Nucleosides and Nucleotides. 182. Synthesis of Branched Oligodeoxynucleotides with Pentaerythritol at the Branch Point and Their Thermal Stabilization of Triplex Formation[1]

Yoshihito Ueno, Masako Takeba, Mai Mikawa, and Akira Matsuda*

Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan

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To find an oligodeoxynucleotide (ODN) with triplex stabilization capability, we designed and synthesized novel branched ODNs **1** and **2** with pentaerythritols at their branch points. Branched ODNs **1** and **2** were synthesized on a solid support using bis(phosphoramidite) **11**. The stability of the triplexes formed by branched ODNs **1** and **2** with $(dA)_{21}$ or $(dT)_{21}$ was studied by thermal denaturation. Branched ODN **1** formed a stable parallel T·AT-type triplex with $(dA)_{21}$ (T_m = 38.9 °C) in a buffer of 0.01 M sodium phosphate (pH 7.0) containing 0.5 M NaCl and 0.02 M $MgCl_2$, while branched ODN **2** formed a stable antiparallel A·AT-type triplex with $(dT)_{21}$ (T_m = 44.2 °C) in the same buffer. The formation of these triplexes was also confirmed by circular dichroism (CD) measurements and a gel retardation assay.

Introduction

Recently, chemically synthesized oligodeoxynucleotides (ODNs) and their analogues have been used in studies of triplex formation and in biological and biochemical studies, such as in the site-specific cleavage of DNA or the inhibition of DNA−protein binding.[2] In intermolecular triplexes, an oligopyrimidine·oligopurine sequence of a DNA duplex is bound by a third-strand ODN in the major groove. To date, two major classes of triplexes have been identified based on the orientation of the third strand.[2] When the third strand consists mainly of pyrimidines, Hoogsteen-type Py·PuPy base triplets (T·AT and C^+·GC) are formed, in which the third strand is parallel to the purine strand of the target duplex. On the other hand, when the third strand is predominantly purines, Pu·PuPy-type base triplets (G·GC and A·AT) are formed, in which the third strand is antiparallel to the purine strand of the target duplex. However, in general, the binding of a third-strand ODN to a target DNA duplex is thermodynamically weaker than duplex formation itself. Thus, many efforts have been made to increase the affinity of the third strand for its target.[2] These include the synthesis of backbone-, sugar-, and/or base-modified ODNs as well as ODN-intercalator conjugates.[3]

On the other hand, since branched RNAs containing vicinal 2′-5′ and 3′-5′ internucleotidic phosphodiester linkages have been discovered in the splicing products of eukaryotic mRNAs, considerable attention has been paid to the chemical synthesis of this unique structure.[4−6] The chemical synthesis of branched RNAs in a solution phase has been reported in several laboratories.[4] On the other hand, the first automated solid-phase synthesis of

* To whom reprint requests should be addressed. Phone: +81-11-706-3228. Fax: +81-11-706-4980. E-mail: matuda@pharm.hokudai.ac.jp.

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(2) Thuong, N. T.; Hélène, C. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 666−690.

(3) (a) Matteucci, M.; Lin, K. Y.; Butcher, S.; Moulds, C. *J. Am. Chem. Soc.* **1991**, *113*, 7767−7768. (b) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497−1500. (c) Escudé, C.; Giovannangeli, C.; Sun, J.-S.; Lloyd, D. H.; Chen, J.-K.; Gryaznov, S. M.; Garestier, T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4365−4369. (d) Shimizu, M.; Konishi, A.; Shimada, Y.; Inoue, H.; Ohtsuka, E. *FEBS Lett.* **1992**, *302*, 155−158. (e) Cuenoud, B.; Casset, F.; Hüsken, D.; Natt, F.; Wolf, R. M.; Altmann, K.-L.; Martin, P.; Moser, H. E. *Angew. Chem., Int. Ed.* **1998**, *37*, 1288−1291. (f) Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 3059−3061. (g) Ono, A.; Ts'o, P. O. P.; Kan, L. S. *J. Am. Chem. Soc.* **1991**, *113*, 4032−4033. (h) Krawczyk, S. H.; Milligan, J. F.; Wadwani, S.; Moulds, C.; Froehler, B. C.; Matteucci, M. D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3761−3764. (i) Griffin, L. C.; Kiessling, L. L.; Beal, P. A.; Gillespie, P.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 7976−7982. (j) Verma, S.; Miller, P. S. *Bioconjugate Chem.* **1996**, *7*, 600−605. (k) Lehmann, T. E.; Greenberg, W. A.; Liberles, D. A.; Wada, C. K.; Dervan, P. B. *Helv. Chem. Acta* **1997**, *80*, 2002−2022. (l) Zhou, B.-W.; Puga, E.; Sun, J.-S.; Garestier, T.; Hélène, C. *J. Am. Chem. Soc.* **1995**, *117*, 10425−10428. (m) Silver, G. C.; Sun, J.-S.; Nguyen, C. H.; Boutorine, A. S.; Bisagni, E.; Hélène, C. *J. Am. Chem. Soc.* **1997**, *119*, 263−268.

(4) (a) Sekine, M.; Hata, T. *J. Am. Chem. Soc.* **1985**, *107*, 5813−5815. (b) Sekine, M.; Heikkilä, J.; Hata, T. *Tetrahedron Lett.* **1987**, *28*, 5691−5694. (c) Sekine, M.; Heikkilä, J.; Hata, T. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 588−601. (d) Damha, M. J.; Pon, R. T.; Ogilvie, K. K. *Tetrahedron Lett.* **1985**, *26*, 4839−4842. (e) Kierzek, R.; Kopp, D. W.; Edmonds, M.; Caruthers, M. H. *Nucleic Acids Res.* **1986**, *14*, 4751−4764. (f) Fourrey, J. L.; Varenne, J.; Fontaine, C.; Guittet, E.; Yang, Z. W. *Tetrahedron Lett.* **1987**, *28*, 1769−1772. (g) Huss, S.; Gosselin, G.; Imbach, J.-L. *Tetrahedron Lett.* **1987**, *28*, 145−418. (h) Huss, S.; Gosselin, G.; Imbach, J.-L. *J. Org. Chem.* **1988**, *53*, 499−506. (i) Hayakawa, Y.; Nobori, T.; Noyori, R.; Imai, J. *Tetrahedron Lett.* **1987**, *28*, 2623−2626. (j) Remaud, G.; Vial, J.-M.; Nyilas, A.; Balgobin, N.; Chattopadhyaya, J. *Tetrahedron* **1987**, *43*, 947−958. (k) Zhou, X.-X.; Remaud, G.; Chattopadhyaya, J. *Tetrahedron* **1988**, *44*, 6471−6489. (l) Balgobin, N.; Földesi, A.; Remaud, G.; Chattopadhyaya, J. *Tetrahedron* **1988**, *44*, 6929−6939. (m) Sund, C.; Földesi, A.; Yamakage, S.; Agback, P.; Chattopadhyaya, J. *Tetrahedron* **1991**, *47*, 6305−6336. (n) Sund, C.; Agback, P.; Chattopadhyaya, J. *Tetrahedron* **1991**, *47*, 9659−9674. (o) Sund, C.; Agback, P.; Chattopadhyaya, J. *Tetrahedron* **1993**, *49*, 649−668. (p) Agback, P.; Glemarec, C.; Yin, L.; Sandström, A.; Plavec, J.; Sund, C.; Yamakage, S.; Viswanadham, G.; Rousse, B.; Puri, N.; Chattopadhyaya, J. *Tetrahedron Lett.* **1993**, *34*, 3929−3932.

