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DESIGN AND SYNTHESIS OF G-QUADRUPLEX LIGANDS BEARING MACROCYCLIC HEXAOXAZOLES WITH FOUR-WAY SIDE CHAINS†

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Abstract – Macrocyclic hexaoxazole compounds bearing four-way side chains L2H2-(4M)6OTD derivatives **3** and **4** were synthesized as new G-quadruplex ligands. Stabilization of G-quadruplex forming oligonucleotides in human telomeres, *c-kit*, *bcl-2*, and *c-myc* with those compounds was evaluated by the FRET (fluorescence resonance energy transfer) assay. These compounds showed less stabilizing ability compared to the ligand possessing two-way side chain of L2H2-(4M)6OTD (**2d**).

G-quadruplex, located throughout the human genome, is one of the secondary structures of DNA consisting of G-quartets, which comprises a coplanar structure associated with four guanine residues forming Hoogsteen-type hydrogen bonds (Figure 1).[1] Recently, G-quadruplex has been realized to play a critical role in the regulation of biological processes.[2] Since G-quadruplex structure is conformationally flexible, an artificial stabilization of the G-quadruplex is recognized as one of the promising approaches for treatment of G-quadruplex related diseases. For example, telomeric G-quadruplex[3] is known to inhibit telomerase,[3h] a specific enzyme in cancer cells, as well as dissociate the telomere related proteins including TRF2 and Pot1, and stabilization of the G-quadruplex results in the induction of certain cancer cell lines into apoptosis.[4] Therefore, a number of small compounds for stabilizing G-quadruplex structures (G-quadruplex ligands)[5] have been developed, and some of them show telomerase inhibitory activity and/or transcriptional inhibition of oncogenes (e.g., *c-kit*,[6] *bcl-2*[7] and

c-myc[8]) *in vitro* as well as *in vivo*. These molecules usually have aromatic rings of similar size to the G-quartet plane, and interact with it through π-π stacking. One of the representative G-quadruplex ligands in nature is telomestatin (**1**), which was reported by Seto and Shin-ya in 2001.[9] Telomestatin (**1**) has a macrocyclic polyoxazole structure, and shows very potent telomeric G-quadruplex stabilization activity by binding the DNA with an end-stacking mode.[10] Over the past decade, telomestatin (**1**) has widely been used for the discovery and elucidation of telomeric G-quadruplex functions.[11] Recently, we have designed and synthesized macrocyclic hexaoxazoles of 6OTDs (6 oxazoles telomestatin derivatives; **2**) as G-quadruplex ligands.[12] The 6OTDs (**2**) have the same size and similar planarity with **1**, and are supposed to interact with the G-quartet through π-π interaction with end-stack mode (Figure 1). The 6OTDs (**2**) also have two side chains with amine, guanidine, or hydroxyl related functional groups, and these side chains were also expected to contribute to the stabilization of G-quadruplex by interacting with the backbone of the DNA, specifically with phosphate (Figure 2). Indeed, L2H2-(4M)6OTD; **2d**[12d] shows potent stabilization activity with G-quadruplex forming oligonucleotides (GFOs). With these results, we examined further structural development of the **2d**; i.e., introduction of four-way side chains in the structure of **3** was planned. Since G-quadruplex structure consists of four backbones as shown in Figure 1, each of the four side chains in **3** was expected to interact with these backbones efficiently,[13] and increment of the G-quadruplex stabilization activity was anticipated. In this paper, we describe the synthesis of 6OTD derivatives with four-way side chains of **3** and **4** bearing hydroxyethyl group on the oxazole at C9 and C21, and C6 and C18 positions, respectively (Figure 2). The stabilizing ability of the G-quadruplexes with these new ligands was examined by fluorescence resonance energy transfer (FRET) melting assays.

