Novel Phosphoramidite Monomer for the Site-Selective Incorporation of a Diastereochemically Pure Phosphoramidate to Oligonucleotide

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Diastereochemically pure dithymidine phosphoramidates have been site-selectively incorporated into synthetic oligonucleotides by a phosphoramidite technique. By using the terminal amino residues bound to the chiral phosphoramidates, various functional residues have been attached to the oligonucleotides in stereospecific ways. No racemization takes place during these procedures. The dependence of the duplex- and triplex-forming activities of these tethered and functionalized oligonucleotides on the diastereochemistry of the phosphoramidate is shown.

Introduction

Recently, chemical modifications of DNA have been widely attempted.[1,2] Various functional residues have been tethered to either the nucleic acid bases, the deoxyriboses, or the phosphorus atoms in natural and synthetic oligonucleotides. The goals involve the preparation of various functionalized DNA molecules for catalysis and photoreactions,[2−5] nonradioactive labeling of DNA,[1,6,7] and others. An application to the regulation of gene expression by antisense techniques has also been proposed.[8−10]

Of these modifications, that at the phosphorus atoms is advantageous in that the resultant perturbation in the physicochemical properties of DNA is intrinsically small (especially when only one or two phosphates are modified, as is the case in the present paper). The phosphate residues, in most cases, do not directly participate in the interactions with other DNA.

One of the key factors in the modification is regulation of the chirality (either *R* or *S*) around the phosphorus atoms. The diastereomers have different orientations of the tethered functional group (see Figure 1). However, all of the previous methods for phosphate modification (H-phosphonate, phosphotriester, and phosphorothioate methods) produced mixtures of diastereomers, which are usually difficult to separate.[1,11,12] New procedures for site-selective incorporation of chiral phosphorus atoms to oligonucleotides are required for further development of the field.

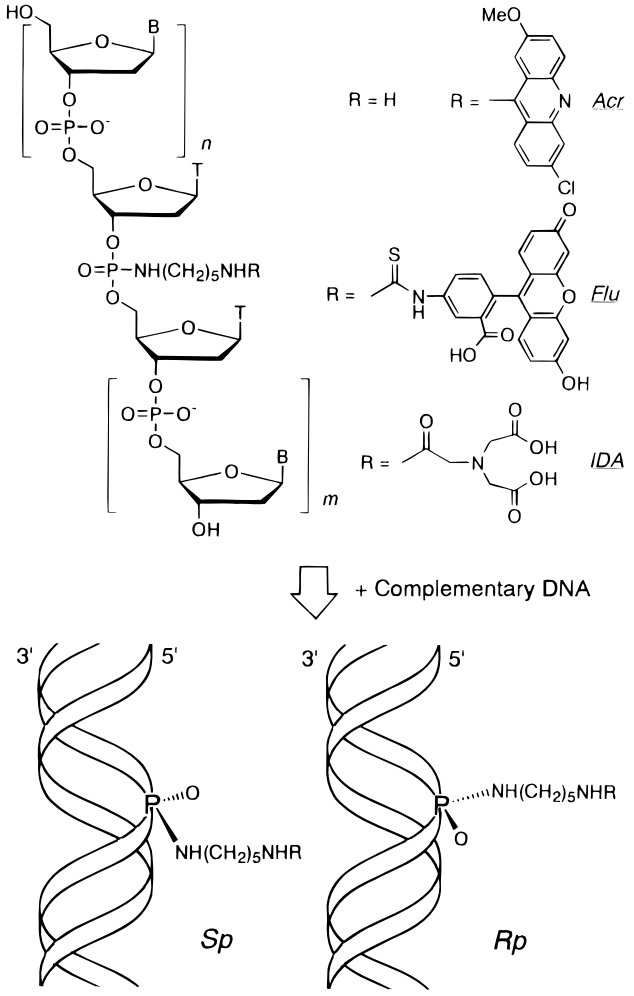

Figure 1. Oligonucleotides containing diastereochemically pure phosphoramidate units and their duplexes with the complementary DNA: the orientations of the tethered functional residues are schematically depicted.

Previously, diastereochemically pure phosphorothioates[12] and phosphonates[13] were introduced to oligonucleotides. However, site-selective incorporation of chiral

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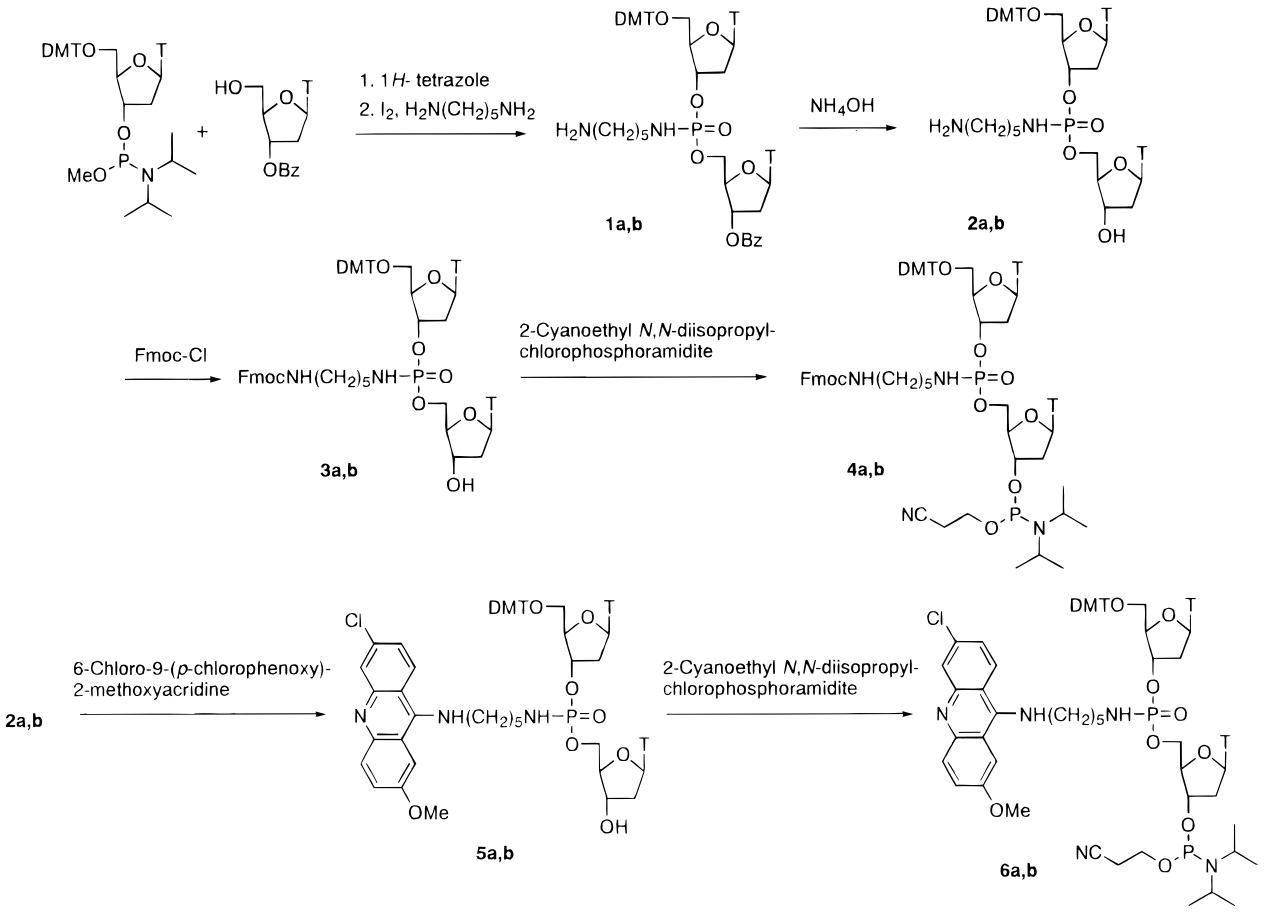
Scheme 1



