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Synthesis of Branched Oligonucleotides with Three Different Sequences Using an Oxidatively Removable Tritylthio Group

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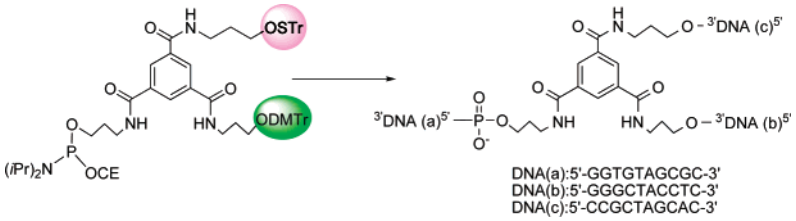
Received June 3, 2007



We synthesized a three-way branched oligodeoxynucleotide (ODN) 30-mer using a new branch unit with acid-labile DMTr and oxidatively cleavable TrS groups as orthogonal protecting groups. The branched ODN was successfully synthesized using 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole and (2*R*,8a*S*)-(+)-(camphorylsulfonyl)oxaziridine as the activator of phosphoramidite units and the oxidizing reagent, respectively. We also found that the TrS group was orthogonal to the Lev, TBDMS, and Fmoc groups. These results indicate the possibility of the synthesis of more complex four- and five-way branched ODNs by the combined use of DMTr, TrS, Lev, TBDMS, and Fmoc groups.

Introduction

Recently, much interest has been paid to synthetic branched oligodeoxynucleotides (ODNs).[1] For example, branched ODNs have shown high affinity for single-stranded ODNs to form alternate-strand triplexes.[2,3] Another compound with three different ODNs was used as an efficient template for the straightforward synthesis of trimeric structures.[4] In addition, central building units with branching points were used for the synthesis of dendrimeric ODNs as multilabeled DNA probes to increase the sensitivity of hybridization experiments.[5−7]

Branched ODNs have also been used in the construction of programmed nanostructures. In particular, branched ODNs containing different sequences have often been used as the vertices of such nanostructures including tetrahedral and cubic forms.[8−14]

In general, central phosphoramidite building units having two hydroxyl groups protected with different protecting groups, PRO^1 and PRO^2, have been used to synthesize three-way branched ODNs, as depicted in Figure 1. For example, first, the DNA (a) is synthesized in the usual manner on solid supports. Next, the DMTr group is removed, and a central phosphoramidite building unit is linked to the resulting 5′-terminal hydroxyl group of DNA (a). After removing one of the two hydroxyl protecting groups (PRO^1), similar chain elongation is continued at this branching site until a new DNA

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10.1021/jo071173a CCC: $37.00
Published on Web 09/27/2007

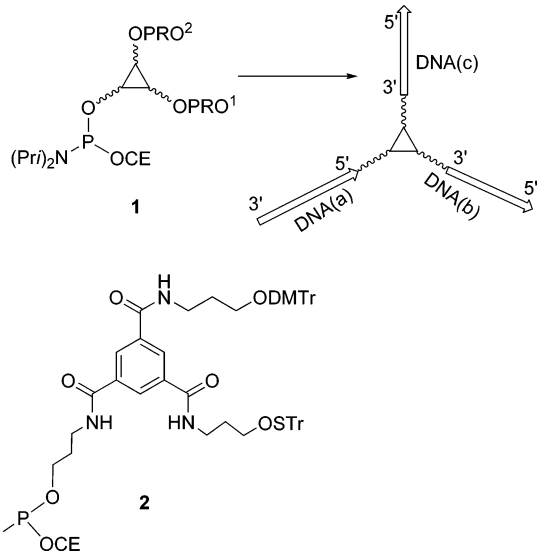
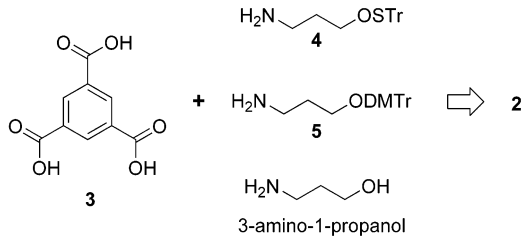


FIGURE 1. General structure of branched building unit **1** and a designed branched building unit **2**.

SCHEME 1. Synthetic Route to 2



chain (b) is elongated. Finally, the remaining protecting group PRO^2 at the branching center is removed, and the third DNA chain (c) is constructed in a similar manner. In this synthesis of branched ODN, the PRO^1 and PRO^2 groups must be cleaved independently under mild conditions, and the selection is quite important. Previously, DMTr/Fmoc,[15,16] DMTr/levulinyl,[17−21] and DMTr/TBDMS[22−24] have been reported as PRO^1/PRO^2 in the literature.

In this paper, we report a new set of protecting groups, PRO^1/PRO^2, for the synthesis of three-way branched molecules with three different ODNs. As PRO^1 and PRO^2 on the branched building unit, we chose the tritylthio (TrS) and DMTr groups, respectively, as shown in compound **2** of Figure 1 and proved

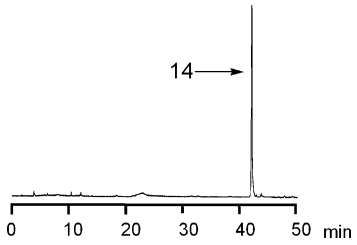


FIGURE 2. Reversed-phase HPLC profile of the mixture obtained after treatment of **13** with 3% TCA.

the usefulness of this branch unit in the synthesis of a branched ODN 30-mer. Although we previously reported the use of the methoxytritylthio (MMTrS) group, which can be removed by mild oxidation as a protecting group for the 5′-hydroxyl group during ODN synthesis,[25−28] we chose the TrS group in this study instead of the MMTrS group because it was stable under the acidic conditions for the removal of the DMTr group. Since the DMTr and TrS groups can be removed under mild acidic and oxidative conditions, respectively, base-labile protecting groups can remain intact. Moreover, because the TrS group was proved to be stable toward various basic reagents such as piperidine, hydrazine, and TBAF, the possibility of using the TrS group in combination with other protecting groups, such as Fmoc, Lev, and TBDMS, was indicated as discussed later.

Results and Discussion

Branch Unit Synthesis. As a branch unit, we designed **2** which can be synthesized from trimesic acid (**3**) and two O-protected 3-amino-1-propanol derivatives **4** and **5** (Scheme 1).

