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DESIGN AND SYNTHESIS OF TELOMESTATIN DERIVATIVES CONTAINING METHYL OXAZOLE AND THEIR G-QUADRUPLEX STABILIZING ACTIVITIES†

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Abstract – Telomestatin derivatives of L2A2-6M(2)OTD (**3b**) and L2A2-6M(4)OTD (**3c**), which have a macrocyclic hexaoxazole skeleton (6OTD) containing bis- and tetra-methyl oxazoles, were synthesized as a novel G-quadruplex ligand. This new class of "methyl oxazole containing" ligands was revealed to stabilize various G-quadruplex forming oligonucleotides more potently than "no methyl oxazole containing" L2A2-6OTD (**3a**).

INTRODUCTION

Guanine-rich DNA sequences can adopt a specific secondary structure called G-quadruplexes,[1] which exist in telomeric DNA sequences[2] as well as in promoter regions of some oncogenes in vitro. In telomeric DNA sequences, G-quadruplex structures not only inhibit telomerase activity, but also dissociate the telomere related proteins including TRF2[3] and Pot1[4] from 3'-overhang chromosomes and thus induce certain cancer cell lines into apoptosis. On the other hand, guanine-rich base sequences in the promoter region of *c-kit*,[5] *bcl-2*[6] and *c-myc*[7] (involved in cell cycle and apoptosis) fold G-quadruplex structures, and suppress the transcriptional level of their mRNA. Thus, a stabilization of the G-quadruplex is recognized as one of the promising approaches for cancer treatments, and a number of small compounds for stabilizing G-quadruplex structures (G-quadruplex ligands) have been developed.[8] In 2001, telomestatin (**1**) was reported as one of the most potent G-quadruplex stabilizing ligands by Seto

and Shin-ya.[9] Telomestatin (**1**) binds to the G-quadruplex in telomere with an end-stacking mode and shows potent telomerase inhibitory activity. Over the past decade, telomestatin (**1**) has widely been applied to the discovery of telomeric G-quadruplex functions.[10] Recently, we have designed and synthesized macrocyclic hexaoxazoles of 6OTDs (**2**) as a G-quadruplex ligand.[11] The 6OTD (**2**) has the same size and similar planarity with **1**, and believed to end-stack with a G-quartet through the π-π interaction. The macrocyclic oxazole structure in **1** and **2** contributes to stacking with a G-quartet, however, the effects of characteristic "methyl oxazole" moiety in **1** (at C6 and C9) has not gained much attention. In this paper, we describe the synthesis of L2A2-6M(2)OTD (**3b**) and L2A2-6M(4)OTD (**3c**) containing bis- and tetra- "methyl oxazoles", respectively, inspired from the skeleton of **1** (Figure 1). Furthermore, the stabilizing ability of the G-quadruplexes by these new ligands was evaluated by fluorescence resonance energy transfer (FRET) melting assays.[12]

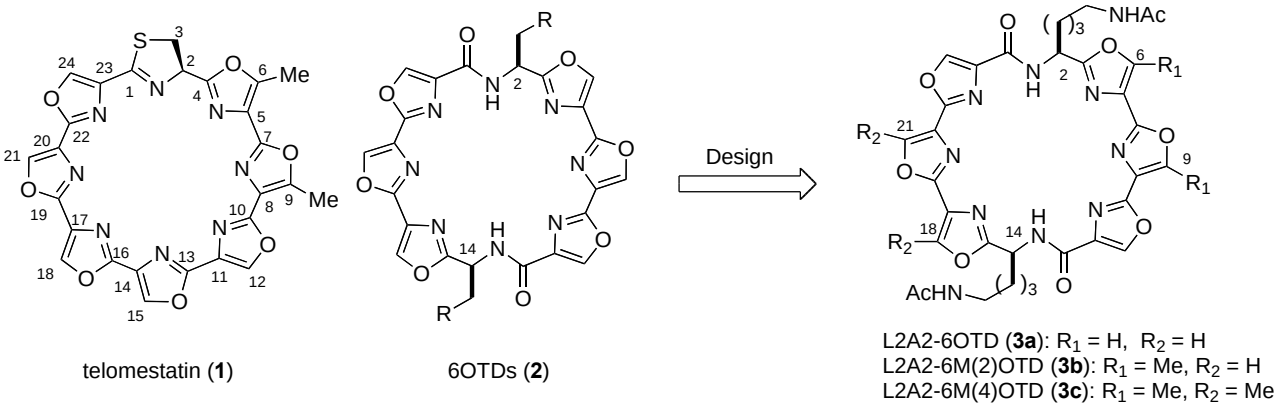
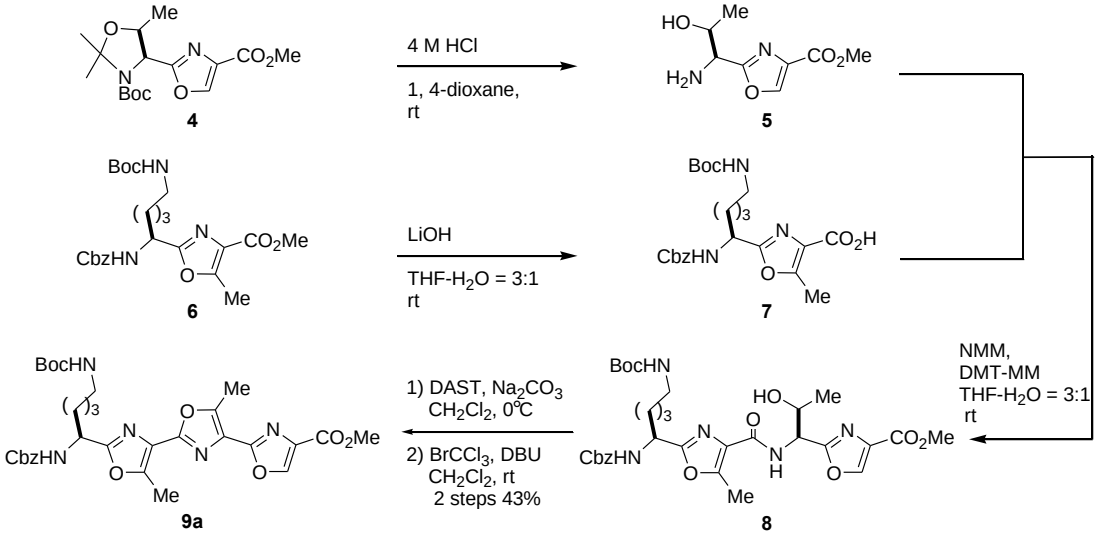