phosphoramidates has not yet been accomplished. Although a tri- or tetranucleotide containing only one chirally pure phosphoramidates was prepared by Hecht et al.,[11a] the target diastereomer was separated by column chromatography from a mixture of the diastereomers. Apparently, a straightforward synthetic method, which is applicable to introduction of the desired number of chiral phosphoramidates at selected sites, is required.

In this study, phosphoramidite monomers (**4** and **6** in Scheme 1) containing chirally pure phosphoramidate linkages were synthesized and site-selectively incorporated into oligonucleotides. In these monomers, latent amino residues were attached to the phosphoramidates so that various functional residues could be tethered to the oligonucleotides when necessary. The stabilities and structures of the duplexes and triplexes of the diastereoselectively modified oligonucleotides were investigated by melting-temperature measurements as well as by circular dichroism, absorption, and fluorescence spectroscopy.

Results and Discussion

Synthesis of Phosphoramidite Monomers 4a,b and 6a,b. The phosphoramidites of dithymidine phosphoramidates (**4a**,**b** and **6a**,**b**) were synthesized according to Scheme 1. The letters **a** and **b** refer to the chirality around the phosphorus atom. First, the dithymidine phosphoramidate **1** was prepared by coupling 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-(*O*-methyl *N*,*N*-diisopropylphosphoramidite) with 3′-*O*-benzoylthymidine using 1*H*-tetrazole, followed by oxidation with iodine in the presence of 1,5-diaminopentane.[11a] Diastereomers **1a** and **1b** were completely separated by silica gel column chromatography, which is the key step for the present synthesis. The chiralities remained totally intact throughout the DNA synthesis (*vide infra*).

After the 3′-benzoyl groups were removed by concentrated NH_4OH, the amino terminates of **2a**,**b** were reacted with either 9-fluorenylmethyl chloroformate (Fmoc-Cl) or 6-chloro-9-(*p*-chlorophenoxy)-2-methoxyacridine. Finally, **3a**,**b** and **5a**,**b** were phosphitylated to phosphoramidites **4a**,**b** and **6a**,**b** using 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite. All of the products were completely characterized by ^{1}H- and ^{31}P-NMR spectroscopy.

Preparation of Diastereoselectively Modified Oligonucleotides. By using synthetic phosphoramidites **4a**,**b** and **6a**,**b**, the following oligonucleotides were synthesized:

5′-TTCTCTTCTCT-p(N5N)-TCCTTTT-3′
(oligonucleotide-*A* and -*B*)

5′-TTCTCT-p(N5N)-TCTCT-p(N5N)-TCCTTTT-3′
(oligonucleotide-*AA* and -*BB*)

5′-TTCTCT-p(N5N)-TCTCT-p(N5N)-TCCT-p-(N5N)-TTT-3′ (oligonucleotide-*AAA* and -*BBB*)

5′-TTCTCTTCTCT-p(N5N-Acr)-TCCTTTT-3′
(oligonucleotide-Acr(*A*) and -Acr(*B*))

5′-TTCTCTTCTCT-p(N5N-Flu)-TCCTTTT-3′
(oligonucleotide-Flu(*A*) and -Flu(*B*))

5′-ATGCT-p(N5N-IDA)-TCCAGGGCTCTAGT-3′
(oligonucleotide-IDA(*A*) and -IDA(*B*))

