Preparation of a Decadeoxyribonucleotide Helix for Studies by Nuclear Magnetic Resonance†

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ABSTRACT: A self-complementary decadeoxyribonucleotide d-CpCpApApGpCpTpTpGpG was chemically synthesized by a procedure based on the phosphotriester approach. This procedure was carefully monitored and appropriately modified to ensure the purity of oligomer components at each step of the synthetic scheme. Extensive use was made of both analytical and preparative high-pressure liquid chromatography to purify and characterize the decamer and its constituent oligonucleotides. The final product (1318 A_{257} units or 16.5 μmol) was obtained in high purity and sufficient quantity for extensive physical studies by UV, CD, and NMR spectroscopy. Our preliminary results show that at a strand concentration of 1.3×10^{-5} M and in 0.10 M sodium chloride and 0.01 M sodium phosphate buffer, pH 7.0, the decamer duplex has a T_m at 47 °C. The CD spectrum of this decamer duplex is similar to that of B-form DNA. All the resonances of the nonexchangeable base protons of the decamer are well resolved in the ^{1}H NMR spectrum, when the single-stranded form was examined by using a 360-MHz spectrometer and when the duplex form was examined by using a 600-MHz spectrometer. These base proton resonances have been tentatively assigned by using the incremental assignment technique. Although the decamer duplex serves as a substrate for *Alu*I restriction endonuclease, it is not cleaved by *Hin*dIII endonuclease.

Oligonucleotides of defined sequence have served as important models for the study of nucleic acid structure and function in both single- and double-stranded forms. In previous studies from this laboratory, we have described extensive proton nuclear magnetic resonance studies on a self-complementary ribooligonucleotide, r-ApApGpCpUpU,[1] which forms a stable duplex in aqueous solution (Borer et al., 1975; Kan et al., 1975). These studies revealed that this short duplex exists in an A′-type conformation and thus serves as a good model for double-helical RNA.

A detailed understanding of deoxyribonucleic acid structure in solution could be obtained by studying an oligonucleotide duplex which represents one full turn of the DNA helix. To this end, we have prepared and examined by a variety of physical methods a self-complementary decadeoxyribonucleotide, d-CpCpApApGpCpTpTpGpG. The decanucleotide was prepared by chemical synthesis using a phosphotriester approach. This synthetic procedure has been used extensively by others to prepare long oligodeoxyribonucleotides for various biochemical experiments (Itakura et al., 1975; Bahl et al., 1976; Heyneker et al., 1976; Scheller et al., 1977; Ullrich et al., 1977) and for the preparation of synthetic genes (Itakura et al., 1977; Crea et al., 1978; Hsiung et al., 1979; Sung et al., 1979). For these studies, the requirements for quantity and purity of the oligonucleotides are not as demanding as for physicochemical studies. In particular, for NMR studies rather large quantities of very pure materials are required. These requirements necessitated modification of the literature procedures in order to obtain material satisfactory for the NMR experiments. The present paper gives a detailed description of the synthetic procedures used to prepare d-CpCpApApGpCpTpTpGpG and a preliminary description of its physical properties in solution. Subsequent publications will present, in detail, studies on the conformation of this molecule.

Experimental Section

Materials. Nucleosides were purchased from P-L Biochemicals and Calbiochem and were checked for purity by paper chromatography. *N*-Benzoyldeoxycytidine, *N*-benzoyldeoxyadenosine, *N*-isobutyryldeoxyguanosine, and the 5′-*O*-(dimethoxytrityl) derivatives of these compounds and of thymidine were prepared according to published procedures (Schaller et al., 1963; Büchi & Khorana, 1972). 1,2,4-Triazole (Aldrich) and *p*-chlorophenol and benzenesulfonic acid (both from Eastman) were used without further purification. Hydroacrylonitrile (Eastman) was dried over 4-Å molecular sieves. Triethylamine was refluxed for 7 h with calcium hydride and then distilled onto 4-Å molecular sieves. *p*-Chlorophenyl phosphorodichloridate was prepared by the method of Cramer & Winter (1959). Mesitylenesulfonyl tetrazolide was prepared as previously described (Miller et al., 1979) and was stored at −80 °C. Anhydrous pyridine was prepared by refluxing reagent-grade pyridine (3 L) with chlorosulfonic acid (40 mL) for 7 h, followed by distillation onto sodium hydroxide pellets (40 g). After being refluxed for 7 h, the pyridine was distilled onto 4-Å molecular sieves and stored in the dark. Reagent-grade chloroform was distilled by using a rotary evaporator.

