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Polymer Support Synthesis of Oligodeoxyribonucleotide with an Aminoethyl or Aminohexyl Group at the 5′ End by the Phosphite-Triester Approach

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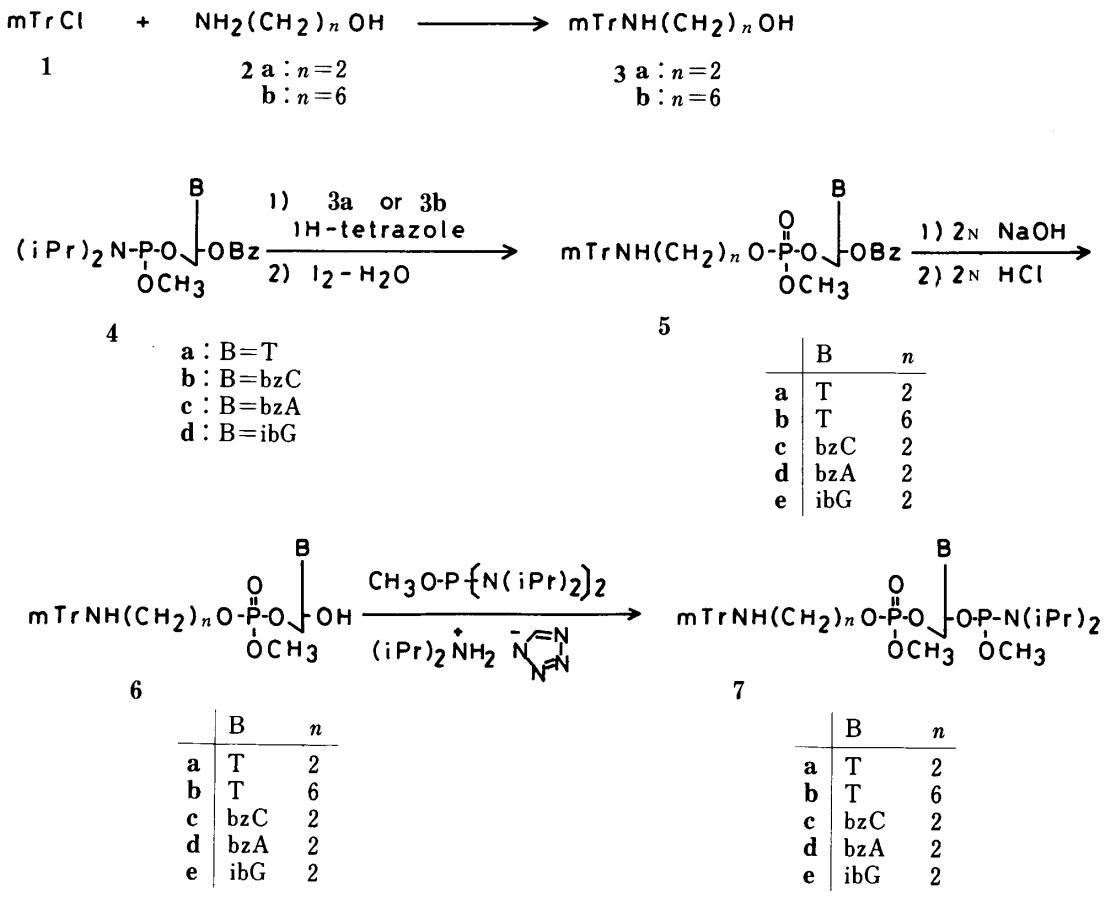
An *N*-monomethoxytrityloxyethyl or-hexyl group was introduced onto the 5′-phosphoryl group of *N*-protected-3′-benzoyldeoxynucleosides. After 3′-*O*-debenzoylation and phosphitylation, they were converted 3′-phosphoramidite derivatives. These were used at the last coupling reaction in the synthesis of octadecadeoxynucleotides with an aminoethyl or aminohexyl group at the 5′ end by the phosphite-triester method on long-chain alkylamine controlled pore glass beads. Partially deblocked oligomers, after thiophenol and ammonia treatment, were separated rapidly on a reversed-phase C-18 silica gel column by utilizing the hydrophobic nature of the monomethoxytrityl group. After AcOH treatment, the octadecamers with an aminoethyl or aminohexyl group at the 5′ end were obtained and then coupled with a fluorescent compound. The ultraviolet-temperature profiles were measured after hybridization with a complementary oligonucleotide.