Terms *A* and *B* show the diasterochemistry of the

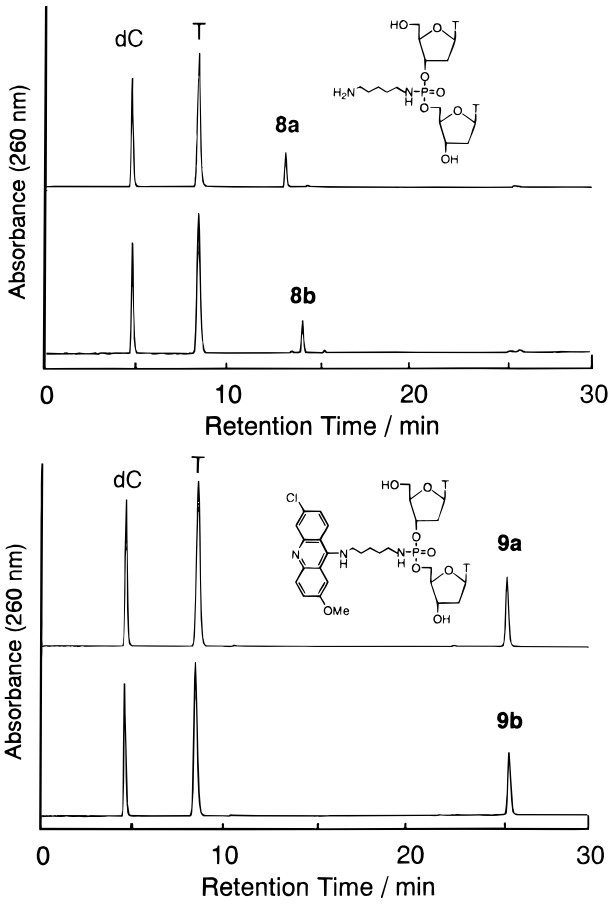


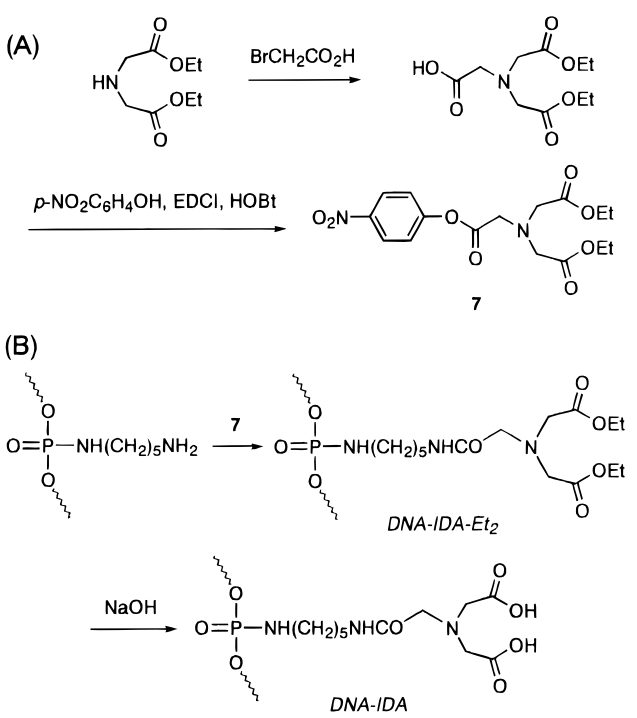
Figure 2. HPLC profiles for the enzymatic digests of oligonucleotides-*A* and -*B* (top) and oligonucleotide-Acr(A) and -Acr-(B) (bottom). The HPLC conditions are presented in the Experimental Section.

phosphoramidite monomers used (**a** and **b**, respectively), whereas p(N5N) denotes 5-aminopentyl phosphoramidate. In p(N5N-Acr), p(N5N-Flu), and p(N5N-IDA), respectively, 9-amino-6-chloro-2-methoxyacridin-9-yl, fluorescein isothiocyanate, and iminodiacetic acid moieties were attached to the terminal amino residues of the phosphoramidates (see Figure 1).

The oligonucleotides-*A*, -*B*, -*AA*, -*BB*, -*AAA*, -*BBB*, -Flu-(*A*), -Flu(*B*), -IDA(*A*), and -IDA(*B*) were synthesized using the conventional phosphoramidite agents, together with the phosphoramidites **4**. In order to synthesize the oligonucleotide-Acr(*A*) and -Acr(*B*) from **6**, however, Expedite reagents had to be used in place of the conventional monomers. Otherwise, most of the acridine residues were removed from the oligonucleotides during the deprotection procedures (treatment by concd NH_4OH at 55 °C for 12 h). With Expedite agents, the deprotection procedures could be achieved under mild conditions (at room temperature for 1 h),[14] so that the target-modified oligonucleotides were successfully prepared without any loss of the dye molecules.

Figure 2 depicts the HPLC profiles for enzymatic digests of the oligonucleotides. For the oligonucleotide-*A*, -*B*, -Acr(*A*), and -Acr(*B*), dC, T, and the corresponding dithymidine phosphoramidate are formed in a ratio of 6:10:1. The ratio is 6:8:2 for the oligonucleotide-*AA* and -*BB*, and the value is 6:6:3 for the oligonucleotide-*AAA* and -*BBB*. These results are totally consistent with the structures of the oligonucleotides. The phosphoramidate linkages remain intact against enzymatic digestion.

Scheme 2



Quite significantly, the dithymidine phosphoramidate, formed by enzymatic digestion, has the same diastereochemistry as does the phosphoramidite monomer used (**a** or **b**). The diasteromeric counterpart is not perceived at all, as confirmed by coinjection with an authentic sample. Thus, no racemization of the phosphoramidate took place during the synthesis of the modified oligonucleotides. Here, the authentic samples of the diastereochemically pure dithymidine phosphoramidates **8a**,**b** and **9a**,**b** were synthesized by the detritylation of **2a**,**b** and **5a**,**b** with acetic acid.

The incorporation of the IDA residue to DNA was carried out using the activated ester **7** (Scheme 2). The alkaline hydrolysis of the ethyl esters in DNA-IDA-Et_2 successfully provided DNA-IDA. The HPLC profiles are shown in Figure 3. Lanthanide complexes of these modified oligonucleotides are expected to show site-specific hydrolysis of RNA and DNA.[16]

Duplex Formation of Diastereoselectively Modified Oligonucleotides. The melting temperatures (T_m's) of the duplexes between the diastereoselectively modified oligonucleotides and their complementary DNA are listed in Table 1. The oligonucleotides involving the phosphoramidates of *A*-type diastereochemistry show higher T_m's than do the corresponding diastereochemical counterparts (*B*-type phosphoramidates). The difference monotonously increases along with the number of phosphoramidate units in the oligonucleotide (2.3 °C for one, 6.8 °C for two, and 9.4 °C for three). A similar stereochemical effect of phosphorus atoms on the duplex-forming activity was reported for the phosphonates.[13] The *A*-type diastereomers of the acridine- or fluorescein-attached

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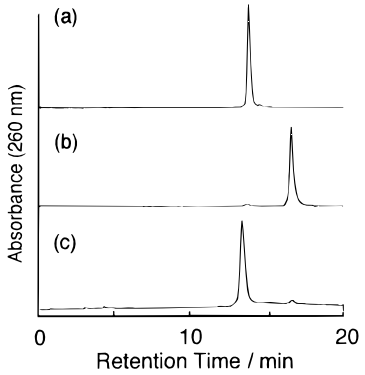

Figure 3. HPLC profiles for the IDA-attached oligonucleotides (*A*-type diastereomer). (a) 5′-ATGCT-p(N5N)-TCCAGGGCTCTAGT-3′. (b) Oligonucleotide attached with a diethyl iminodiacetate residue (DNA-IDA-Et_2). (c) IDA-attached oligonucleotide (DNA-IDA). HPLC conditions, linear gradient 5−20% (20 min), acetonitrile/water (50 mM ammonium formate).

