[Chem. Pharm. Bull. 34(9)3688—3694(1986)]

# A Large-Scale Synthesis of a Nonadecadeoxyribonucleotide Duplex Having a Sequence Identical to That of Phage $\phi$80 $O_R2$ by the Phosphoro-*p*-anisidate Method[1)]

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(Received March 10, 1986)

A nonadecanucleotide duplex, dCATCACCATAATGTTCATT·dAATGAACATTATGGTGATG, having a sequence identical to that of phage $\phi$80 $O_R2$ has been synthesized by the phosphoro-*p*-anisidate method. 5′-*O*-Dimethoxytrityl-N-protected deoxynucleotide 3′-(*o*-chlorophenyl)phosphates and N-protected deoxynucleoside 3′-(*o*-chlorophenyl)phosphoro-*p*-anisidates were used to prepare di- and trinucleotide blocks. Oligomers were elongated either in the 5′-direction by dedimethoxytritylation or in the 3′-direction by treatment with isoamyl nitrite, and isolated by chromatography on silica gel or alkylated silica gel. The deblocked nonadecamers were purified by reversed phase and ion-exchange chromatography and the duplex was isolated by gel filtration.

**Keywords**——phosphoro-*p*-anisidate triester method; operator DNA; mini DNA duplex high pressure liquid chromatography; gel filtration; melting temperature

Regulation of lysogeny of bacteriophage $\lambda$ has been characterized and shown to involve the interaction of repressors with a specific site.[3)] Bacteriophage $\phi$80 is known to have properties similar to those of $\lambda$ phage, and its regulatory proteins and the base sequences of their binding regions have been determined.[4)] To investigate these interactions physicochemically, large quantities of deoxyribonucleic acid (DNA) duplexes are required. We have previously synthesized a heptadecamer duplex corresponding to the sequence of $\lambda$ $O_R3$, which is a binding site for $\lambda$ cro protein.[5)] High-resolution nuclear magnetic resonance (NMR) spectroscopy was used to investigate the structural properties of the duplex.[6)] In this paper we describe a synthesis of a nonadecadeoxyribonucleotide duplex which is identical to an operator $O_R2$ or $\phi$80, which is a binding site for $\phi$80 cro repressor. Figure 1 shows the base


Fig. 1. The Sequences of $\phi$80 $O_R2$ and $\lambda$ $O_R3$

Identical sequences are surrounded by solid lines and common purine or pyridine parts are shown by dotted lines.

sequence of $\phi$80 $O_R2$ together with that of $\lambda$ $O_R3$. The nonadecadeoxyribonucleotides were synthesized by the phosphoro-*p*-anisidate method in solution.[7)]

## Synthesis of Protected Nonadecadeoxyribonucleotides (28 and 53)

N-Protected deoxyribonucleoside 3′-(*o*-chlorophenyl)phosphoro-*p*-anisidates and 5′-*O*-dimethoxytrityl-N-protected deoxyribonucleoside 3′-(*o*-chlorophenyl)phosphates were used to prepare dinucleotide blocks as described[7,8)] except that 1-(mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT)[9)] was used as the condensing reagent. Fully protected dinucleotides

were isolated by silica gel[7] or C-18 silica gel[8] chromatography. The 3′-terminal blocks were prepared by condensing the 3′-*O*-benzoylnucleosides. Schemes for the synthesis of the two nonadecamers are shown in Charts 1 and 2. For elongation of the chain, the 5′-*O*-