Silica gel column chromatography was carried out at atmospheric pressure with Baker 3405 silica gel (60–200 mesh) for protected mono- and dideoxyribonucleotides and Merck silica gel 60 (70–230 mesh) for protected oligodeoxyribonucleotides. Thin-layer silica gel chromatography (TLC) was performed on Merck silica gel 60 F_{254} plastic-backed TLC sheets (0.2 mm thick). Chromatography was carried out in chambers lined with filter paper saturated with solvent. To ensure reproducible results, it is extremely important to charge the chamber with fresh solvent before each run. High-pressure liquid chromatography (high-pressure LC) was carried out by using either a Laboratory Data Control instrument or a Varian

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[1] Abbreviations used: d-NpN, a deoxyribooligonucleotide *p*-chlorophenyl phosphotriester; MST, mesitylenesulfonyl tetrazolide; bz, benzoyl; bu, butyryl; Tr, trityl. The symbols used to represent oligonucleotides and their protected derivatives follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (1970).

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5000 liquid chromatograph. Analytical silica gel high-pressure LC was performed on columns (2.1 mm × 1 m) packed with HC Pellosil (Whatman). Analytical ion-exchange high-pressure LC was carried out on columns (2.1 mm × 50 cm) packed with Pellionex AL WAX (Whatman) while preparative work was carried out on 0.8 × 60 cm columns (Leutzinger et al., 1978). Reversed-phase high-pressure LC was carried out on Whatman Partisil PXS 10/25 ODS-2 (analytical) and Partisil M9 10/50 ODS-2 (preparative) columns. The conditions used to elute the analytical columns are given in the footnotes in Tables III and V.

Ultraviolet spectra were recorded on a Cary 14 or Varian 219 ultraviolet spectrophotometer. The following extinction coefficients for the protected monomers at 260 nm in 95% ethanol were used: dT, 9100; d[$(MeO)_2$Tr]T, 10 300; d-bzC, 21 400; d-[$(MeO)_2$Tr]bzC, 22 250; d-bzA, 10 600; d-[$(MeO)_2$Tr]bzA, 12 500; d-ibuG, 16,700; d-[$(MeO)_2$Tr]ibuG, 17 400. Circular dichroism spectra were measured on a Cary 60 spectrophotometer with CD attachment.

Preparation of Fully Protected 5′-O-(Dimethoxytrityl)-deoxyribonucleoside 3′-(p-Chlorophenyl β-cyanoethyl phosphates), d-[(MeO)$_2$Tr]NṗCE. The following general synthetic procedure was used. The conditions given are for a 10 mmol scale reaction. Reactions using different amounts of d-[$(MeO)_2$Tr]N can be directly scaled up or down based on the amounts of reagents given here. The preparation scales, yields, and column chromatography conditions for these reactions are described in Table I. d-[$(MeO)_2$Tr]N (10 mmol) was dried by evaporation with anhydrous pyridine (3 × 10 mL). Dry nitrogen was admitted into the flask after each evaporation. In a separate flask, benzenesulfonic acid (40 mmol) was dried by evaporation with anhydrous pyridine (3 × 10 mL) and then dissolved in 10 mL of pyridine. Triethylamine (40 mmol) was added to this solution.

The remaining operations were carried out in a glove bag filled with dry nitrogen. Triazole (30 mmol) in dry tetrahydrofuran (10 mL) was reacted with triethylamine (30 mmol) followed by *p*-chlorophenyl phosphorodichloridate (15 mmol) for 10 min. The resulting triethylammonium chloride was removed by filtration on a sintered-glass filter and washed with tetrahydrofuran (12 mL). The pyridine solution of d-[$(MeO)_2$Tr]N was then added to the filtrate. The phosphorylation reaction was checked after 1 h by withdrawing a small aliquot and treating it with 50% aqueous pyridine. After removal of the solvents by evaporation at room temperature, the residue was dissolved in 10% MeOH–$CHCl_3$ and examined by TLC using 5% MeOH–$CHCl_3$. Disappearance of the nucleoside and appearance of a trityl-containing spot at the origin of the chromatogram indicate the reaction is complete. If the reaction is not complete, additional phosphorylating reagent may be prepared and added to the reaction mixture.