Keywords——oligodeoxyribonucleotide synthesis; phosphite-triester method; polymer support; aminoalkylated oligonucleotide

Recently, deoxyoligoribonucleotides with an amino group at the 5′ end were found to be useful as probes for deoxyribonucleic acid (DNA) sequence determination when the amino group was linked to a fluorescent compound.[1] Such a compound could also be used as a hybridization probe for a certain DNA when the terminal amino group was coupled with biotin.[2] These procedures are very promising since a radioisotope, such as ^{32}P, is not needed. Therefore, an easy method for preparation of deoxyribonucleotides with an amino group at the 5′ end is required. On the other hand, the synthesis of oligonucleotides with define sequence can easily be performed by the phosphite-triester method using an automatic DNA synthesizer. In this paper, we report the synthesis of a deoxyribooligonucleotide with an amino group at the 5′ end on the polymer support by the phosphite-triester method. During the synthesis, a lipophilic trityl group was used for the protection of the terminal amino group to facilitate separation of the partially deblocked oligonucleotide on a reversed-phase C-18 column.[3]

Results and Discussion

Aminoethyl or aminohexyl alcohol (**2a** or **2b**) was treated with monomethoxytrityl chloride in dimethylformamide in the presence of triethylamine to afford the *N*-monomethoxytritylaminoalkyl alcohol[4] (**3a** or **3b**). This was purified on a silica gel column to give a yellow oil in a yield of 82% or 89%, respectively. A 3′-*O*-benzoylthymidine-5′-*O*-diisopropylaminomethoxyphosphoramidite (**4a**) was prepared from 3′-*O*-benzoylthymidine by phosphitylation using diisopropylamine hydrotetrazolide and bisdiisopropylamino-methoxyphosphine.[5] The yield obtained was 95%. This compound was condensed with **3a** or **3b** and 1*H*-tetrazole in acetonitrile followed by oxidation with I_2–H_2O to give **5a** or



	B	n
a	T	2
b	T	6
c	bzC	2
d	bzA	2
e	ibG	2

mTr=monomethoxytrityl Bz or bz=benzoyl ib=isobutyryl iPr=isopropyl

Chart 1

TABLE I. ^{31}P-NMR Spectra Data for **7a—e**

Compound	Chemical shift (ppm)			
7a	147.18	146.60	−2.15	−2.28
7b	147.15	146.61	−2.42	
7c	147.39	146.66	−2.09	−2.28
7d	147.08	146.84	−2.29	−2.32
7e	146.62	146.51	−2.80	−2.91
	146.42	146.33	−2.96	−3.07

The chemical shifts are downfield with respect to trimethylphosphate in $CDCl_3$ as an external standard.

5b in 59% or 50% yields, respectively. Then selective de-benzoylation from the 3′-hydroxyl group of **5a** or **5b** was carried out by treatment with 2 N NaOH at 0 °C for 10 min. During this treatment, the methyl phosphate protecting group was stable. After neutralization with 2 N HCl, **6a** or **6b** was isolated on a silica gel column in 80% or 79% yield, respectively.

