while the c-myc[3] and c-kit[4] promoter regions have a parallel-type mode, and the bcl-2 promoter region has a mixed antiparallel/parallel mode.[5] These DNA sequences also play significant roles in the inhibition of transcriptional activity.[6] Therefore, sequence- and mode-selective G-quadruplex ligands would be candidate anticancer agents as well as biological tools. There have been a number of studies,[7] but only a few compounds, such as 9944[8a] and 115405,[8a] have been reported as selective binders to the telomeric G-quadruplex.[8b,c] Herein, we describe the development of 6-OTD (**2**; Scheme 1) derivatives with a macrocyclic hexaoxazole skeleton as sequence- and mode-selective G-quadruplex binders.



Scheme 1. Structure of telomestatin (**1**) and designed G-quadruplex binders **2a**–**2d**.

Telomestatin (**1**, Scheme 1), which has a macrocyclic structure containing five oxazole rings, two methyl oxazole rings, and a thiazoline ring, is a natural product which was isolated from *Streptomyces anulatus* 3533-SV4 after screening with the telomeric repeat amplification protocol (TRAP).[9] Telomestatin (**1**) shows potent telomerase inhibitory activity, with an IC_{50} value of 5 nM. The macrocyclic polyoxazole structure of **1** interacts with, and strongly stabilizes, the telomere G-quadruplex,[10a] and **1** is widely used as a standard G-quadruplex binder.[10] The total synthesis of **1** was accomplished by Doi et al. in 2006,[11] but further structural development is difficult, because **1** has no convenient functional groups, such as hydroxy and/or amine groups. Recently, we reported the synthesis of S2A2-6OTD (**2a**), which is a macrocyclic hexaoxazole with a bis(hydroxymethyl acetate) moiety.[12] One of the stereoisomers, (2*S*,14*S*)-**2a**, was found to show moderate telomerase-inhibitory activity by the TRAP assay. On this basis, we have designed the amine and guanidine derivatives (2*S*,14*S*)-L2H2-6OTD (**2b**) and (2*S*,14*S*)-L2G2-6OTD (**2c**) with cationic functional groups. These derivatives have a planar macrocyclic hexaoxazole pharmacophore, which is expected to bind to guanine tetrads through π–π interactions. The cationic functional groups of **2b** and **2c**, which are directed toward the G-quadruplex grooves, are expected to interact with phosphate groups to effectively stabilize the G-quadruplex structure; consequently these compounds should exhibit potent telomerase-inhibitory activity.

The amine and guanidine derivatives **2b** and **2c** were synthesized as shown in Scheme 2. Briefly, the trioxazole **3**, which was synthesized from L-lysine and L-serine,[12] was converted into the carboxylic acid **4** and amine **5**. These two segments were coupled to give the linear hexaoxazole **6**. After

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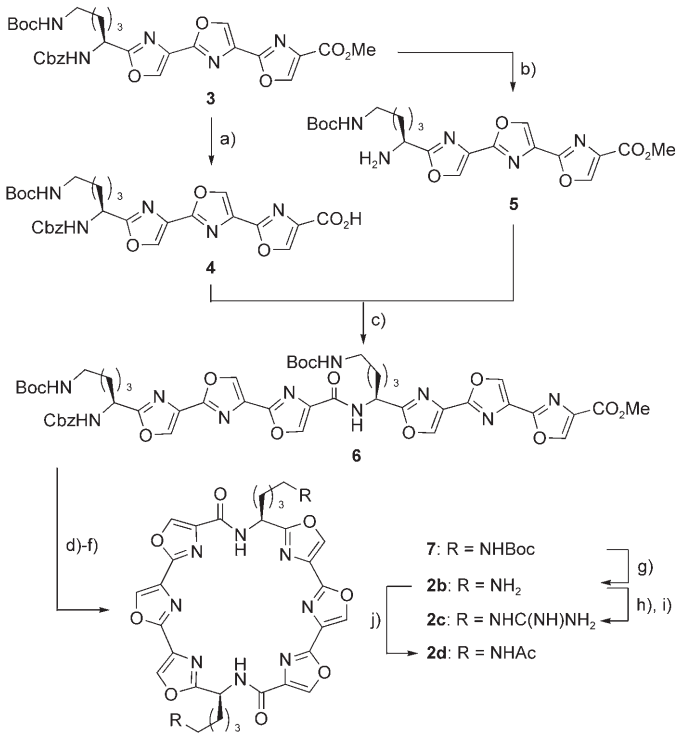