(5) (a) Damha, M. J.; Zabarylo, S. *Tetrahedron Lett.* **1989**, *30*, 6295−6298. (b) Damha, M. J.; Ganeshan, K.; Hudson, R. H. E.; Zabarylo, S. V. *Nucleic Acids Res.* **1922**, *20*, 6565−6573. (c) Hudson, R. H. E.; Damha, M. J. *J. Am. Chem. Soc.* **1993**, *115*, 2119−2124. (d) Hudson, R. H. E.; Uddin, A. H.; Damha, M. J. *J. Am. Chem. Soc.* **1995**, *117*, 12470−12477. (e) Braich, R. S.; Damha, M. J. *Bioconjugate Chem.* **1997**, *8*, 370−377. (f) Hudson, R. H. E.; Robidoux, S.; Damha, M. J. *Tetrahedron Lett.* **1998**, *39*, 1299−1302. (g) Damha, M. J.; Braich, R. S. *Tetrahedron Lett.* **1998**, *39*, 3907−3910.

(6) (a) Sproat, B. S.; Beijer, B.; Grøtli, M.; Ryder, U.; Morand, K. L.; Lamond, A. I. *J. Chem. Soc., Perkin Trans. 1* **1994**, 419−431. (b) Grøtli, M.; Sproat, B. S. *J. Chem. Soc., Chem. Commun.* **1995**, 495−497. (c) Grøtli, M.; Eritja, R.; Sproat, B. S. *Tetrahedron* **1997**, *53*, 11317−11346.

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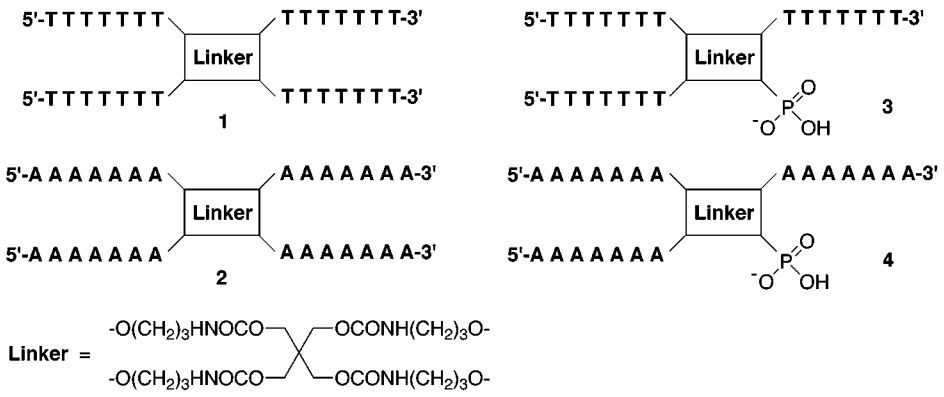


Figure 1. Structures of the branched ODNs.

branched RNA was reported by Damha et al.[5a,b] They constructed branched RNAs and ODNs with adenosine at the branch point by reacting adenosine 2′,3′-*O*-bis-(phosphoramidite) with the 5′-hydroxyl groups of two adjacent RNAs or ODNs on a solid support. Furthermore, they also showed that branched poly(thymidine) ODNs with adenosine at the branch point can form stable antiparallel T·AT triplexes.[5d] More recently, several kinds of branched ODNs with 3′-deoxy-β-D-psicothymidine, 1-(2-methyl-β-D-arabinofuranosyl)uracil, or 4′-*C*-(hydroxymethyl)thymidine at their branch points have been synthesized, and some of these have been shown to form stable triplexes with single-stranded ODNs.[7,8] Branched (dendrimeric) ODNs have also been used to amplify radioactive or fluorescent signals in hybridization studies.[9]

On the basis of these findings, we designed new branched ODNs **1** and **2** with pentaerythritol **5** at the branch point (Figure 1), which form a stable parallel T·AT-type triplex with $(dA)_{21}$ and an antiparallel A·AT-type triplex with $(dT)_{21}$, respectively (Figure 2). In this paper, we report the synthesis of branched ODNs **1** and **2** and their thermal stabilization of triplex formation.

Results and Discussion

Synthesis. Damha et al. constructed branched ODNs with adenosine at the branch point by reacting a low concentration of adenosine 2′,3′-*O*-bis(phosphoramidite) with the 5′-hydroxyl groups of two adjacent ODNs on a solid support.[5a−d] We also adopted this so-called "convergent" method (Scheme 1). For the synthesis of branched ODNs **1** and **2**, we synthesized a bis(phosphoramidite) **11**. In the convergent method, the reaction of bis-(phosphoramidite) **11** with the 5′-hydroxyl groups of two adjacent ODNs on a solid support is a crucial step. Thus,

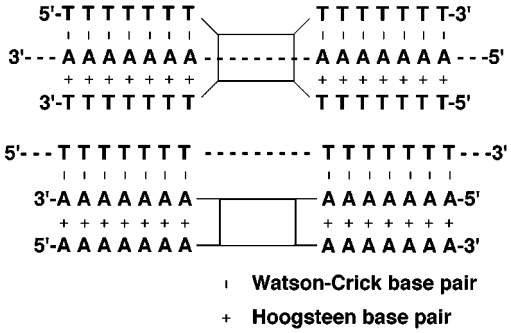


Figure 2. Structures of the triplexes between the branched ODNs and $(dT)_{21}$ or $(dA)_{21}$.

pentaerythritol **5** was modified with a propylcarbamoyl tether for efficient coupling on a solid support and for stable triplex formation.

First, pentaerythritol **5** was treated with 2 equiv of *tert*-butylchlorodiphenylsilane (TBDPSCl) in the presence of imidazole in DMF to give mono-, bis-, and tris(TBDPS) derivatives **6a**,[10] **6b**, and **6c** in 13, 55, and 31% yields, respectively (Scheme 2). Compound **6b** was converted into a carbonylimidazolide, which was reacted with 3-amino-1-propanol to afford **7** in 88% yield. The primary hydroxy groups of **7** were protected with a DMTr group. After removal of the TBDPS groups, **9** was converted into carbonylimidazolide, which was reacted with 3-amino-1-propanol again to give **10** in 91% yield. Compound **10** was phosphitylated by a standard procedure[11] to afford the bis(phosphoramidite) **11** in 65% yield.