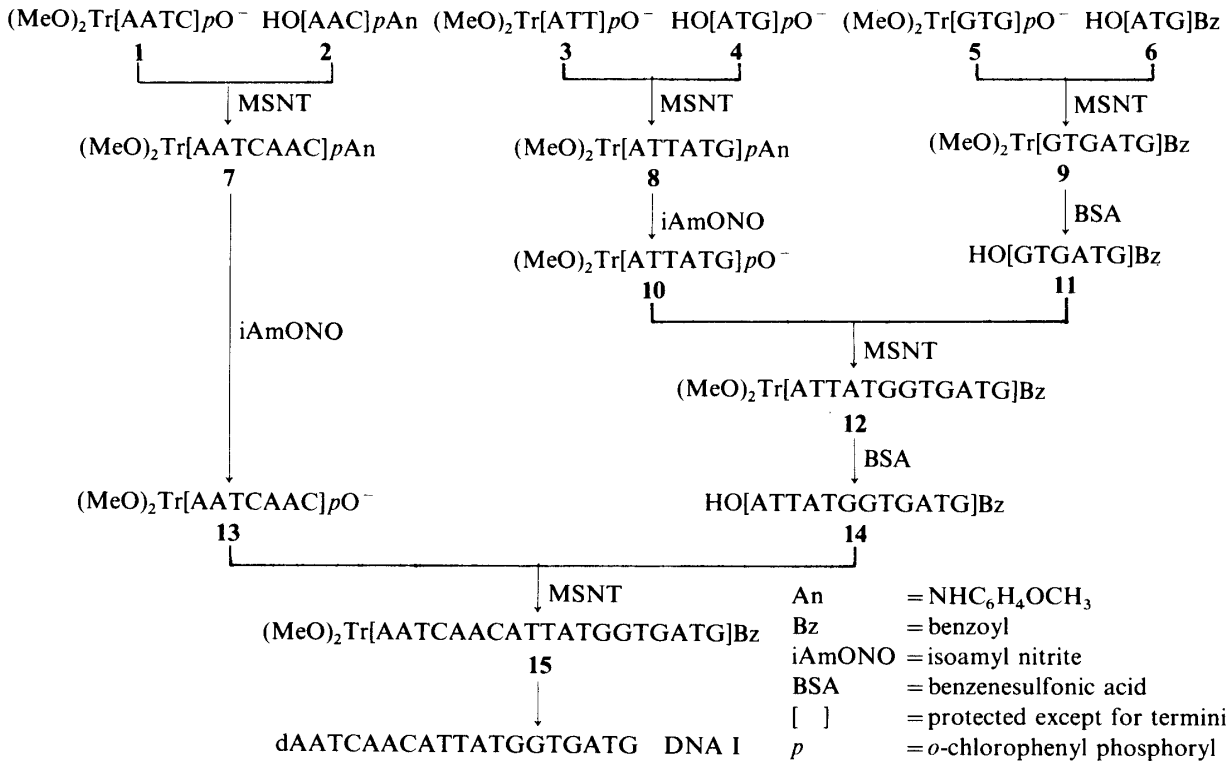


Chart 1

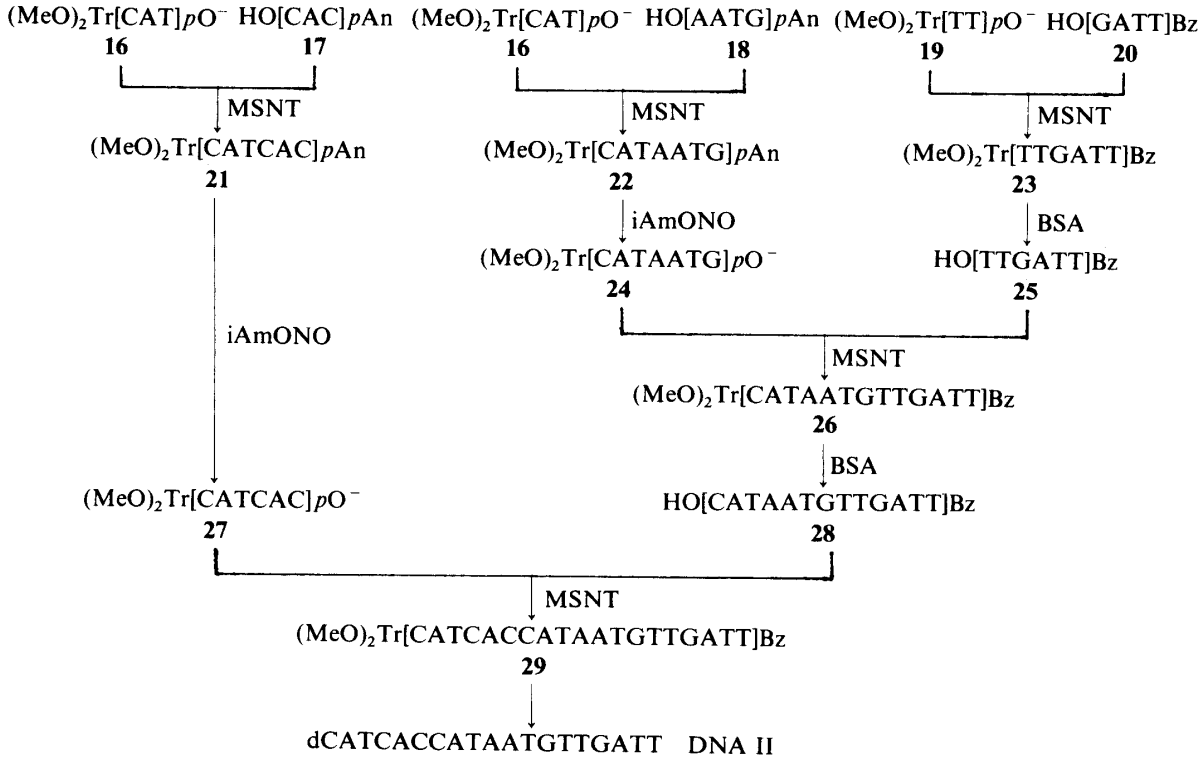


Chart 2

TABLE I. Reaction Conditions for the Synthesis of Protected Oligonucleotides

| 3′-Phosphodiester component (mmol) | 5′-OH component | MSNT (mmol) | Product (mmol) | Chain length | Yield (%) |
|---|---|---|---|---|---|
| **1** (0.831) | **2** (1.05) | 1.66 | **7** (0.700) | 7 mer | 83.8 |
| **3** (1.03) | **4** (0.800) | 2.06 | **8** (0.645) | 6 mer | 80.7 |
| **5** (1.22) | **6** (1.04) | 2.44 | **9** (0.800) | 6 mer | 77.0 |
| **10** (0.645) | **11** (0.800) | 1.29 | **12** (0.373) | 12 mer | 57.8 |
| **13** (0.466) | **14** (0.373) | 0.932 | **15** (0.354, crude) | 19 mer | — |
| **16** (1.20) | **17** (0.810) | 2.40 | **21** (0.516) | 6 mer | 67.3 |
| **16** (0.874) | **18** (0.728) | 1.75 | **22** (0.278) | 7 mer | 32.8 |
| **19** (1.14) | **20** (0.912) | 2.28 | **23** (0.633) | 6 mer | 69.4 |
| **24** (0.278) | **25** (0.336) | 0.556 | **26** (0.185) | 13 mer | 66.5 |
| **27** (0.276) | **28** (0.185) | 0.552 | **29** (0.092) | 19 mer | 49.8 |

dimethoxytrityl and 3′-phosphoro-*p*-anisidate were treated with a five-fold excess of benzenesulfonic acid and a fifty-fold excess of isoamyl nitrite, respectively. These reactions became slower with increasing chain length, so larger amounts of reagents were used as compared with the amounts described for shorter oligonucleotides. MSNT was used as the condensing reagent at 38 °C for 20 min. Reaction conditions and yields of protected oligonucleotides are summarized in Table I. Condensation products were isolated by chromatography on silica gel and/or C-18 silica gel. Dedimethoxytritylated products, *e.g.*, **2** and isoamyl nitrite- treated product **3** were condensed without purification. However, the unpurified heptamer (**13**) caused contamination of the nonadecamer (**15**) during isolation by reversed phase chromatography. For the synthesis of the other protected nonadecamer (**29**), the phosphodiester component (**27**) was purified by chromatography on C-18 silica gel before condensation with the 5′-hydroxy component (**28**).