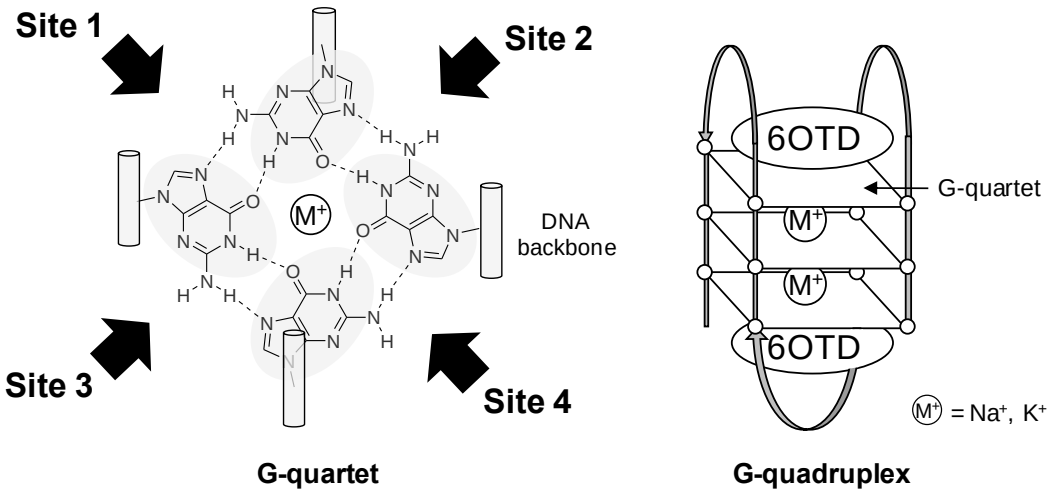


Figure 1. Schematic picture for G-quadruplex interaction with 6OTDs

