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Nucleosides and nucleotides. Part 226: Alternate-strand triple-helix formation by 3′-3′-linked oligodeoxynucleotides composed of asymmetrical sequences ☆

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Abstract—In this paper, we describe the synthesis of the 3′-3′-linked oligonucleotides connected with pentaerythritol composed of asymmetrical sequences. Stability of the triplexes between these oligonucleotides and the DNA targets involving the adjacent oligopurine domains on alternate strands was investigated using the electrophoretic mobility shift assay (EMSA) and DNase I footprinting experiment. It was found that the 3′-3′-linked oligonucleotides composed of asymmetrical sequences formed the stable antiparallel triplexes with the DNA targets as compared with the *unlinked* oligonucleotides. Thus, oligonucleotides linked with pentaerythritol would be useful as antigene oligonucleotides for DNA targets consisting of the alternating oligopyrimidine–oligopurine sequences.
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Synthetic oligonucleotide-directed triple-helix (triplex) formation represents a promising way to interfere with gene expression and has become an area of intense investigation.[1] Triplex-forming oligonucleotides (TFOs) bind in the major groove of duplex DNA containing oligopurine/oligopyrimidine stretches and form hydrogen bonds with the target oligopurine sequence. Pyrimidine-rich TFOs bind parallel to the purine strand of the duplex and form T·AT and C^+·GC base triplets by Hoogsteen hydrogen bonds,[2,3] while purine-rich TFOs bind antiparallel to the purine strand of the duplex and form A·AT (or T·AT) and G·GC base triplets by reverse Hoogsteen hydrogen bonds.[4,5] However, target sequences in the triplex strategy are quite restricted. Since the thermal stability of the triplexes is generally lower than that of the duplexes under physiological conditions, an oligopurine cluster with long chain lengths is required for stable triplex formation.

Several approaches have been attempted to expand the repertory of potential DNA targets.[6–24] If short oligopurine sequences appear adjacently and alternately on the two strands of the DNA target, the alternate sequences can be recognized by TFOs that simultaneously and cooperatively bind to the adjacent oligopurine domains by cross-over in the major groove (Fig. 1). Recently, we reported the synthesis of the 3′-3′-linked TFOs connected with pentaerythritol, that were composed of symmetrical sequences.[25,26] We found that the 3′-3′-linked TFOs formed the thermally stable parallel (Fig. 1a) and antiparallel triplexes (Fig. 1b) with the DNA targets. Additionally, we showed that the 3′-3′-linked TFOs inhibited the cleavages of the DNA targets by restriction enzymes more effectively than the *unlinked* decamers. These results prompted us to examine the synthesis and properties of the 3′-3′-linked TFOs connected with pentaerythritol, that were composed of asymmetrical sequences (Fig. 1c).

In this paper, we report the synthesis of the 3′-3′-linked TFOs consisting of asymmetrical sequences that can form the antiparallel triplexes with a DNA target (Fig. 1c). The stabilities of the alternate-strand triplexes between these TFOs and the DNA targets were investigated with the electrophoretic mobility shift assay (EMSA) and DNase I footprinting experiment.

Synthesis. The controlled pore glass (CPG) with pentaerythritol was synthesized as shown in Scheme 1.

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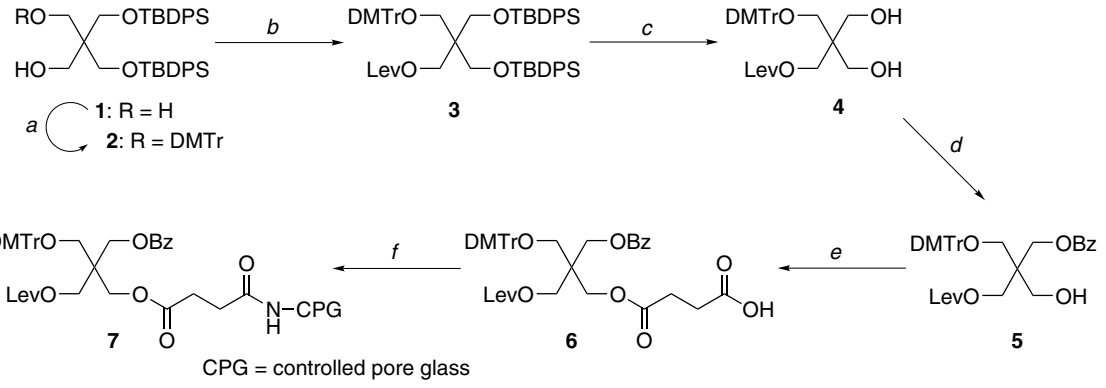
Figure 1. Schematic presentation of 'alternate-strand triplex' formation by the 3′-3′-linked TFOs.