The 3′-hydroxyl group of **6a** or **6b** was then phosphitylated using diisopropylamine hydrotetrazolide and bisdiisopropylaminomethoxyphosphine to give **7a** or **7b** in 80% or 89% yield, respectively, as a solid after purification on a silica gel column. In order to prepare 5′-aminoethylated oligonucleotides with any desired sequence, the nucleotide phosphoramidites with four different bases (T, C, A, G) are required. Therefore, by the same procedure, **7c—e** were prepared from *N*,3′-*O*-dibenzoyldeoxycytidine, *N*-3′-*O*-dibenzoyldeoxyadenosine and

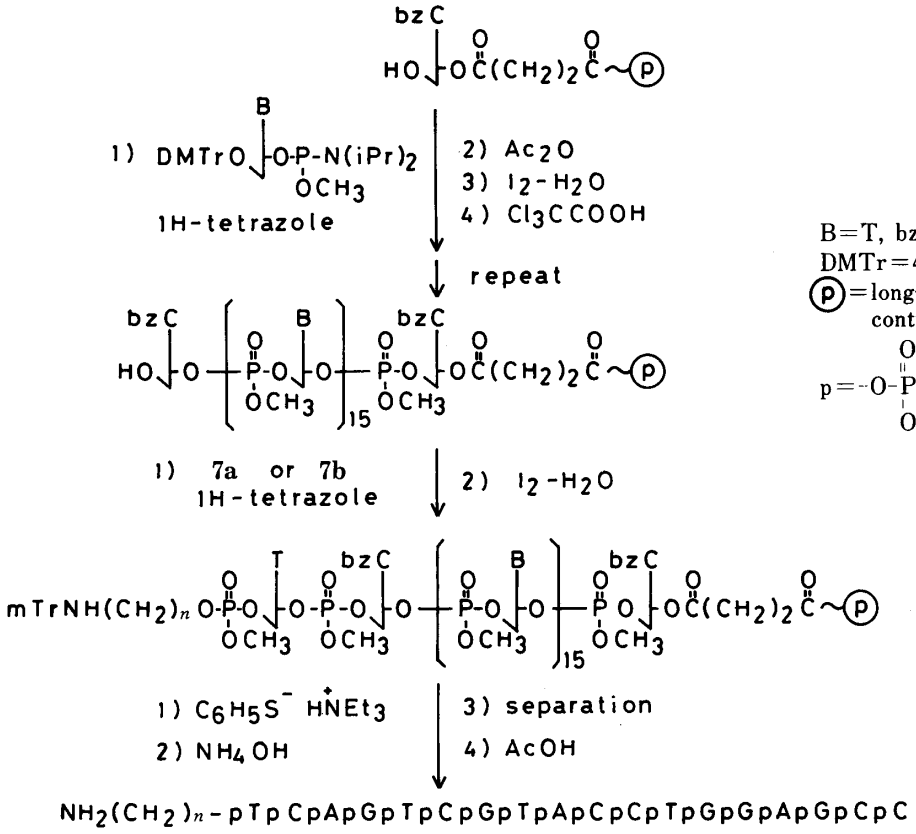


Chart 2

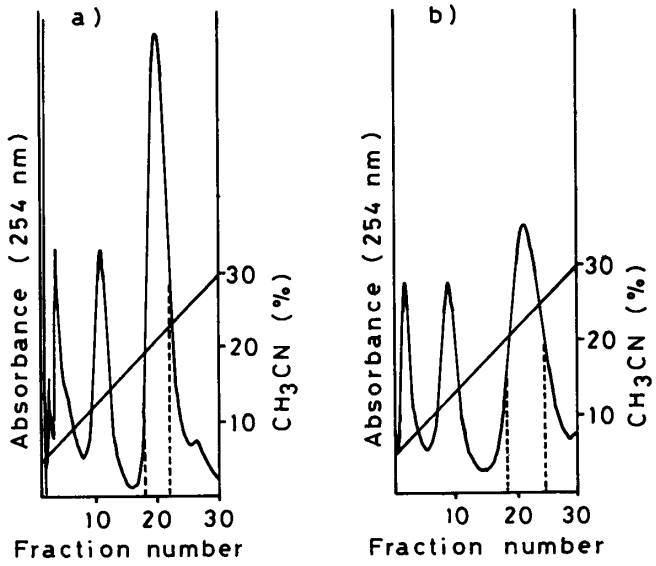


Fig. 1. Isolation of $mTrNH(CH_2)_nOpTCAGTCGTACCTGGAGCC$ (a, $n=2$; b, $n=6$) on a Reversed-Phase (C-18) Silica Gel Column (0.7×8 cm) with a Linear Gradient of Acetonitrile (5—30%) in 100 mM Triethylammonium Acetate (Total 100 ml)