Figure 1. Structure of telomestatin (**1**) and its analogue of macrocyclic hexaoxazoles (**2**, **3a-c**)

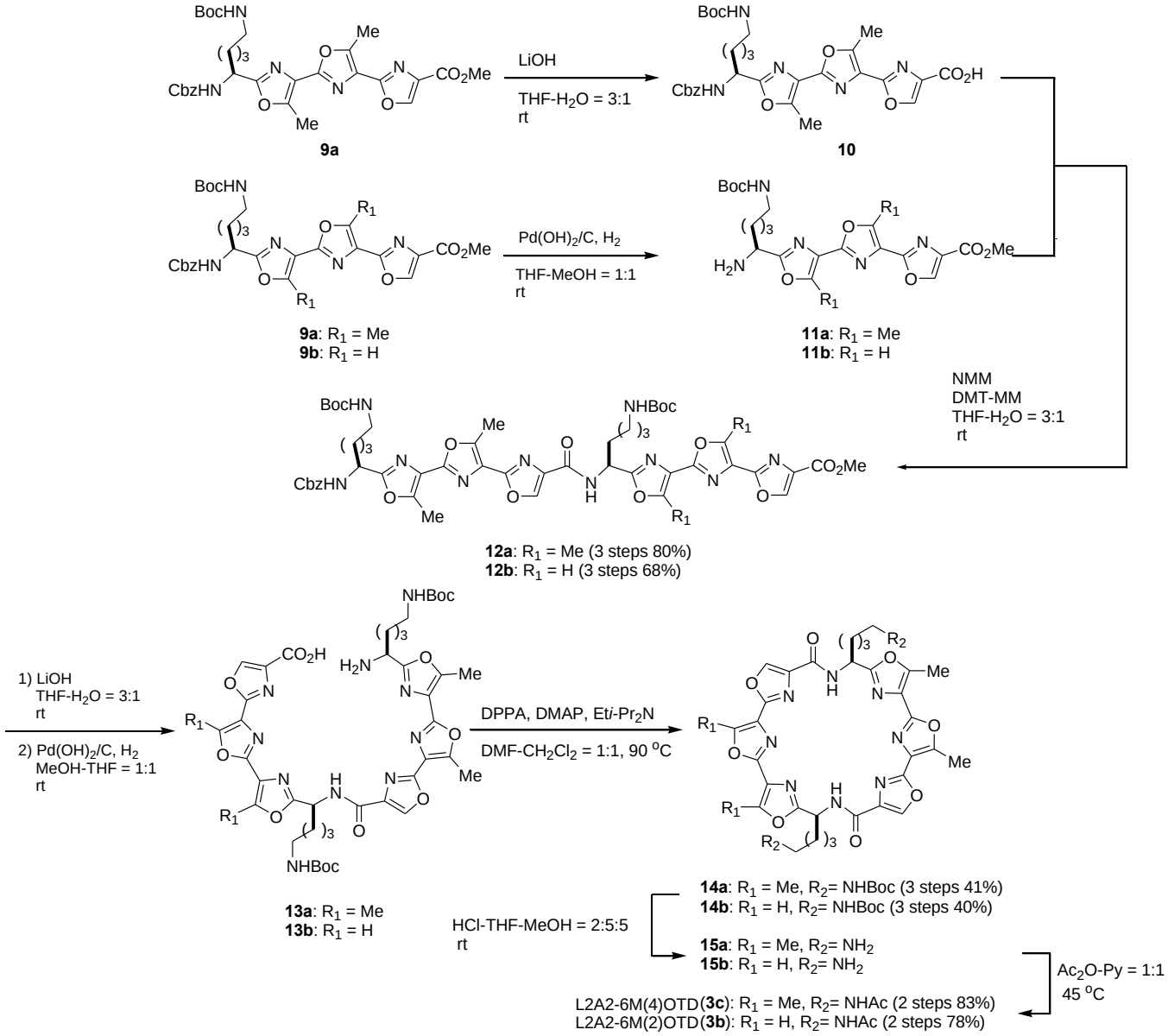
RESULTS AND DISCUSSION

A novel class of 6OTD, i.e., L2A2-6M(2)OTD (**3b**) and L2A2-6M(4)OTD (**3c**), was synthesized from oxazoles **4**[9b] and **6**[11a] (Scheme 1). Deprotection of *N,O*-acetonide and the Boc group in **4** was employed with 4 M HCl to give amine **5**. Methyl ester of **6** was hydrolyzed with lithium hydroxide to give carboxylic acid **7**. Then, the amine **5** was reacted with the carboxylic acid **7** in the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM)[13] and *N*-methylmorpholine (NMM) to give β-hydroxy amide **8** in 85% from **6**. Cyclodehydration of **8** with diethylaminosulfurtrifluoride (DAST)[14] followed by oxazole formation with bromotrichloromethane in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)[15] gave trioxazole **9a** in 43% from **8**.

Hydrolysis of the methyl ester **9a** was conducted with lithium hydroxide to give carboxylic acid **10**, which was subsequently reacted with amine **11b** obtained from **9b**[11b] to give **12b** by using DMT-MM and NMM in 68% yield from **9a**. Deprotection of the Cbz group in **12b** followed by hydrolysis of methyl



Scheme 1. Synthesis of trioxazole **9a**



Scheme 2. Synthesis of L2A2-6M(2)OTD (**3b**) and L2A2-6M(4)OTD (**3c**).

ester gave amino acid **13b**, which was further subjected to macrocylization using diphenylphosphoryl azide (DPPA) and Et*i*-Pr$_2$N in the presence of 4-dimethylaminopyridine (DMAP) under high dilution conditions (3 mM) in CH_2Cl_2-DMF (2:1) to give **14b** in 40% yield from **12b**. After conversion of the Boc group in **14b** into amine, the resulting amine **15b** was reacted with acetic anhydride to give L2A2-6M(2)OTD (**3b**) in 78% yield from **14b**. A tetra-methyl oxazole derivative of L2A2-6M(4)OTD (**3c**) was also synthesized from **9a** and **11a** with a similarity to **3b** via **12a**, **13a**, and **14a**.

The G-quadruplex stabilizing ability of L2A2-6M(2)OTD (**3b**) and L2A2-6M(4)OTD (**3c**) was examined by FRET melting assays.[16] 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) in the presence of **3a-c** at a concentration of 2 μM are summarized in Table 1. The ΔT_m value of telo21 in the presence of **3a-c** were 11.7, 14.0, 16.7 ºC, respectively i.e., **3c** showed most potent G-quadruplex stabilizing activity among them. The other GFOs of kit22, bcl27 and myc22 showed similar tendency with these ligands. Thus, it was revealed that with a higher increment of "methyl-oxazole" moieties in macrocyclic hexaoxazole, G-quadruplex stabilizing activity was increased. Thus, incorporation of the "methyl oxazoles" into a macrocyclic skeleton in 6OTD was realized to be an effective strategy on augmenting the interaction with the G-quadruplex. Furthermore, no significant interactions between the double-stranded DNA of ds-26 and **3a-c** were observed even at high concentrations (Table 1 and Figure 2). Thus, **3b** and **3c** are selective ligands for GFOs likewise **3a**.

Table 1. ΔT_m (ºC) by FRET melting assays

Ligand	ΔT_m in 2 μM / ºC				
	telo21	kit22	bcl27	myc22	ds26
3a*	11.7	14.7	10.0	10.3	0.0
3b	14.0	17.7	12.0	12.3	0.0
3c*	16.7	19.3	14.3	13.0	0.0

The ΔT_m values are an average of three independent measurements.
*In the case of all GFOs, **3a** and **3c** showed significant differences regarding the activity to stabilize G-quadruplex structures (*P*-value < 0.025).