Scheme 1. Reagents and conditions: (a) (1) Bu_2SnO, benzene, 80 °C; (2) DMTrCl, benzene, rt; (b) levulinic acid, DMAP, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), CH_2Cl_2, rt; (c) TBAF, AcOH, THF, rt, 46% from **1**; (d) (1) Bu_2SnO, benzene, 80 °C; (2) BzCl, benzene, rt, 84%; (e) succinic anhydride, Et_3N, DMAP, CH_3CN, rt, 63%; (f) aminopropyl controlled pore glass, EDC·HCl, DMF, rt.

2,2-Bis[[(*tert*-butyldiphenylsilyl)oxy]methyl]-1,3-propanediol (**1**)[27] was treated with Bu_2SnO in benzene and then reacted with 4,4′-dimethoxytrityl chloride (DMTrCl) to produce the *O*-DMTr derivative **2**. After protection of the other hydroxyl group of **2** with a levulinyl (Lev) group, the *tert*-butyldiphenylsilyl (TBDPS) groups were removed by treatment with tetrabutylammonium fluoride (TBAF) to give **4** in 46% yield from **1**. One of the two hydroxyl groups of **4** was protected with a benzoyl (Bz) group to afford the *O*-mono(Bz) derivative **5** in 84% yield. Compound **5** was succinated to give **6**, which was further reacted with CPG to afford the solid support **7** bearing **6** (32 μmol/g).

The 3′-3′-linked TFOs **10**–**13** (Fig. 2a), which consist of 2′-deoxyguanosine (dG) and thymidine (T), dG and 2′-deoxyadenosine (dA), or dG, T and dA were synthesized on a DNA synthesizer using the CPG **7** by the phosphoramidite method. One strand was elongated in the usual manner. After capping the 5′-end of the strand and removing the Lev group by treatment with 0.5 M hydrazine in pyridine–CH_3CO_2H (4:1, v/v), the other strand was elongated. The fully protected TFOs (each 1 μmol) linked to the solid support were treated with concentrated NH_4OH at 55 °C for 16 h. The released TFOs were purified by denaturing with 20% polyacrylamide gel electrophoresis (20% PAGE) to give the deprotected TFOs **10**–**13**, in 40, 24, 29, and 36 OD_{260} units, respectively. These TFOs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and the observed molecular weights supported their structures.[28]

Studies of triplex formation by the electrophoretic mobility shift assay. The stabilities of the triplexes were investigated using the electrophoretic mobility shift assay (EMSA). The EMSA detects the difference in electrophoretic mobility of the double-stranded DNA and the triplex formed.

The ^{32}P-labeled DNA **14** was incubated in the presence of increasing concentrations of a 1:1 mixture of the *unlinked* decamers **8** and **9** or the 3′-3′-linked TFO **10** in a buffer of 20 mM Tris–HCl (pH 7.5) containing 10 mM $MgCl_2$. The solutions were analyzed by non-denaturing 20% polyacrylamide gel electrophoresis (PAGE) at 4 °C. As shown in Figure 3, the less mobile bands corresponding to the triplexes were observed for the TFO **10**, whereas the band corresponding to the triplex was not detected up to 100 μM for the mixture of the TFOs **8** and **9**. Table 1 summarizes the dissociation constants (K_ds) of the TFOs bound to the duplex DNA target. The K_d value of the TFO **10** to the DNA **14** was 160 ± 10 nM, whereas that of the *unlinked* decamers **8** and **9** was more