Branched ODNs **1** and **2** were synthesized on a DNA synthesizer by the phosphoramidite method.[12] A low concentration (0.025 M) of bis(phosphoramidite) **11** was used in the branching step. The coupling yield of **11** was 112% using a 0.025 M solution of **11** in CH_3CN with a coupling time of 600 s. The fully protected ODNs (1 μmol) were concomitantly cleaved from the solid support and deprotected by treatment with concentrated NH_4OH at 55 °C for 16 h. Removal of the ammonia solution furnished the crude ODNs. The crude ODNs were purified by 20% polyacrylamide gel electrophoresis (20% PAGE), followed by reversed-phase HPLC. In 20% PAGE,

(7) (a) Azhayeva, A.; Guzaev, A.; Hovinen, J.; Azhayeva, E.; Lönnberg, H. *Tetrahedron Lett.* **1993**, *34*, 6435−6438. (b) Azhayeva, E.; Azhayeva, A.; Guzaev, A.; Hovinen, J.; Lönnberg, H. *Nucleic Acids Res.* **1995**, *23*, 1170−1176. (c) Azhayeva, E.; Azhayeva, A.; Guzaev, A.; Lönnberg, H. *Nucleic Acids Res.* **1995**, *23*, 4255−4261.

(8) (a) Brandenburg, G.; Petersen, G. V.; Rasmussen, K.; Wengel, J. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 791−794. (b) von Buren, M.; Petersen, G. V.; Rasmussen, K.; Brandenburg, G.; Wengel, J. *Tetrahedron* **1995**, *51*, 8491−8506. (c) Thrane, H.; Fensholdt, J.; Regner, M.; Wengel, J. *Tetrahedron* **1995**, *51*, 10389−10402. (d) Meldgaard, M.; Nielsen, N. K.; Bremner, M.; Pedersen, O. S.; Olsen, C. E.; Wengel, J. *J. Chem. Soc., Perkin Trans. 1* **1997**, 1951−1955.

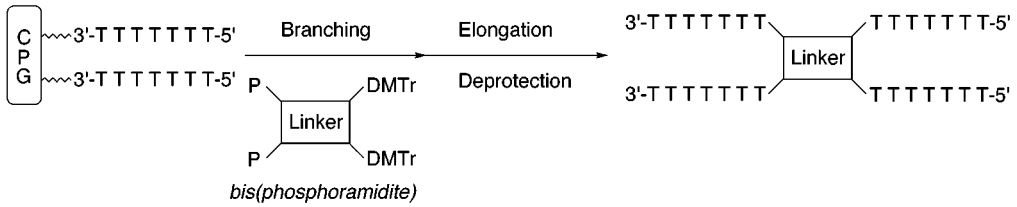
(9) (a) Shchepinov, M. S.; Udalova, I. A.; Bridgman, A. J.; Southern, E. M. *Nucleic Acids Res.* **1997**, *25*, 4447−4454. (b) Horn, T.; Chang, C.-A.; Urdea, M. S. *Nucleic Acids Res.* **1997**, *25*, 4835−4841. (c) Horn, T.; Chang, C.-A.; Urdea, M. S. *Nucleic Acids Res.* **1997**, *25*, 4842−4849.

(10) The same compound was recently synthesized by Hanessian et al.: Hanessian, S.; Prabhanjan, H.; Qiu, D.; Nambiar, S. *Can. J. Chem.* **1996**, *74*, 1731−1737.

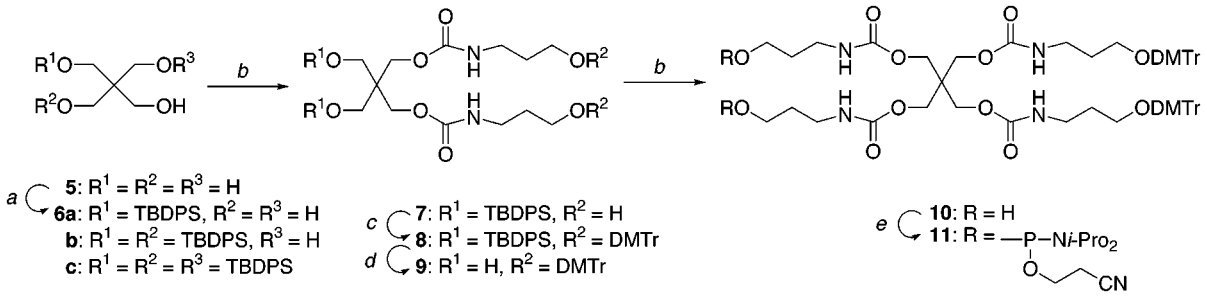
(11) Gait, M. J., Ed. In *Oligonucleotides Synthesis*: *a Practical Approach*; IRL Press: Oxford, UK, 1984.

(12) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859−1862.

Scheme 1



Scheme 2[a]



[a] (a) TBDPSCl, Imidazole, DMF; (b) (1) *N*,*N*-carbonyldiimidazole, DMAP, DMF, (2) 3-amino-1-propanol, DMF; (c) DMTrCl, pyridine; (d) 0.5 M TBAF, THF; (e) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, *N*,*N*-diisopropylethylamine, CH_2Cl_2.

two major products were observed. In the synthesis of branched ODN **1**, ODN **1** and ODN **3** (Figure 1), which would be derived from the reaction of **11** with one of the 5′-hydroxyl groups of the ODNs on a solid support, were obtained in 19 and 21 OD_{260} units, respectively. Similarly, in the synthesis of branched ODN **2**, ODN **2** and ODN **4** were obtained in 17 and 16 OD_{260} units, respectively.

The structures of ODNs **1**−**4** were confirmed by electrospray ionization (ESI) mass spectrometry. The observed molecular weights supported their structures (see Experimental Section).

Studies of Triplex Formation by Thermal Denaturation. Branched ODN **1** was designed to form a parallel T·AT-type triplex with $(dA)_{21}$, whereas branched ODN **2** was designed to form an antiparallel A·AT-type triplex with $(dT)_{21}$ (Figure 2). To study the effect of the linker groups on triplex formation, we examined the stability of the triplexes formed by these branched ODNs with $(dA)_{21}$ or $(dT)_{21}$ by thermal denaturation. Their melting transitions were monitored at both 260 and 284 nm. UV absorbances of A−T Watson−Crick duplexes do not change when their melting transitions are monitored at 284 nm, since this wavelength (284 nm) is an isosbestic point of an A−T Watson−Crick duplex.[13] On the other hand, the UV absorbances of triplexes change at both 260 and 284 nm. Thus, a melting transition of a triplex can be detected independently of a transition of an A−T Watson−Crick duplex by measuring the melting profiles at 284 nm.