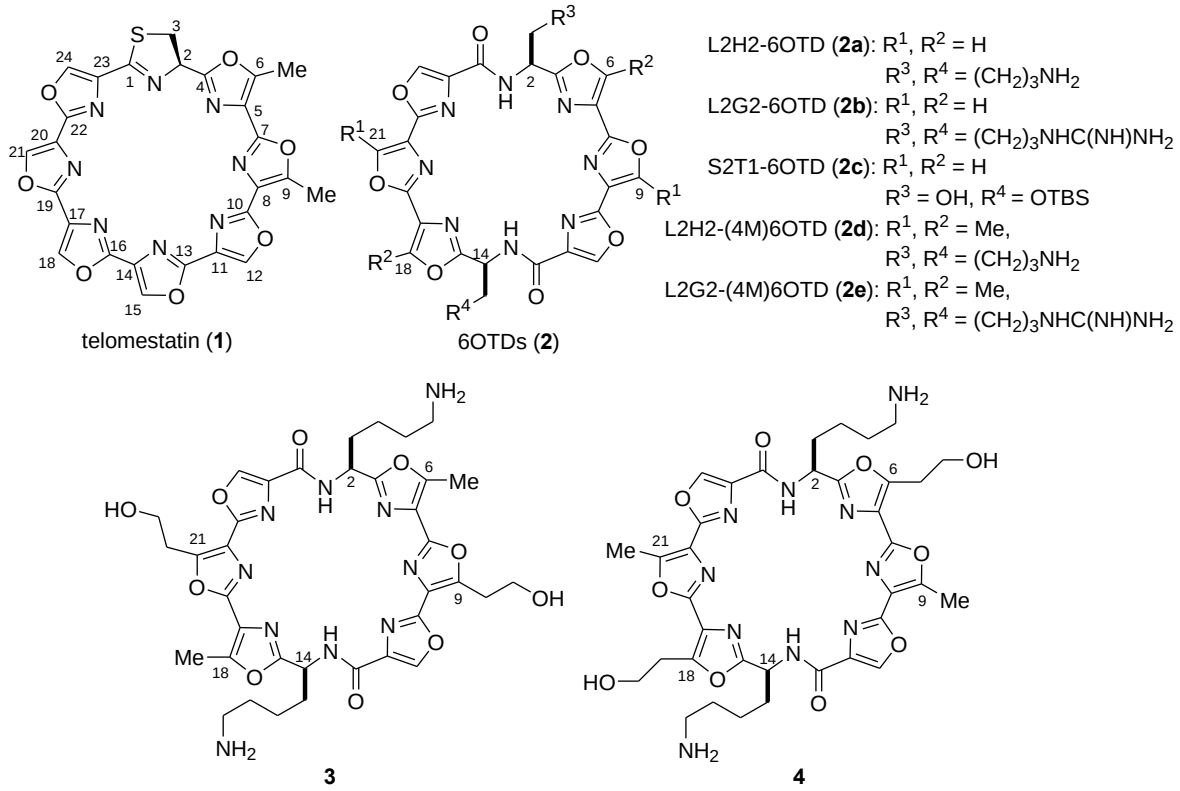
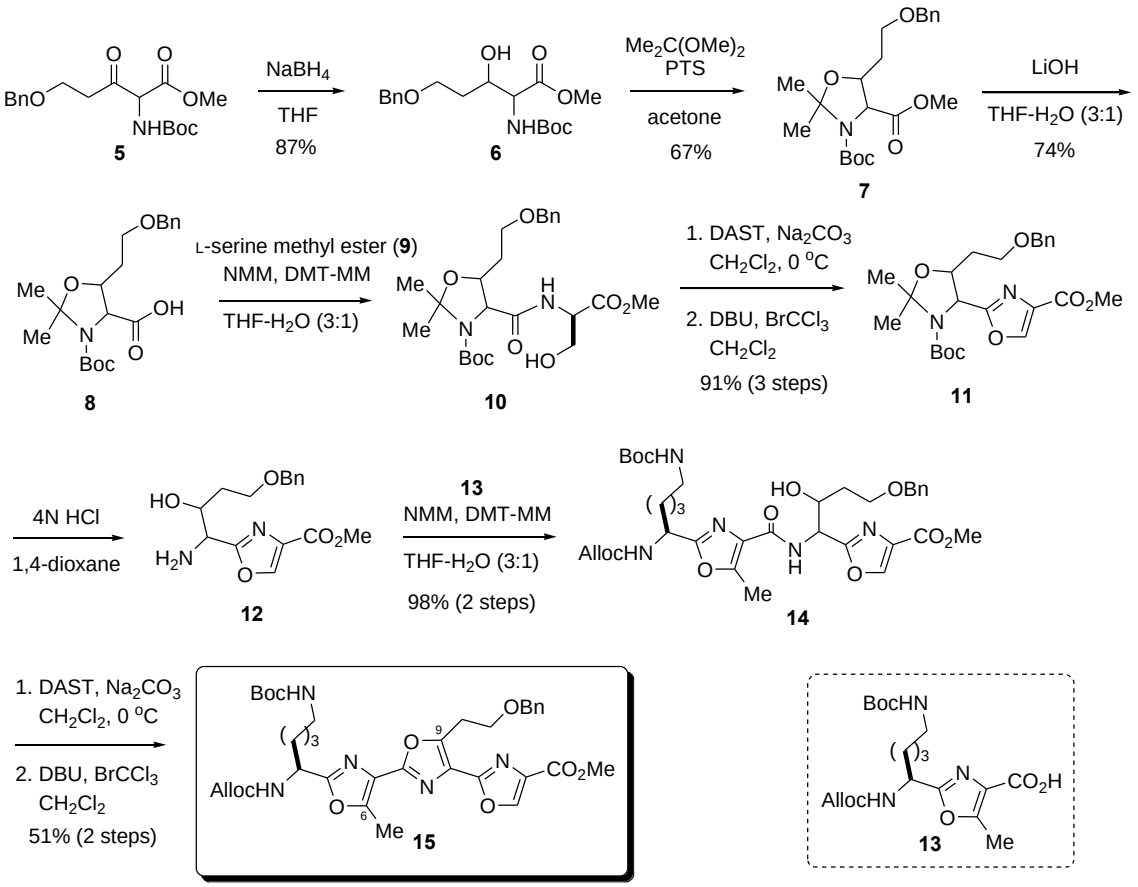