Table 1. Melting Temperatures of the Duplexes of the Diastereoselectively Modified Oligonucleotides at pH 7.0[a−c]

modified DNA	T_m of the duplex with AAAAGGAAGAGAAGAGAA	difference between A and B diastereomers
oligonucleotide-A	55.7	2.3
-B	53.4	
-AA	54.9	6.8
-BB	48.1	
-AAA	47.9	9.4
-BBB	38.5	
-Acr(A)	59.1	3.8
-Acr(B)	55.3	
-Flu(A)	53.4	2.9
-Flu(B)	50.5	

[a] $[DNA]_0 = 1\ \mu M$. [b] The change of the absorbance at 260 nm in 10 mM sodium cacodylate (pH 7.0), 100 mM NaCl, and 10 mM $MgCl_2$ was measured at a rate of 0.5 °C/min. [c] The value for the unmodified DNA oligomer is 56.8 °C.

oligonucleotides also formed more stable duplexes than did the corresponding *B*-type isomers.

Figure 4 shows the circular dichroism spectra of duplexes of the modified oligonucleotides. The spectra are positive at 260-300 nm and negative at 220−260 nm. Apparently, all of the duplexes have typical B-DNA conformations.[17] Neither the diasterochemistry of the phosphoramidates nor their number causes any significant effect on the spectra (thus, on the conformations of the duplexes).

Interactions of the Tethered Dyes with Duplex DNA. When the acridine-tethered DNA forms a duplex with the complementary DNA, the chromophore strongly interacts with the duplex, as is clearly evidenced by both the significant hypochromicity and the red shift in the UV spectra (Figure 5). Consistently, the fluorescence from the acridine is remarkably quenched by duplex formation (Figure 6).

The interaction between the acridine and the DNA duplex was further confirmed by the fact that the T_m's of the duplexes of the oligonucleotide-Acr(*A*) and -Acr(*B*) were higher than those of the oligonucleotide-*A* and -*B* (Table 1). In addition, the difference in T_m between the diastereomers (3.8 °C) for the acridine-tethered oligonucleotides was larger than the value for those without them (2.3 °C). The strong stabilization of DNA duplex

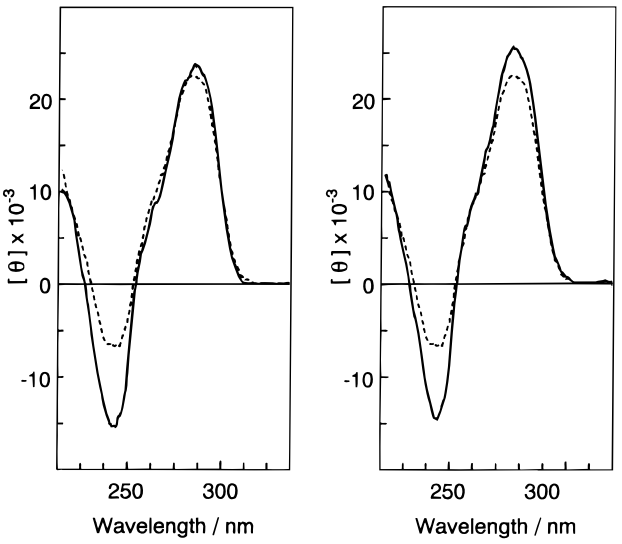

Figure 4. Circular dichroism spectra of the duplexes of oligonucleotide-*A* (left) and -*B* (right) at pH 7.0 and 20 °C. The solid lines denote the spectra of the modified DNA duplexes, and the dotted lines are for those of unmodified DNA duplex. $[DNA]_0 = 5\ \mu M$; [sodium cacodylate]$_0$ = 10 mM, $[NaCl]_0$ = 100 mM, and $[MgCl_2]_0$ = 10 mM.

due to the intercalation of acridine derivatives, tethered to the ends of the oligonucleotides, was reported previously.[18] The smaller stabilization in the present case is associated with the restricted rotation of the tethers around the phosphoramidate linkage. A similar argument has been made for the modified oligonucleotides, in which a pyrene is attached to an internal phosphorothioate.[19]

The hypochromicity of the oligonucleotide-Acr(*A*) on duplex formation (31% at 424 nm when the ratio of the two DNAs is 1:1) is larger than the value of the *B*-diastereomer (22%). The red shift (5 nm) of the former is also larger than that (2 nm) for the latter (see Figure 5). Furthermore, the shift in the fluorescence-emission maximum for the *A*-type diastereomer (497 to 491 nm) is greater than that for the *B*-type one (497 to 496 nm) (Figure 6a). Apparently, the tethered dye in the *A*-type diastereomer interacts more strongly with the duplex than the dye in the *B*-type one does. The difference in the interactions for these two diastereomers is also clearly evidenced by the fluorescence excitation spectra (Figure 6b). The significant fluorescence quenching may indicate that the acridine ring intercalates to one of the adjacent GC base pairs.[20,21]

In contrast, the duplexes of the oligonucleotide-Flu(*A*) and -Flu(*B*) are less stable than those of the oligonucleotides without the dyes (Table 1). An electrostatic repulsion between the negative charges of the DNA and the negatively charged chromophore is responsible for this destabilization (the pK_a values of fluorescein are 4.3 and 6.5).