Scheme 2. Synthesis of L2H2-6OTD (**2b**), L2G2-6OTD (**2c**), and L2A2-6OTS (**2d**). a) LiOH, THF/H_2O; b) $Pd(OH)_2/C$, H_2, THF/MeOH; c) DMT-MM, *N*-methylmorpholine, THF/H_2O/MeOH, 63% over 3 steps from **3**; d) LiOH, THF/H_2O; e) $Pd(OH)_2/C$, H_2, THF/MeOH; f) $EtiPr_2N$, DMAP, BOPCl, DMF-CH_2Cl_2, 51% over 3 steps from **6**; g) TFA, CH_2Cl_2 99%; h) Et_3N, $HgCl_2$, 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea; i) TFA, CH_2Cl_2, 81% over 2 steps from **2b**; j) Ac_2O, CH_2Cl_2 60%. Boc = *tert*-butyloxycarbonyl, Cbz = benzyloxycarbonyl, DMT-MM = 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, DMAP = 4-dimethylaminopyridine, BOPCl = bis(2-oxo-3-oxazolidinyl)phosphinic chloride, TFA = trifluoroacetic acid.

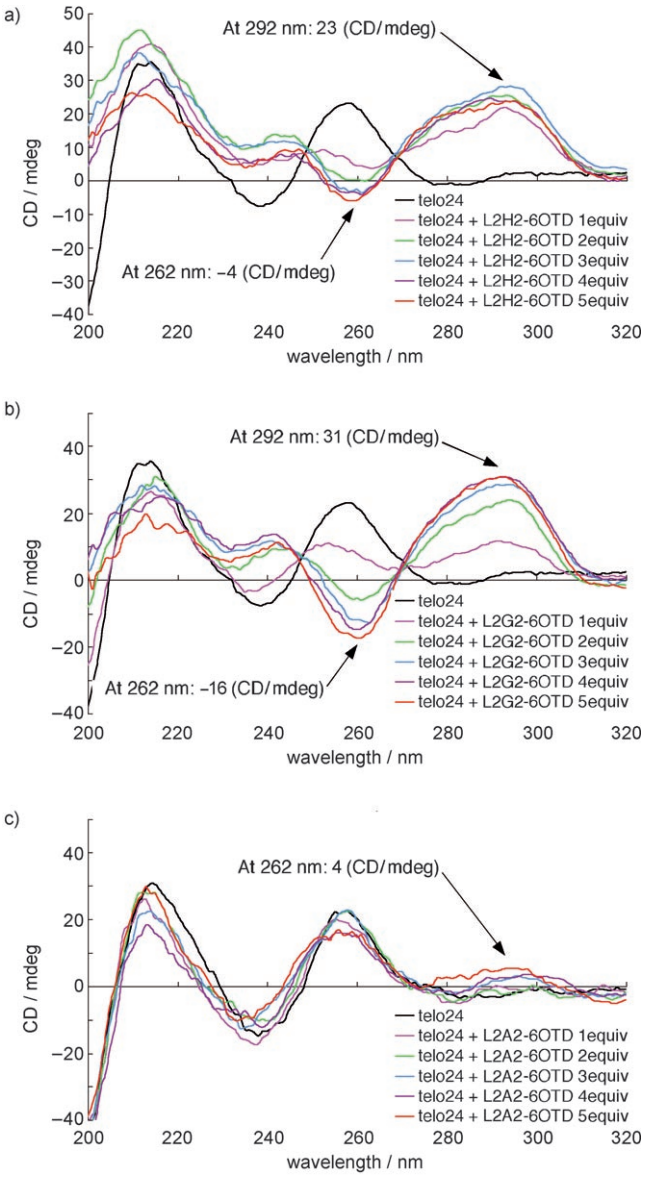

Figure 1. CD spectra of 10 μM telo24 in Tris-HCl buffer (50 mM, pH 7.6) in the presence of a) L2H2-6OTD (**2b**), b) L2G2-6OTD (**2c**), and c) L2A2-6OTD (**2d**) (0–50 μM).