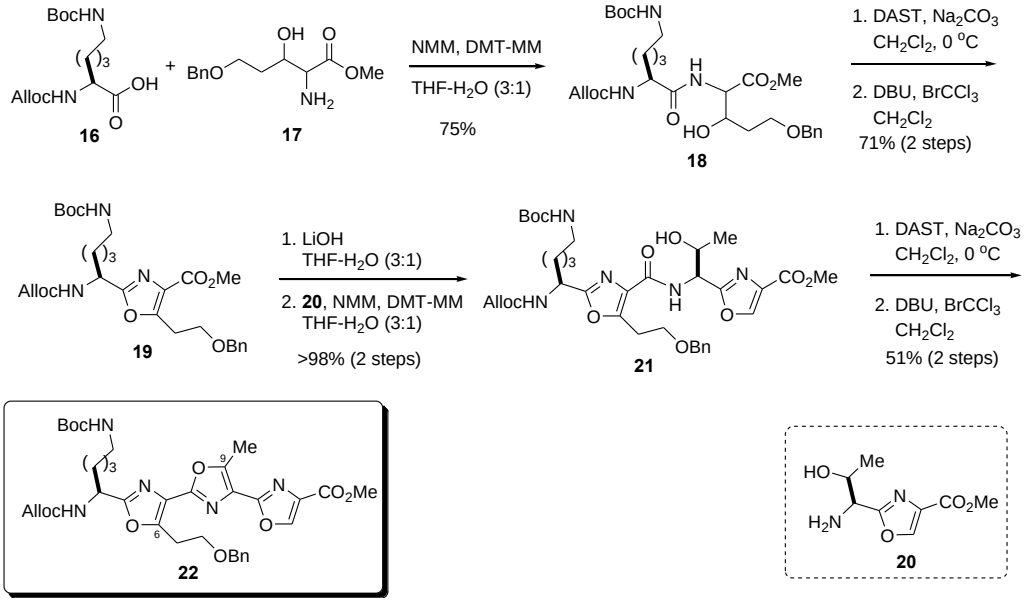
Figure 2. Structure of telomestatin (**1**) and its analogue of 6OTDs