N-isobutyryl-3′-*O*-benzoyldeoxyguanosine, respectively. They were more than 95% pure based on a ^{31}P-nuclear magnetic resonance (NMR) analysis. The ^{31}P-NMR spectral data of **7a**—**e** are summarized in Table I. Each compound has four diastereoisomers which arise from the 5′-*O*-phosphate and the 3′-*O*-phosphite. Good separation of the four diastereoisomers were seen in the case of compound **7e**. Four signals around 146 ppm (phosphite) and four peaks around −3 ppm (phosphate) were observed. However, **7a, c, d** showed two phosphite and two phosphate signals. In the case of **7b**, only one phosphate signal was seen as well as two phosphite signals.

As outlined in Chart 2, two octadecadeoxyribonucleotides [$NH_2(CH_2)_nOpTCAGT$-CGTACCTGGAGCC ($n=2$ or 6)] were synthesized by the stepwise addition of mononucleotide 3′-methoxyphosphoramidite using an automatic DNA synthesizer.[6] At the last coupling, **7a** or **7b** was condensed. After the synthesis, the nucleotide resin was treated with thiophenol–triethylamine–dioxane to remove methyl phosphate protecting groups followed by treatment with concentrated NH_4OH to remove *N*-acyl protecting groups and release the nucleotidic compounds from the resin. Then the oligonucleotides, which still possessed the lipophilic monomethoxytrityl group at the terminal amino group were quickly purified on a reversed-phase C-18 silica gel short column. As shown in Fig. 1, the monomethoxytritylated oligomers (between the dotted lines) were eluted slowly and were easily separated from the reagents and truncated oligonucleotides. Then the monomethoxytrityl group was removed by treatment with 80% aqueous AcOH for 1 h to give the unprotected octadecadeoxyribonucleotides with an aminoalkyl group at the 5′ end; these products were isolated by the reversed-phase C-18 high pressure liquid chromatography (HPLC) (Fig. 2).

Both the oligonucleotides were treated with fluoresein isothiocyanate (FITC) in buffer

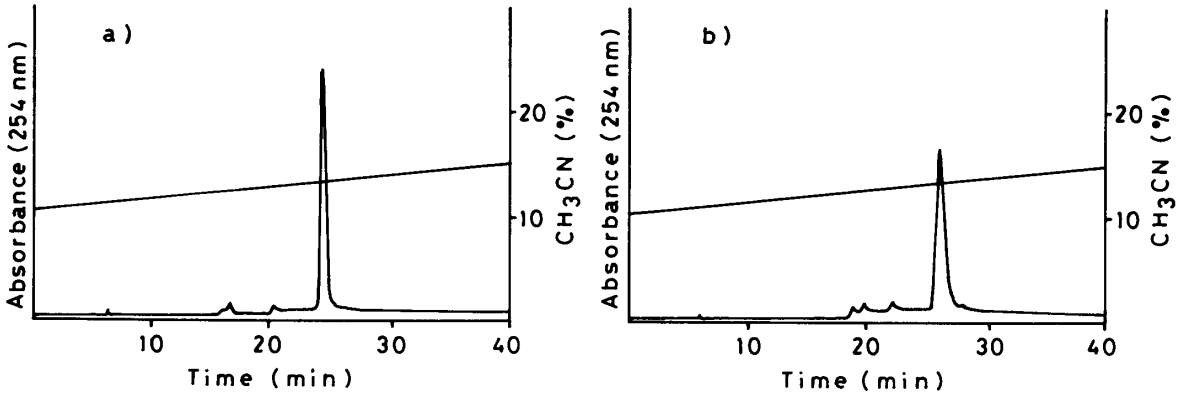


Fig. 2. Reversed-Phase HPLC Analysis of $NH_2(CH_2)_nOpTCAGTCGTACCTG$-GAGCC (a, $n=2$; b, $n=6$) with a Linear Gradient of Acetonitrile (11—15%, 40 min) in 0.1 M Triethylammonium Acetate at a Flow Rate of 0.7 ml/min