hydrolysis of the ester group of **6** followed by cleavage of the Cbz group, the resulting amino acid was subjected to macrocyclization to afford the bisamide **7**. The Boc group was removed with TFA to give **2b**.[13] The guanidine **2c** was synthesized from **2b** in 57% yield by reaction with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea, followed by cleavage of the Boc groups with TFA. The *N*-acetyl derivative of L2A2-6OTD (**2d**) was synthesized from **2b** in 60% yield.

The ability of compounds **2b–d** to form G-quadruplexes was investigated by circular dichroism (CD) by using telo24.[14] Telo24 forms an intermolecular G-quadruplex (Figure 1, black line) in the absence of a G-quadruplex binder, and forms an intramolecular G-quadruplex in the their presence (Figure 1 a,b). Telomeric antiparallel intramolecular G-quadruplexes have characteristic CD spectra consisting of a positive signal at 292 nm and a negative signal at 262 nm.[2b] The, intensity of the signals at 292 and 262 nm reflect the ratios of the inter- and intramolecular G-quadruplexes.[15] Telo24 interacts with **1** to form an antiparallel G-quadruplex.[15] Figure 1 shows the CD spectra of telo24 in the presence of **2b–d** at various molar ratios. Compound **2b** efficiently increased the intensity of the signals at 292 and 262 nm (the formation of a steady state required two equivalents of **2b**), while **2c** was less effective (three equivalents of **2c** were required to reach a steady state). However, the **2c**–telo24 complex was more stable than that with **2b**. To examine these differences in the stabilities of the **2b**–telo24 and **2c**–telo24 complexes, the melting temperatures T_m were evaluated from the CD melting curves at 292 nm. The T_m values of the complexes were found to be 38.1 and 53.2 °C for the complexes with **2b** and **2c**, respectively. Since the T_m value of **1** is 47.8 °C, the guanidine derivative **2c** is a stronger stabilizer than **1** of the antiparallel form of telo24.

The DNA sequence selectivity of **2b–d** was next investigated. For this purpose the polymerase chain reaction (PCR) stop assay[8a] was conducted using the DNA sequences of telo24, telo24-mutant, c-kit, and c-kit-mutant.[14] Telomestatin (**1**) was also evaluated for comparison, and the results

Table 1: DNA sequence selectivity of 6OTDs **2b–d**, and **1** by the PCR stop assay.

G-quadruplex binders	**2b**	**2c**	**2d**	**1**
	IC_{50} [μM]			
telo24	0.71 ± 0.03	0.64 ± 0.01	> 75	0.65 ± 0.35[a]
telo24 mut	5.5 ± 0.2	20.4 ± 1.1	> 75	40[a]
c-kit	1.9 ± 0.1	2.1 ± 0.3	> 75	4.1 ± 0.1
c-kit mut	9.1 ± 0.7	14.9 ± 4.5	> 75	7.2 ± 0.3

[a] The inhibitory activities using the TRAP G4 assay are shown.[8a]

are summarized in Table 1. L2H2-6OTD (**2b**) strongly inhibited the chain extension reaction of telo24 by a factor of 7.7-fold compared with the telo-24-mutant DNA (IC_{50} value of 0.71 μM versus 5.5 μM). In the case of L2G2-6OTD (**2c**), the selectivity for telo24 versus the telo24-mutant was increased 31-fold. However, L2A2-6OTD (**2d**) showed no inhibitory activity on the chain extension reaction with either telo24 or its mutant. L2H2-6OTD (**2b**) and L2G2-6OTD (**2c**) also showed selective inhibitory activity toward the chain extension reaction of c-kit (4.8-fold and 7.1-fold, respectively, versus the c-kit-mutant oligonucleotides), although the IC_{50} values of **2b** and **2c** for c-kit were only moderate (1.9 and 2.1 μM, respectively). Thus, **2b** and **2c** were more selective for the telo24 DNA sequence than for the c-kit DNA sequence.