Syntheses of 6OTD derivatives **3** and **4** were described in Schemes 1-4. Firstly, trioxazoles **15** and **22**, key synthetic intermediates for **3** and **4**, were synthesized (Schemes 1 and 2). α-Amino-β-ketoester **5**[15] was reduced with sodium borohydride to give alcohol, which was then subjected with 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid to give ester **7**. Hydrolysis of ester **7** with lithium hydroxide gave carboxylic acid **8** in 74% yield. Then, the carboxylic acid **8** was reacted with L-serine methylester (**9**) in the presence of *N*-methylmorpholine (NMM) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM)[16] to give β-hydroxy amide **10**, which was subsequently reacted with diethylaminosulfurtrifluoride (DAST)[17] followed by treatment with bromotrichloromethane in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)[18] to give oxazole **11** in 91% yield from **10**. Deprotection of both *N,O*-acetonide and the Boc group in **11** was employed with 4N HCl, and the resulting amine **12** was reacted with the carboxylic acid **13**[12d] in the presence of NMM and DMT-MM to give β-hydroxyamide **14** in 98% yield from **12**. Cyclodehydration of **14** with DAST followed by oxazole formation with bromotrichloromethane in the presence of DBU

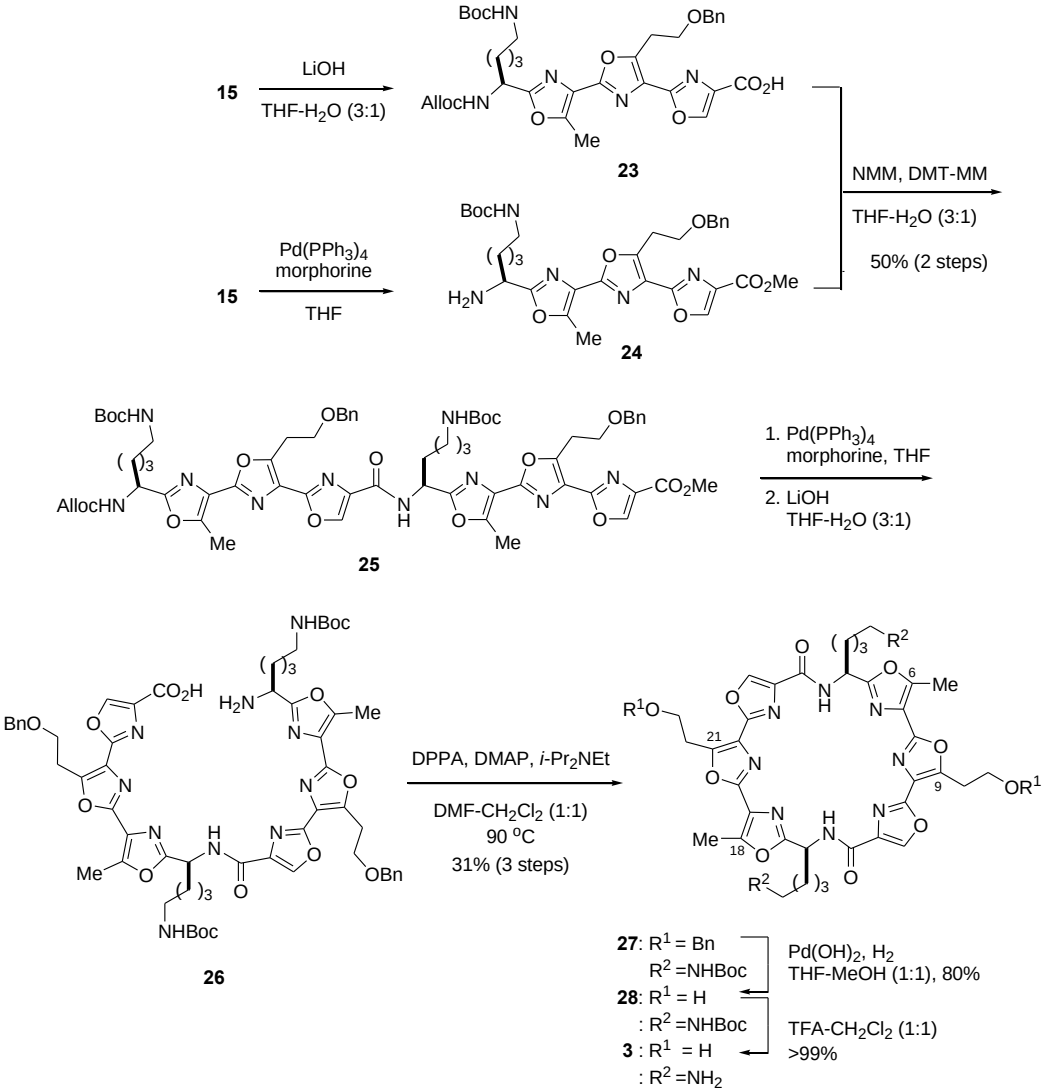
gave trioxazole **15** bearing methyl and hydroxyethyl group at C6 and C9, respectively, in 51% yield from **14**. Synthesis of trioxazole **22** was also performed in a similar way with **15** from carboxylic acid **16** and amine **17** (Scheme 2). β-Hydroxyamide **18** was synthesized from carboxylic acid **16** and amine **17** in the presence of NMM and DMT-MM in 75% yield. Oxazole ring formation of **18** was carried out by treatment with DAST followed by bromotrichloromethane in the presence of DBU, and **19** was obtained in 71% yield. After hydrolysis of the ester of **19** with lithium hydroxide, the resulting carboxylic acid was reacted with amine **20** in the presence of NMM and DMT-MM, and oxazole ring was constructed from β-hydroxyamide **21** to give trioxazole **22** bearing methyl and hydroxyethyl group at C9 and C6 positions, respectively, in 51% yield.