First, the synthesis of O-protected 3-amino-1-propanol derivatives **4** and **5** was examined. Compound **4** was synthesized from 1,3-propanediol in 57% yield in three steps (see Supporting Information). Compound **5** has already been reported in the literature.[29] The synthesis of the branch unit **2** is shown in Scheme 2. Condensation of the amine **5** with trimesic acid dimethyl ester[30,31] (**6**) in the presence of 1,1′-carbonyldiimidazole (CDI) gave compound **7** in 90% yield, as shown in Scheme 2. Treatment of **7** with 1 M NaOH gave the monocarboxylic acid **8** as the triethylammonium salt after purification by column chromatography using the solvent system containing triethylamine. Similarly, the amine **4** was condensed with compound **8** to give the diamide **9** in a quantitative yield, which, *in turn*, was converted to the monocarboxylate **10** in 87% yield by a similar alkaline treatment as mentioned above. Finally, compound **10** was coupled with 3-amino-1-propanol to give compound **11** in 94% yield. The alcohol **11** was converted in the usual manner to the phosphoramidite derivative **2** in 63% yield.

Stability of the TrS Group Toward Detritylation Conditions. To confirm the stability of the TrS group toward

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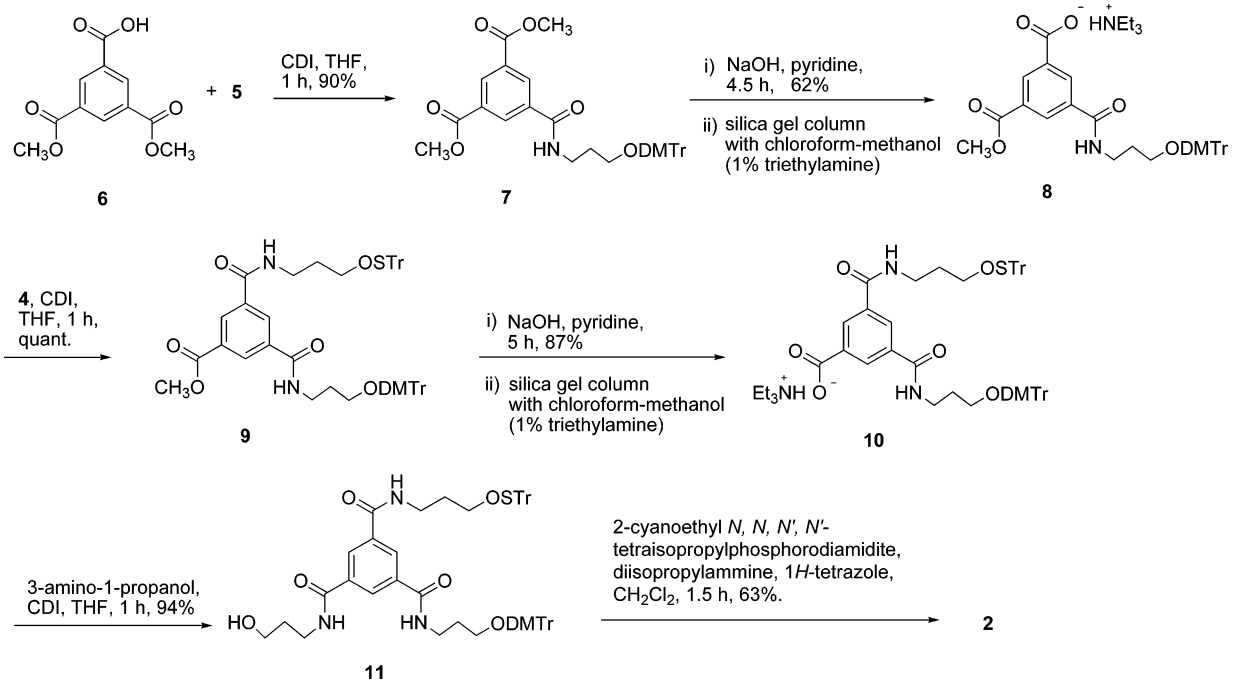
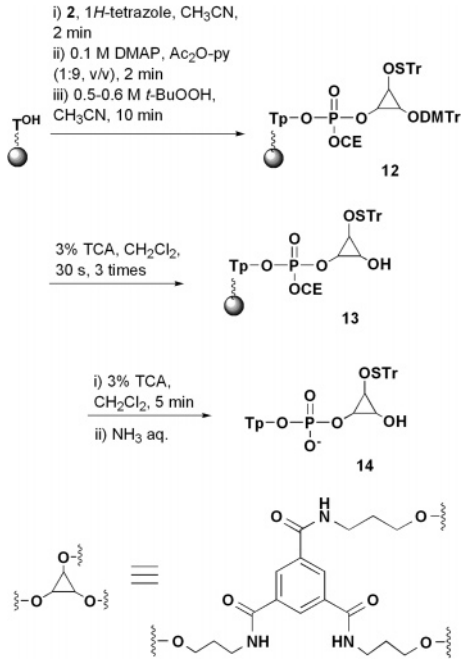
SCHEME 2. Synthesis of the Branch Unit 2



SCHEME 3. Stability of the TrS Group under Detritylation Conditions



deprotection conditions of the DMTr group on solid supports, several experiments were conducted using the solid support **12**, as shown in Scheme 3. This support was prepared by condensation of the branch unit **2** with the 5′-hydroxyl group of thymidine-loaded CPG followed by successive capping and oxidation reactions. First, the DMTr group was removed from **12** by treatment with 3% trichloroacetic acid (3% TCA, 30 s × 3). Subsequently, the support was exposed again to 3% TCA at room temperature for 5 min. We chose such a two-step treatment with 3% TCA in order to simplify the reaction conditions by removing the still reactive DMTr cation. The product **13** was treated with aq NH_3, and the desired compound **14** was analyzed by reversed-phase HPLC. As shown in Figure 2, a new compound with the TrS group intact was obtained in high purity at the retention time of 42 min. This result suggested that the TrS group was sufficiently stable under the conditions for the removal of the DMTr group. In addition to the HPLC analysis, we confirmed the structure of **14** by MALDI-TOF mass analysis.

Synthesis of Branched ODN 9-mer. Next, we determined the best conditions for the synthesis of branched ODNs by synthesizing a simple model compound **15** with three trithymidylates at the three positions, as shown in Scheme 4. In this structure, two of the three TpTpT strands of **15** are attached to the trimesic acid branch point at their 3′-ends, whereas the other TpTpT is attached at its 5′-end. To avoid the cleavage of the sulfenyl ester by iodine oxidation, the oxidizer should be changed to other reagents during chain elongation of pTpTpT in the presence of the TrS group. In this study, we tested two oxidizers, *tert*-butyl peroxide (*t*-BuOOH)[32] and (2*R*,8a*S*)-(+)-(camphorylsulfonyl)oxaziridine (CSO).[33]

In addition, the activating reagents were also optimized by testing three kinds of activating agents, 1*H*-tetrazole (Tet), 5-benzylthio-1*H*-tetrazole (BTT),[34] and 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole (Activator 42: Act42),[35] because the coupling yield was expected to be lowered by the synthesis of oligonucleotides with unusual structures, such as branched ODNs.