**Deblocking of Nonadecamers (15 and 29) and Isolation of DNA I and DNA II**

The protecting groups for phosphodiesters (*o*-chlorophenyl) and the 3′-hydroxyl group (benzoyl) were removed by treatment with 1,1,3,3-tetramethylguanidinium 2-pyridine aldoximate.[9] After removal of the N-benzoyl groups with ammonia, the dimethoxytritylated nonadecamer was separated from other non-lipophilic oligonucleotides by reversed phase chromatography on C-18 silica gel as shown in Fig. 2a. Fractions containing the dimethoxytrityl oligonucleotides were treated with 80% acetic acid and the completely deblocked product (DNA I) was subjected to reversed-phase chromatography as shown in Fig. 2b. However, analysis by high-pressure liquid chromatography (HPLC) showed contamination in the nonadecamer. DNA I was purified by ion-exchange chromatography on diethylaminoethyl (DEAE)-Toyopearl as shown in Fig. 2c. The product was further purified by reversed phase chromatography with a shallower gradient of acetonitrile than used in Fig. 1b.

To improve the procedure for isolation of DNA II, a shallower gradient of acetonitrile was used for reversed-phase chromatography of the dimethoxytrityl nonadecamer. As shown in Fig. 3, the product was separated from impurities with higher lipophilicity. The compound in Table I was dedimethoxytritylated and subjected to the same chromatography with a much shallower gradient. The product (DNA II) obtained was homogeneous on reversed-phase and ion-exchange HPLC (Fig. 4).

**Properties of the Duplex**

The two nonadecamers, DNA I and DNA II, were annealed at 90 °C and the duplex was separated by gel filtration on Sephadex G-75 as shown in Fig. 5. Triethylammonium ions in the product were converted to sodium ions. The temperature–ultraviolet (UV) absorption

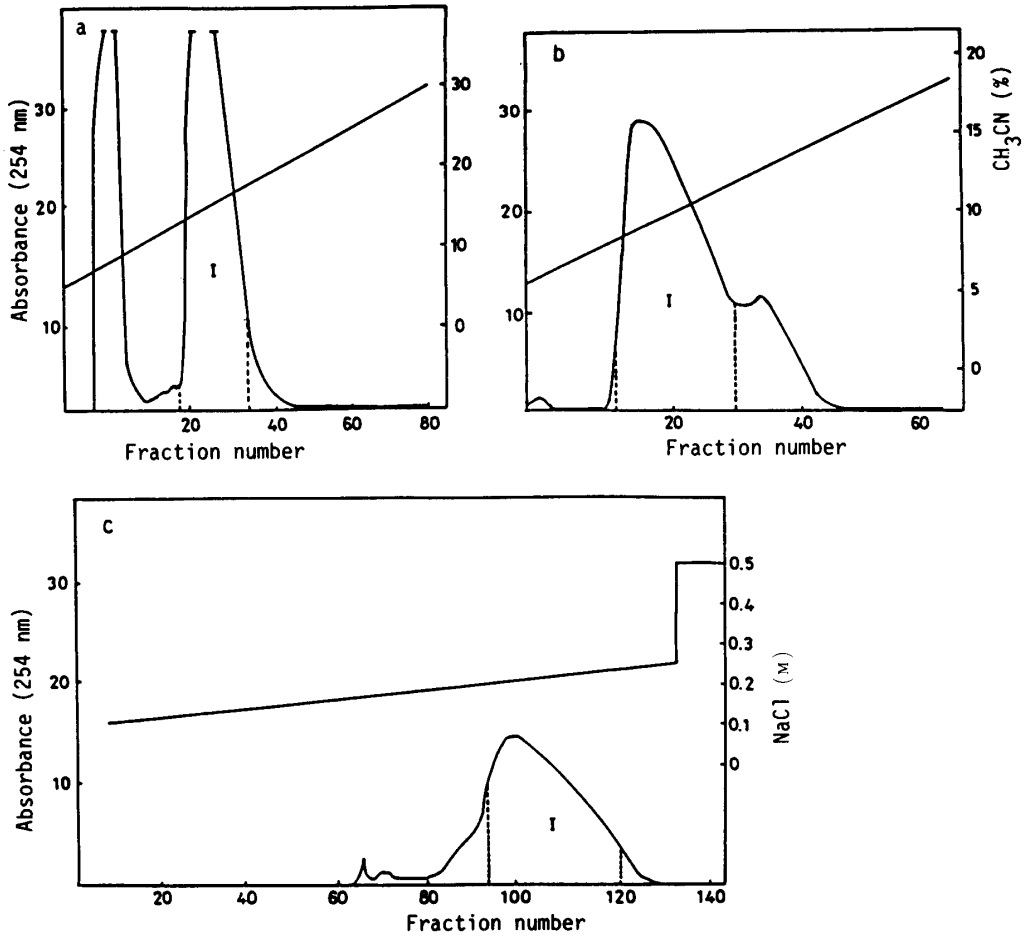


Fig. 2. (a) Reversed-Phase Chromatography of Dimethoxytritylated DNA I on a Column (1.5 × 23.0 cm) of C-18 Silica Gel