Scheme 1. Synthesis of trioxazole **15**

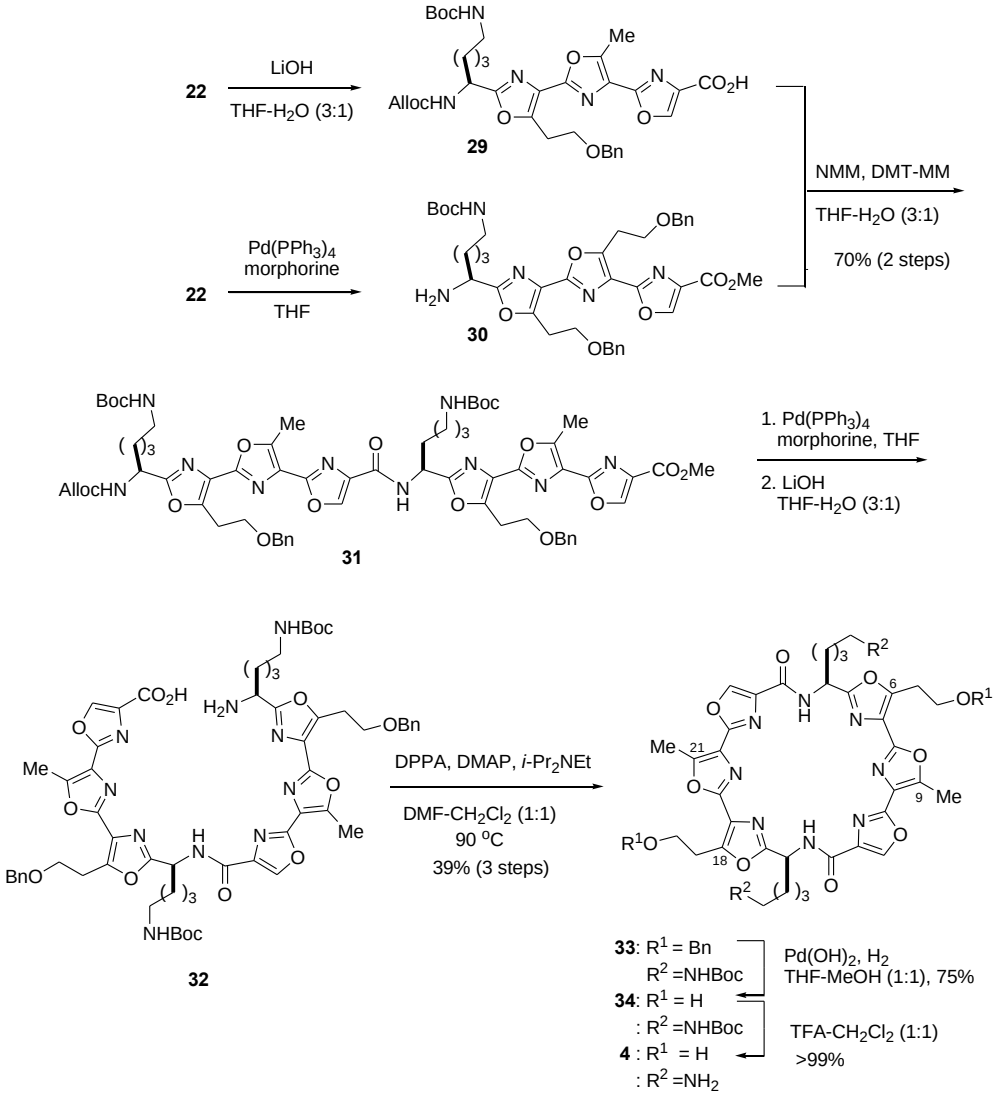


Scheme 2. Synthesis of trioxazole **22**



Scheme 3. Synthesis of **3**

Trioxazoles **15** and **22** in hand, synthesis of **3** and **4** was carried out. Methyl ester group in trioxazole **15** was hydrolyzed with lithium hydroxide to give carboxylic acid **23**, which was subsequently reacted with amine **24**, obtained from **15** by deprotection of Alloc group in the presence of $Pd(PPh_3)_4$ and morphorine, by using NMM and DMT-MM to give **25** in 50% yield from **15**. Deprotection of the Alloc group in **25** with catalytic amount of $Pd(PPh_3)_4$ followed by hydrolysis of methyl ester with lithium hydroxide gave amino acid **26**, which was further subjected to macrocyclization using diphenylphosphoryl azide (DPPA) and *i*-Pr_2NEt in the presence of 4-dimethylaminopyridine (DMAP) under high dilution conditions (3 mM) in CH_2Cl_2-DMF (2:1) to give **27** in 31% yield from **25**. Finally, deprotection of benzyl group and Boc group in **27** was conducted with hydrogenolysis in the presence of $Pd(OH)_2$ followed by treatment with TFA to give **3** in 80% yield from **27** (Scheme 3). 6OTD derivative **4** was also synthesized from **29** and **30** with a similarity to **3** *via* **31**, **32**, and **33** (Scheme 4).[19]



Scheme 4. Synthesis of **4**

The G-quadruplex stabilizing ability of **3** and **4** was examined by FRET melting assay,[20] and these abilities were evaluated by comparison with **2d**. In this assay, a ligand-induced stabilization of a folded G-quadruplex is evaluated by an incremental measurement of the melting temperature as ΔT_m. The ΔT_m values of the four kinds of GFOs (0.2 μM); i.e., telo21, kit22, bcl27, and myc22, in the presence of **2d, 3, 4** at a concentration of 2 μM are summarized in Table 1.[21] The ΔT_m value of telo21 in the presence of **2d, 3, 4** were 21.1, 18.1, 17.8 ºC, respectively, and **3** and **4** showed less potent G-quadruplex stabilizing activity than **2d**. Similar tendencies with telo21 were obtained to the other GFOs of kit22, bcl27 and myc22 by these ligands (Table 1).[22]

As shown in these results, newly designed four-way type 6OTD derivatives did not show significant improvement of G-quadruplex stabilizing ability. Since the planarity of macrocyclic structure of 6OTDs, which is one of the important factor for interacting with GFOs, of **3** and **4** are the same with that of **2d**, it is obvious that the newly introduced side chains in **3** and **4** have no effect on the interaction with G-quadruplex DNA backbone. Since the slight decline of ΔT_m values of G-quadruplex stabilizing activity of **3** and **4** were observed, newly introduced hydroxyethyl group only affected *via* steric hindrance. A functional group for stronger interaction with phosphate should be required in the four-way type 6OTD derivatives for the improvement of stabilization of G-quadruplex structure.

Table 1. ΔT_m values by FRET melting assay

Ligand	ΔT_m values (°C)			
	telo21	kit22	bcl27	myc22
2d	22.1±0.8	22.6±0.5	17.6±0.9	14.3±1.5
3	17.8±0.8	18.4±0.3	16.8±0.8	11.4±0.7
4	18.1±1.5	17.5±0.1	17.7±0.6	12.1±0.3

The ΔT_m values are an average of three independent measurements.

In conclusion, we have designed new G-quadruplex ligands of **3** and **4** bearing four-way side chains with amine and hydroxyl groups based upon the interaction mode of G-quadruplex with 6OTDs, our previously reported two-way type of G-quadruplex ligands. Unfortunately, these new ligands did not show significant improvement of stabilizing ability with some G-quadruplex forming DNA sequences compared to **2d**. This might be due to the lower interaction of phosphate with hydroxyl group than amine. Further structural development of four-way type 6OTD derivatives, focusing on the functional groups in the side chain, is in progress.