The branch unit **2** (40 equiv) was condensed with the phosphate-protected trithymidylate **16**, which was synthesized in advance on the CPG support using one of the above-mentioned activating reagents, by use of 80 equiv of Tet, BTT, or Act42 for 2 min. After the capping of the remaining hydroxyl

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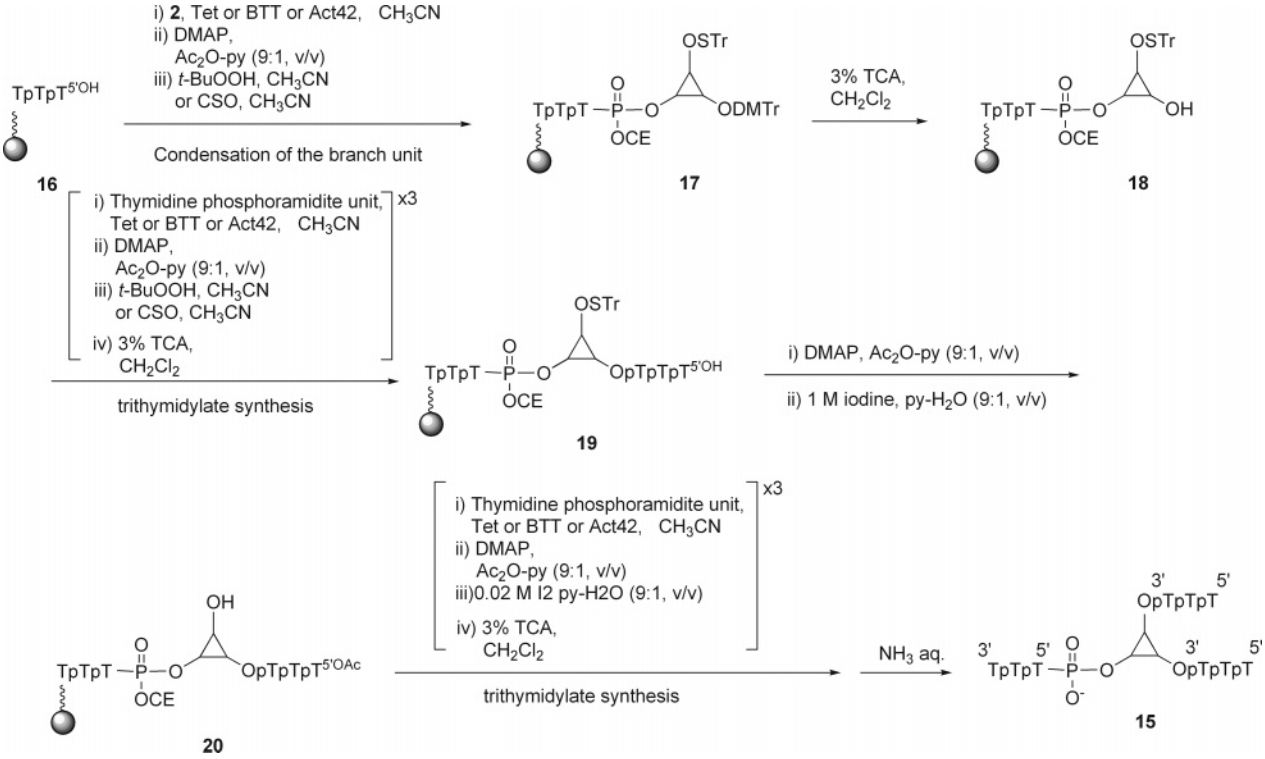
SCHEME 4. Synthesis of the Branched ODN 15 with Three Trithymidylates



function by treatment with Ac_2O/pyridine (9:1, v/v) in the presence of 0.1 M DMAP for 2 min, the oxidation reactions were carried out using either *t*-BuOOH (0.5−0.6 M in CH_3CN, 10 min) or CSO (0.5 M in CH_3CN, 3 min) to give **17**. After the detritylation of the branch unit **17** by treatment with 3% TCA/CH_2Cl_2 (30 s × 3), similar stepwise condensations of a thymidine phosphoramidite unit to the resulting hydroxyl component **18** (three times) gave compound **19**. In this synthesis, Tet, BTT, or Act42 and either *t*-BuOOH or CSO were also tested as condensing and oxidizing agents, respectively. The 5′-hydroxyl group of the terminal thymidine residue of **19** was capped by acetylation, and then the TrS group on the branch unit was removed by treatment with a 1 M iodine in pyridine/H_2O (9:1, v/v) for 2 min to give compound **20**. The third trithymidylate chain was synthesized using an activating reagent, Tet, BTT, or Act42, and iodine oxidation. Thus, compound **15** was obtained after NH_3 treatment.

The resulting product was analyzed by reversed-phase HPLC, as shown in Figure 3. When Tet was used as the activator in combination with *t*-BuOOH as the oxidizer, shorter sequences resulting from failure coupling were observed, as shown in Figure 3a. This result indicated that the activation of phosphoramidite by Tet was not enough for the synthesis of the ODNs with an unusual branch structure. Although changing the activating reagent from Tet to BTT slightly decreased the ratio of failure sequences to **15** (data not shown), a stronger activating reagent Act42 gave a better result, as shown in Figure 3b. These results indicated that the increase of coupling efficiency could be an essential factor that improves the synthesis of the branched ODN. Further improvement was achieved by changing the oxidizer from *t*-BuOOH to CSO. By this combination, the target material was synthesized in much better purity. Since CSO is a stronger oxidizer than *t*-BuOOH, it could oxidize the phosphite intermediate more completely and in a shorter reaction time (3 min for CSO, 10 min for *t*-BuOOH). In these experiments, structure of **15** was characterized by MALDI-TOF mass analyses.

We also checked the stability of the TrS group toward CSO (3 min × 10) in a protocol similar to that described in Scheme 3. Briefly, **12** was prepared on solid phase by use of 0.5 M CSO/CH_3CN (3 min × 10), which corresponded to the oxidation time required for the synthesis of oligodeoxynucleotide 10-mers, in place of the *t*-BuOOH treatment. After the detritylation by 3% TCA (30 s × 3) and the aqueous ammonia treatment, the resulting mixture containing **14** was analyzed by reversed-phase HPLC. Although small amounts of byproducts were observed at around 10 min, the HPLC profile showed the presence of **14** having the intact TrS group with good purity (Figure S20). The