A gradient of acetonitrile in 0.1 M ammonium acetate was prepared by using 5% and 35% acetonitrile (300 ml, each). Fractions of 7 ml were collected every 3.5 min.

(b) Reversed-Phase Chromatography of DNA I

Conditions were the same except that 25% acetonitrile was used instead of 35% and 5.5 ml fractions were collected.

(c) Anion-Exchange Chromatography of DNA I on a Column (2.5 × 31.0 cm) of DEAE-Toyopearl

Elution was performed with 0.02 M Tris–HCl (pH 7.5) and 7 M urea containing a gradient of NaCl (0.1—0.25 M, total 1000 ml). Fractions of 4.3 ml were collected every 7 ml.

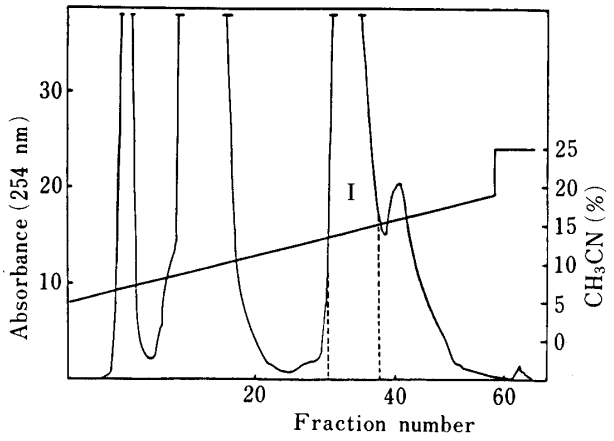


Fig. 3. Reversed Phase Chromatography of Dimethoxytritylated DNA II

Conditions were the same as in Fig. 2b. Fractions of 7.3 ml were collected every 7 min.

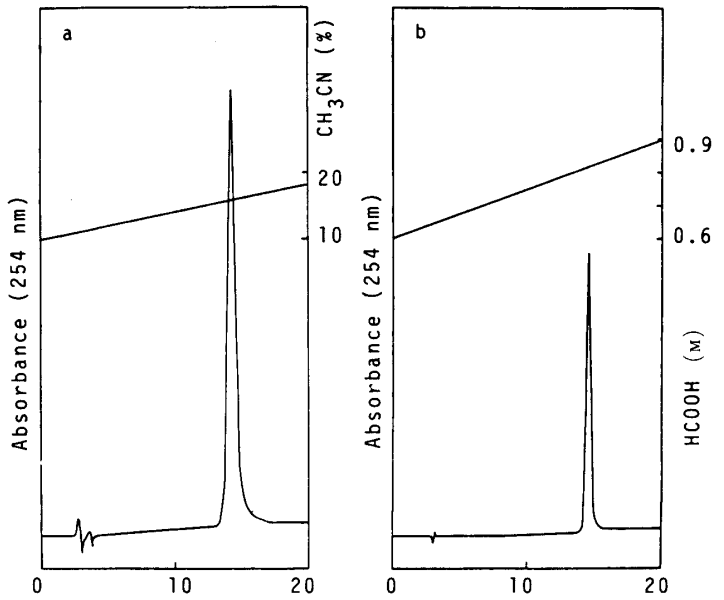


Fig. 4. Analysis of DNA II by Reversed-Phase (a) and Anion-Exchange (b) HPLC

(a) A column (4 × 250 mm) of TSKgel DDS-120T was used with a gradient of acetonitrile in 0.1 M triethylammonium acetate. The flow rate was 1 ml/min. (b) A column (4 × 250 mm of TSKgel DEAE-2SW was used with a gradient of ammonium formate in 20% acetonitrile. The flow rate was 1 ml/min.

