G-quadruplex recognition by macrocyclic hexaoxazole (6OTD) dimer: greater selectivity than monomer†

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Macrocyclic hexaoxazole (6OTD) dimers were designed as candidates for potent G-quadruplex binders and synthesized.

Human telomeres are located at the ends of chromosomes and consist of repeating sequences of (TTAGGG)n.[1] This guanine-rich DNA sequence forms G-quadruplex structures in the presence of monovalent cations.[2] The G-quadruplex structure of telomeres promotes dissociation of telomere-related proteins, such as Pot1 and TRF2, and induces apoptosis of cancer cells.[3] Therefore, G-quadruplex binders, which stabilize the telomeric G-quadruplex structure, are candidate cancer therapeutic agents.[4] In 2001, Seto and Shin-ya reported that the macrocyclic polyoxazole-thiazoline telomestatin (TMS; **1**) from *Streptomyces annulatus* 3533-SV4 is one of the most potent G-quadruplex binders yet discovered (Fig. 1).[5,6] The interaction with the telomeric G-quadruplex was reported to involve two molecules of TMS.[7] Based on a docking study, Hurley *et al.* proposed an end-stacked binding mode, *i.e.*, interaction of the terminal G-quartet flat surfaces through π–π stacking.[8]

We recently reported a synthetic TMS derivative, 6OTD, which has C_2-symmetrical macrocyclic hexaoxazole structure, as a new G-quadruplex binder (Fig. 1).[9,10] Taking into consideration the proposed stacking model of TMS with telomeric G-quadruplex, we hypothesized that a 6OTD dimer connected through an appropriate linker would show cooperative interaction of the two monomer moieties with telomeric DNA, and would therefore bind more selectively than the monomer. Since the distance between the terminal G-quartets in the telomeric G-quadruplex structure is known to be *ca.* 8 Å (l_1 in Fig. 2),[11] the distance between two 6OTD structures in the dimer is required to be *ca.* 15 Å (l_2 in Fig. 2) for the interaction with G-quadruplex as depicted in Fig. 2. Hence, we designed 6OTD dimers **9–11** having three different length of linkers. These dimers were calculated by molecular dynamics using AMBER9, and dimer **10** was suggested to have the appropriate length for binding to the G-quadruplex (Fig. S1 in ESI†). In this paper, we describe the synthesis of 6OTD dimers **9–11**, and evaluation of their interaction abilities with telomeric G-quadruplex.

The 6OTD dimers **9–11** were synthesized starting from the reported macrocyclic bis-amide **4** (Scheme 1).[9c] Briefly, the Boc group of **4** was deprotected with 5% TFA to give amine **5**. Reaction of the resulting amine **5** with triphosgene followed by deprotection of the TIPS ether with TBAF gave an alcohol, whose hydroxyl group was converted into acetate with acetic anhydride to afford dimer **9** in 29% yield. The dimers **7** and **8** were also synthesized from the common intermediate of **5**. Thus, the amino group of **5** was reacted with the dipentafluorophenyl adipic diester and 1,12-dodecanedicarboxylate to afford dimers **7** and **8**, respectively.[12] The TIPS ether groups of **7** and **8** were converted into acetate with TBAF followed by treatment with acetic anhydride to give dimers **10** and **11** in 57 and 61% yield, respectively. LSA2-6OTD (**3**), which corresponds to a monomer derivative of **9–11**, was also prepared from **4** in 93% yield.

Induction and structural stabilization of the G-quadruplex structure of telomeric DNA by the newly synthesized 6OTD dimers **9–11** and LSA2-6OTD (**3**) was quantitatively evaluated by fluorescence resonance energy transfer (FRET) melting assay using fluorescence-labeled, single-stranded human telomeric oligonucleotide Flu-ss-telo21.[13,14] The ΔT_m values of **9–11** and **3** at a concentration of 2 μM, which corresponds to 10 equivalents with respect to labeled oligonucleotide

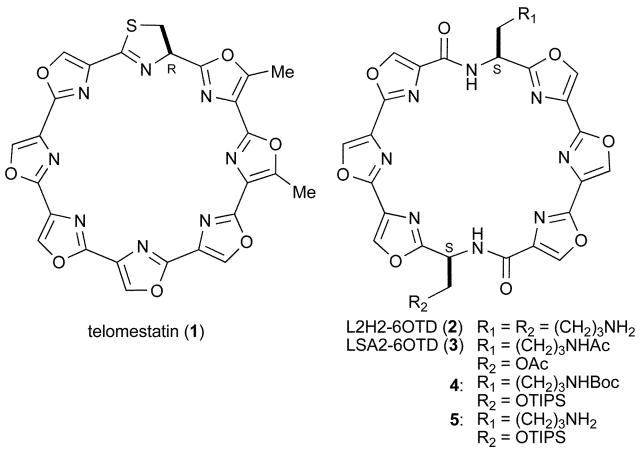

Fig. 1 Chemical structures of TMS (**1**) and 6OTDs **2–5**.