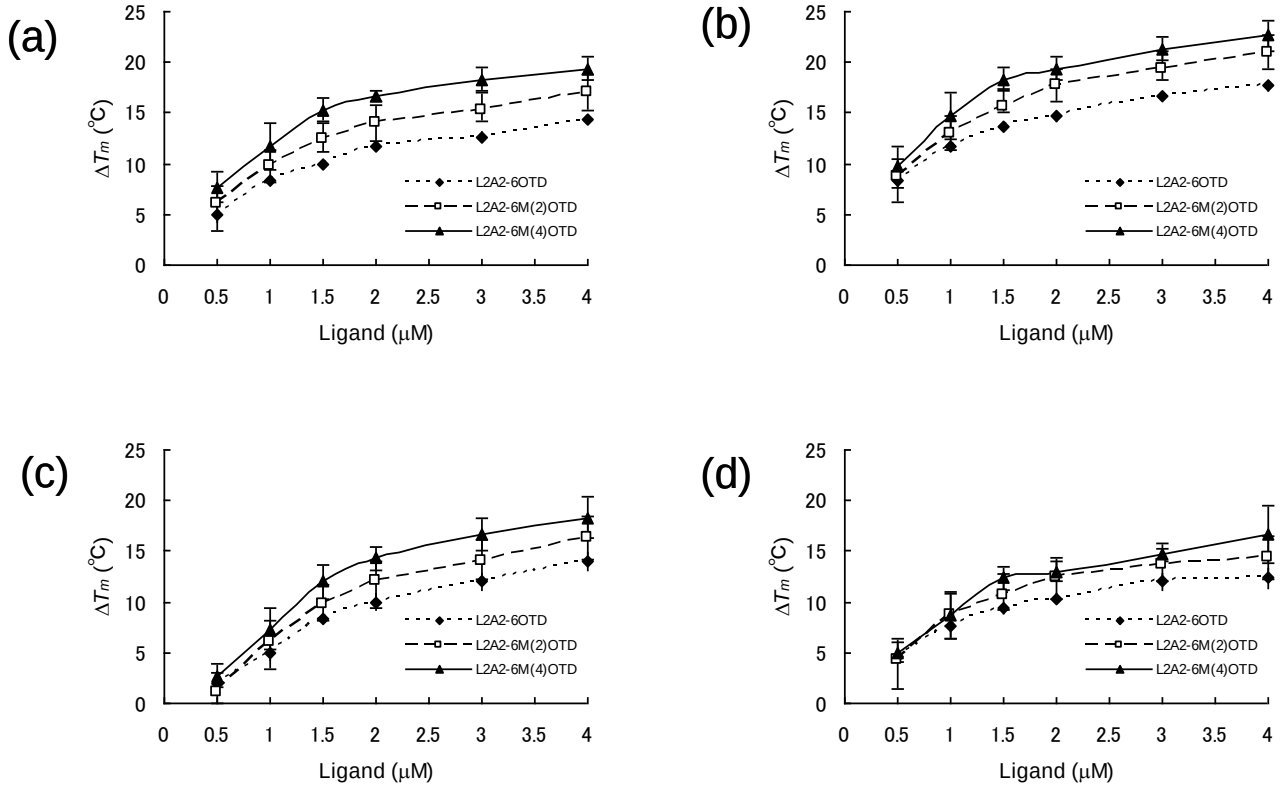


Figure 2. ΔT_m values of (a) telo21, (b) kit22, (c) bcl27 and (d) myc22 (0.2 μM) obtained by FRET melting assays in the presence of 60 mM KCl and various concentrations of **3a-c**. All ΔT_m values were obtained by means of triplicate assay and error bars indicate +/- standard deviations.

In conclusion, we report a synthesis of a novel G-quadruplex ligand of L2A2-6M(2)OTD (**3b**) and L2A2-6M(4)OTD (**3c**), a macrocyclic polyoxazole containing methyl oxazoles. It was revealed that the "methyl oxazole" moiety in 6M(2)OTD and 6M(4)OTD has an effect on the stabilizing activity for G-quadruplexes.

EXPERIMENTAL

General

Flash chromatography was performed on silica gel 60 (spherical size 0.040~0.100 mm; Kanto). Optical rotations were measured on a JASCO P 2200 polarimeter, using the sodium D line. ^{1}H NMR 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 = 77.0ppm), CD_3OD (^{1}H NMR; δ = 3.30 ppm, ^{13}C NMR; δ = 49.0 ppm) 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; t, triplet; m, multiplet; br, broad), integration, coupling constant (Hz). Mass spectra were recorded on JEOL JMS-T100X spectrometer with ESI-MS mode using method as solvent. All oligonucleotides purified were obtained from Sigma Genosys and dissolved in double-distilled water to be used without further purification. FRET melting assays were 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 (Bio-Rad).

Synthesis of **5**: To a solution of **4** (3.4 g, 9.9 mmol) in dioxane (100 mL) was added 12 M HCl (33 mL), and the mixture was stirred for 20 min at rt. The reaction mixture was concentrated *in vacuo* to give **5**, which was used without further purification for the synthesis of **8**.

Synthesis of **7**: To a solution of **6** (3.8 g, 8.0 mmol) in THF-H_2O (3:1, 80 mL) was added lithium hydroxide (662 mg, 15.8 mmol) at rt. After stirring at rt for 4 h, to the reaction mixture was added 1 M HCl. The resultant mixture was concentrated *in vacuo* to give **7**, which was used without further purification for the synthesis of **8**.