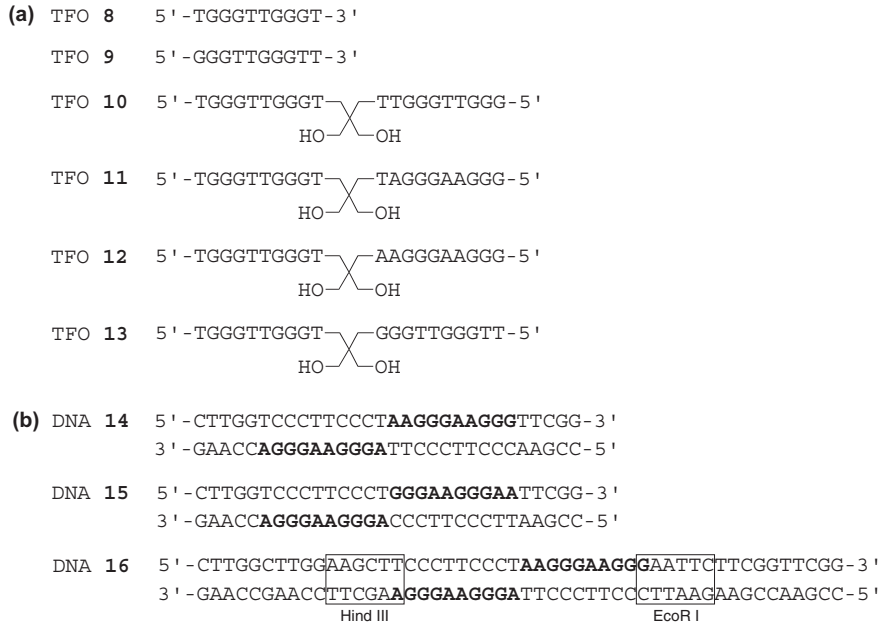


Figure 2. Sequences of TFOs and the target duplexes. The binding sites of TFOs are written in boldface.

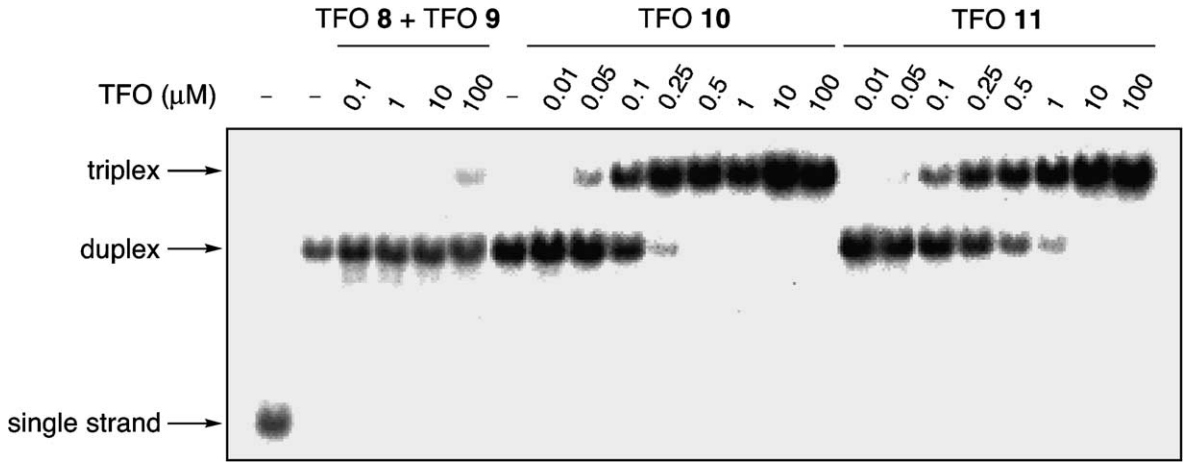


Figure 3. Quantitative EMSA to detect binding of TFOs **8** and **9**, TFO **10**, and TFO **11** to the DNA target **14**. DNA concentration: 10 nM. TFO concentrations are indicated.

Table 1. Summary of dissociation constants (K_d) of the TFOs bound to the DNA targets

TFO	Duplex	K_d (nM)
TFO **8** + **9**	DNA **14**	>100,000
TFO **10**	DNA **14**	160 (±10)
TFO **11**	DNA **14**	260 (±20)
TFO **12**	DNA **14**	1100 (±100)
TFO **13**	DNA **15**	>100,000

than 100 μM. Thus, this result proved that the triplex was significantly stabilized by connecting the TFO fragments with pentaerythritol.