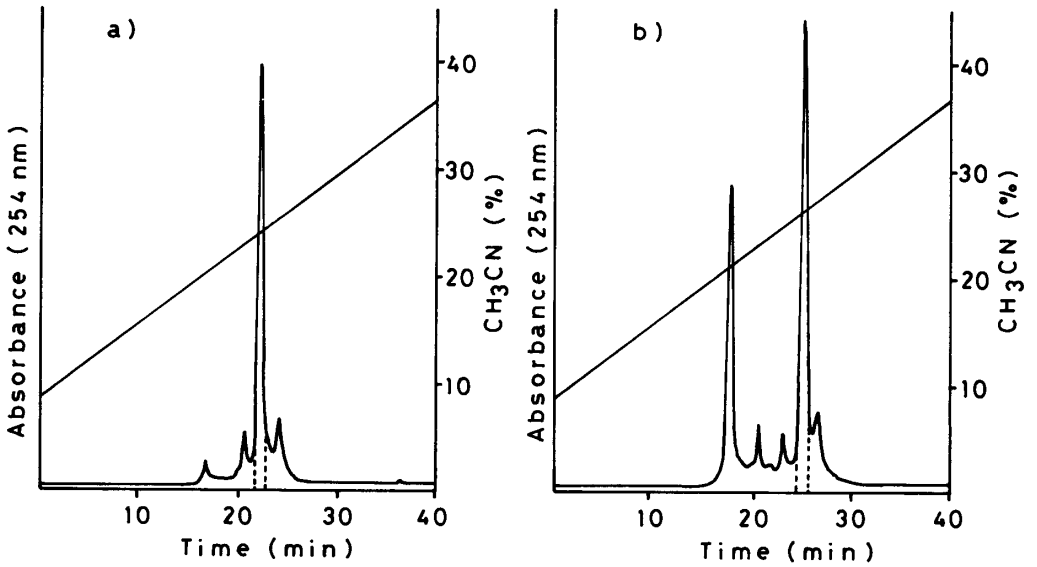


Fig. 3. Reversed-Phase HPLC Analysis of (FITC) $NH(CH_2)_nOpTCAGTCGTA$-CCTGGAGCC (a, $n=2$; b, $n=6$) with a Linear Gradient of Acetonitrile (9.5—36.5%, 40 min) in 0.1 M Triethylammonium Acetate at a Flow Rate of 0.7 ml/min