The pyridine solution of benzenesulfonic acid triethylammonium salt (including precipitate) was added, followed by addition of hydroacrylonitrile (20 mmol). The reaction mixture was concentrated to 100 mL and kept at room temperature overnight. The reaction mixture was poured into 230 mL of 5% sodium bicarbonate, and the mixture was extracted with chloroform (3 × 120 mL). The combined chloroform extracts were dried over anhydrous sodium sulfate. After filtration, the chloroform solution was evaporated to a thick syrup at <35 °C by using a water aspirator. The residual pyridine was removed by an oil pump. The resulting gum, which still contained some pyridine, was dissolved in a minimum volume of chloroform (10 mL), and the solution was chromatographed on a silica gel column. The column was operated at a flow rate of between 5 and 10 mL/min. Table I gives specific elution conditions. Fractions (15–20 mL) were collected by using a fraction collector and monitored by TLC. Fractions containing the desired material were pooled, the solvents were evaporated, and the resulting gum was evaporated several times with tetrahydrofuran. The resulting foam was dissolved in 10 mL of tetrahydrofuran. The solution was added dropwise to 500 mL of hexane with stirring. The resulting precipitate was collected by filtration on a medium-porosity sintered-glass filter, washed with hexane, and dried under vacuum. The monomer was checked for purity by silica gel TLC and high-pressure LC (Table III) and UV spectroscopy (Table IV). The monomer may be stored indefinitely as the solid at 0 °C or less in a desiccator.

Preparation of Deoxyribonucleoside 3′-(p-Chlorophenyl β-cyanoethyl phosphates), d-NṗCE. The following general procedure was used. The conditions given are for a theoretical 10 mmol scale reaction. Specific conditions and yields are given in Table I. d-[$(MeO)_2$Tr]NṗCE (10 mmol) was dissolved in 38 mL of 75% MeOH–$CHCl_3$. The solution was treated at 0 °C with 145 mL of ice-cold 2% benzenesulfonic acid in chloroform for 4 min. The solution was diluted with 500 mL of chloroform and extracted with 500 mL of 5% sodium bicarbonate. The chloroform extract was dried over anhydrous sodium sulfate. After filtration and evaporation, the resulting gum was chromatographed on silica gel as described above and in Table I. Fractions containing the desired d-NṗCE were collected and precipitated from hexane. The purity of each monomer was checked by silica gel TLC and high-pressure LC (Table III) and by UV spectroscopy (Table IV).

Preparation of Fully Protected Oligodeoxyribonucleotides. The following general procedure for a theoretical 1 mmol scale reaction was used. Specific conditions for each reaction are listed in Table II. The mono- or oligodeoxyribonucleotide component bearing the 3′-(*p*-chlorophenyl-β-cyanoethyl phosphoryl) group, –NṗCE (1.25–1.5 mmol), was treated with a solution containing 25 mL of pyridine, 8.4 mL of water, and 8.4 mL of triethylamine for 30 min at room temperature. The reaction was checked for complete removal of the cyanoethyl group by TLC (5% or 10% methanol–chloroform), as indicated by the appearance of a trityl-containing spot at the origin and disappearance of the starting material. The solvents were evaporated and the residue was evaporated with pyridine (4 × 2 mL). Dry nitrogen was admitted into the flask after each evaporation. The component bearing the 5′-OH group (1.0 mmol) was then added, followed by further evaporations with anhydrous pyridine (4 × 2 mL). The resulting foam was dissolved in 5 mL of anhydrous pyridine, and the solution was treated with 4 mmol of mesitylenesulfonyl tetrazolide for 3.5 h at room temperature. The reaction mixture was cooled to 0 °C, and 5 mL of ice-cold 50% aqueous pyridine was added. The solution was kept at room temperature for 20 min and then diluted with 20 mL of 5% sodium bicarbonate solution. The mixture was extracted with chloroform (2 × 20 mL), and the combined chloroform extracts were dried over anhydrous sodium sulfate. The chloroform extract was then treated exactly as described above for d[$(MeO)_2$Tr]NṗCE. The specific chromatography conditions are given in Table II. The precipitated oligonucleotides were checked for purity by TLC and high-pressure LC (Table III) and by UV spectroscopy (Table IV).