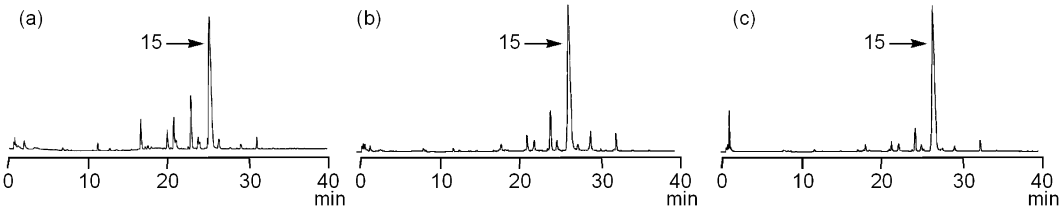
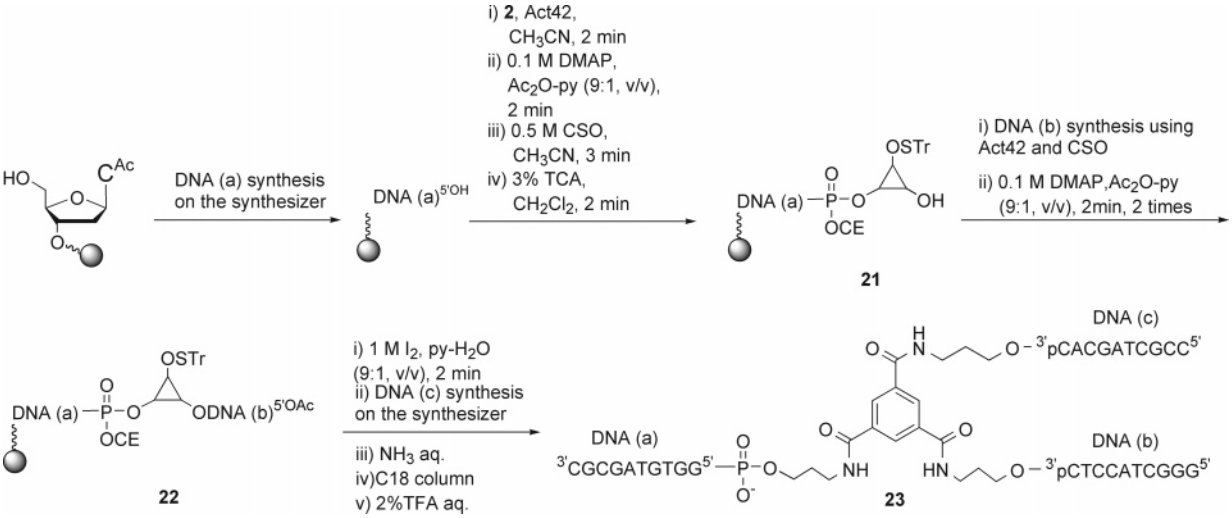


FIGURE 3. Reversed-phase HPLC profiles of the branched ODN oligomer **15**. Panel (a): Tet and *t*-BuOOH were used as the activator and the oxidizing agent, respectively. Panel (b): Act42 and *t*-BuOOH were used. Panel (c): Act42 and CSO were used. See Experimental Section.

SCHEME 5. Synthesis of 23



partial elimination of the TrS group during the CSO treatment indicated the possible formation of the side products during the synthesis of the second DNA fragments such as the DNA (b) described below. However, the byproducts generated by such side reactions could be easily separated from the target molecule by a simple DMTr-On purification on C18 reversed-phase column after the elongation of the third DNA fragments such as the DNA (c). Considering these results of CSO totally, we decided to use the protocol using CSO to synthesize a longer branched DNA 30-mer.

Synthesis of Branched ODN 30-mer. Finally, the synthesis of a branched ODN 30-mer **23** was achieved from the CPG support by the combined use of the conventional protocol of DNA synthesis on an automated DNA synthesizer and the manual synthesis using Act42 and CSO instead of Tet and aqueous iodine, respectively, as shown in Scheme 5. In this structure, DNA (a), DNA (b), and DNA (c) should have the sequences that did not interact with each other. For this purpose, we chose a set of three sequences, 5′-GGTGTAGCGC-3′, 5′-GGGCTACCTC-3′, and 5′-CCGCTAGCAC-3′, which were reported by Endo et al. in the development of programmable DNA nanostructures.[13] Similarly to **15**, DNA (a) was attached to the branch point at its 5′-end, and DNAs (b) and (c) were attached at their 3′-ends.

The DNA (a):5′-GGTGTAGCGC-3′ was elongated on a DNA synthesizer, and the branch unit **2** (40 equiv) was condensed with the 5′-terminal hydroxyl group of DNA (a) in the presence of Act42 (80 equiv) to give **21**. After the elongation of DNA (b):5′-GGGCTACCTC-3′ using Act42 and CSO under the conditions identical to those used for the synthesis of **15**, the 5′-end of the terminal G residue was capped using acetic anhydride to give **22**. Finally, the TrS group was removed by treatment with 1 M iodine solution, and the last strand DNA (c):5′-CCGCTAGCAC-3′ was elongated on an automated DNA synthesizer. To simplify the purification procedure, the DMTr group on the last DNA (c) sequence was not removed before exposing the aqueous ammonia solution. After the cleavage and deprotection of the phosphate and base protecting groups by treatment with aqueous ammonia, the compound with the DMTr group was isolated from the crude material by reversed-phase HPLC. After its isolation, the DMTr group was removed by treatment with aqueous 2% TFA to give the target material **23**. Shown in Figure 4 is the anion-exchange HPLC profile of the branched 30-mer **23**, which showed high purity even after isolation. The structure of **23** was characterized by the MALDI-TOF mass analysis.

Hybridization Properties of the Branched ODN 30-mer 23. Hybridization properties of **23** with the complementary DNA strands were studied. Here, the complementary strand to the DNA (a) strand of **23** is named DNA (a′):5′-GCGCTACACC-3′; the one complementary to DNA (b) is DNA (b′):5′-GAGGTAGCCC-3′; and the one complementary to DNA (c) is DNA (c′):5′-GTGCTAGCGG-3′. The complementary strands of DNA (a′), (b′), and (c′) were separately hybridized to **23**. Each oligonucleotide was dissolved in 10 mM sodium cacodylate (pH 7.0) containing 0.5 M NaCl and 10 mM $MgCl_2$ so that the final concentration of each ODN became 1.5 μM. The melting curves of the complexes formed between **23** and its complementary ODNs showed two inflection points (Figure 5A−C and Figure S21A−C). The T_m values are listed in Table 1. In all cases, melting curves at higher temperatures (around 75 °C) were commonly observed. In contrast, those at lower temperatures were specific in each case. The DNA (a)/(a′) duplex showed the lowest melting point (25 °C) compared to the other duplexes (50 and 51 °C). Interestingly, the branched ODN 30-mer **23** in the absence of any complementary strands showed an inflection point at around 75 °C (Figures 4D and S21D). From those experiments, we presumed that **23** formed a higher-order structure and the higher inflection point corresponds to the melting point of the higher-order structure. The lower melting points would be those of the duplexes formed between **23** and the complementary strands. It should be noted that the DNA duplexes having the sequences identical to those of DNA (a)/DNA (a′), DNA (b)/DNA (b′), and DNA (c)/ DNA (c′) had similar T_m values of 53, 48, and 53 °C, respectively

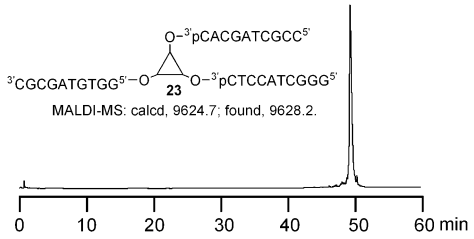


FIGURE 4. Anion exchange HPLC profile of the branched ODN 30-mer **23**.