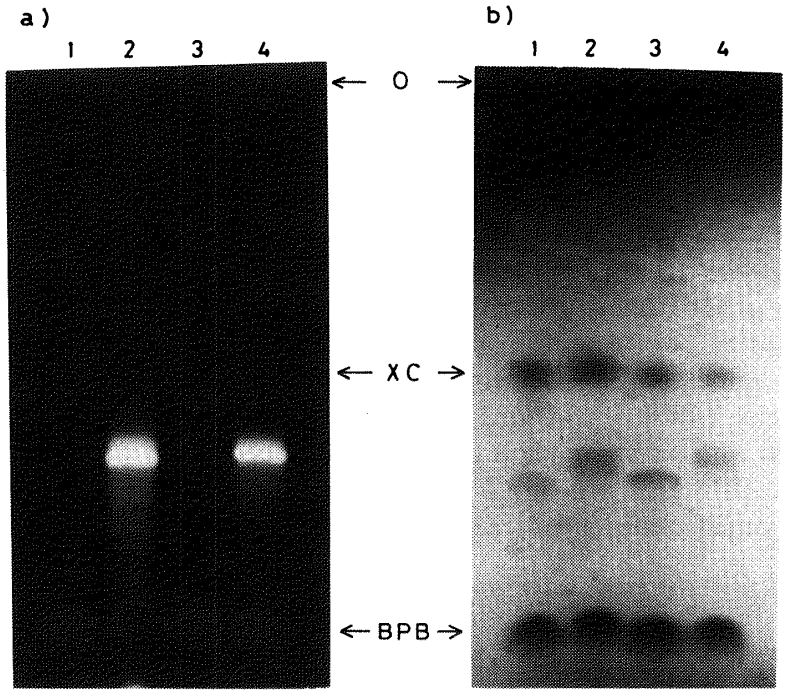


Fig. 4. 20% Polyacrylamide Gel Electrophoregram of $NH_2(CH_2)_nOpTCAGTCG-TACCTGGAGCC$ (Lane 1, $n=2$; Lane 3, $n=6$) and (FITC) $NH(CH_2)_nOpTC-AGTCGTACCTGGAGCC$ (Lane 2, $n=2$; Lane 4, $n=6$) under UV Light (a, 302 nm; b, 260 nm)

O, origin; XC, xylene cyanol; BPB, bromophenol Blue.

TABLE II. The Melting Temperatures (T_m) of the Duplexes

Duplex	T_m (°C)
(5′) p-T-C-A-G-T-C-G-T-A-C-C-T-G-G-A-G-C-C (3′) (3′) G-A-G-T-A-A-G-T-C-A-G-C-A-T-G-G-A-C-C-T-C-G-G (5′)	35.5
(5′) FITC-$NH(CH_2)_2O$-p-T-C-A-G-T-C-G-T-A-C-C-T-G-G-A-G-C-C (3′) (3′) G-A-G-T-A-A-G-T-C-A-G-C-A-T-G-G-A-C-C-T-C-G-G (5′)	33.5
(5′) FITC-$NH(CH_2)_6O$-p-T-C-A-G-T-C-G-T-A-C-C-T-G-G-A-G-C-C (3′) (3′) G-A-G-T-A-A-G-T-C-A-G-C-A-T-G-G-A-C-C-T-C-G-G (5′)	365.5

Each strand ($0.5\,A_{260}$ unit) was dissolved in 3 ml of 0.1 M NaCl in the presence of 10 mM Tris–HCl (pH 8) and 1 mM ethylenediaminetetraacetic acid (EDTA).

(pH 9) for 10 h in the dark.[7] After separation on the Sephadex G25 column to remove the unreacted FITC, the oligonucleotides with FITC at the 5′-terminal were isolated by reversed-phase C-18 silica gel HPLC (Fig. 3, between the dotted lines). Then, oligonucleotides labeled with FITC were analyzed by 20% polyacrylamide gel electrophoresis containing 7 M urea (Fig. 4). From the ultraviolet (UV) shadowing, oligonucleotides labeled with FITC have less mobility on the gel than those labeled with an aminoalkyl group. Under irradiation at 302 nm, only oligonucleotides labeled with FITC were seen on the gel.

To measure the UV absorption-temperature profile, a 23 mer (GGCTCCAGGTAC-GACTGAATGAG) with a complementary sequence to the 18 mer and 5 bases protruding at the 3′ end was prepared by the phosphite-triester approach. For comparison, the oc-

tadecadeoxyribonucleotide which has the same nucleotide sequence but a phosphoryl group at the 5′ end was prepared by the reported procedure.[8] Three types of 18 mer (one has FITC bound *via* an aminoethyl linker, one has FITC bound *via* an aminohexyl linker, and the third has phosphate) and 23 mer were hybridized in 0.1 M NaCl and their UV absorption-temperature profiles were measured to obtain the melting temperature (T_m) values. The results are summarized in Table II. From the T_m measurments, considerable differences in hybridization to the complementary 23 mer were seen; octadecamer labeled with FITC *via* the ethyl linker had the lowest T_m value. If a bulkier group than FITC were attached to the amino group, the aminohexyl linker would probably be preferable to the aminoethyl linker.[9]