Effect of Diasterochemically Pure Oligonucleotide on Triple Helix Formation. When a homopy-

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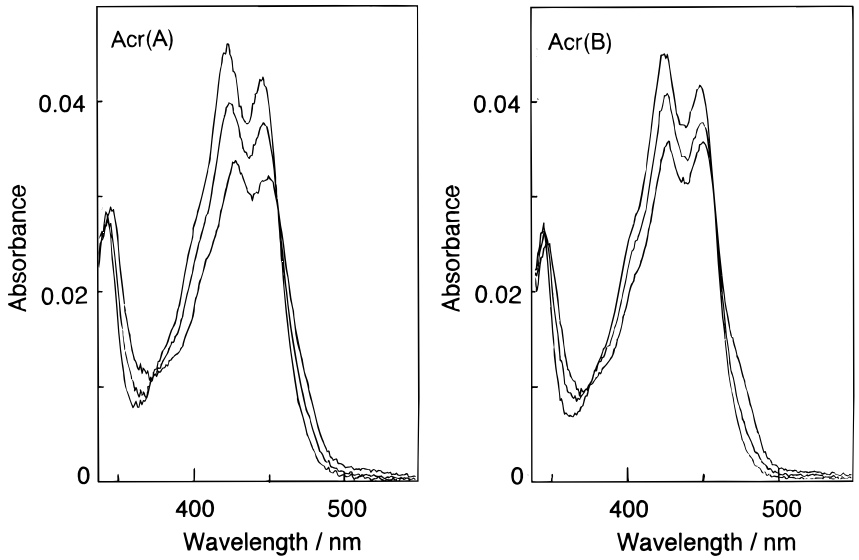

Figure 5. UV titration spectra of the oligonucleotide-Acr(A) (left) and -Acr(B) (right) in the presence of their complementary DNA (0, 3.5, and 8.7 μM from top) at pH 7.0 and 4 °C; [modified DNA]$_0$ = 8.7 μM; the solvent is identical to that described in Figure 4.

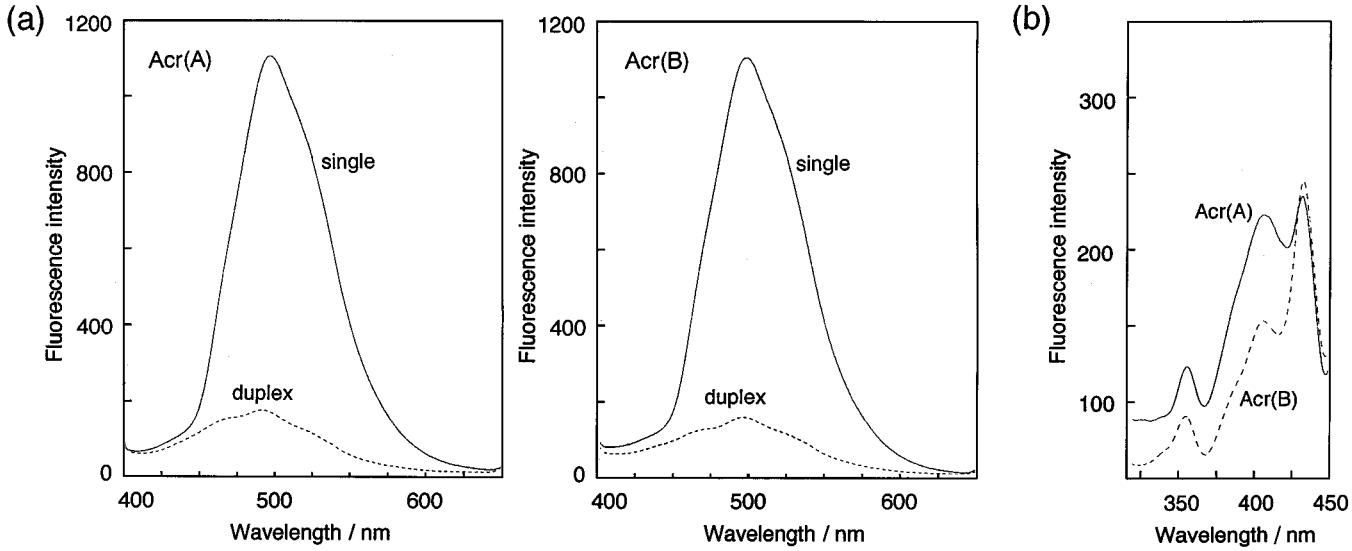

Figure 6. Fluorescence emission spectra (a: excitation at 345 nm) of the single-stranded and duplex-forming oligonucleotide-Acr(*A*) (left) and -Acr(*B*) (right). The excitation spectra (emission at 470 nm) of the modified oligonucleotides in the presence of their complementary DNA at pH 7.0 and 15 °C are presented in (b). The solvent is identical to that described in Figure 4. The excitation wavelength (345 nm) in (a) is the isosbestic point in the absorption spectra.

rimidine, 5′-TTTTCCTTCTCTTCTCTT-3′, is added to a 1:1 mixture of diastereochemically pure oligonucleotides and their complementary DNA, dually sigmoidal melting profiles are obtained. Here, triple helixes are formed in addition to the duplexes. The T_m's for the triple helix formation are shown in Table 2. All of the B-type isomers show higher T_m's than do the corresponding A-type isomers. Quite interestingly, the stability of the triple helix gradually increases along with an increase in the number of phosphoramidate units (from one to three). This is mainly ascribed to a reduction of the electrostatic repulsion between the duplex and the third strand, due to a replacement of the negatively charged phosphate(s) with neutral phosphoramidate(s). Positive charges at the terminal ammonium ions (only in the case of the oligonucleotides without dyes) should further increase the stability.

The T_m for the triplex formation of the oligonucleotide-Flu(*A*) (27.0 °C) is considerably lower than that for the oligonucleotide-Flu(*B*) (30.8 °C). Similarly, the oligonucleotide-Acr(*A*) forms a less stable triple helix than does its diasteromeric counterpart. Thus, triple-helix

Table 2. Melting Temperatures of the Triplexes of the Diastereoselectively Modified Oligonucleotides at pH 7.0[a–c]

modified DNA	T_m of the triplex with AAAAGGAAGAGAAGAGAA and TTTTCCTTCTCTTCTCTT	difference between A and B diastereomers
oligonucleotide-A	28.0	−2.0
-B	30.0	
-AA	28.8	−0.9
-BB	31.2	
-AAA	30.3	−1.5
-BBB	31.8	
-Acr(A)	23.3	−2.6
-Acr(B)	25.7	
-Flu(A)	27.0	−2.2
-Flu(B)	30.8	

[a] [DNA]$_0$ = 1 μM. [b] The experimental conditions are identical as described in Table 1, except for the fact the rate of temperature increase is 0.2 °C/min. [c] The value for the unmodified DNA oligomer is 29.7 °C.

formation is suppressed more explicitly by the dyes in *A*-type diastereochemistry than by the dyes in *B*-type diastereochemistry. This is probably associated with a difference in the orientations of the tethers around the phosphoramidate linkages.

A direct determination of the absolute configurations of the phosphoramidates by NOESY and ROESY techniques[11b,22] was unsuccessful. For **1a**,**b** and **2a**,**b**, no measurable NOEs were detected between the protons of the deoxyribose and the methylene protons adjacent to the phosphoramidate.