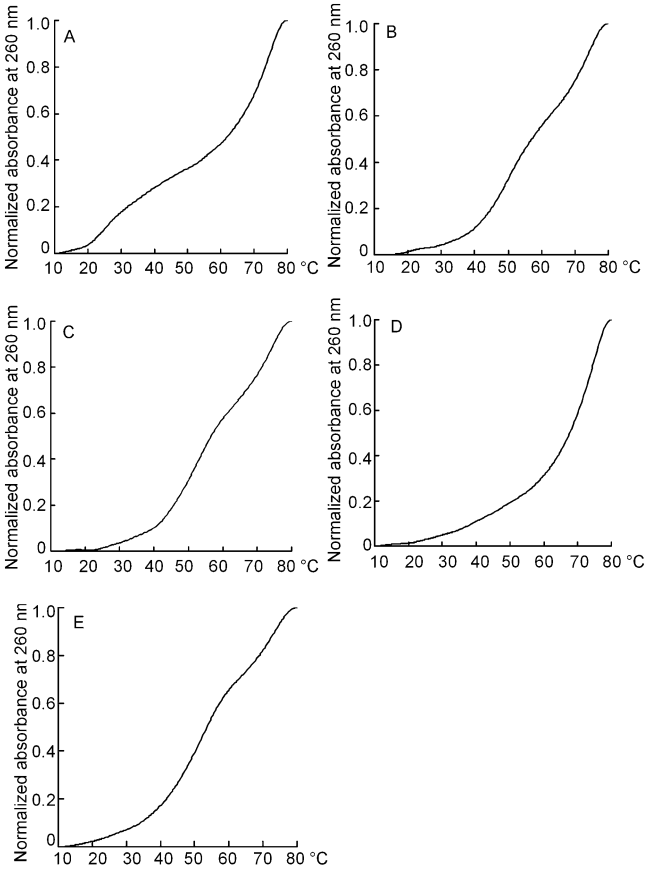


FIGURE 5. Melting profile of the complexes formed. Panel (A): between **23** and DNA (a′). Panel (B): between **23** and DNA (b′). Panel (C): between **23** and DNA (c′). Panel (D): melting profile of **24**. Panel (E): **23** and DNA (a′) + DNA (b′) + DNA (c′).

TABLE 1. Melting Temperatures at 260 nm and Sequences of Complementary Strands of DNA (a′), (b′), and (c′)[a]

	lower T_m (°C)	higher T_m (°C)
DNA (a′) and **23**	25	75
DNA (b′) and **23**	50	75
DNA (c′) and **23**	51	75
23	n.d.	75
DNA (a′ + b′ + c′) and **23**	26, 53	73

[a] DNA (a′) 5′-GCGCTACACC-3′; DNA (c′) 5′-GTGCTAGCGG-3′; DNA (b′) 5′-GAGGTAGCCC-3′.

(data not shown). The reasons of the large differences in the T_m value between DNA (a)/DNA (a′) and the other duplexes in the branched complexes are not clear. Because the DNA (a) was attached to the branch point at its 5′-end and the other strands were attached at their 3′-ends, such structural variations could be one of the reasons.

We also measured the T_m values of **23** in the presence of all three complementary strands DNA (a′) + DNA (b′) + DNA (c′), as shown in Figure 5E and the bottom row of Table 1. The differentiation of the UV melting curve suggested the presence of three inflection points at 26, 53, and 73 °C (Figure S21E), which indicated that the melting of the three duplexes and the high-order structure occurred independently.

Stability of the TrS Group Under Various Basic Conditions. As described in the Introduction, combinations of protecting groups, such as DMTr/Lev, DMTr/TBDMS, and DMTr/Fmoc, have been used for the previous branch units reported in the literature. If the TrS group is stable under the conditions required for the selective removal of the Lev, TBDMS, or Fmoc group, it could be used to synthesize branched ODNs in combination with these protecting groups. Therefore, we checked the stability of the TrS group under the deprotection conditions of Lev, TBDMS, and Fmoc groups separately.

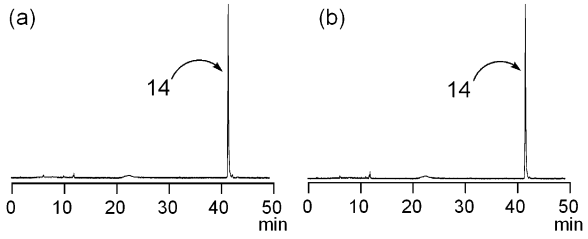


FIGURE 6. Reversed-phase HPLC profiles of **14** obtained by the successive treatment of **12** with various reagents. Panel (a): (i) 0.5 M hydrazine monohydrate, Py/Ac_2O (4:1, v/v), 20 min; (ii) 3% TCA, CH_2Cl_2, 30 s, 3 times; (iii) aq NH_3. Panel (b): (i) 1 M TBAF, THF, 10 min; (ii) 3% TCA, CH_2Cl_2, 30 s, 3 times; (iii) aq NH_3.

Two conditions were tested using the branch unit **2** on solid supports: The reagents, NH_2NH_2 and TBAF, used for the deprotection of the Lev[36,37] and TBDMS groups,[38] respectively, were examined. First, compound **12** was exposed to NH_2NH_2 and TBAF for 20 and 10 min, respectively. The DMTr group was removed by treatment with 3% TCA. The resulting nucleotidic materials were cleaved from the solid support by treatment with aqueous ammonia and then analyzed by reversed-phase HPLC. As shown in Figure 6a and b, the peak corresponding to compound **14** was clearly observed. These results suggested that the TrS group was stable under the above conditions.

The stability of the TrS group to piperidine used for the deprotection of the Fmoc group[16] was also tested in the liquid phase by treatment of **11** with piperidine/DMF (1:5, v/v). As a result, the TrS group proved to be stable for over 48 h by TLC analysis.

Conclusion

We synthesized the branch unit **2** with the DMTr and TrS groups as orthogonal protecting groups. Because the TrS group was unstable to iodine used for the oxidation of phosphite intermediates during the usual DNA synthesis, a peroxide-type oxidizer was tested for the synthesis of the branched ODN. As a result, CSO proved to be efficient. In addition, Act42 was more effective than the conventional Tet for the synthesis of the branched ODN, probably because of its higher acidity.