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† Electronic supplementary information (ESI) available: Further experimental details, NMR spectra, FRET-melting, PCR stop assay and molecular dynamics simulation experiment. See DOI: 10.1039/b910242f

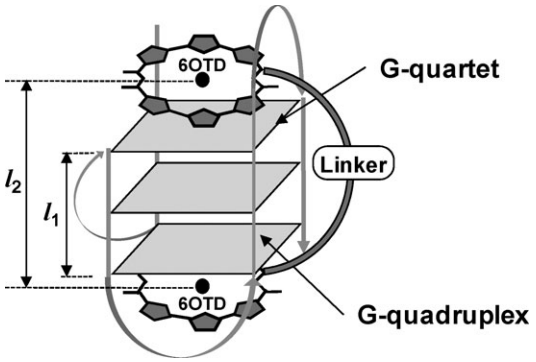
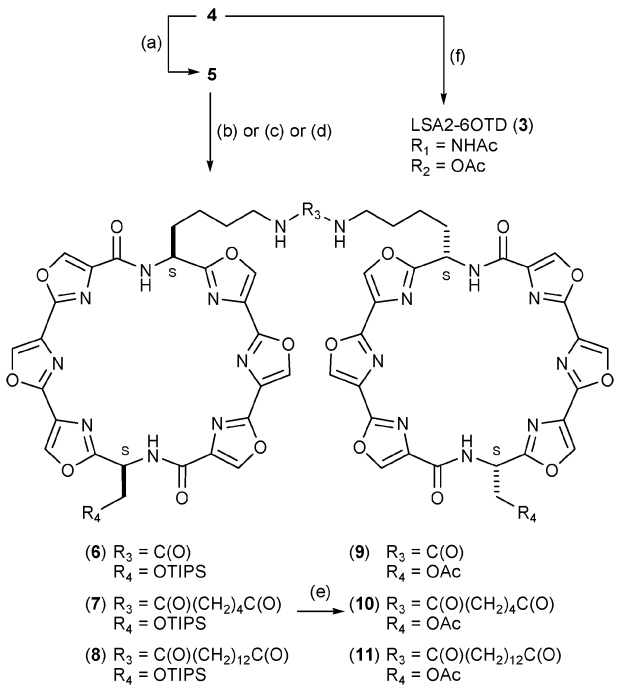


Fig. 2 Design concept of 6OTD dimers.



Scheme 1 Synthesis of 6OTD dimers **9–11**. *Reagents and conditions*: (a) TFA, CH_2Cl_2, r.t. for 2 h; (b) triphosgene, DIPEA, MeCN, r.t. for 18 h (**6**: 31%); (c) dipentafluorophenyl ester of adipic acid, DIPEA, MeCN, reflux 3 h (**7**: 67%); (d) dipentafluorophenyl ester of 1,12-dodecanedicarboxylic acid, DIPEA, MeCN, reflux 3 h (**8**: 51%); (e) TBAF, Ac_2O, THF, r.t. for 0.5–1 h (29–61%); (f) HCl, MeCN, 0.5 h, then Ac_2O, Py, 70 °C for 1 h (93%).

Flu-ss-telo21, are summarized in Table 1. The ΔT_m values were dependent upon the linker length, *i.e.*, the dimers **9** and **11** showed ΔT_m values of 17.2 and 8.5 °C, respectively. On the other hand, the ΔT_m value of **10** was found to be 25.1 °C, the same as that of the monomer **3**. Therefore, dimer **10** appears to afford the same degree of stabilization to the G-quadruplex structure of telomeric DNA as the monomeric 6OTD derivative LSA2-6OTD (**3**) (Fig. S2 in ESI†).

Then, the selectivity of the interactions of ligands **9–11** with single-stranded telomeric DNA (Flu-ss-telo21) and the duplex form, Flu-ds-26mer,[14] was investigated by the same method described above (Table 1).[15] No significant interaction between the double-stranded DNA of Flu-ds-26mer and ligands **9–11** was observed even at high concentrations. Thus, these ligands **9–11** were highly selective for single-stranded telomeric DNA

Table 1 ΔT_m by FRET melting and IC_{50} by PCR stop assays

	ΔT_m (at 2 μM)/°C		IC_{50}[a]/μM	
Ligand	Flu-ss-telo21	Flu-ds-26mer	ss-telo24	ss-telo24-mut
3	25.0	−0.9	2.9 ± 0.5	243 ± 13
9	17.2	−0.1	23.3 ± 3.7	1150 ± 36
10	25.1	−0.8	3.0 ± 1.2	>2500
11	8.5	−0.2	35.1 ± 3.8	1000 ± 41

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

over double-stranded DNA, in accordance with the reported character of 6OTD derivatives.[9b] These results clearly showed that the macrocyclic hexaoxazole structure of 6OTD was important for the interaction with G-quadruplex DNA, even in the case of the 6OTD dimers **9–11**.