Synthesis of **8**: To a solution of carboxylic acid **7** in THF-H_2O (3:1, 160 mL) was added amine **5**, NMM (1.75 mL, 16 mmol) and DMT-MM (3.2 g, 11.8 mmol), and the mixture was stirred at rt for 14 h. To the reaction mixture was added EtOAc, and the organics were washed with sat-$NaHCO_3$, 1 M HCl and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified on silica gel (hexane-EtOAc = 1:2) to give peptide **8** (4.4 g, 85%, 2 steps from **6**). Spectral data for **8**: $[\alpha]^{25}_D$ -32.3 (*c* 1.1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19 (s, 1H), 7.70 (m, 1H), 7.34 (m, 5H), 5.48 (s, 2H), 5.30 (δ, *J* = 6.4 Hz, 1H), 5.11 (s, 2H), 4.87 (br, 1H), 4.56 (br, 2H), 3.92 (s, 3H), 3.09 (br, 2H), 2.59 (s, 3H), 1.91-1.74 (m, 4H), 1.48-1.41 (m, 13H), 1.28 (d, *J* = 6.4 Hz); ^{13}C NMR (400 MHz, $CDCl_3$) δ 163.7, 162.1, 161.3, 160.8, 156.1, 155.8, 153.9, 144.2, 136.1, 133.0, 128.4, 128.1, 79.1, 77.2, 67.7, 67.0, 52.2, 48.9, 40.0, 33.3, 29.3, 28.3, 22.4, 19.3, 11.6; HRMS (ESI, M+Na) calcd for $C_{31}H_{41}N_5O_{10}Na$ 666.2751, found 666.2759.

Synthesis of **9a**: To a solution of peptide **8** (4.3g, 6.7 mmol) in CH_2Cl_2 (150 mL) was added DAST (0.87 mL, 6.6 mmol) in the presence of Na_2CO_3 (698 mg, 6.6 mmol) and the reaction mixture was stirred at 0 °C under N_2 for 30 min. To the reaction mixture was added H_2O, and organic layer was extracted with CH_2Cl_2. The extracts were washed with H_2O and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified on silica gel (hexane-EtOAc = 1:2) to give oxazoline (3.5 g, 83%). The oxazoline was dissolved in CH_2Cl_2 (80 mL), and $BrCCl_3$ (1.10 mL, 11.2 mmol) and DBU (1.67mL,

11.2 mmol) was added to the solution. The resulting mixture was stirred at 0 °C for 18 h. To the reaction mixture was added 1 M HCl, and organic layer was extracted with CH_2Cl_2. The extracts were washed with H_2O and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified on silica gel (hexane-EtOAc = 1:1) to give **9a** (1.9 g, 43%, in 2 steps from **8**). Spectral data for **9a**: $[\alpha]^{25}_D$ -26.7 (*c* 1.35, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (s, 1H), 7.35 (m, 5H), 5.52 (d, J = 8.3 Hz, 1H), 5.12 (s, 2H), 4.96 (br, 1H), 4.56, (br, 1H), 3.93 (s, 3H), 3.08 (br, 2H), 2.79 (s, 3H), 2.70 (s, 3H), 2.00-1.85 (m, 2H), 1.50-1.35 (m, 13H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 162.7, 161.5, 156.6, 156.0, 155.7, 154.7, 150.9, 150.6, 143.3, 136.1, 134.1, 128.4, 128.0, 125.4, 124.5, 79.0, 67.0, 52.1, 49.2, 39.9, 33.6, 29.4, 28.3, 22.3, 11.8, 11.7; HRMS (ESI, M+Na) calcd for $C_{31}H_{37}N_5O_9Na$ 646.2489, found 646.2469.

Synthesis of **12b**: To a solution of **9a** (840 mg, 1.4 mmol) in THF-H_2O (3:1, 120 mL) was added lithium hydroxide (122 mg, 2.9 mmol) at rt, and the resulting mixture was stirred for 30 min. To the reaction mixture was added 1 M HCl, and resultant was concentrated *in vacuo* to give carboxylic acid **10**, which was used without further purification. To a solution of **9b** (950 mg, 1.5 mmol) in MeOH-THF (1:1, 100 mL) was added 20% $Pd(OH)_2$/C (190 mg), and the mixture was stirred at rt for 1 h under an atmosphere of hydrogen (balloon). The reaction mixture was diluted with MeOH and filtered through a pad of celite. The organics were concentrated *in vacuo* to give amine **11b**, which was used without further purification. To a solution of carboxylic acid **10** in THF-H_2O (3:1, 100 mL) was added amine **11b**, NMM (0.33 mL, 3.0 mmol) and DMT-MM (410 mg, 2.3 mmol), and the mixture was stirred at rt for 7 h. The reaction mixture was concentrated *in vacuo*, and the resulting mixture was extracted with EtOAc. The organic layer was washed with 1 M HCl, sat-$NaHCO_3$, and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified on silica gel (hexane-EtOAc = 1:4) to give bis-trioxazole **12b** (1.0 g, 68%, 3 steps). Spectral data for **12b**: $[\alpha]^{25}_D$ +6.8 (*c* 1.4, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.34 (s, 2H), 8.29 (s, 1H), 8.28 (s, 1H), 7.50 (d, J = 9.2 Hz, 1H), 7.50 (m, 5H), 5.57 (br, 1H), 5.43 (m, 1H), 5.12 (s, 2H), 5.03 (br, 1H), 4.57 (br, 2H), 3.93 (s, 3H), 3.10 (br, 4H), 2.80 (s, 3H), 2.72 (s, 3H), 2.18-1.71 (m, 4H), 1.72-1.41 (m, 26H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 165.5, 161.9, 161.5, 159.6, 156.7, 156.2, 156.0, 155.9, 155.8, 154.7, 154.4, 151.0, 150.8, 143.4, 141.6, 139.6, 139.1, 136.7, 136.0, 134.1, 130.8, 129.6, 128.4, 128.1, 125.4, 124.6, 79.1, 79.0, 67.2, 52.1, 49.3, 46.7, 40.2, 39.8, 33.6, 33.5, 29.5, 29.4, 28.3, 22.7, 22.3, 11.8, 11.8; HRMS (ESI, M+Na) calcd for $C_{51}H_{60}N_{10}O_{15}Na$ 1075.4137, found 1075.4151.