In conclusion, we have prepared protected nucleoside phosphoramidite derivatives for the synthesis of oligonucleotides with an aminoalkyl group at the 5′ end. By using the lipophilic monomethoxytrityl group for the protection of the amino group, the partially deblocked oligonucleotide could be isolated easily with high purity on a reversed-phase C-18 silica gel column. Nucleoside phosphoramidites with four different bases were required and prepared. Recently Connolly reported[10] a phosphitylating agent, *N*-monomethoxytrityl-*O*-methoxydiisopropylaminophosphinyl 3-aminopropan(1)ol ($mTrNH(CH_2)_3P(OCH_3)$-$[N(iPr)_2]$). This method is also very useful, except that the phosphitylating reagent is an oil. On the other hand, nucleoside phosphoramidites obtained here are solids, which are easier to handle.

Materials and Methods

Thin-layer chromatography (TLC) was performed on plates of Kieselgel $60F_{254}$ (Merck). For column chromatography, Kieselgel 60 (Merck) or alkylated silica gel (C-18, 55—105 μ, Waters Associates Inc.) was used.

HPLC was performed on an Altex 322Mp chromatography system. For the reversed-phase HPLC, Nucleosil C-18 (5 μ) was packed under 500 kg/cm^2 in a stainless steel column (i.d. 0.6 × 20 cm).

UV and visible spectra were measured with a Hitachi model 200-10 instrument. UV-temperature profiles in 0.1 M NaCl were recorded on a Beckman DU-8B spectrophotometer with a Tm accessory.

Preparation of 3a, b——Monomethoxytrityl chloride (1.55 g, 5 mmol) was added slowly to the aminoalkyl alcohol (**2a** or **b**) (7 mmol) in CH_2Cl_2 (10 ml) and diisopropylethylamine (1.3 ml, 7 mmol) with vigorous stirring. After 10 min at room temperature, the mixture was diluted with CH_2Cl_2 (20 ml) and washed with 0.1 M triethylammonium bicarbonate (TEAB, pH 7.5) (30 ml × 3). The organic layer was evaporated to a small volume, which was chromatographed on a silica gel column (30 g) using a stepwise gradient of MeOH in CH_2Cl_2. After evaporation of the appropriate fractions, **3a** or **b** was obtained in a yield of 82% or 88%, respectively, as a yellow oil.

Preparation of 4a, b——Diisopropylamine hydrotetrazolide (86 mg, 0.5 mmol) and bisdiisopropylaminomethoxyphosphine (341 mg, 1.3 mmol) were added to a solution of *N*-protected-3′-*O*-benzoyldeoxynucleoside (1 mmol) in CH_2Cl_2 (5 ml) and the mixture was stirred at 30 °C. After 1 h, TLC analysis showed the reaction was complete. The mixture was diluted with ethyl acetate (15 ml) and washed successively with saturated aqueous $NaHCO_3$ (20 ml × 3) then H_2O (20 ml × 3). The organic layer was evaporated and the residue was applied to a silica gel column and eluted with ethyl acetate containing triethylamine (1%). The fractions containing **4** were pooled and evaporated to give a solid. Yields were 95% for **4a**, 92% for **4b**, 83% for **4c** and 93% for **4d**.

Preparation of 5a—e——Compounds **4** (0.4 mmol) and **3** (0.4 mmol) were co-evaporated with pyridine then toluene, dissolved in CH_3CN and again evaporated. The residue was dissolved in CH_3CN (10 ml) and treated with 1*H*-tetrazole (280 mg, 4 mmol) for 5 min at room temperature. A solution of 0.1 M I_2 in tetrahydrofuran–pyridine–H_2O (8 : 1 : 1, v/v/v) (10 ml) was added to the mixture and the whole was allowed to stand for 30 min at room temperature. The mixture was diluted with CH_2Cl_2 (30 ml), washed with 0.1 M TEAB (30 ml × 4) and evaporated. The residue was applied to a silica gel column (20 g) and eluted with a stepwise gradient of MeOH in CH_2Cl_2. The fractions containing **5** were pooled and evaporated. The residue was dissolved in CH_2Cl_2 (3 ml) and precipitated with *n*-hexane (50 ml). The white precipitate was collected and dried. Yields were 59% for **5a**, 50% for **5b**, 65% for **5c**, 45% for **5d**, and 55% for **5e**.