Conclusions

Phosphoramidites of dithymidine phosphoramidates have been synthesized in diastereochemically pure forms and have been incorporated into oligonucleotides at desired sites. The separation of the diastereomers of the oligonucleotide (with respect to the phosphorus atoms) is not required at all, since no racemization of the chiral phosphoramidates takes place during the preparation and subsequent treatment. In the previous synthesis of chiral phosphoramidates,[23] however, after mixtures of a number of diastereomers were obtained, the desired diastereomers were separated from the mixtures. The separation was troublesome, especially when the degree of polymerization of the oligonucleotide was large.

Various functional residues were attached to the diastereochemically pure oligonucleotides by using the terminal amino residues at the phosphoramidates. The procedures are highly effective for the site-selective and diastereoselective incorporation of versatile functional residues to synthetic oligonucleotides.

The duplex- and triplex-forming activities of these modified oligonucleotides are dependent on the diastereochemistry of the phosphoramidates, since the chiralities govern the orientations of the functional residues, and thus their interactions with the duplexes and triplexes. The application of the present method to the precise fixation of various functional groups (e.g., DNA-cleaving molecules and photoreactive agents) at the target positions in DNA is currently being attempted in our laboratory.

Experimental Section

General Procedures. 6-Chloro-9-(*p*-chlorophenoxy)-2-methoxyacridine was synthesized from 6,9-dichloro-2-methoxyacridine (Aldrich), as reported.[11a] 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, 9-fluorenylmethyl chloroformate, 1,5-diaminopentane, 1*H*-tetrazole, *p*-nitrophenol, *N*-hydroxybenzotriazole (HOBt), 1-ethyl-3-[3′-(dimethylamino)-propyl]carbodiimide hydrochloride (EDCI) (Tokyo Kasei), and fluorescein isothiocyanate (FITC: Molecular Probes) were commercially obtained. Pyridine and 1,4-dioxane were distilled from potassium hydroxide and metallic sodium, respectively, while acetonitrile and THF were dried over trap-pak (MilliGen/Biosearch) and 4A molecular sieves, respectively. Silica gel for column chromatography was obtained from Merck (Kisel gel 60, 70−230 mesh) or from Wako (Wakogel C-200, 75−150 μm; C-300, 45−75 μm). TLC was performed on precoated silica gel plates (Merck Kisel gel 60 F254). Alkaline phosphatase and snake venom phosphodiesterase were from Boehringer Mannheim. Automated DNA synthesis was performed on a MilliGen/Biosearch Cyclone Plus DNA synthesizer. The reagents for DNA synthesis were purchased from MilliGen/Biosearch. ^{1}H-NMR spectra (with TMS as internal standard) and ^{31}P-NMR spectra (with 85% H_3PO_4 in D_2O as external standard) were recorded at 400 (or 270) MHz and 161.9 (or 109.4) MHz.

***P*-[(5-Aminopentyl)amino]-*P*-deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidylyl-(3′−5′)-3′-*O*-benzoylthymidine (1a and 1b).** 1*H*-Tetrazole (263 mg, 3.8 mmol) was dissolved in 20 mL of dry acetonitrile and was added to an acetonitrile solution of 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-(*O*-methyl-*N*,*N*-diisopropylphosphoramidite) (1.06 g, 1.5 mmol) and 3′-*O*-benzoylthymidine (415 mg, 1.2 mmol). All of the reagents were dried beforehand by coevaporation with acetonitrile (three times). The reaction mixture was stirred at room temperature for 1 h and then treated with iodine (381 mg, 1.5 mmol) for 20 min in a THF/1,5-diaminopentane mixture (3 mL/1.5 mL) under nitrogen. The excess amine and the salts were removed by a short silica gel column (eluent: CH_2Cl_2:MeOH = 4:1); the final purification was made by silica gel column chromatography (CH_2Cl_2:MeOH:Et_3N = 100:12−20:1): **1a** (397 mg, 32% yield) and **1b** (524 mg, 42% yield). R_f = 0.24 for **1a** and 0.12 for **1b** (silica gel TLC, CH_2Cl_2:MeOH:Et_3N = 90:10:1). The structures of both isomers were concretely confirmed by ^{1}H-NMR spectroscopy.

***P*-[(5-Aminopentyl)amino]-*P*-deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidylyl-(3′−5′)-thymidine (2a and 2b).** Concentrated aqueous ammonia solution (10 mL) was poured into 10 mL of an ethanol solution of **1a** (370 mg, 0.36 mmol); the mixture was kept in a sealed vessel at 50 °C for 12 h. After the solvent was removed, the product was purified by silica gel column chromatography ($CHCl_3$:MeOH:Et_3N = 70:30:1), giving 282 mg of **2a** (85% yield). The yield of **2b** was 317 mg (72%). R_f = 0.16 for **2a** and 0.10 for **2b** ($CHCl_3$:MeOH:Et_3N = 70:30:1).

***P*-[[5-[[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino]pentyl]amino]-*P*-deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidylyl-(3′−5′)-thymidine (3a and 3b).** **2a** (162 mg, 0.17 mmol) in a chloroform/*N*,*N*-diisopropylethylamine mixture (5 mL/91 μL) was cooled in an ice bath under a nitrogen atmosphere, to which a chloroform solution of 9-fluorenylmethyl chloroformate (68 mg, 0.26 mmol) was added. After 30 min at room temperature, the reaction was quenched by adding 1 mL of 2-propanol and 30 mL of CH_2Cl_2. The mixture was washed twice with aqueous $NaHCO_3$ and once with brine and was then dried over anhydrous Na_2SO_4. Silica gel column chromatography (CH_2Cl_2:*i*-PrOH:Et_3N = 100:5−10:1) afforded 194 mg of **3a** (97% yield). The yield of **3b** was 250 mg (78%). R_f = 0.49 for **3a** and 0.47 for **3b** (CH_2Cl_2:MeOH:Et_3N = 90:10:1).