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19. Spectral data for **15**: $[\alpha]^{25}_{D}$ -26.4 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3Cl) δ8.27 (s, 1H), 7.29-7.21 (m, 5H), 5.98-5.87 (m, 1H), 5.52-5.50 (m, 1H), 5.30 (d, *J* = 5.0 Hz, 1H), 5.20 (d, *J* = 5.0, Hz 1H), 4.95-4.94 (m, 1H), 4.58 (d, *J* = 4.8 Hz, 2H), 4.54 (s, 2H), 4.58-4.50 (m, 1H), 3.92 (s, 3H), 3.82 (t, *J* = 6.5 Hz, 2H), 3.53 (t, *J* = 6.5 Hz, 2H), 3.09 (br, 2H), 2.65 (s, 3H), 2.15-1.81 (m, 2H). 1.52-1.25 (m, 4H), 1.41 (s, 9H), ^{13}C NMR (400 MHz, $CDCl_3$) δ162.8, 161.5, 156.4, 156.0, 155.7, 155.5, 155.1, 151.7, 150.8, 143.4, 137.9, 134.2, 132.5, 128.2, 127.5, 126.2, 124.6, 117.8, 79.1, 72.7, 67.0, 65.9, 52.1, 49.1, 40.0, 33.8, 29.5, 28.3, 26.6, 22.4, 11.7, HRMS (ESI, M+Na) calcd for $C_{35}H_{43}N_5O_{10}Na$ 716.2908, found 716.2904. Spectral data for **22**: $[\alpha]^{25}_{D}$ -33.9 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz,CD_3Cl) δ8.28 (s, 1H), 7.29-7.21 (m, 5H), 5.98-5.87 (m, 1H), 5.52-5.50 (m, 1H), 5.30 (d, *J* = 5.0 Hz, 1H), 5.20 (d, *J* = 5.0 Hz 1H), 4.97-4.96 (m, 1H), 4.58 (d, *J* = 4.8 Hz, 2H), 4.53 (s, 2H), 4.58-4.50 (m, 1H), 3.93 (s, 3H), 3.83 (t, *J* = 6.5 Hz, 2H), 3.44 (t, *J* = 6.5 Hz, 2H), 3.05 (br, 2H), 2.77 (s, 3H), 2.16-1.81 (m, 2H). 1.54-1.29 (m, 4H)1.41 (s, 9H), ^{13}C NMR (400 MHz, $CDCl_3$) δ163.0, 161.3, 156.4, 155.8, 155.5, 154.2, 151.4, 150.8, 143.2, 137.7, 133.9, 132.3, 128.0, 127.3, 127.2, 125.2, 125.0, 117.5, 78.7, 72.4, 66.8, 65.7, 52.0, 49.0, 40.0, 33.6, 29.2, 28.2, 26.4, 22.2, 11.7, HRMS (ESI, M+Na) calcd for $C_{35}H_{43}N_5O_{10}Na$ 716.2908, found 716.2909. Spectral data for **25**: $[\alpha]^{25}_{D}$ 0.8 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, CD_3Cl) δ8.26 (s, 2H), 7.48 (d, *J* = 9.2 Hz, 1H), 7.27-7.22 (m, 10H), 5.95-5.88 (m, 1H), 5.53-5.52 (m, 1H), 5.47-5.41 (m, 1H), 5.32 (d, *J* = 5.0 Hz, 1H), 5.21 (d, *J* = 5.0 Hz, 1H), 4.96-4.95 (m, 1H), 4.59 (d, *J* = 4.8 Hz, 2H), 4.54 (s, 4H), 4.59-4.53 (m, 2H), 3.93 (s, 3H), 3.90-3.86 (m, 4H), 3.54 -3.48 (m, 4H), 3.11 (br, 4H), 2.67 (s, 3H), 2.68 (s, 3H), 2.17-1.87 (m, 4H), 1.54-1.29 (m, 8H), 1.41 (s, 18H),^{13}C NMR (500 MHz, $CDCl_3$) δ162.1, 161.5, 159.9, 156.5, 155.9, 155.7, 155.4, 151.7, 151.4, 151.0, 143.4, 141.2, 138.0, 137.8, 136.5, 134.2, 132.5, 128.3, 127.7, 127.5, 126.2, 124.7, 124.5, 117.9, 79.1, 72.9, 67.1, 65.9, 52.1, 49.2, 46.6, 40.2, 40.0, 38.0, 33.8, 29.7, 29.5, 28.4, 26.8, 22.4, 11.8, HRMS (ESI, M+Na) calcd for $C_{65}H_{78}N_{10}O_{17}Na$ 1293.5444, found 1293.5431. Spectral data for **31**: $[\alpha]^{25}_{D}$ -7.0 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz,CD_3Cl) δ8.28 (s, 1H), 8.25 (s, 1H), 7.49 (d, *J* = 9.2 Hz, 1H), 7.30-7.21 (m, 10H), 5.98-5.87 (m, 1H), 5.50-5.49 (m, 1H), 5.47-5.42 (m, 1H), 5.31 (d, *J* = 5.0 Hz, 1H), 5.21 (d, *J* = 5.0 Hz, 1H), 4.97-4.96 (m, 1H), 4.58 (d, *J* = 4.8 Hz, 2H), 4.53 (s, 4H), 4.58-4.50 (m, 2H), 3.93 (s, 3H), 3.84 (t, *J* = 6.5 Hz, 4H), 3.46 (t, *J* = 6.5 Hz, 4H), 3.07 (br, 4H), 2.77 (s, 3H), 2.76 (s, 3H), 2.16-1.81 (m, 4H). 1.54-1.29 (m, 8H)1.41 (s, 18H), ^{13}C NMR (500 MHz, $CDCl_3$) δ163.1, 162.5, 161.3, 159.8, 156.5, 155.9, 155.8, 155.5, 154.5, 154.3, 151.5, 150.6, 143.2, 140.9, 137.7, 136.3, 132.4, 128.1, 127.4, 127.3, 125.3, 125.2, 125.0, 117.6, 78.8, 72.5, 66.8, 65.7, 52.0, 49.0, 46.5, 40.0, 33.6, 33,4 29.2, 28.2, 26.4, 22.6, 22.2, 11.7, HRMS (ESI, M+Na) calcd for $C_{65}H_{78}N_{10}O_{17}Na$ 1293.5444, found 1293.5446. Spectral data for **3**: $[\alpha]^{25}_{D}$ 12.9 (*c* 0.3, CH_3OH); ^{1}H NMR (400 MHz, DMSO-d_6) δ8.89 (s, 2H), 8.26