A melting profile of the triplex between ODN **1** and $(dA)_{21}$ in a buffer of 0.01 M sodium phosphate (pH 7.0) containing 0.5 M NaCl and 0.02 M $MgCl_2$ is shown in Figure 3b, whereas a melting profile of a natural triplex composed of $(dA)_{21}$:$2(dT)_{14}$ in the same buffer is shown in Figure 3a. In the melting curve of the natural triplex at 260 nm, two transitions are observed (Figure 3a). The transition at around 30 °C was also observed in a melting curve at 284 nm. Thus, we presumed that the transition with the higher T_m (47.7 °C) at 260 nm was due to melting of the A−T Watson−Crick duplex, while that with the lower T_m (30.1 °C) corresponded to dissociation of the third strand from the triplex. On the other hand, in the melting profile of the triplex between ODN **1** and $(dA)_{21}$, only one transition was observed at each wavelength (T_m = 38.9 °C). Thus, we supposed that the transition was due to both dissociation of the third strand from the triplex and melting of the A−T Watson−Crick duplex. Based on these results, it was found that the thermal stability of the triplex between branched ODN **1** and $(dA)_{21}$ is greater than that of the natural triplex [$\Delta T_{m\ triplex}$ [T_m of the triplex between branched ODN **1** and $(dA)_{21}$ minus T_m of the triplex of the mixture of $(dA)_{21}$:$2(dT)_{14}$] was +8.8 °C], although the thermal stability of the A−T Watson−Crick duplex between the branched ODN and A_{21} was less than that of the natural duplex [$\Delta T_{m\ duplex}$ [T_m of the Watson−Crick duplex between branched ODN **1** and $(dA)_{21}$ minus T_m of the duplex between $(dT)_{14}$ and $(dA)_{21}$] was −8.8 °C].

Melting profiles of the triplex between ODN **2** and $(dT)_{21}$, and a solution of $(dT)_{21}$:$2(dA)_{14}$ in a buffer of 0.01 M sodium phosphate (pH 7.0) containing 0.5 M NaCl and 0.02 M $MgCl_2$, are shown in Figure 3, parts d and c, respectively. The $(dT)_{21}$:$2(dA)_{14}$ solution had a monophasic transition (T_m = 48.1 °C) at 260 nm, but no observable transition at 284 nm (Figure 3c). This result indicates that the mixture of $(dT)_{21}$:$2(dA)_{14}$ forms a A−T Watson−Crick duplex, but not an antiparallel A·AT-type triplex under these conditions.[13a,14] On the other hand, the mixture of branched ODN **2** and $(dT)_{21}$ had a monophasic transition (T_m = 44.2 °C) at each wavelength. Thus, branched ODN **2** formed a thermally stable triplex with $(dT)_{21}$ under these conditions. These results show that the thermal stabilities of triplexes increase due to linking

(13) (a) Pilch, D. S.; Levenson, C.; Shafer, R. H. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1942−1946. (b) Pilch, D. S.; Brousseau, R.; Shafer, R. H. *Nucleic Acids Res.* **1990**, *18*, 5743−5750. (c) Durand, M.; Peloille, S.; Thuong, N. T.; Maurizot, J. C. *Biochemistry* **1992**, *31*, 9197−9204. (d) Fossella, J. A.; Kim, Y. J.; Shih, H.; Richards, E. G.; Fresco, J. R. *Nucleic Acids Res.* **1993**, *21*, 4511−4515.

(14) Dagneaus, C.; Gousset, H.; Shchyolkina, A. K.; Ouali, M.; Letellier, R.; Liquier, J.; Florentiev, V. L.; Taillandier, E. *Nucleic Acids Res.* **1996**, *24*, 4506−4512.

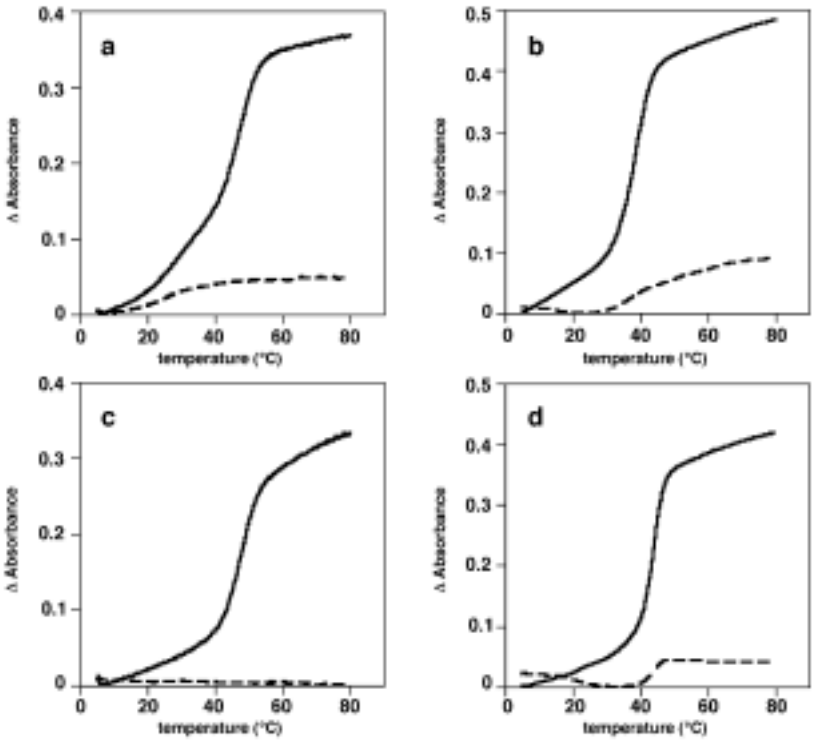


Figure 3. UV melting profiles monitored at 260 nm (—) and 284 nm (- - -). (a) $(dA)_{21}$:2$(dT)_{14}$. (b) ODN **1**:$(dA)_{21}$. (c) $(dT)_{21}$:2$(dA)_{14}$. (d) ODN **2**:$(dT)_{21}$.