***P*-[[5-[[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino]pentyl]amino]-*P*-deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidylyl-(3′−5′)-thymidine 3′-*O*-(2-Cyanoethyl *N*,*N*-diisopropylphosphoramidite) (4a and 4b).** To **3a** (194 mg, 0.17 mmol) in a methylene chloride/*N*,*N*-diisopropylethylamine mixture (5 mL/117 μL) was slowly added 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (113 μL, 0.50 mmol) dropwise under a nitrogen atmosphere. After the mixture was stirred at room temperature for 1 h, the reaction was quenched by 1 mL of ethanol. The usual workup, followed by silica gel column chromatography (CH_2Cl_2:*i*-PrOH:Et_3N = 100:3:1), gave 157 mg of the phosphoramidite monomer **4a** (76% yield). The yield of **4b** was 263 mg (98%).

4a: ^{1}H-NMR ($CDCl_3$) δ 7.75 (d, J = 7.5 Hz, 2H), 7.59 (d, J = 7.5 Hz, 2H), 7.54 (s, 1H), 7.18−7.43 (m, 14H), 6.82 (d, J = 8.8 Hz, 4H), 6.42 (t, J = 6.6 Hz, 1H), 6.23 (t, J = 4.4 Hz, 1H), 5.12 (m, 1H), 4.61 (m, 1H), 4.39 (d, J = 6.6 Hz, 2H), 4.13−4.23 (m, 5H), 3.63−3.90 (m, 2H), 3.77 (s, 6H), 3.59 (m, 2H), 3.50 (brd, J = 10.2 Hz, 1H), 3.35 (brd, J = 9.5 Hz, 1H), 3.14 (m, 2H), 2.77 (m, 2H), 2.67 (m, 1H), 2.65 (t, J = 5.9 Hz, 2H), 2.38−2.53 (m, 2H), 2.19−2.35 (m, 1H), 1.89 (s, 3H), 1.40 (m, 4H), 1.37 (s, 3H), 1.24 (m, 2H), 1.17 (t, J = 6.2 Hz, 12H); ^{31}P-NMR ($CDCl_3$) δ 12.82, 153.00, 153.18; R_f = 0.42 (CH_2Cl_2:*i*-PrOH:Et_3N = 100:5:1).

4b: ^{1}H-NMR ($CDCl_3$) δ 7.75 (d, J = 7.4 Hz, 2H), 5.59 (d, J = 7.4 Hz, 2H), 7.58 (s, 1H), 7.20−7.45 (m, 14H), 6.82 (d, J = 8.7 Hz, 4H), 6.43 (brt, 1H), 6.17 (brt, 1H), 5.14 (m, 1H), 4.54 (brs, 1H), 4.40 (m, 2H), 4.13−4.29 (m, 5H), 3.64−3.97 (m, 2H), 3.78 (s, 6H), 3.68 (m, 2H), 3.60 (m, 2H), 3.49 (brd, 1H), 3.41

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(brd, 1H), 3.16 (m, 2H), 2.92 (brs, 2H), 2.62 (t, J = 6.0 Hz, 2H), 2.60 (m, 1H), 2.43 (m, 2H), 2.21 (m, 1H), 1.88 (s, 3H), 1.49 (m, 4H), 1.36 (s, 3H), 1.34 (m, 2H), 1.17 (t, J = 4.7 Hz, 12H); ^{31}P-NMR ($CDCl_3$) δ 13.00, 152.94, 153.12. R_f = 0.43.

***P*-[[5-[(6-Chloro-2-methoxyacridin-9-yl)amino]pentyl]-amino]-*P*-deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidylyl-(3′−5′)-thymidine (5a and 5b).** **2a** (504 mg, 0.54 mmol) in 10 mL of anhydrous pyridine was treated with 6-chloro-9-(*p*-chlorophenoxy)-2-methoxyacridine (906 mg, 2.5 mmol) at 60 °C for 12 h. Silica gel column chromatography (CH_2Cl_2:MeOH:Et_3N = 100:7−10:1) afforded 360 mg of **5a** in 60% yield. The yield of **5b** was 310 mg (45%). R_f = 0.13 for **5a** and 0.14 for **5b** (CH_2Cl_2:MeOH = 90:10).

***P*-[[5-[(6-Chloro-2-methoxyacridin-9-yl)amino]pentyl]-amino]-*P*-deoxy-5′-*O*-(4,4′-dimethoxytrityl)thymidylyl-(3′−5′)-thymidine 3′-*O*-(2-Cyanoethyl *N*,*N*-diisopropyl-phosphoramidite) (6a and 6b).** **5a** (285 mg, 0.26 mmol) was dried by coevaporation with dry acetonitrile and then suspended in a dioxane/acetonitrile/*N*,*N*-diisopropylethylamine mixture (10 mL/5 mL/178 μL). 2-Cyanoethyl *N*,*N*-diisopropyl-chlorophosphoramidite (172 μL, 0.77 mmol) was slowly added under a nitrogen atmosphere. After 1 h at room temperature, the reaction was quenched by 1 mL of ethanol and the solvent was removed. The residue was dissolved in 100 mL of chloroform, washed with aqueous $NaHCO_3$ and brine, and then dried over anhydrous Na_2SO_4. The product was purified by silica gel column chromatography (CH_2Cl_2:*i*-PrOH:Et_3N = 100:3−7:1). The yields of **6a** and **6b** were 169 mg (50%) and 245 mg (57%).

6a: ^{1}H-NMR ($CDCl_3$) δ 8.01 (d, J = 9.2 Hz, 1H), 7.91 (s, 1H), 7.82 (d, J = 9.2 Hz, 1H), 7.16−7.62 (m, 14H), 6.82 (d, J = 8.6 Hz, 4H), 6.45 (brt, 1H), 6.19 (brt, 1H), 5.14 (m, 1H), 4.61 (m, 1H), 4.10−4.43 (m, 4H), 3.93 (s, 3H), 3.77 (s, 6H), 3.70−3.95 (m, 2H), 3.61 (m, 2H), 3.35−3.54 (m, 2H), 2.75−3.00 (m, 4H), 2.66 (t, J = 5.9 Hz, 2H), 2.18−2.74 (m, 4H), 1.87 (s, 3H), 1.82 (m, 2H), 1.40 (m, 4H), 1.35 (s, 3H), 1.19 (m, 12H); ^{31}P-NMR ($CDCl_3$) δ 13.18, 153.02, 153.24; R_f = 0.40 (CH_2Cl_2:*i*-PrOH:Et_3N = 100:5:1).