Where required, the dimethoxytrityl groups were removed in a manner analogous to that described above for the mononucleotides. The oligomers were purified either by column

chromatography or by direct precipitation from hexane and were obtained in the following yields: d-bzAṗbzAṗCE (71%, column), d-ibuGṗibuGOAc (76%, column), d-ibuGṗbzCṗCE (95%, precipitation), and d-TṗTṗibuGṗibuGOAc (88%, precipitation). The chromatographic mobilities and UV spectral properties of these oligomers are given in Tables III and IV, respectively.

Characterization of Oligodeoxyribonucleotides. For purposes of characterization, the protecting groups were removed from the fully protected tetramers d-[$(MeO)_2$Tr]-bzCṗbzCṗbzAṗbzAṗCE and d-TṗTṗibuGṗibuGOAc and from the hexamer d-[$(MeO)_2$Tr]bzCṗbzCp-bzAṗbzAṗibuGṗbzCṗCE by the following procedure. (Deprotection of the decamer is described under Preparation of d-CpCpApApGpCpTpTpGpG). Several milligrams of the protected oligomer was treated with 1 mL of a solution containing 0.017 M tetra-*n*-butylammonium fluoride in tetrahydrofuran–pyridine–water (8:1:1 v/v) for 48 h at room temperature. The solution was diluted with 2 mL of 60% aqueous ethanol and passed through a DEAE-cellulose column (0.5 × 2 cm) in the bicarbonate form. The column was washed with 6 mL of 60% ethanol, 4 mL of 0.02 M triethylammonium bicarbonate in 60% ethanol, and 2 mL of 2 M triethylammonium bicarbonate in 60% ethanol. The latter solution was evaporated and the buffer was removed by repeated evaporations with 60% ethanol. The residue was treated with 3 mL of 60% concentrated ammonium hydroxide in pyridine for 3 h at 50 °C after which the solvents were evaporated and the residue was treated with 80% acetic acid for 30 min at room temperature. After evaporation of solvents, the residue was dissolved in 1 mL of water and the solution was extracted with ethyl acetate (2 × 1 mL). The resulting oligomers, d-TpTpGpG, d-CpCpApAp, and d-CpCpA-pApGpCp, were purified by preparative high-pressure LC on Pellionex AL WAX or ODS-2. The spectral characteristics and high-pressure LC chromatographic mobilities of these oligomers are listed in Table V.

The 3′-terminal phosphate groups were removed from d-CpCpApAp and d-CpCpApApGpCp by treatment of the oligomer (15 A_{254} units) with bacterial alkaline phosphatase (5 μg) in 0.5 mL of 0.02 M Tris, pH 8.2, at 37 °C for 1.5 h. The dephosphorylated oligomers were separated from the enzyme by chromatography on a small DEAE-cellulose column (0.5 × 1 cm). The spectral and chromatographic properties of the oligomers are given in Table V.

The oligonucleotides were completely digested to mononucleotides with snake venom phosphodiesterase under the following conditions. The oligomer (0.2 A_{254} unit) in 22 μL of 0.01 M Tris, pH 8.2, and 2.0 mM $MgCl_2$ was treated with 3 μg of snake venom phosphodiesterase for 60 min at 37 °C. The solution was heated for 1 min at 75 °C and then cooled. Aliquots of the reaction mixture were then analyzed by reversed-phase high-pressure LC using a Partisil 10/25 ODS-2 column with the following gradient: 1% acetonitrile in 0.10 M ammonium acetate, pH 5.8 (0–5 min), and 1–15% acetonitrile in 0.10 M ammonium acetate, pH 5.8 (5–15 min), at a flow rate of 2.5 mL/min. Under these conditions, the monomers have the following retention times: dpC, 1.5 min; dpT, 3.0 min; dpG, 3.9 min; dC, 5.4 min; dpA, 9.3 min; dT and dG, 9.9 min. The base ratios were determined by integration of the peaks using the following extinction coefficients at 254 nm: dpT, 7250; dpC, 6260; dpA, 13 200; dpG, 13 600. The base ratios are given in Table VI.