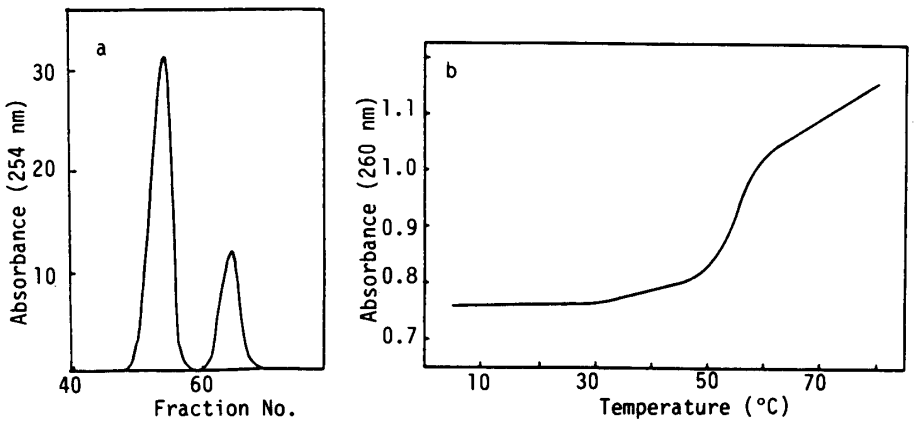


Fig. 5. (a) Gel Filtration of the Nonadecamer Duplex on a Column (1.8 × 131 cm) of Sephadex G-75 in 0.05 M Triethylammonium Bicarbonate

Fractions of 2.5 ml were collected every 11.6 min. The first peak contain the duplex.

(b) Temperature–UV Absorption Profile of the Duplex in 0.01 M Phosphate Buffer (pH 7.1) in the Presence of 0.1 M NaCl

profile of the duplex ($\phi$80 $O_R2$) was measured as shown in Fig. 5 and the mid point was found to be 55.5 °C, which is lower than that of the heptadecamer duplex (Tm 59 °C) corresponding to $\lambda$ $O_R3$. This may be due to different base compositions of the duplexes, although similar sequences are found, as shown in Fig. 1. The AT-rich region in the middle of $\phi$80 $O_R2$ may destabilize the nonadecamer duplex. The sequence may play an important role in recognition by repressors. A preliminary experiment on binding of $\lambda$ cro to DNA containing $\phi$80 $O_R3$ showed low shielding of the protein from digestion by deoxyribonuclease (DNase). NMR studies on the duplex will be reported in a separate paper.

## Experimental

**General Methods**——Thin layer chromatography (TLC) was performed on pre-coated Kiesel gel 60 $F_{254}$ or

HPTLC 60 $F_{254}$ (Merck) using chloroform (or dichloromethane) and methanol, or benzene and ethyl acetate. For reversed phase TLC, Kiesel gel 60 $F_{254}$ silanisiert or C-8 (Merck) was used in a mixture of acetone–water. Nucleosides or nucleotides were detected and identified by spraying 30% sulfuric acid followed by heating.

For column chromatography, Kiesel gel 60 or 60H (Merck) was used. Alkylated silica gel (C-18, 35—100 $\mu$, Waters) was used for reversed-phase column chromatography at low pressure with increasing amounts of acetone in water in the presence of 0.2% pyridine. For unprotected oligonucleotides, 0.1 M triethylammonium acetate was used with acetonitrile.

HPLC was performed with a column of C-18 silica gel on Altex MP or Spectra Physics apparatus. UV and circular dichroism (CD) spectra were measured with a Hitachi 200-10 spectrophotometer and a JASCO J500A spectropolarimeter, respectively. UV-temperature profile were measured with a Beckman DU-8B spectrophotometer. Mobility shift analysis[9] was carried out using Homo mixture I.[10]

Protected nucleoside and nonanucleotides were prepared as described.[8,9] Removal of the dimethoxytrityl and anisidate groups from shorter oligonucleotides was performed by the reported procedures.[8]