Synthesis of **12a**: To a solution of **9a** (850 mg, 1.4 mmol) in THF-H_2O (3:1, 100 mL) was added lithium hydroxide (120 mg, 2.8 mmol) at rt, and the resulting mixture was stirred for 1 h. To the reaction mixture was added 1 M HCl, and resultant was concentrated *in vacuo* to give carboxylic acid **10**, which

was used without further purification. To a solution of **9a** (1.1 g, 1.9 mmol) in MeOH-THF (1:1, 100 mL) was added $Pd(OH)_2/C$ (190 mg), and the mixture was stirred at room temperature for 1 h under an atmosphere of hydrogen (balloon). The reaction mixture was diluted with MeOH and filtered through a pad of celite. The filtrate was concentrated *in vacuo* to give amine **11a**, which was used without further purification. To a solution of carboxylic acid **10** in THF-H_2O (3:1, 100 mL) was added amine **11a**, NMM (300 μL, 2.8 mmol) and DMT-MM (575 mg, 2.1 mmol), and the mixture was stirred at rt for 17 h. The reaction mixture was concentrated *in vacuo* and the resulting mixture was extracted with EtOAc. The organic layer was washed with 1 M HCl and H_2O, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified on silica gel ($CHCl_3$-EtOAc = 1:1) to give bis–trioxazole **12a** (1.5 g, 95%, 3 steps). Spectral data for **12a**: $[\alpha]^{25}_D$ +3.7 (*c* 1.8, $CHCl_3$); ^{1}H NMR (400 Mz, CD_3Cl) δ 8.29 (s, 1H), 8.27 (s, 1H), 7.48 (d, *J* = 9.2 Hz, 1H), 7.34 (m, 5H), 5.52 (d, *J* = 8.7 Hz, 1H), 5.43 (m, 1H), 5.12 (s, 2H), 4.96 (br, 1H), 4.56 (br, 2H), 3.93 (s, 3H), 3.10 (br, 4H), 2.79 (s, 3H), 2.78 (s, 3H), 2.72 (s, 3H), 2.70 (s, 3H), 2.17-1.84 (m, 4H), 1.50-1.40 (m, 26H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 172.1, 167.6, 162.8, 162.6, 162.1, 161.5, 160.0, 156.7, 156.0, 155.9, 155.8, 154.9, 154.7, 150.9, 150.8, 150.6, 143.4, 141.1, 136.4, 136.1, 134.1, 128.4, 128.1, 125.4, 124.6, 124.4, 79.1, 67.4, 52.1, 49.2, 48.9, 46.6, 40.1, 39.9, 33.7, 33.5, 29.6, 29.5, 28.3, 22.8, 22.4, 14.0, 11.8; HRMS (ESI, M+Na) calcd for $C_{53}H_{64}N_{10}O_{15}Na$ 1103.4450, found 1103.4463.

Synthesis of **14b**: To a solution of bis-trioxazole **12b** (580 mg, 0.55 mmol) in THF-H_2O (3:1, 60 mL) was added lithium hydroxide (46 mg, 1.1 mmol), and the mixture was stirred at rt for 4 h. To the reaction mixture was added 1 M HCl, and resultants were concentrated *in vacuo* to give carboxylic acid, which was used without further purification. To the carboxylic acid solution in MeOH-THF (1:1, 50 mL) was added 20% $Pd(OH)_2/C$ (116 mg), and resulting mixture was stirred for 3 h under hydrogen (balloon). The reaction mixture was filtered through a pad of celite and the filtrate was concentrated *in vacuo* to give amino acid **13b**, which was used without further purification. To a solution of the crude amino acid **13b** in DMF-CH_2Cl_2 (1:2, 180 mL) was added DMAP (134 mg, 1.1 mmol), Et*i*-Pr_2N (187 μL, 1.1 mmol), and DPPA (237 μL, 1.1 mmol). The resulting mixture was stirred for 24 h at 90 °C under a nitrogen atmosphere. To the reaction mixture was added H_2O and the organic layer was extracted with EtOAc. The extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified on silica gel ($CHCl_3$-EtOAc = 1:1) to give **14b** (194 mg, 40%, 3 steps). Spectral data for **14b**: $[\alpha]^{25}_D$ -5.4 (*c* 0.63, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (m, 2H), 8.25 (s, 1H), 8.22 (s, 1H), 8.18 (s, 2H) 5.44 (m, 1H), 5.35 (m, 1H), 4.58 (br, 2H), 3.07 (br, 4H), 2.74 (s, 3H), 2.68 (s, 3H), 2.23-1.84 (m, 4H), 1.57-1.17 (m, 26H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.8, 162.2, 160.0, 159.8, 156.0,