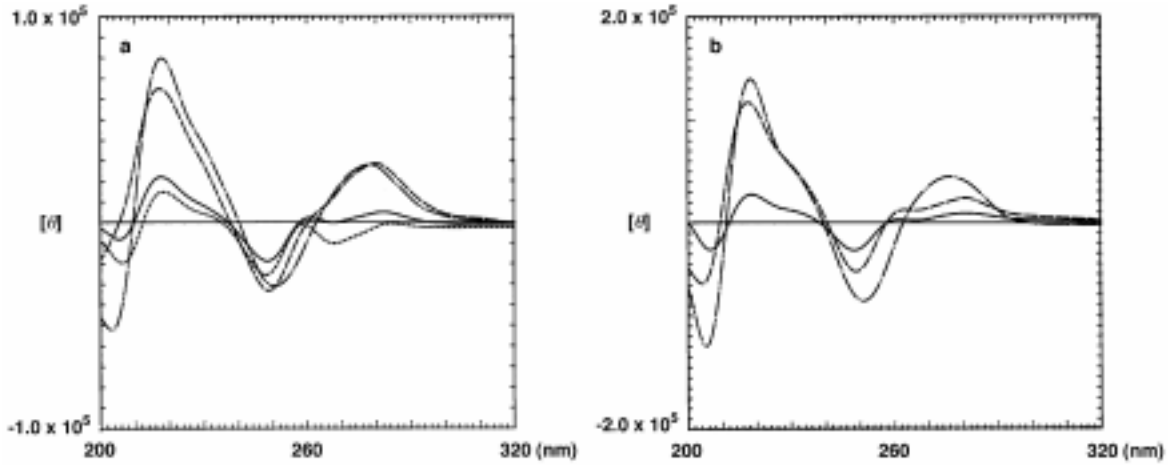


Figure 4. CD spectra of the triplexes at 5 °C in a buffer of 0.01 M sodium phosphate (pH 7.0) containing 0.5 M NaCl and 0.02 M $MgCl_2$. (a) An ODN **1**:$(dA)_{21}$ solution (—); the normalized sum of a ODN **1** plus $(dA)_{21}$ solutions (—•—); the normalized sum of a $(dT)_{14}$ plus $(dA)_{21}$:$(dT)_{14}$ solutions (— —); a $(dT)_{14}$:$(dA)_{21}$:$(dT)_{14}$ solution (- - -). (b) An ODN **2**:$(dT)_{21}$ solution (—); the normalized sum of a ODN **2** plus $(dT)_{21}$ solutions (—•—); the normalized sum of a $(dA)_{14}$ plus $(dA)_{21}$:$(dT)_{14}$ solutions (- - -).

between the third strand and one strand of the Watson–Crick duplex by linker groups.

Circular Dichroism. The formation of triplexes was confirmed by circular dichroism (CD) measurements. The CD spectra of the mixture of a 1:1 molar ratio of branched ODN **1**:$(dA)_{21}$ in a buffer of 0.01 M sodium phosphate (pH 7.0) containing 0.5 M NaCl and 0.02 M $MgCl_2$ at 5 °C were compared with the appropriately normalized summed spectra of branched ODN **1** and $(dA)_{21}$, and $(dT)_{14}$ and $(dA)_{21}$:$(dT)_{14}$, and the spectrum of a mixture of a 1:1:1 molar ratio of $(dT)_{14}$:$(dA)_{21}$:$(dT)_{14}$ (Figure 4a).

The CD spectrum (solid line) of a branched ODN **1**:$(dA)_{21}$ solution had positive CD bands at 217 and 283 nm and negative CD bands at 206 and 248 nm. The CD spectrum of a $(dT)_{14}$:$(dA)_{21}$:$(dT)_{14}$ solution had a positive CD band at 217 nm and negative CD bands at 206, 248, and 267 nm. The shapes of these CD spectra were similar to each other. On the other hand, the normalized summed spectra of branched ODN **1** and $(dA)_{21}$, and $(dT)_{14}$ and $(dA)_{21}$:$(dT)_{14}$, exhibited large positive CD bands at around 216 and 280 nm and negative CD bands at around 250 nm. The shapes of these spectra were obviously different from that of the branched ODN **1**:$(dA)_{21}$ solution. The positive CD bands at around 215 and 280 nm for the branched ODN **1**:$(dA)_{21}$ solution were clearly less intense than those in the normalized summed spectra from the branched ODN **1** and $(dA)_{21}$, and $(dT)_{14}$ and $(dA)_{21}$:$(dT)_{14}$ solutions. Recently, Pilch et al. reported that the positive CD band at around 280 nm of a $(dT)_{10}$:$(dA)_{10}$:$(dT)_{10}$-type triplex was less intense than that of the normalized

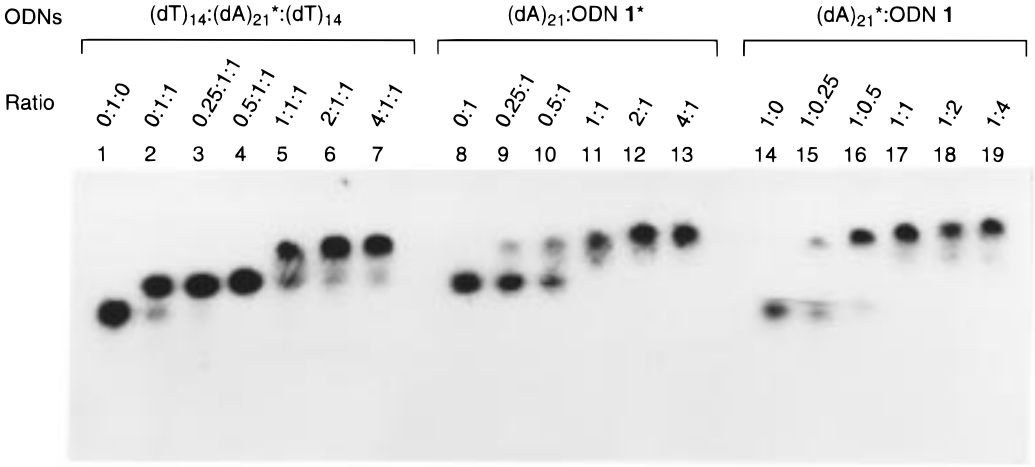


Figure 5. Gel retardation assays were carried out in 90 mM tris-borate buffer (pH = 7.8) containing 10 mM $MgCl_2$ at room temperature. The stoichiometry of ODNs is indicated by ratio. ^{32}P-Labeled ODNs are indicated by an asterisk.

summed spectra from $(dT)_{10}$ and $(dA)_{10}$:$(dT)_{10}$ solutions.[13a] Therefore, these CD spectral data support the existence of a triplex between branched ODN **1** and $(dA)_{21}$.

The CD spectra of the mixture of a 1:1 molar ratio of branched ODN **2**:$(dT)_{21}$ in a buffer of 0.01 M sodium phosphate (pH 7.0) containing 0.5 M NaCl and 0.02 M $MgCl_2$ at 5 °C was next compared with the appropriately normalized summed spectra of branched ODN **2** and $(dT)_{21}$, and $(dA)_{14}$ and $(dA)_{14}$:$(dT)_{21}$ (Figure 4b). The CD spectrum (solid line) of the branched ODN **2**:$(dT)_{21}$ solution had positive CD bands at 218 and 281 nm, shoulders near 235 and 260 nm, and negative CD bands at 206 and 248 nm. The shape of the spectrum was obviously different from that of the normalized summed spectrum of branched ODN **2** and $(dT)_{21}$. Furthermore, the positive CD bands at around 218 and 280 nm and the negative CD bands at around 206 and 248 nm for the branched ODN **2**:$(dT)_{21}$ solution were clearly less intense than those in the normalized summed spectra from $(dA)_{14}$ and $(dA)_{14}$:$(dT)_{21}$, although the shapes of these spectra were similar to each other. Recently, Howard et al. reported that the CD spectrum of a $(dT)_n$:$(dA)_n$:$(dA)_n$-type triplex had maxima at 218 and 282 nm, a shoulder near 260 nm, and a minimum at 248 nm.[15] Therefore, these CD spectral data also support the existence of a triplex between branched ODN **2** and $(dT)_{21}$.