Preparation of 6a—e——Aqueous 2 N NaOH solution (1.2 ml) was added to a solution of the compound **5** (0.2 mmol) in MeOH–pyridine (6 ml–1 ml) at 0 °C. After 10 min, the completion of the reaction was confirmed by TLC. The mixture was neutralized with 2 N HCl, diluted with CH_2Cl_2 (20 ml) then washed with 0.1 M TEAB (20 ml × 4). The organic layer was evaporated. The residue was applied to a silica gel column (10 g) and eluted with a stepwise gradient of MeOH in CH_2Cl_2. The fractions containing **6** were pooled and evaporated. The residue was

dissolved in CH_2Cl_2 (3 ml) and precipitated with *n*-hexane (50 ml). The white precipitate was collected and dried. Yields were 80% for **6a**, 79% for **6b**, 70% for **6c**, 75% for **6d** and 86% for **6e**.

Preparation of 7a—e——Diisopropylamine hydrotetrazolide (13 mg, 0.075 mmol) and bisdiisopropylamino-methoxyphosphite (59 mg, 0.225 mmol) were added to a solution of compound **6** (0.15 mmol) in CH_2Cl_2 (5 ml). The mixture was stirred at 30 °C for 1 h, and completion of the reaction was confirmed by TLC. The mixture was diluted with ethyl acetate (20 ml) and washed with saturated aqueous $NaHCO_3$ (20 ml × 3) then H_2O (20 ml × 3). The organic layer was evaporated and the residue was chromatographed on a silica gel column (6 g) with ethyl acetate containing triethylamine (1%). The fractions containing **7** were pooled and evaporated to give a solid. Yields were 80% for **7a**, 89% for **7b**, 97% for **7c**, 77% for **7d** and 70% for **7e**.

Synthesis of Octadecadeoxyribonucleotides with an Aminoalkyl Group at the 5′ End——The synthesis was performed on an Applied Biosystems DNA synthesizer, model 381A, by the methoxy phosphoramidite method. At the last coupling, **7a** or **7e** was used. After the synthesis, the nucleotide resin was treated with a mixture of thiophenol–triethylamine–dioxane (1 : 2 : 2, 0.5 ml) at room temperature for 1 h. The resin was washed with acetonitrile and ether then dried. It was treated with concentrated NH_4OH (2 ml) and pyridine (0.5 ml) at 55 °C for 12 h. The resin was filtered off and the solution was evaporated. The residue was chromatographed on a reversed-phase C-18 silica gel column (i.d. 0.7 × 7 cm) with a linear gradient of acetonitrile in 50 mM triethylammonium acetate (TEAA, pH 7). The fractions between the dotted lines in Fig. 1 were pooled and evaporated to a small volume, which was treated with 80% aqueous AcOH (2 ml) at room temperature for 1 h. The mixture was quenched with H_2O (1 ml) and washed with ether (6 ml × 2). The aqueous layer was evaporated to afford 40 A_{260} units in both cases. An aliquot of each sample was analyzed by reversed-phase C-18 HPLC as shown in Fig. 2.

FITC Labeling of the Amino Group of Oligonucleotide——Fluorescein isothiocyanate (1 mg) in 1 M Na_2CO_3–$NaHCO_3$ (pH 9) (50 μl), H_2O (80 μl) and dimethylformamide (20 μl) was added to an aminoalkylated octadecamer (5 A_{260} unit) in H_2O (150 μl). The mixture was shaken at room temperature in the dark for 18 h then applied to a Sephadex G-25 column (i.d. 1.8 × 10 cm) and eluted with 0.1 M TEAB. The fraction eluted at the void volume was collected and evaporated to a small volume, which was subjected to reversed-phase C-18 HPLC. The fraction between the dotted lines in Fig. 3 was collected. UV (H_2O) peaks were seen at 258, 460 (sh) and 490 nm for both oligonucleotides.

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