G-Quadruplex binders generally show telomerase inhibitory activities. Since **2b** and **2c** selectively interacted with telo24, we next examined the telomerase inhibitory activities of **2b** and **2c** by the TRAP assay, which readily evaluates telomerase activity of G-quadruplex binders using cancer cells. In this experiment, **2b** and **2c** inhibited the telomerase activity of Namalwa cells at a concentration of 20 nM by 49 and 42%, respectively.[16] Consistent with the telomerase inhibitory activities, both **2b** and **2c** inhibited the growth of HeLa cells (which are well-known as telomerase-positive cells) after 6 days incubation, with IC_{50} values of 7.4 and 0.5 μM, respectively.[10j,n] These results also reflect the G-quadruplex-stabilizing abilities obtained from the CD analysis.

In conclusion, we have developed the macrocyclic hexaoxazole 6OTD-type G-quadruplex binders L2H2-6OTD (**2b**) and L2G2-6OTD (**2c**). These new binders are selective for the telo24 DNA sequence, and strongly stabilize telo24 in the antiparallel form. These compounds also showed potent telomerase-inhibitory activity in both cell-free and cell-based assay systems. The G-quadruplex stabilizing ability and the cell-based assay results for the G-quadruplex binders were discussed for the first time using 6OTD derivatives. Further structure–activity relationship studies of 6OTD derivatives and investigations of the biological activities are in progress.

Experimental Section

CD spectroscopy: The CD titration experiment was performed with a modification of the reported procedure.[15] Telo24[14] oligonucleotide was dissolved in Tris-HCl buffer (50 mM, pH 7.6) and the solution was heated to 90 °C for 5 min, then slowly cooled to 25 °C. L2H2-6OTD (**2b**) and L2G2-6OTD (**2C**) were diluted with water from 10 mM stock solutions to give a concentration of 1 mM, and titrated into the oligonucleotide samples at 1 mol equiv up to 5 mol equiv (the 10 mM stock solutions of **2b** and **2c** were made in 50% MeOH). The DNA concentrations were 10 μM, and the CD spectra are representative of three averaged scans taken at 25 °C. The CD melting curves of the telo24 G-quadruplex were determined from measurements of the CD intensity at 292 nm. The heating rate was 2.0 °C min^{-1}. The results are presented in the Supporting Information.

PCR stop assay: The PCR stop assay was performed by means of a modification of the reported protocol.[8a] Oligonucleotides[14] and the corresponding complementary sequence of telo24 d[TCTCGTCTTCCCTA-A] (telo24 rev) and c-kit d[TATATATA-TACCCTCCTC] (c-kit rev) were used. The chain-extension reaction was performed in PCR buffer containing 0.2 mM dNTP, 5 U Taq polymerase, 7.5 pmol oligonucleotides, and various concentrations of **2b–d** and **1**. The mixtures were incubated in a thermocycler with the following cycling 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 fluorescent intensity of ethidium bromide was measured and quantified on a phosphorimager (Typhoon 8600, Molecular Dynamics). The results are summarized in the Supporting Information.

TRAP assay: Inhibitory effects against telomerase, which was semipurified from cell lysates of human B lymphoma Namalwa cells, were estimated by the telomeric repeat amplification protocol (TRAP), which is a modified version of the method developed by Kim et al.,[17] as described in detail previously.[18]

Cell culture: HeLa cells were cultivated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 50 μg mL^{-1} streptmycin, and 5 units ml^{-1} penicillin at 37 °C in a 5% CO_2 atmosphere. Cells were plated onto 96-well multiwell plates at a density of 2×10^3 cells per well. After the cells had been treated with compounds (**2b** and **2c**) for 6 days, 10 μL of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) (MTT) solution was added to each well. After further incubation for 4 h, the cell viability was estimated from the optical density at 520 nm.

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[14] The DNA oligonucleotide sequences used are shown below.

DNA oligomer	DNA sequences
telo24	5'-TTAGGGTTAGGGTTAGGGTTAGGG-3'
telo24 mut	5'-TTAGAGTTAGAGTTAGAGTTAGGG-3'
c-kit	5'-AGGGAGGGCGCTGGGAGGAGGG-3'
c-kit mut	5'-AGAAAGAACGCTGGGAGGAGGG-3'

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