(*1*) *Preparation of d-CpCpApApGpCpTpTpGpG.* The protected decamer (estimated 0.079 mmol) was deblocked in several portions. In a typical reaction, 0.035 mmol of protected decamer was dissolved in 3.2 mL of dioxane, and the solution was treated with 26 mL of a dioxane solution containing 1 M pyridine-2-aldoximate tetramethylguanidinium salt. After 4 h, an additional 3.17 mL of tetramethylguanidine were added and the solution was incubated for 16 h. The reaction mixture was diluted with 643 mL of 60% ethanol, and the solution was passed through a DEAE-cellulose column (3 × 11 cm). The column was washed with 120 mL of 60% ethanol and 120 mL of 0.05 M triethylammonium bicarbonate (TEAB) and then eluted with 120 mL of 2 M TEAB in 60% ethanol. The washes were combined and recycled through the DEAE column. The 2 M TEAB eluates were evaporated, and the residue was treated with a solution containing 21 mL of pyridine and 42 mL of concentrated ammonium hydroxide for 3 days at room temperature. The solvents were evaporated and the residue was treated with 8 mL of 80% acetic acid for 30 min at room temperature. After evaporation of the solvents, the residue was dissolved in 10 mL of water, and the solution was extracted with three 5-mL portions of ethyl acetate. The aqueous solution was diluted with ethanol to give a 60% solution, and the decamer was purified by preparative high-pressure LC on a Pellionex AL WAX column (0.8 × 60 cm). Portions of the decamer solution (up to 1100 A_{254} units) were pumped onto the column; the column was washed briefly with 0.001 M ammonium acetate in 60% ethanol until the recorder pen returned to the base line, and the column was then eluted with 450 mL of a linear gradient of 0.001–2.5 M ammonium acetate in 60% ethanol (pH 6.5) at a flow rate of 5 mL/min. Fractions containing the decamer, which eluted between 1.6 and 1.8 M ammonium acetate, were pooled. The total decamer fractions (380 mL) were diluted with 3300 mL of 10% ethanol, and the solution was passed through a DEAE-cellulose column (1.5 × 4.5 cm) at a flow rate of 150 mL/h. The column was washed with 250 mL of 0.02 M ammonium bicarbonate and the decamer was eluted with 1.0 M ammonium bicarbonate. The buffer was removed by evaporation and the decamer was lyophilized from water. A total of 1318 A_{257} units (0.0165 mmol, 21% yield) of d-CpCpA-pApGpCpTpTpGpG (measured in water, pH 7.0) was obtained. A small sample of the decamer was digested with snake venom phosphodiesterase, and the base ratio was determined as described under Characterization of Oligodeoxyribonucleotides (see Table VI). The extinction coefficient of the decamer [$\epsilon_{257}(H_2O$, pH 7) = 79 980; ϵ_{257}(10 mM Tris and 2 mM $MgCl_2$, pH 8.6) = 72 610] was determined by digesting the decamer to mononucleotides with snake venom phosphodiesterase.

(*2*) *Hydrolysis of d-CpCpApApGpCpTpTpGpG with AluI.* The decamer (0.42 A_{254} unit) was treated with 7 units of *Alu*I restriction endonuclease (P-L Biochemicals) in 40 μL of buffer containing 6 mM Tris, pH 8.2, 6 mM $MgCl_2$, and 6 mM β-mercaptoethanol at 22 or 31 °C for 24 h. Aliquots of the reaction mixture were analyzed by high-pressure LC on Pellionex AL WAX and ODS-2 columns. The decamer was partially hydrolyzed (~50%) at 22 °C to two pentanucleotides, d-CpCpApApG and d-pCpTpTpGpG, and was completely hydrolyzed at 31 °C. Further treatment with bacterial alkaline phosphatase gave two pentanucleotides, d-CpCpApApG and d-CpTpTpGpG, whose chromatographic mobilities were identical with those of authentic samples (E. E. Leutzinger et al., unpublished results).

(*3*) *Proton Magnetic Resonance Studies.* The ^{1}H NMR spectra were recorded on two different high-resolution NMR spectrometers: (1) a Bruker WH-360 located at the University