**The Protected Nonadecanucleotide (15)**——The heptadecanucleotide (**7**, 1.85 g, 0.466 mmol) was dissolved in pyridine–acetic acid (10.3 ml–8.3 ml) and treated with isoamyl nitrite (3.8 ml, 28 mmol) at 32 °C for 4 h. A mixture of pyridine (46 ml) and 0.2 M triethylammonium bicarbonate (96 ml) was added to the reaction mixture in an ice bath and the whole was washed three times with 1 : 1 pentane–ether (240 ml). The product was extracted with dichloromethane (180 ml), backextracted, washed three times with 0.2 M triethylammonium bicarbonate (200 ml), and dried by evaporation with pyridine, then **13** was precipitated with 1 : 1 pentane–ether containing 1% triethylamine from its solution in dichloromethane.

The dodecamer (**12**, 2.27 g, 0.373 mmol) was treated with 2% benzenesulfonic acid in a mixture of dichloromethane–methanol (7 : 3, 26 ml) at 0 °C for 30 min. Saturated sodium bicarbonate solution (26 ml) was added and the soution was washed with 5% aqueous pyridine. The product (**14**) was dried by coevaporation with pyridine. The nonadecamer (**15**) was synthesized by condensation of the heptamer (**13**) and dodecamer (**14**) with MSNT (276 mg, 0.932 mmol) in pyridine (5 ml) for 30 min. The reaction was stopped by the procedure described for **13**, then **15** was applied to a column (3.2 × 5.7 cm) of C-18 silica gel. However, **15** was not eluated with a mixture of acetone and 1 M ammonium acetate or with methanol, and it was recovered by washing the column with pyridine. The yield was 3.418 g, 0.354 mmol (crude).

**The Protected Nonadecamer (29)**——The hexamer (**21**, 0.950 g, 0.276 mmol) was treated with isoamyl nitrite (16.5 mmol) by the procedure used for the preparation of 13, and 27 was purified by reversed-phase chromatography on a column (3.2 × 5.2 cm) of C-18 silica gel.

The hexamer (**27**) and tridecamer (**28**) obtained from the dimethoxytritylated tridecamer (**26**, 1.19 g, 0.185 mmol) were dried by evaporation with pyridine, then condensed by using MSNT (0.163 g, 0.552 mmol) at 31 °C for 30 min. The mixture was worked up as described for **13** and applied to a column (3.2 × 5.5 cm) of C-18 silica gel. The yield of **29** was 49% (0.87 g, 0.92 mmol).

**Deblocking of the Nonadecamers**——The nonadecamer **15** (0.254 g, *ca.* 0.025 mmol) was dissolved in dioxane (24 ml) containing 1 M $N,N,N^3,N^3$-tetramethylguanidinium *syn* pyridine-2-aldoximate. Water (24 ml) was added and the mixture was shaken at room temperature for 84 h. The solvent was removed and the residue was dissolved in pyridine (8 ml), then treated with conc. ammonia (80 ml) at 55 °C for 6 h. Volatile materials were removed and an aqueous solution of the residue was passed through a column (1.8 × 21.2 cm) of pyridinium Dowex 50 × 2. The column was washed with 30% aqueous pyridine (200 ml) and the concentrated solution was washed 3 times with ethyl acetate. The dimethoxytrityl nonadecamer was isolated by reversed-phase chromatography, as shown in Fig. 2a, and treated with 80% acetic acid (20 ml) for 20 min. The residue was dissolved in water (30 ml) and washed 3 times with ethyl acetate (30 ml). Conditions for further purification of DNA I are shown in Fig. 2b and 2c. The yield was 1000 $A_{260}$ units.

DNA II (1440 $A_{260}$ units) was obtained from the protected nonadecamer (**29**) by the same procedure. Reversed-phase chromatography of the dimethoxytritylated nonadecamer was performed as shown in Fig. 3.

**Preparation of the Duplex**——DNA I (1000 $A_{260}$ units) and DNA II (1440 $A_{260}$ units) were dissolved in water (50 ml). The solution was heated at 90 °C for 5 min and left in the water bath, then concentrated to *ca.* 15 ml. An aliquot (2 ml) was subjected to gel filtration (Fig. 5) and the duplex was separated from the single strands. The total yield of the duplex was 1620 $A_{260}$ units.

## References and Notes

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