Gel Retardation Assay. The formation of triplexes was further confirmed by a gel retardation assay. Gel retardation assays were carried out in a 90 mM Tris-borate buffer (pH = 7.8) containing 10 mM $MgCl_2$ at room temperature. The experiments presented in Figure 5 show the formation of the $(dT)_{14}$:$(dA)_{21}$*:$(dT)_{14}$ triplex (lanes 1−7), the $(dA)_{21}$:ODN **1*** triplex (lanes 8−13), and the $(dA)_{21}$*:ODN **1** triplex (lanes 14−19). The asterisks indicate ^{32}P-labeled ODNs.

As can be seen in Figure 5, lanes 1−7, as the molar ratio of $(dT)_{14}$ increased, the bands corresponding to the double strand [$(dA)_{21}$*:$(dT)_{14}$] (Figure 5, lanes 2−4), which has a slower mobility than $(dA)_{21}$*, and those corresponding to the triple strand [$(dT)_{14}$:$(dA)_{21}$*:$(dT)_{14}$], which have slower mobilities than $(dA)_{21}$* and $(dA)_{21}$*:$(dT)_{14}$, became more intense. On the other hand, when $(dA)_{21}$ was added to a solution containing ODN **1***, bands with mobilities similar to that of the $(dT)_{14}$:$(dA)_{21}$*:$(dT)_{14}$ triplex were observed (Figure 5, lanes 9−13). We presumed that the bands were due to the formation of a $(dA)_{21}$:ODN **1*** triplex. Similarly, when ODN **1** was added to a solution containing $(dA)_{21}$*, bands with mobilities similar to that of the $(dT)_{14}$:$(dA)_{21}$*:$(dT)_{14}$ triplex were also observed (Figure 5, lanes 15−19).

Conclusion. To find ODNs with triplex stabilization capability, we designed and synthesized novel branched ODNs **1** and **2** with pentaerythritols at their branch points. Branched ODNs **1** and **2** were synthesized on a solid support using bis(phosphoramidite) **11**. The stability of the triplexes formed by branched ODNs **1** and **2** with $(dA)_{21}$ or $(dT)_{21}$ was studied by thermal denaturation. Branched ODN **1** formed a stable parallel T·AT-type triplex with $(dA)_{21}$ (T_m = 38.9 °C) in a buffer of 0.01 M sodium phosphate (pH 7.0) containing 0.5 M NaCl and 0.02 M $MgCl_2$, and branched ODN **2** formed a stable antiparallel A·AT-type triplex with $(dT)_{21}$ (T_m = 44.2 °C) in the same buffer. The formation of these triplexes was also confirmed by circular dichroism (CD) measurements and a gel retardation assay.

Recently, several kinds of branched ODNs with 3′-deoxy-β-D-psicothymidine, 1-(2-methyl-β-D-arabinofuranosyl)uracil, or 4′-*C*-(hydroxymethyl)thymidine at their branch points have been synthesized, and some of these have been shown to form stable triplexes with single-stranded ODNs.[7,8] Branched ODNs can target single-stranded ODNs or RNAs. Therefore, we believe that branched ODNs will be a new class of antisense ODNs.

Experimental Section

NMR spectra were recorded at 270 MHz (^{1}H), at 125 MHz (^{13}C), and at 202 MHz (^{31}P) and are reported in ppm downfield from TMS or 85% H_3PO_4. *J* values are given in hertz. Mass spectra were obtained by fast atom bombardment (FAB) method. Thin-layer chromatography was done on Merck coated plates $60F_{254}$. The silica gel or the neutralized silica gel used for column chromatography were Merck silica gel 5715 or ICN silica 60A, respectively.

2-[[(*tert*-Butyldiphenylsilyl)oxy]methyl]-2-(hydroxymethyl)-1,3-propanediol (6a), 2,2-Bis[[(*tert*-butyldiphenylsilyl)oxy]methyl]-1,3-propanediol (6b), and 3-*O*-(*tert*-Butyldiphenylsilyl)-2,2-bis[[(*tert*-butyldiphenysilyl)oxy]-methyl]-1,3-propanediol (6c). A mixture of pentaerythritol **5** (4.08 g, 30.0 mmol), TBDPSCl (17.2 mL, 66.1 mmol), and imidazole (8.99 g, 132 mmol) in DMF (500 mL) was stirred at

(15) Howard, F. B.; Miles, H. T.; Ross, P. D. *Biochemistry* **1995**, *34*, 7135−7144.

room temperature. After 30 min, EtOH (10 mL) was added to the mixture, and the whole was stirred for 10 min. The mixture was concentrated in vacuo and was taken in AcOEt, which was washed with saturated aqueous $NaHCO_3$ and brine. The separated organic phase was dried (Na_2SO_4) and concentrated to dryness. The residue was purified by column chromatography (SiO_2, 0−5% MeOH in $CHCl_3$) to give **6a**[10] (1.48 g, 13%), **6b** (10.1 g, 55%), and **6c** (7.95 g, 31%). The physical data of **6a**: mp 69−72 °C; ^{1}H NMR ($CDCl_3$) δ 7.67−7.37 (m, 10H), 3.72 (d, 6H, J = 4.8), 3.64 (s, 2H), 2.60 (t, 3H, J = 4.8, D_2O exchangeable), 1.07 (s, 18H); ^{13}C NMR ($CDCl_3$, ^{13}C signals were assigned on the basis of DEPT experiment) δ 135.59 (CH), 132.64 (C), 130.03 (CH), 127.91 (CH), 65.32 (CH_2), 64.83 (CH_2), 45.62 (C), 26.91 (CH_3), 19.21 (C); HRMS (FAB) calcd for $C_{21}H_{31}O_4Si$ 375.1990, found 375.1974. Anal. Calcd for $C_{21}H_{30}O_4Si$: C, 67.34; H, 8.07. Found: C, 67.44; H, 7.90. The physical data of **6b**: mp 85−86 °C; ^{1}H NMR ($CDCl_3$) δ 7.62−7.32 (m, 20H), 3.75 (d, 4H, J = 6.0), 3.67 (s, 4H), 2.26 (t, 2H, J = 6.0, D_2O exchangeable), 1.02 (s, 18H); ^{13}C NMR ($CDCl_3$, ^{13}C signals were assigned on the basis of DEPT experiment) δ 135.6 (CH), 132.8 (C), 129.9 (CH), 127.8 (CH), 64.8 (CH_2), 64.6 (CH_2), 46.4 (C), 26.9 (CH_3), 19.2 (C); HRMS (FAB) calcd for $C_{37}H_{49}O_4Si_2$ 613.3167, found 613.3141. Anal. Calcd for $C_{37}H_{48}O_4Si_2$: C, 72.50; H, 7.89. Found: C, 72.49; H, 7.84. The physical data of **6c**: mp 92−94 °C; ^{1}H NMR ($CDCl_3$) δ 7.58−7.25 (m, 30H), 3.81 (d, 2H, J = 6.0), 3.74 (s, 6H), 2.78 (t, 1H, J = 6.0, D_2O exchangeable), 0.96 (s, 27H); ^{13}C NMR ($CDCl_3$, ^{13}C signals were assigned on the basis of DEPT experiment) δ 135.6 (CH), 132.6 (C), 130.0 (CH), 127.9 (CH), 65.3 (CH_2), 64.7 (CH_2), 64.6 (CH_2), 45.6 (C), 25.9 (CH_3), 19.2 (C); HRMS (FAB) calcd for $C_{53}H_{67}O_4Si_3$ 851.4343, found 851.4391. Anal. Calcd for $C_{53}H_{66}O_4Si_3$: C, 74.77; H, 7.81. Found: C, 74.76; H, 7.97.