156.0, 155.9, 155.1, 154.6, 150.3, 150.1, 140.8, 140.6, 139.0, 138.3, 136.9, 136.6, 130.9, 129.6, 125.7, 124.6, 78.9, 47.7, 47.6, 40.3, 34.6, 31.9, 31.5, 29.6, 29.5, 28.4, 22.6, 21.9, 14.1, 11.8; HRMS (ESI, M+Na) calcd for $C_{42}H_{50}N_{10}O_{12}Na$ 909.3507, found 909.3492.

Synthesis of **14a**: To a solution of bis-trioxazole **12a** (1.5 g, 1.33 mmol) in THF-H_2O (3:1, 60 mL) was added lithium hydroxide (110 mg, 2.66 mmol), and the mixture was stirred at rt for 1 h. To the reaction mixture was added 1 M HCl, and resulting mixture was concentrated *in vacuo* to give carboxylic acid, which was used without further purification. To the carboxylic acid solution in MeOH-THF (1:1, 60 mL) was added $Pd(OH)_2$/C (250 mg), and resulting mixture was stirred for 2 h under a hydrogen atmosphere (balloon). The reaction mixture was filtered through a pad of celite and the filtrates were concentrated *in vacuo* to give amino acid **13a**. To a solution of the amino acid **13a** in DMF-CH_2Cl_2 (1:2, 400 mL) was added DMAP (650 mg, 5.32 mmol), Et*i*-Pr_2N (452 μL, 2.66 mmol), and DPPA (1.15 mL, 5.32 mmol), and the resulting mixture was stirred at 90 °C for 3 h under a nitrogen atomosphere. To the reaction mixture was added H_2O and the organic layer was extracted with EtOAc. The extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified on silica gel ($CHCl_3$-EtOAc = 3:1) to give **14a** (506 mg, 0.55 mmol, 41%, 3 steps). Spectral data for **14a**: $[\alpha]^{25}_D$ +4.3 (*c* 2.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (d, J = 7.5 Hz, 2H), 8.22 (s, 2H), 5.34 (m, 2H), 4.61 (br, 2H), 3.05 (m, 4H), 2.71 (s, 6H), 2.65 (s, 6H), 2.06 (m, 2H), 1.91 (m, 2H), 1.53-1.19 (m, 26H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 162.2, 160.0, 155.9, 155.0, 150.1, 149.9, 140.5, 136.6, 125.7, 124.6, 78.9, 47.4, 40.3, 34.6, 29.5, 28.3, 21.9, 11.7; HRMS (ESI, M+Na) calcd for $C_{44}H_{54}N_{10}O_{12}Na$ 937.3820, found 937.3825.

Synthesis of **15b**: To a solution of **14b** (130 mg, 0.146 mmol) in THF-MeOH (1:1, 20 mL) was added 12 M HCl (4 mL), and the mixture was stirred for 10 min at rt. The reaction mixture was concentrated *in vacuo* to give **15b** (95 mg, 86%). Spectral data for **15b**: $[\alpha]^{25}_D$ -34.6 (*c* 2.6, MeOH); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.13 (s, 1H), 9.11 (s, 1H), 8.92 (s, 1H), 8.89 (s, 1H), 8.31 (d, J = 7.3, 1H), 8.28 (d, J = 7.3, 1H), 7.78 (br 4H), 5.45 (m, 1H), 5.35 (m, 1H), 2.75 (s, 3H), 2.71 (s, 3H), 2.07 (m, 2H), 1.92 (m, 2H), 1.65-0.98 (m, 8H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 164.4, 162.0, 158.9, 158.7, 158.1, 157.8, 155.6, 155.6, 154.5, 154.5, 154.4, 151.4, 151.1, 142.5, 142.3, 141.8, 141.1, 136.0, 135.9, 129.7, 128.5, 124.7, 123.7, 69.8, 47.2, 47.1, 33.3, 26.7, 20.9, 11.5; HRMS (ESI, M+Na) calcd for $C_{32}H_{35}N_{10}O_8Na$ 687.2639, found 687.2601.