From the DNA 10-mer on the CPG support, we synthesized the three-way branched ODN **23** using the branch unit **2**. The three-way branched ODN with three different 10-mers at each branch point could form duplexes with the complementary strands. These results clearly demonstrated the usefulness of the TrS group for the synthesis of the branched ODN in combination with the DMTr group. Moreover, we also revealed

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that the TrS group is orthogonal to the Lev, TBDMS, or Fmoc group. These results indicated that, by the combined use of DMTr, TrS, and/or Lev, TBDMS, and Fmoc, we could synthesize more complex four- and five-way branched ODNs, which can be used as the building units of DNA nanostructures.

Experimental Section

Dimethyl 5-[3-(4,4′-Dimethoxytrityloxy)propylcarbamoyl]-benzene-1,3-dicarboxylate (7). To a solution of 3,5-bis(methoxycarbonyl)benzoic acid (**6**) (2.3 g, 9.5 mmol) in THF (20 mL) was added CDI (2.3 g, 14 mmol). The resulting mixture was stirred at room temperature for 1 h, and then the solution of (4,4′-dimethoxytrityl)oxypropan-1-amine (**5**) (5.4 g, 14 mmol) in THF (30 mL) was added. After being stirred at room temperature for 1 h, the reaction mixture was concentrated under reduced pressure. During this concentration, care should be taken not to render the mixture to complete dryness. The residue was diluted with ethyl acetate (80 mL) and washed three times with water (80 mL). The organic layer was collected, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was crystallized from hexane/ethyl acetate (4:1, v/v), and the crystals were collected by filtration to give **7** (3.6 g, 64%). The filtrate was concentrated under reduced pressure, and the residue was chromatographed on an NH silica gel column (15 g) with hexane/ethyl acetate (3:1, v/v). The eluted solution was concentrated under reduced pressure. The residue was crystallized from hexane/ethyl acetate (4:1, v/v), and the crystals were collected by filtration to give **7** (1.5 g, 26%; total 5.1 g, 90%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.91 (m, 2H), 3.31 (t, J = 5.3 Hz, 2H), 3.60 (dd, J = 5.7, 12 Hz, 2H), 3.74 (s, 6H), 3.91 (s, 6H), 6.76 (d, J = 8.5 Hz, 4H), 6.83 (br, 1H), 7.16−7.30 (m, 9H), 7.39 (d, J = 8.1 Hz, 2H), 8.46 (s, 2H), 8.76 (s, 1H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 29.0, 39.2, 52.5, 55.1, 62.4, 86.5, 113.1, 126.8, 127.9, 128.0, 129.8, 131.0, 132.1, 132.9, 136.0, 144.7, 158.4, 165.4, 165.8. Anal. Calcd for $C_{35}H_{35}NO_8 \cdot 0.1H_2O$: C, 70.13; H, 5.92; N, 2.34. Found: C, 69.90; H, 5.93; N, 2.70.

Triethylammonium 3-{3-(4,4′-Dimethoxytrityloxy)propylcarbamoyl}-5-(methoxycarbonyl)benzoate (8). Compound **7** (3.6 g, 6.1 mmol) was dissolved in pyridine (30 mL). To the solution was added 1 M aq NaOH (7.3 mL). The resulting mixture was stirred at room temperature for 3 h, and then the reaction mixture was concentrated under reduced pressure. Pyridine was removed by coevaporation three times with toluene. The residue was chromatographed on a silica gel column (80 g) with chloroform/methanol/triethylamine (100:1:1, v/v/v) to give **8** (2.6 g, 62%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.33 (t, J = 7.3 Hz, 9H), 1.89 (m, 2H), 3.14 (dd, J = 7.3, 15 Hz, 6H), 3.22 (t, J = 5.6 Hz, 2H), 3.57 (dd, J = 6.1, 6.3 Hz, 2H), 3.74 (s, 6H), 3.88 (s, 3H), 6.57 (t, J = 5.4 Hz, 1H), 6.78 (d, J = 8.8 Hz, 4H), 7.16 (t, J = 7.2 Hz, 1H), 7.22−7.32 (m, 6H), 7.41 (d, J = 7.3 Hz, 2H), 8.43 (s, 1H), 8.53 (s, 1H), 8.83 (s, 1H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 8.6, 29.5, 38.4, 45.0, 52.1, 55.1, 61.6, 86.2, 113.1, 126.7, 127.8, 128.1, 129.9, 130.2, 130.3, 131.7, 133.1, 134.9, 136.2, 137.8, 144.9, 158.3, 166.4, 166.7, 170.9. ESI-MS: calcd for $C_{40}H_{49}N_2O_8$ $(M + H)^+$, 685.3483; found, 685.3473.

Methyl 3-[3-(4,4′-Dimethoxytrityloxy)propylcarbamoyl]-5-(3-tritylsulfenyloxypropylcarbamoyl)benzoate (9). To a solution of **8** (1.9 g, 2.8 mmol) in THF (6 mL) was added CDI (690 mg, 4.3 mmol). The resulting mixture was stirred at room temperature for 1 h, and then the solution of **4** (1.5 g, 4.3 mmol) and THF (3 mL) was added. After being stirred at room temperature for 1 h, the reaction mixture was concentrated under reduced pressure. Care should be taken not to render the residue to complete dryness. The solution was diluted with ethyl acetate (70 mL) and washed three times with water (70 mL). The organic layer was collected, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (70 g) with hexane/ethyl acetate (2:3, v/v) to give **9** (2.6 g, quant): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.60 (t, J = 6.3 Hz, 2H), 1.90 (m, 2H), 3.22−3.29 (m, 4H), 3.36 (t, J = 5.6 Hz, 2H), 3.58 (dd, J = 6.1, 12 Hz, 2H), 3.74 (s, 6H), 3.87 (s, 3H), 6.18 (t, J = 5.6 Hz, 1H), 6.72−6.78 (m, 5H), 7.15−7.41 (m, 24H), 8.23 (s, 1H), 8.38 (s, 1H), 8.44 (s, 1H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 29.1, 30.1, 37.8, 38.9, 52.5, 55.1, 62.1, 72.0, 76.6, 86.4, 113.1, 126.8, 127.3, 127.9, 128.0, 129.8, 129.8, 129.9, 130.3, 130.4, 130.9, 135.5, 135.8, 136.0, 142.7, 144.7, 158.4, 165.5, 165.6, 165.8. ESI-MS: calcd for $C_{56}H_{54}N_2O_8SNa$ $(M + Na)^+$, 937.3493; found, 937.3939.