1,3-*O*-Bis(*tert*-butyldiphenysilyl)-2,2-bis[[[*N*-(3-hydroxypropyl)carbamoyl]oxy]methyl]-1,3-propanediol (7). *N*,*N*-Carbonyldiimidazole (9.73 g, 60.0 mmol) and DMAP (370 mg, 3.00 mmol) were added to a solution of **6b** (9.19 g, 15.0 mmol) in DMF (50 mL), and the mixture was stirred at room temperature. After 40 min, 3-amino-1-propanol (17.2 mL, 225 mmol) was added to the mixture, and the whole was stirred at room temperature for 1 h. The mixture was concentrated in vacuo and was taken in AcOEt, which was washed with H_2O and brine. The separated organic phase was dried (Na_2SO_4) and concentrated to dryness. The residue was purified by column chromatography (SiO_2, 1% MeOH in $CHCl_3$) to give **7** (10.9 g, 88%): mp 148−149 °C; ^{1}H NMR ($CDCl_3$) δ 7.64−7.31 (m, 20H), 4.69 (br s, 2H, J = 5.8, D_2O exchangeable), 4.09 (s, 4H), 3.67 (s, 4H), 3.62 (m, 4H), 3.28 (m, 4H), 2.80 (br s, 2H, D_2O exchangeable), 1.65 (m, 4H), 1.02 (s, 18H); ^{13}C NMR ($CDCl_3$, ^{13}C signals were assigned on the basis of DEPT experiment) δ 157.2 (C), 135.7 (CH), 133.2 (C), 129.7 (CH), 127.7 (CH), 65.9 (CH_2), 61.4 (CH_2), 59.4 (CH_2), 50.5 (CH_3), 45.8 (C), 37.4 (CH_2), 32.5 (CH_2), 26.9 (CH_3), 19.3 (C); HRMS (FAB) calcd for $C_{45}H_{63}N_2O_8Si_2$ 815.4119, found 815.4087. Anal. Calcd for $C_{45}H_{62}N_2O_8Si_2$: C, 66.30; H, 7.67; N, 3.44. Found: C, 66.32; H, 7.68; N, 3.66.

2,2-Bis[[[*N*-[3-*O*-(4,4′-dimethoxytrityloxy)propyl]carbamoyl]oxy]methyl]-1,3-propanediol (9). A mixture of **7** (10.0 g, 12.3 mmol) and DMTrCl (9.17 g, 27.1 mmol) in pyridine (50 mL) was stirred at room temperature for 1 h. EtOH (15 mL) was added to the mixture, and the whole was stirred for 10 min. The mixture was evaporated under reduced pressure. The residue was taken in AcOEt, which was washed with saturated aqueous $NaHCO_3$ and brine. The separated organic phase was dried (Na_2SO_4) and concentrated to dryness. The residue was dissolved in THF (50 mL). TBAF (1 M in THF, 50 mL, 50 mmol) was added to the solution, and the resulting mixture was stirred overnight at room temperature. The mixture was evaporated under reduced pressure and was taken in AcOEt, which was washed with H_2O and brine. The separated organic phase was dried (Na_2SO_4) and concentrated to dryness. The residue was purified by column chromatography (SiO_2, 0−2% MeOH in $CHCl_3$) to give **9** (9.88 g, 85%): mp 148−149 °C; ^{1}H NMR (DMSO-d_6) δ 7.37−7.21 (m, 18H), 6.97 (br s, 2H, D_2O exchangeable), 6.89−6.86 (m, 8H), 4.44 (br s, 2H, D_2O exchangeable), 3.88 (s, 4H), 3.72 (s, 12H), 3.36 (m, 4H), 3.06 (m, 4H), 2.98 (t, 4H), 1.67 (br s, 4H); ^{13}C NMR ($CDCl_3$, ^{13}C signals were assigned on the basis of DEPT experiment) δ 158.4 (C), 157.1 (C), 144.8 (C), 136.1 (C), 129.9 (CH), 128.0 (CH), 127.8 (CH), 126.8 (CH), 113.1 (CH), 86.3 (C), 62.4 (CH_2), 61.7 (CH_2), 61.5 (CH_2), 55.2 (CH_3), 46.1 (C), 39.4 (CH_3), 29.6 (CH_2). Anal. Calcd for $C_{55}H_{62}N_2O_{12}\cdot H_2O$: C, 68.73; H, 6.71; N, 2.91. Found: C, 68.74; H, 6.51; N, 2.93.

1,3-*O*-Bis[[[*N*-[3-*O*-(4,4′-dimethoxytrityloxy)propyl]carbamoyl]-2,2-bis[[*N*-(3-hydroxypropyl)carbamoyl]oxy]-methyl]-1,3-propanediol (10). *N*,*N*-Carbonyldiimidazole (6.49 g, 40.0 mmol) and DMAP (120 mg, 1.00 mmol) were added to a solution of **9** (9.43 g, 10.0 mmol) in DMF (40 mL), and the mixture was stirred at room temperature. After 40 min, 3-amino-1-propanol (11.5 mL, 150 mmol) was added to the mixture, and it was stirred at room temperature for 1 h. The mixture was concentrated in vacuo and was taken in AcOEt, which was washed with H_2O and brine. The separated organic phase was dried (Na_2SO_4) and concentrated to dryness. The residue was purified by column chromatography (SiO_2, 1−3% MeOH in $CHCl_3$) to give **10** (10.4 g, 91%): ^{1}H NMR (DMSO-d_6) δ 7.37−7.20 (m, 18H), 7.09 (br s, 4H, D_2O exchangeable), 6.89−6.86 (m, 8H), 4.39 (t, 2H, J = 5.0, D_2O exchangeable), 3.94 (br s, 8H), 3.72 (s, 12H), 3.39 (m, 4H), 3.05−2.97 (m, 12H), 1.66 (m, 4H), 1.54 (m, 4H); ^{13}C NMR ($CDCl_3$, ^{13}C signals were assigned on the basis of DEPT experiment) δ 158.4 (C), 145.0 (C), 136.2 (C), 129.9 (CH), 128.1 (CH), 127.8 (CH), 126.8 (CH), 113.1 (CH), 86.2 (C), 61.6 (CH_2), 59.6 (CH_2), 55.2 (CH_3), 39.2 (CH_2), 38.0 (CH_2), 32.3 (CH_2), 29.7 (CH_2); HRMS (FAB) calcd for $C_{63}H_{77}N_4O_{16}$ 1145.5330, found 1145.5410. Anal. Calcd for $C_{63}H_{76}N_4O_{16}\cdot{}^3/_4H_2O$: C, 65.30; H, 6.74; N, 4.83. Found: C, 65.23; H, 6.60; N, 4.90.