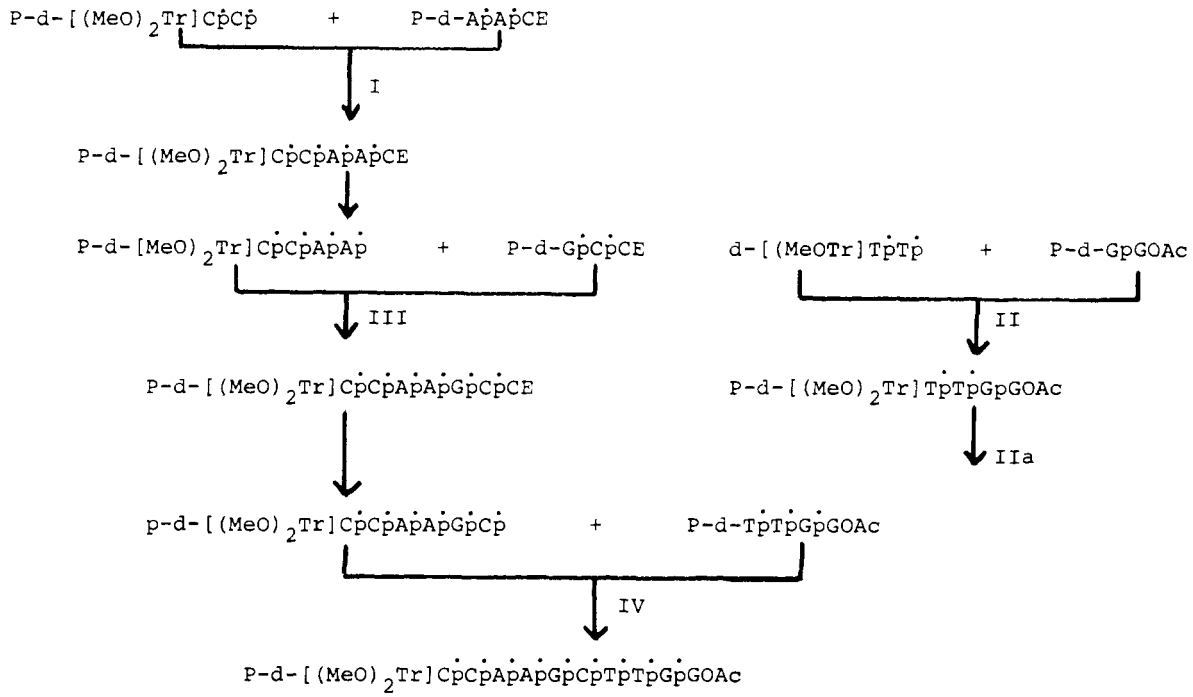

FIGURE 1: Synthetic scheme for the preparation of protected oligodeoxyribonucleotides. The letter P before the oligonucleotide indicates the base groups are protected; C = bzC, A = bzA, G = ibuG, and T = T. The symbol p indicates a *p*-chlorophenyl phosphotriester internucleotide linkage. The roman numerals refer to the steps in Table IV.

of Pennsylvania, Philadelphia, PA, which is equipped with fast FT and variable-temperature accessories; (2) a 600-MHz instrument operated at 14.1-T magnetic field strength located at Carnegie-Mellon University, Pittsburgh, PA. This spectrometer is operated with a probe temperature of 20 °C in the fast correlation spectrum mode using the HDO signal from the sample as a lock. A solution of the decamer (200 A_{257} units) dissolved in autoclaved water was passed through a Chelex column to remove paramagnetic ions. After evaporation, the decamer was lyophilized twice from D_2O and finally dissolved in 0.4 mL of 99.8% D_2O containing 1×10^{-4} M EDTA and 0.01 M phosphate buffer, pH 7.0, to give a 6.3 mM solution. All chemical shift values were measured in parts per million by using 2,2-dimethyl-2-silapentanesulfonate as the reference standard.

Results

The overall synthetic scheme for preparation of the decamer is shown in Figure 1. The approach involves condensation of protected dimers to give a hexamer, d-[$(MeO)_2$Tr]-bzCpbzCpbzApbzApibuGpbzCpCE, and a tetramer, d-TpTpibuGpibuGOAc. These two oligomers were then joined to give the protected decamer. The protected dimers were prepared as shown in Figure 2.

The key intermediates in this synthesis are the fully protected deoxyribonucleoside 3′-*p*-chlorophenyl β-cyanoethyl phosphotriesters, d-[$(MeO)_2$Tr]NpCE, which are required in large quantities and high purity. These protected monomers were synthesized by using a modification of an approach originally described by Agarwal & Riftina (1978). This procedure involves phosphorylation of suitably protected 5′-*O*-(dimethoxytrityl)deoxyribonucleosides with a small excess of *p*-chlorophenyl bis(triazolide), followed by esterification with hydroacrylonitrile. As indicated in Table I, this two-part reaction proceeds in 58–82% overall yield. The fully protected mononucleoside phosphotriesters were carefully purified by silica gel column chromatography and were isolated as amorphous white solids after precipitation from hexane.