6b: ^{1}H-NMR ($CDCl_3$) δ 7.94 (d, J = 8.9 Hz, 1H), 7.72−7.88 (m, 2H), 7.03−7.62 (m, 14H), 6.83 (d, J = 8.9 Hz, 4H), 6.45 (brt, 1H), 6.19 (brt, 1H), 5.18 (m, 1H), 4.53 (m, 1H), 4.08−4.35 (m, 4H), 3.90 (s, 3H), 3.78 (s, 6H), 3.35−3.85 (m, 6H), 2.75−3.00 (m, 4H), 2.70−2.92 (m, 1H), 2.62 (t, J = 5.9 Hz, 2H), 2.32−2.54 (m, 2H), 2.23 (m, 1H), 1.84 (s, 3H), 1.84 (m, 2H), 1.50 (m, 4H), 1.26 (s, 3H), 1.16 (m, 12H); ^{31}P-NMR ($CDCl_3$) δ 13.06, 153.04, 153.20. R_f = 0.33.

DNA Synthesis. Automated DNA synthesis was performed by the phoshoramidite method on a DNA synthesizer, according to the recommended protocol. For DNA synthesis using phosphoramidites **6a** and **b**, Expedite agents (MilliGen/Biosearch) were used to achieve the subsequent deprotection procedures under mild conditions (*vide ante*). The protecting groups of the nucleic acid bases in these agents were easily removed, compared with those in the conventional phosphoramidite agents.[14]

After the coupling reactions, the oligonucleotides were deprotected by concentrated NH_4OH, either at 55 °C for 12 h (for **4a** and **b**) or at room temperature for 1 h (for **6a** and **b**). The deprotected oligonucleotides (with the terminal 4,4′-dimethoxytrityl (DMT) residues on) were purified by reversed-phase HPLC and then treated with a 4:1 acetic acid/acetonitrile mixture for 1 h at room temperature to remove the DMT. The final purification of the products was also achieved by HPLC.

Structural analyses of the oligonucleotides were carried out after they had been digested by alkaline phosphatase and snake-venom phosphodiesterase at pH 7.0 (50 mM HEPES buffer) and 37 °C for 2 h. The digests were analyzed by HPLC [linear gradient 5-50% (30 min), acetonitrile/water (50 mM ammonium formate), Shiseido capcell pak C18 SG120 ODS column (4.6 mm × 250 mm), 1.0 mL/min: see Figure 2].

Post-treatment of the Oligonucleotides for Tethering a Fluorescein. The oligonucleotides containing the chiral phosphoramidates were reacted with fluorescein isothiocyanate (FITC) in a 13:2 mixture of pH 9.0 carbonate buffer and DMF for 60 h at room temperature in the dark.[15] The functionalized oligonucleotides were purified by HPLC.

***N*,*N*-Bis[2-(ethoxycarbonyl)methyl]glycine.** An activated ester for the synthesis of the iminodiacetic acid (IDA)-attached oligonucleotides was prepared according to Scheme 2A. Iminodiacetic acid diethyl ester (5.5 mL, 33.2 mmol) and 2-bromoacetic acid (2.18 g, 16.6 mmol) were reacted in 20 mL of dry dioxane for 24 h at 70 °C. The solvent was removed under reduced pressure, and the reaction mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure, and the resultant brown oil was used without further purification: 3.52 g (91% yield); ^{1}H-NMR ($CDCl_3$) δ 4.22 (q, J = 5.1 Hz, 4H), 3.59 (s, 4H), 3.53 (s, 2H), 1.29 (t, J = 5.1 Hz, 6H).

***N*,*N*-Bis[2-(ethoxycabonyl)methyl]glycine *p*-Nitrophenyl Ester (7).** To a mixture of *N*,*N*-bis[2-(ethoxycarbonyl)methyl]glycine (2.47 g, 10 mmol), *p*-nitrophenol (1.39 g, 10 mmol), and *N*-hydroxybenzotriazole (1.35 g, 10 mmol) in 10 mL of DMF on ice was added 1-ethyl-3-[3′-(dimethylamino)-propyl]carbodiimide hydrochloride (EDCI) (1.92 g, 10 mmol); the reaction mixture was then stirred for 12 h at room temperature. The mixture was concentrated and dissolved in ethyl acetate. After being washed with saturated aqueous $NaHCO_3$ and NaCl, the organic layer was dried over anhydrous Na_2SO_4. The activated ester **7** (1.02 g, 28% yield) was obtained by silica gel column chromatography (hexane:ethyl acetate = 4:1): ^{1}H-NMR ($CDCl_3$) δ 8.28 (d, J = 9.1 Hz, 2H), 7.34 (d, J = 9.1 Hz, 2H), 4.20 (q, J = 7.1 Hz, 4H), 3.98 (s, 2H), 3.74 (s, 4H), 1.20 (t, J = 7.1 Hz, 6H); R_f = 0.21 (hexane:ethyl acetate = 4:1).

Synthesis of Iminodiacetic Acid (IDA)-Attached Oligonucleotides (Oligonucleotide-IDA). The synthetic scheme and HPLC profiles are presented in Scheme 2B and Figure 3c, respectively. To the oligonucleotide containing the phosphoramidate (5 nmol) in a mixture of 15 μL of 100 mM sodium borate buffer (pH 8.6) and 25 μL of DMF, 5 μL of DMF solution of **7** (460 mM) was introduced. After 4 h at room temperature, DNA-IDA-Et_2 was purified by HPLC. The solution was concentrated, and its pH was adjusted to 12.5 by NaOH. After 2 h at room temperature, the mixture was neutralized by hydrochloric acid.

Melting Temperature Measurements. The melting profiles of the DNA duplexes and triplexes were measured at 260 nm. A quartz cell of 1 cm path length was fitted with a Teflon stopper. The concentration of each DNA oligomer was 1 μM. The heating rates were 0.5 °C/min for duplex formation and 0.2 °C/min for triplex formation. The experimental error in T_m was estimated to be ±0.5 °C for the duplex and ±1.0 °C for the triplex.

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Supporting Information Available: ^{1}H NMR spectra for **1a**,**b**, **2a**,**b**, **3a**,**b**, **4a**,**b**, **5a**,**b**, **6a**,**b**, *N*,*N*-bis[2-(ethoxycarbonyl)-methyl]glycine, and **7**, ^{1}H−^{1}H COSY spectra for **1a**,**b**, **2a**,**b**, **3a**,**b**, and **4a**,**b**, and ^{31}P NMR spectra for **3a**,**b** **4a**,**b**, **5a**,**b**, and **6a**,**b** (18 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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