The antiparallel triplexes are formed by the A·AT, T·AT, or G·GC base triplet. In order to examine the effect of the composition of a third strand on the alternate-strand triplex formation, the ability of the 3′-3′-linked TFO **11** comprised of T, dG, and dA to bind to the DNA **14** was also examined. The K_d value of the TFO **10** to the DNA **14** was 160 ± 10 nM, whereas that of the TFO **11** was 260 ± 20 nM. Thus, it was found that the TFO consisting of T and dG stabilized the alternate-strand triplex slightly more efficiently than that involving dA.

It has been reported that the sequences of the junction regions of the alternate-strand triplexes critically influence the stabilities of the triplexes.[13] Thus, the ability of the TFO **12** with dA and the TFO **13** with dG at the junction regions to bind to the DNAs **14** and **15** was also examined. The K_d values of the TFOs **12** and **13** to the DNAs **14** and **15** were 1100 ± 100 nM and more than 100 μM, respectively. Thus, it turned out that the TFO with T at the junction region stabilized the alternate-strand triplex more efficiently than those with dA or dG.

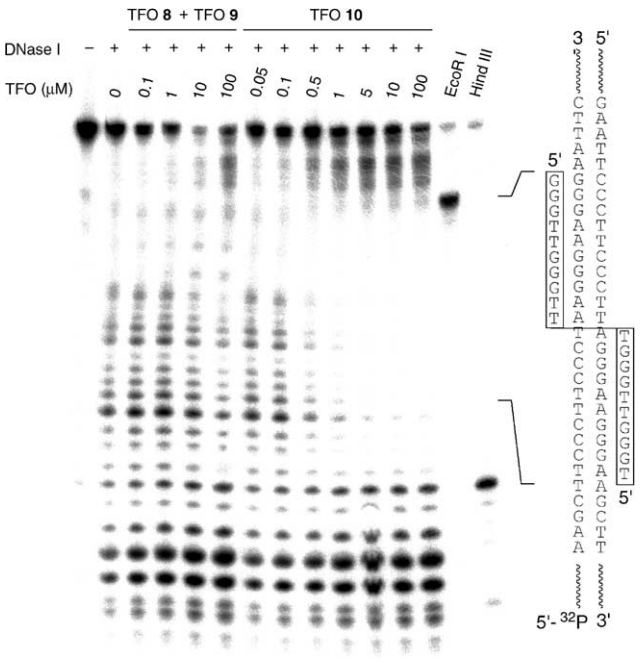


Figure 4. DNase I footprinting experiment. DNA concentration: 100 nM. TFO concentrations are indicated.

DNase I footprinting experiment. We next performed a DNase I footprinting experiment to analyze the structure of the triplexes. The ^{32}P-labeled DNA **16**, which has the oligopurine domains between the Hind III and EcoR I recognition sites, was digested by DNase I at 25 °C in the presence of increasing concentrations of the mixture of the *unlinked* decamers **8** and **9** or the 3′-3′-linked TFO **10** in a buffer of 20 mM Tris–HCl (pH 7.5) containing 10 mM $MgCl_2$. The solutions were analyzed by denaturing 20% PAGE. As shown in Figure 4, the 3′-3′-linked TFO **10** gave a clear footprint between the Hind III and EcoR I recognition sites, whereas the *unlinked* decamers **8** and **9** did not exhibit the binding to the DNA target. Thus, it was revealed that the 3′-3′-linked TFO **10** binds simultaneously to both the adjacent oligopurine domains of the DNA **16**.

In conclusion, we have synthesized the 3′-3′-linked TFOs connected with pentaerythritol, that were composed of asymmetrical sequences. We found that 3′-3′-linked TFOs form stable antiparallel triplexes with the DNA target consisting of the adjacent oligopurine domains on alternate strands as compared with the *unlinked* TFOs. Thus, the TFOs linked with pentaerythritol would be useful as antigene oligonucleotides for DNA targets consisting of the alternating oligopyrimidine–oligopurine sequences.

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