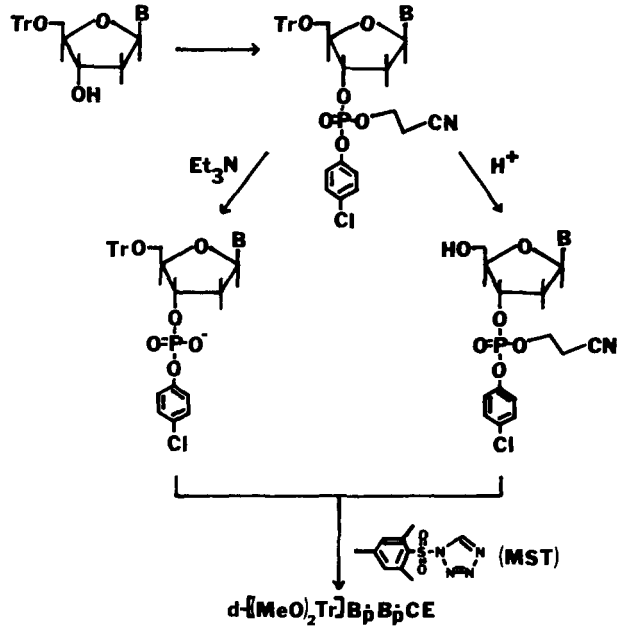

FIGURE 2: General synthetic scheme for the preparation of protected dideoxyribonucleoside *p*-chlorophenyl phosphotriesters.

The dimethoxytrityl protecting groups were selectively removed from the monomers by treatment with 2% benzenesulfonic acid in chloroform (Stawinski et al., 1977). The resulting d-NpCE was separated from dimethoxytrityl alcohol by silica gel column chromatography or by direct precipitation of the crude monomer from hexane. As shown in Table I, the latter procedure results in a higher yield of the desired product; however, this material is contaminated with traces (<5%) of dimethoxytrityl alcohol. This contamination does not interfere with subsequent condensation reactions as recently pointed out by Gilham and co-workers (Gough et al., 1979a).

The cyanoethyl group was selectively and quantitatively removed by treatment of d-[$(MeO)_2$Tr]NpCE with aqueous

Table I: Preparation of Protected Mononucleotides

monomer (mmol)	product (mmol)	silica gel column: size (cm)	silica gel column: % methanol in chloroform [vol (L)]	yield (%)
	Phosphorylation			
d-[(MeO)$_2$Tr] T (8.5)	d-[(MeO)$_2$Tr] TpCE (6.6)	4 × 40	0 (1.5) 3 (1.5)	78
d-[(MeO)$_2$Tr] bzC (45)	d-[(MeO)$_2$Tr] bzCpCE (36.7)	5 × 50	0 (2) 5 (2) 15 (2)	82
d-[(MeO)$_2$Tr] bzA (33)	d-[(MeO)$_2$Tr] bzApCE (19.3)	5 × 33	0 (1.3) 3 (0.8) 4 (2)	58
d-[(MeO)$_2$Tr] ibuG (18)	d-[(MeO)$_2$Tr] ibuGpCE (13.6)	4 × 45	0 (1.0) 4 (1.0) 5 (1.0)	72
	Detritylation			
d-[(MeO)$_2$Tr] TpCE (3.21)	d-TpCE (2.35)	3 × 26	0 (0.4) 3 (0.4) 6 (0.8)	73
d-[(MeO)$_2$Tr] bzCpCE (15.21)	d-bzCpCE (12.46)	3.5 × 34	0 (1.5) 4 (1.0) 4 (1.0)	82
d-[(MeO)$_2$Tr] bzApCE (3.79)	d-bzApCE (2.66)	2.7 × 35	0 (1.5) 5 (1.0)	70
d-[(MeO)$_2$Tr] bzApCE (5.59)	d-bzApCE (5.26)	–	–	94[a]

[a] The product was purified by direct precipitation from hexane.