Synthesis of **15a**: To a solution of **14a** (270 mg, 0.295 mmol) in THF-MeOH (1:1, 10 mL) was added 12 M HCl (2 mL), and the mixture was stirred for 2 h at rt. The reaction mixture was concentrated *in vacuo*

to give **15a** (200 mg, 86%). Spectral data for **15a**: $[\alpha]^{25}_{D}$ +38.9 (*c* 0.5, MeOH); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.89 (s, 2H), 8.24 (d, J = 6.9, 2H), 7.82 (br, 4H), 5.36 (br, 2H), 3.16 (br, 4H), 2.75 (br, 6H), 2.71 (br, 6H), 2.05 (br, 2H), 1.91 (br, 2H), 1.68-0.58 (m, 8H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 162.0, 158.9, 158.1, 157.8, 155.6, 154.4, 151.3, 151.1, 142.3, 135.8, 124.7, 123.7, 48.6, 46.9, 45.6, 33.4, 26.7, 20.9, 11.5, 8.9; HRMS (ESI, M+Na) calcd for $C_{34}H_{39}N_{10}O_8$ 715.2952, found 715.2962.

Synthesis of L2A2-6M(2)OTD (**3b**): To a solution of **15b** (36 mg, 0.047 mmol) in acetic anhydride (4 mL) was added pyridine (1 mL), and the mixture was stirred at 40 ºC for 15 h. The reaction mixture was concentrated *in vacuo*. The residue was purified on silica gel (EtOAc-MeOH = 4:1) to give L2A2-6M(2)OTD (**3b**) (33 mg, 91%). Spectral data for **3b**: $[\alpha]^{25}_{D}$ +30.9 (*c* 1.5, MeOH); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.11 (s, 1H), 9.08 (s, 1H), 8.89 (s, 1H), 8.86 (s, 1H), 8.35 (d, J = 7.5 Hz, 1H), 8.31 (d, J = 7.5, 1H), 7.82 (br, 2H), 5.41 (dd, J = 5.7, 12.6 Hz, 1H), 5.32 (dd, J = 5.2, 12.6 Hz, 1H), 2.94 (br, 4H), 2.74 (s, 3H), 2.70 (s, 3H), 2.02 (br, 2H), 1.89 (br, 2H), 1.71 (s, 6H), 1.35-1.12 (m, 8H); ^{13}C NMR (400 MHz, DMSO-d_6); δ 170.3, 168.9, 164.6, 162.2, 158.9, 158.7, 155.7, 155.5, 154.5, 154,4, 151.3, 151.0, 142,5, 142,2, 141,7, 141,0, 136,0, 135,8, 129,7, 128,3, 124,6, 123,6, 69.8, 59.7, 47.3, 47,1, 38.3, 33,4, 28,7, 22,5, 21,4, 21,3, 20,7, 14,1, 11,5; HRMS (ESI, M+Na) calcd for $C_{36}H_{38}N_{10}O_{10}Na$ 793.2670, found 793.2687.

Synthesis of L2A2-6M(4)OTD (**3c**): To a solution of **15a** (16 mg, 0.022 mmol) in acetic anhydride (3 mL) was stirred at 40 °C for 7 h. The reaction mixture was concentrated *in vacuo* and the residue was purified on silica gel (EtOAc-MeOH = 4:1) to give L2A2-6M(4)OTD (**3c**) (17 mg, 97%). Spectral data for **3c**: $[\alpha]^{25}_{D}$ +7.0 (*c* 0.5, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.90 (s, 1H), 8.87 (s, 1H), 8.26 (br, 2H), 7.86 (br, 2H), 5.34 (br, 2H), 2.95 (br 4H), 2.73 (s, 6H), 2.69 (s, 6H), 2.02 (br, 2H), 1.89 (br, 2H), 1.73 (s, 6H), 1.36-1.10 (m, 8H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 170.5, 162.7, 160.1, 156.0, 155.2, 150.3, 150.2, 140.7, 136.7, 125.5, 124.4, 47.2, 39.3, 34.2, 29.7, 28.6, 23.2, 21.8, 11.8, 11.7; HRMS (ESI, M+Na) calcd for $C_{38}H_{42}N_{10}O_{10}Na$ 821.2983, found 821.2982.

FRET melting assays: FRET melting assays were performed as previously reported methods.[12] The dual fluorescently labeled oligonucleotides were used in this protocol. The donor fluorophore was 6-carboxyfluorescein, 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 denatured at a concentration of 0.4 μM by heating at 94 ºC for 5 min followed by cooling to rt to anneal.

We added the various concentrations of ligands (**3a-c**) into different samples, using a total reaction volume of 40 μL, with 0.2 μM of labelled oligonucleotide. Following experiments should keep the temperature-procedure in real-time PCR and the procedure was finished as following: 25 °C for 10 minutes, then a stepwise increase of 1 °C every minute from 25 °C to reach 99 °C. During the procedures, the fluorescence of FAM was measured after each the temperature.

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REFERENCES AND NOTES

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16. In FRET melting assays, four representative single stranded G-quadruplex forming oligonucleotides (GFOs), i.e., telo21, kit22, bcl27 and myc22 conjugated with dyes at both 5' and 3' ends were used in the presence of potassium cation. GFOs and double-stranded 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'
ds26	5'-FAM-[TATAGCTATATTTTTTTATAGCTATA]-TAMRA-3'