The selectivity of interaction of ligands **3** and **10**, which showed strong interaction with telomeric DNA, with the single-stranded nucleotide base sequence ss-telo24 and its mutant sequence ss-telo24-mut was investigated using PCR stop assay (Table 1 and Fig. S3 in ESI†).[14,16] The monomer ligand of LSA2-6OTD (**3**) inhibited the extension of ss-telo24 with an IC_{50} value of 2.9 ± 0.5 μM, whereas low inhibitory activity was observed with the mutant sequence of ss-telo24-mut (IC_{50} of 243 ± 13 μM; 83-fold selectivity). In the case of 6OTD dimer **10**, the ss-telo24 extension reaction was inhibited with an IC_{50} of 3.0 ± 1.2 μM, while no inhibitory activity toward ss-telo24-mut was detectable (IC_{50} >2500 μM; >800-fold selectivity).[17] Thus, at least 10-fold higher selectivity for interaction with telomeric DNA was obtained by dimerization of the 6OTD structure.

The binding stoichiometry of 6OTD dimer **10** with telomeric DNA was determined by means of ESI-MS spectral analysis.[18] The mass spectra of the G-quadruplex DNA (ss-telo24) with LSA2-6OTD (**3**) and dimer **10** at a molar ratio of 1 : 4 are shown in Fig. 3. In the case of LSA2-6OTD (**3**), the 2 : 1 stoichiometric complex with ss-telo24 was detected as a major peak (Fig. 3A).[19] On the other hand, only the ion signal of the 1 : 1 complex of dimer **10** and ss-telo24 was observed (Fig. 3B). Thus, dimer **10** was suggested to interact with telo-24 by utilizing two macrocyclic 6OTD moieties (Fig. S5 in ESI†).[20]

In summary, we have synthesized 6OTD dimers having a macrocyclic hexaoxazole structure in which the monomer units are connected with different lengths of linkers, and examined the interaction with the telomeric G-quadruplex structure. Among them, dimer **10** showed potent G-quadruplex stabilizing activity, and was revealed to interact with ss-telo24 more selectively than the corresponding monomer **3**. Since it forms the 1 : 1 complex with DNA, two macrocyclic moieties in dimer **10** were suggested to interact with the G-quadruplex. Further structure development of 6OTD dimers as well as NMR and X-ray analyses are currently underway to clarify the interacting manner of 6OTD dimers with the G-quadruplex.

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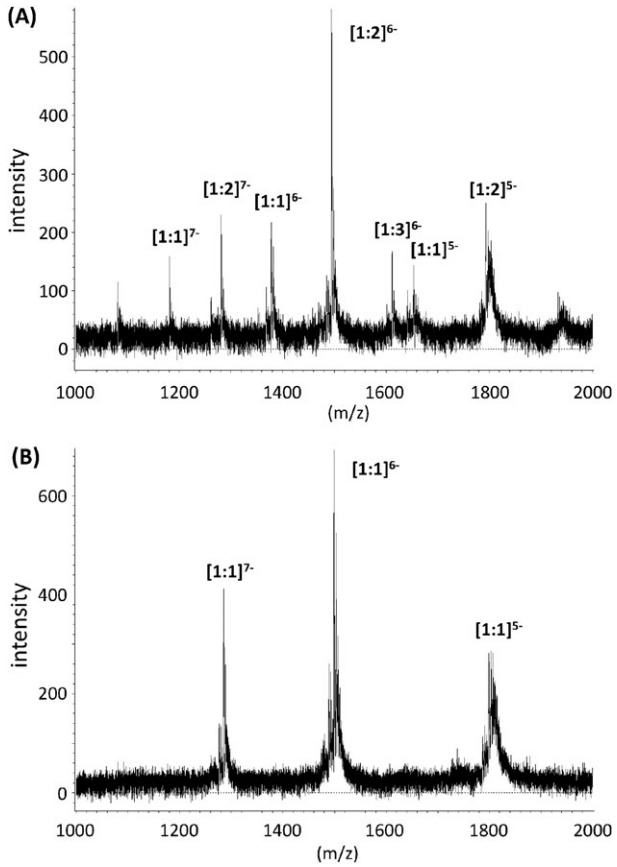

Fig. 3 ESI mass spectra of 10 μM ss-telo24 with 40 μM ligand **3** (A) or **10** (B).

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14 The oligonucleotide sequences are as follows:

DNA oligomer	DNA sequence
Flu-ss-telo21	5' -FAM-$G_3(T_2AG_3)_3$-TAMRA-3'
Flu-ds-26mer	5' -FAM-$(TA)_2GC(TA)_2T_6(TA)_2GC(TA)_2$-TAMRA-3'
ss-telo24	5'-$(TTAGGG)_4$-3'
ss-telo24-mut	5'-$(TTAGAG)_3TTAGGG$-3'

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17 The 6OTD dimers **9** and **11** showed PCR-inhibitory activity with IC_{50} values of 23.3 ± 3.7 μM and 35.1 ± 3.8 μM for telo24 and >1000 μM against ss-telo24-mut, respectively.

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19 Both 1 : 1 and 3 : 1 stoichiometric complexes of **3** and ss-telo24 were also detected as minor peaks.

20 In the case of **9** and **11**, 1 : 1 complexes with DNA were also observed (as a major peak for dimer **9**), although complexations are thought to be different from the case of **10** (see Fig. S4 and S5 in ESI†).