2,2-Bis[[[*N*-[3-*O*-[[*β*-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphino]oxy]propyl]carbamoyl]oxy]methyl]-1,3-bis[*N*-[3-*O*-(4,4′-dimethoxytrityloxy)propyl]carbamoyl]-1,3-propanediol (11). After successive coevaporation with pyridine, **10** (458 mg, 0.40 mmol) was dissolved in CH_2Cl_2 (12 mL) containing *N*,*N*-diisopropylethylamine (0.28 mL, 1.60 mmol). 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.27 mL, 1.20 mmol) was added to the solution, and the reaction mixture was stirred for 10 min at room temperature. Aqueous saturated $NaHCO_3$ and $CHCl_3$ were added to the mixture, and the separated organic layer was washed with aqueous saturated $NaHCO_3$, brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (neutralized SiO_2, AcOEt) to give **11** (400 mg, 65%): ^{1}H NMR ($CDCl_3$) δ 7.42−6.81 (m, 26H), 5.14 (br s, 2H), 4.98 (br s, 2H), 4.03 (s, 8H), 3.78 (s, 12H), 3.89−3.42 (m, 12H), 3.26 (m, 8H), 3.16 (t, 4H), 2.63 (t, 4H), 1.78 (m, 8H), 1.19 (d, 12H), 1.17 (d, 12H); ^{31}P NMR ($CDCl_3$) δ 148.51.

Synthesis of ODNs. ODNs were synthesized on a DNA synthesizer (Applied Biosystem Model 391A) by using a slightly modified 1-μmol-scale cycle in the "trityl off" mode. The 1-μmol-scale cycle supplied by ABI was modified in the following manner: *phosphoramidite coupling*, a 300-s "wait" for 5′-*O*-(dimethoxytrityl)thymidine 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) and N^6-benzoyl-5′-*O*-(dimethoxytrityl)adenosine 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) and a 600-s "wait" for **11**. A 0.025 M solution of **11** in CH_3CN was used in the branching step. Controlled-pore glass (CPG, 500-Å pore size, Funakoshi Co.) was used as a solid support. The ODN (1 μmol) linked to the solid support was treated with concentrated NH_4OH (2 mL) at 55 °C for 16 h. The ammoniacal solutions of ODNs were concentrated under reduced pressure. The crude ODNs were purified by 20% polyacrylamide gel electrophoresis under denaturing conditions (7 M urea), followed by reversed-phase HPLC. The deprotected ODNs **1** and **3** were obtained in 19 and 21 OD_{260} units, respectively. Similarly, ODNs **2** and **4** were obtained in 17 and 16 OD_{260} units, respectively.

Electrospray Ionization Mass Spectrometry. Spectra were obtained on a Quattro II (Micromass, Manchester, UK) triple quadrupole mass spectrometer equipped with an ESI source in the negative ion mode. The HPLC-purified ODN samples were dissolved in aqueous 50% 2-propanol containing 1% triethylamine (10 pmol ODN/μL) and introduced into the

ion source through a loop injector with a carrier solvent, 33% aqueous methanol, flowing at 10 mL/min flow rate. About 15 scans were acquired in approximately 1 min period and combined to obtain smoothed spectra. All molecular masses of the ODNs were calculated from the multiple-charge negative-ion spectra. The observed average molecular masses of **1**, **2**, **3**, and **4** were 9058.6, 9309.0, 7008.9, and 7197.1, respectively, and fit the calculated molecular weights (theoretical average molecular masses) for these compounds, i.e., 9058.0 (for **1**, $C_{301}H_{404}N_{60}O_{208}P_{28}$), 9310.4 (for **2**, $C_{301}H_{376}N_{144}O_{152}P_{28}$), 7008.7 (for **3**, $C_{231}H_{314}N_{46}O_{162}P_{22}$), and 7197.9 (for **4**, $C_{231}H_{293}N_{109}O_{120}P_{22}$) within a commonly accepted error range of ESI MS, 0.01%.

Thermal Denaturation and CD Spectroscopy. Each solution containing each branched ODN (3 μM) and each single stranded DNA (3 μM) in an appropriate buffer was heated at 100 °C for 3 min and then gradually cooled to 4 °C. Thermal-induced transitions of each mixture were monitored at 260 and 284 nm on a Perkin-Elmer Lambda2S. Sample temperature was increased 0.5 °C/min. Extinction coefficients of the branched ODNs were calculated from those of mononucleotides and dinucleotides according to the nearest-neighbor approximation.[16] Samples for CD spectroscopy were prepared by the same procedure used in the thermal denaturation study, and spectra were measured at 5 °C. The ellipticities of triplexes were recorded from 200 to 350 nm in a cuvette with a path length 1 mm. CD data were converted into mdeg·mol of residues^{-1}·cm^{-1}.

Gel Electrophoresis. For gel retardation assays of the triplexes, a nondenaturating 15% polyacrylamide gel (99:1 acrylamide/bisacrylamide) containing 90 mM Tris-borate (pH = 7.8) and 10 mM $MgCl_2$ was prepared and run at room temperature using same buffer. ODNs were labeled with ^{32}P at the 5′-end using [γ-^{32}P]ATP and T4 polynucleotide kinase.[17] A solution containing each ODN in a 90 mM Tris-borate buffer (pH = 7.8) containing 10 mM $MgCl_2$ was heated at 100 °C for 3 min and then gradually cooled to room temperature. The samples were loaded in 15% Ficoll (Pharmacia type 400). Electrophoresis was performed for 20 h with 60 V at room temperature. After electrophoresis, gels were autoradiographed using Kodak X-Omat JB film at −70 °C.

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(16) Puglisi, J. D.; Tinoco, I., Jr. In *Methods in Enzymology*; Dahlberg, J. E., Abelson, J. N., Eds.; Academic Press: San Diego, 1989: Vol. 180, pp 304−325.

(17) Maniatis, T.; Fritsch, E. F.; Sambrook, J. In *Molecular Cloning*: *a Laboratory Manual*; Cold Spring Harbor University Press: Cold Spring Harbor, NY, 1982.