(d, J = 7.2 Hz, 2H), 7.72 (br, 4H), 5.42-5.30 (m, 2H), 4.97 (t, J = 5.0 Hz, 2H), 3.88-3.67 (m, 4H), 3.28-3.21 (m, 4H), 2.75 (br, 10H), 2.11-1.85 (m, 4H), 1.70-0.70 (m, 8H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 162.3, 158.9, 155.6, 154.4, 152.9, 151.0, 142.3, 135.8, 124.7, 124.4, 69.8, 58.7, 46.9, 33.6, 29.5, 26.6, 21.1, 11.5, HRMS (ESI, M+Na) calcd for $C_{36}H_{42}N_{10}O_{10}Na$ 797.2983, found 797.2982. Spectral data for **4**: $[\alpha]^{25}_{D}$ 48.3 (c 0.1, CH_3OH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.89 (m, 8H), 8.23 (d, J = 7.2 Hz, 2H), 7.71 (br, 4H), 5.43-5.32 (m, 2H), 5.00 (t, J = 5.5 Hz, 2H), 3.88-3.76 (m, 4H), 2.72 (br, 10H), 2.14-1.82 (m, 4H), 1.66-0.77 (m, 8H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 162.1, 159.0, 155.8, 154.8, 152.9, 151.7, 142.4, 135.9, 125.5, 123.9, 69.9, 58.8, 47.1, 33.5, 29.6, 26.8, 20.1, 11.6, HRMS (ESI, M+H) calcd for $C_{36}H_{43}N_{10}O_{10}$ 775.3164, found 775.3147.

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21. In FRET melting assays, four representative single stranded G-quadruplex forming oligonucleotides (GFOs), i.e., telo21 (telomere), kit22 (*c-kit*), bcl27 (*bcl-2*) and myc22 (*c-myc*) were used in the presence of potassium cation (60 mM cacodylate buffer, pH 7.0 containing 60 mM KCl). GFOs oligonucleotides used in this paper are as follows.

Oligomer name	Sequence
telo21	5'-FAM-[GGGTTAGGGTTAGGGTTAGGG]-TAMRA-3'
kit22	5'-FAM-[AGGGAGGGCGCTGGGAGGAGGG]-TAMRA-3'
bcl27	5'-FAM-[CGGGCGCGGGAGGAAGGGGGCGGGAGC]-TAMRA-3'
myc22	5'-FAM-[GAGGGTGGGGAGGGTGGGGAAG]-TAMRA-3'

22. No significant interactions between the double-stranded DNA and 6OTDs are observed.