Triethylammonium 3-[(3-(4,4′-Dimethoxytrityloxy)propyl)-carbamoyl}-5-{(3-tritylsulfenyloxypropyl)carbamoyl]-benzoate (10). Compound **9** (1.6 g, 1.7 mmol) was dissolved in pyridine (10 mL). To the solution was added 1 M aq NaOH (2.1 mL). The resulting mixture was stirred at room temperature for 5 h, and then the reaction mixture was concentrated under reduced pressure. The residue was diluted with ethyl acetate (150 mL) and washed three times with brine (100 mL). The organic layer was collected, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (70 g) with chloroform/methanol/triethylamine (100:2:1, v/v/v) to give **10** (1.5 g, 87%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.23 (t, J = 7.3 Hz, 9H), 1.58 (m, 2H), 1.88 (m, 2H), 2.94 (d, J = 6.6 Hz, 6H), 3.21 (m, 4H), 3.31 (t, J = 5.9 Hz, 2H) 3.56 (dd, J = 6.6, 12 Hz, 2H), 3.74 (s, 6H), 6.17 (t, J = 5.9 Hz, 1H), 6.45 (t, J = 5.6 Hz, 1H), 6.79 (d, J = 9.0 Hz, 4H), 7.14−7.42 (m, 24H), 8.19 (s, 1H), 8.48 (s, 1H), 8.51 (s, 1H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 9.4, 29.7, 30.3, 37.2, 38.2, 45.4, 55.1, 61.3, 71.9, 76.1, 86.1, 113.0, 126.7, 127.3, 127.8, 128.0, 128.1, 129.9, 130.0, 130.1, 134.6, 134.8, 136.2, 138.2, 142.8, 144.9, 158.3, 166.7, 166.8, 171.1. ESI-MS: calcd for $C_{61}H_{68}N_3O_8S$ $(M + H)^+$, 1002.4712; found, 1002.4668.

***N*-[3-(4,4′-Dimethoxytrityloxy)propyl]-*N*′-(3-hydroxypropyl)-*N*′′-(3-tritylsulfenyloxypropyl)benzene-1,3,5-tricarboxamide (11).** To a solution of **10** (1.9 g, 2.8 mmol) in THF (6 mL) was added CDI (690 mg, 4.3 mmol). The resulting mixture was stirred at room temperature for 1 h, and then a solution of 3-aminopropan-1-ol (1.5 g, 4.3 mmol) in THF (3 mL) was added. After being stirred at room temperature for 1 h, the reaction mixture was concentrated under reduced pressure but was not dried completely. The solution was diluted with ethyl acetate (70 mL) and washed three times with water (70 mL). The organic layer was collected, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (70 g) with hexane/ethyl acetate (2:3, v/v) to give **11** (1.2 g, 94%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.53 (t, J = 6.1 Hz, 2H), 1.69 (t, J = 5.9 Hz, 2H), 1.83 (t, J = 5.9 Hz, 2H), 3.14−3.19 (m, 4H), 3.27−3.30 (m, 2H), 3.43−3.49 (m, 4H), 3.58−3.61 (m, 2H), 3.69 (s, 6H), 3.79−3.82 (m, 1H), 6.74−6.77 (m, 4H), 6.91−6.93 (m, 1H), 7.15−7.41 (m, 28H), 7.62−7.65 (m, 1H), 7.92 (s, 1H), 8.00 (s, 1H), 8.02 (s, 1H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 29.4, 30.1, 31.8, 37.5, 38.5, 55.1, 59.9, 61.6, 72.0, 76.3, 76.7, 86.2, 113.1, 126.7, 127.3, 127.8, 127.9, 128.0, 129.9, 135.1, 135.3, 135.5, 136.1, 142.7, 144.8, 158.4, 166.1, 166.3, 166.4. ESI-MS: calcd for $C_{58}H_{59}N_3O_8SNa$ $(M + Na)^+$, 980.3921; found, 980.4393.

3-[3-(4,4′-Dimethoxytrityloxy)propylcarbamoyl]-5-(3-tritylsulfenyloxypropylcarbamoyl)benzamido]propyl 2-Cyanoethyl *N*,*N*-Diisopropylphosphoramidite (2). Compound **11** (1.56 g, 1.63 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine, toluene, CH_2Cl_2, and finally dissolved in dry CH_2Cl_2 (8 mL). To this solution were added 2-cyanoethyl *N*,*N*,*N*′*N*′-tetraisopropylphosphorodiamidite (621 μL, 1.96 mmol), diisopropylamine (91 μL, 0.65 mmol), and 1*H*-tetrazole (46 mg, 0.65 mmol). The resulting solution was stirred for 1.5 h. The reaction was quenched by addition of H_2O/CH_3CN (1:1, v/v, 2 mL). The mixture was diluted with Et_2O/AcOEt (3:1, v/v, 40 mL) and washed with 0.2 M aq NaOH (3 × 30 mL). The organic layer was collected, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was chromatographed on an NH silica gel column (40 g) with hexane/ethyl acetate (1:1, v/v) to give **2** (1.2 g, 63%): ^{1}H

NMR (500 MHz, $CDCl_3$) δ 1.14−1.16 (m, 12H), 1.59 (m, 2H), 2.61−2.64 (m, 4H), 3.23 (m, 2H), 3.33 (t, J = 5.9 Hz, 2H), 3.53−3.12 (m, 6H), 3.74−3.89 (m, 10H), 6.31 (t, J = 5.6 Hz, 1H), 6.64 (t, J = 5.6 Hz, 1H), 6.79 (m, 4H), 6.87 (t, J = 5.6 Hz, 1H), 7.15−7.41 (m, 24H), 8.16 (s, 1H), 8.24, 8.25 (s, 2H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 20.4, 24.5, 29.4, 31.2, 31.5, 37.5, 37.9, 38.5, 43.0, 43.1, 55.1, 55.2, 58.0, 58.2, 61.5, 72.0, 76.3, 86.2, 113.1, 113.1, 118.2, 126.8, 127.3, 127.8, 128.0, 129.9, 130.0, 135.3, 135.4, 136.1, 142.8, 144.9, 158.4, 165.7, 165.7, 165.9; ^{31}P NMR (202.33 MHz, $CDCl_3$) δ 148.9. ESI-MS: calcd for $C_{67}H_{76}N_5O_9PSNa$ $(M + Na)^+$, 1180.4994; found, 1180.4628.

Synthesis of 12. Thymidine-loaded CPG (0.5 μmol, 46 μmol/g, succinyl linker) was used in the manual operation: (1) detritylation (3% TCA in CH_2Cl_2, 0.5 mL, 30 s × 3); (2) washing [CH_2Cl_2 (1 mL × 3), CH_3CN (1 mL × 3)]; (3) coupling [phosphoramidite unit (20 μmol), Activator 42 (0.25 M, 160 μL, 40 μmol), and CH_3CN (80 μL), 2 min]; (4) washing [CH_3CN (1 mL × 3)]; (5) capping [Ac_2O/Py (1:9, v/v, 500 μL) in the presence of 0.1 M DMAP for 2 min]; (6) oxidation [0.5 M CSO, CH_3CN (200 μL), 3 min]; and (7) washing [CH_3CN (1 mL × 3)].

The Stability of the TrS Group to 3% TCA. A 3% solution of TCA in CH_2Cl_2 (0.5 mL) was added to **12**, and the reaction mixture of compound **13** was kept at ambient temperature for 5 min. After washing, the oligomer was released from the polymer support by treatment with concd aq NH_3 (1 mL) for 30 min. The product was analyzed by reversed-phase HPLC and ESI-MS: calcd for compound **14**, $C_{47}H_{55}N_5O_{13}PS$ $(M + H)^+$, 960.3249; found, 960.3148.

Synthesis of 15 by Use of Tet as an Activator and *t*-BuOOH as an Oxidizing Agent. A thymidine-loaded CPG (0.5 μmol, 46 μmol/g, succinyl linker) was used. After the usual elongation of trithymidylate by use of Tet as an activator and a 0.02 M I_2 solution as an oxidizing reagent, and the branch unit **2** was coupled with the resin. Furthermore, an additional trithymidylate synthesis was performed on the resin. Each cycle of the additional chain elongation consisted of the following steps: (1) detritylation (3% TCA in CH_2Cl_2, 0.5 mL, 30 s × 3); (2) washing [CH_2Cl_2 (1 mL × 3), CH_3CN (1 mL × 3)]; (3) coupling [phosphoramidite unit (20 μmol), Tet (40 μmol), and CH_3CN (250 μL), 2 min]; (4) washing [CH_3CN (1 mL × 3)]; (5) capping [Ac_2O/Py (1:9, v/v, 500 μL) in the presence of 0.1 M DMAP for 2 min]; (6) oxidation [0.5−0.6 M *t*-BuOOH, CH_3CN (250 μL), 10 min]; (7) washing [CH_3CN (1 mL × 3)]. After the chain elongation was finished, the terminal 5′-hydroxyl group was acetylated by use of acetic anhydride. Then the TrS group was removed by a 1 M I_2 solution (Py/H_2O, 9:1, v/v, 250 μL) for 2 min. From the generated hydroxyl group, another trithymidylate was synthesized by use of Tet and a 0.02 M I_2 solution. After the terminal DMTr group was removed, the oligomer was released from the polymer support by treatment with concd aq NH_3 (1 mL) for 30 min. The polymer support was removed by filtration and washed with water (1 mL × 3). The filtrate was evaporated and analyzed by reversed-phase HPLC. The retention time of the target compound **15** was ca. 25 min. Compound **15** was isolated by use of reversed-phase HPLC, and the structure was characterized by MALDI-MS. MALDI-MS: calcd for compound **15**, $C_{108}H_{145}N_{21}O_{69}P_9$ $(M + H)^+$, 3118.6; found, 3116.8.

Synthesis of 15 by Use of Act42 as the Activator and *t*-BuOOH as the Oxidizing Agent. The activator was changed from Tet to Act42 (0.25 M, 160 μL, 80 μmol) in the method described above.

Synthesis of 15 by Use of Act42 as the Activator and 0.5 M CSO as the Oxidizing Agent. The oxidizing reagent was changed from *t*-BuOOH to CSO (0.5 M, 200 μL, 3 min) during the synthesis of the second trithymidylate from the method described above.

Synthesis of Branched ODN 23. First, DNA (a) sequence was synthesized in an automatic synthesizer by using the standard procedure of the DMTr-On method. After the 10th dG was oxidized, the CPG resin was removed from the column and placed in a glass syringe for the manual operation. Manual synthesis was started from the detritylation step of the 10th dG. Chain elongation cycle was repeated 11 times. The last nucleotide, dG, was detritylated and then capped twice by use of acetic anhydride. After that, the TrS group was removed by treatment with a 1 M I_2 solution (Py/H_2O, 9:1, v/v, 500 μL) for 2 min. Then the solid support was packed back into the column of the automatic synthesizer to extend the third sequence. The third sequence was extended under the usual conditions of the DMTr-On method. After the synthesis, the solid phase support was exposed to an ammonia solution for 20 h. The resulting solution was collected and evaporated. The material having a DMTr group was isolated using reversed-phase HPLC. The fractions were combined and evaporated under reduced pressure. The DMTr group was deprotected on a C-18 cartridge column, and the resulting target material was eluted with CH_3CN/H_2O, (2:8, v/v). The fractions were collected, combined, evaporated under reduced pressure to give **23** in 5.4% yield, and analyzed by anion exchange HPLC. The yield was determined by the absorbance at 260 nm. The ϵ_{260} was estimated as the sum of the ϵ_{260} values of DNA (a), DNA (b), and DNA (c). MALDI-MS: calcd for $C_{307}H_{393}N_{116}O_{187}P_{30}$ $(M + H)^+$, 9624.7; found, 9628.2. The sequences of DNA (a), (b), and (c) were designed according to the literature.[13]

Melting Curve Analysis. Each oligonucleotide was dissolved in 10 mM sodium cacodylate (pH 7.0) containing 0.5 M NaCl and 10 mM $MgCl_2$ so that the final concentration of each oligonucleotide became 1.5 μM. The solutions were separated into quartz cells (10 mm) and incubated at 85 °C. After 10 min, the solutions were cooled to 5 °C at the rate of 0.5 °C/min and then heated until the temperature reached 85 °C at the same rate. During this annealing and melting, the absorptions at 260 nm were recorded and used to draw UV melting curves. The oligonucleotide concentrations of DNA were determined as described in the literature.[39]

The Stability of the TrS Group to Hydrazine. Compound **12** was synthesized by the above procedure. A 0.5 M hydrazine monohydrate solution (1 mL, in pyridine/acetic acid, 4:1, v/v) was added, and the reaction mixture was kept at the ambient temperature for 20 min. After washing, the DMTr group was removed. The oligomer was released from the polymer support by treatment with concd aq NH_3 (1 mL) for 30 min. The product was analyzed by reversed-phase HPLC.

The Stability of the TrS Group to TBAF. Compound **12** was synthesized by the above procedure. A 1 M TBAF solution (1 mL, in THF) was added, and the reaction mixture was kept at the ambient temperature for 10 min. After washing, the DMTr group was removed. The oligomer was released from the polymer support by treatment with concd aq NH_3 (1 mL) for 30 min. The product was analyzed by reversed-phase HPLC.

The Stability of the TrS Group to Piperidine. Compound **11** (4.1 mg, 4.3 μmol) was dissolved in DMF (84 μL). To the solution was added piperidine (21 μL, 0.21 mmol). The reaction was monitored for 48 h by TLC analysis.

Acknowledgment. This work was supported by a Grant-in-Aid to CREST of JST (Japan Science and Technology) and the Ministry of Education, Science, Sports and Culture, Grant-in-aid for Scientific Research (A). This study was also supported by Industrial Technology Research Grant Program in ’05 from New Energy and Industrial Technology Development Organization (NEDO) of Japan.

Supporting Information Available: List of experimental procedures for the synthesis of the products other than those described in the Experimental Section. This material is available free of charge via the Internet at http://pubs.acs.org.

JO071173A

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