Table II: Preparation of Protected Oligodeoxyribonucleotides

starting materials (mmol)	product (mmol)	step[a]	silica gel column: size (cm)	silica gel column: solvents[b]	yield (%)
d-[(MeO)$_2$Tr] Tp (3.2) + d-TpCE (2.26)	d-[(MeO)$_2$Tr] TpTpCE (1.17)		3.5 × 48	0 (1), 4 (1.5)	51
d-[(MeO)$_2$Tr] bzCp (14.6) + d-bzCpCE (9.76)	d-[(MeO)$_2$Tr] bzCpbzCpCE (6.19)		3.5 × 35	0 (1), 1 (1), 2 (1), 4 (2)	63
d-[(MeO)$_2$Tr] bzAp (9.96) + d-bzApCE (7.92)	d-[(MeO)$_2$Tr] bzApbzApCE (5.30)		3.5 × 40	0 (1.5), 2 (1.5), 3 (1.5)	67
d-[(MeO)$_2$Tr] ibuGp (2.82) + d-ibuGOAc (2.0)	d-[(MeO)$_2$Tr] ibuGpibuGO-Ac (1.0)		2.0 × 33	0 (0.3), 4 (0.5), 10 (0.5)	50
d-[(MeO)$_2$Tr] ibuGp (3.69) + d-bzCpCE (2.45)	d-[(MeO)$_2$Tr] ibuGpbzCpCE (1.47)		2.8 × 30	0 (0.5), 1 (0.5), 2 (0.5), 4 (1)	60
d-[(MeO)$_2$Tr] bzCpbzCp (4.63) d-bzApbzApCE (3.75)	d-[(MeO)$_2$Tr] bzCpbzCpbz-ApbzApCE (2.43)	I	3.5 × 34	0 (1), 1 (1), 2 (1), 5 (2)	65
d-[(MeO)$_2$Tr] TpTp (1.02) + d-ibuGpibuGOAc (0.76)	d-[(MeO)$_2$Tr] TpTpibuGp-ibuGOAc (0.22)	II	3.0 × 34	0 (0.75), 5 (0.75), 7.5 (0.75), 10 (0.75)	29
d-[(MeO)$_2$Tr] bzCpbzCpbzAp-bzAp (1.68) + d-ibuGpbz-CpCE (1.39)	d-[(MeO)$_2$Tr] bzCpbzCpbz-ApbzApibuGpbzCpCE (0.58)	III	3.0 × 34	0 (0.6), 2 (0.5), 4 (0.5), 6 (0.5), 8 (0.5)	43
d-[(MeO)$_2$Tr] bzCpbzCpbzAp-bzApibuGpbzCp (0.21) + d-TpTpibuGpibuGOAc (0.17)	d-[(MeO)$_2$Tr] bzCpbzCpbz-ApbzApibuGpbzCpTpTp-ibuGpibuGOAc (0.079)	IV	2.0 × 28	0 (0.2), 4 (0.2), 6 (0.2), 8 (0.2), 10 (0.2)	46

[a] Refer to Figure 1. [b] Percent methanol in chloroform (volume in liters is in parentheses).

triethylamine in pyridine solution at room temperature. This reaction is complete in less than 30 min. The resulting acrylonitrile and the excess triethylamine were removed by evaporation.

The syntheses of the dimers and longer oligonucleotides were carried out in anhydrous pyridine solution using mesitylenesulfonyl tetrazolide (Stawinski et al., 1977) as the condensing agent. The nucleotide component bearing the 3′-(*p*-chlorophenyl phosphoryl) group was employed in 1.25–1.5-fold excess over the nucleotide component bearing the free 5′-hydroxyl group. A fourfold excess of activating agent was used, and the reactions were run for 3.5 h at room temperature. After workup, the fully protected oligonucleotides were purified by silica gel column chromatography and were isolated as solids after precipitation from hexane. The conditions employed in each condensation reaction, the conditions used to chromatograph the oligomers, and the yields obtained are given in Table II.

The purity of the protected mono- and oligodeoxyribonucleotides was checked by conventional silica gel thin-layer chromatography (TLC) and by high-pressure LC using columns packed with pellicular silica gel. The chromatographic mobilities of the monomers and oligomers are given in Table III. In some cases, the protected mono- and oligonucleotides appeared as two closely moving spots or peaks. This phenomenon was attributed to the partial resolution of diastereoisomers.

In all cases, except the protected decamer, the purity of the protected mono- and oligonucleotides was estimated to be 95% or greater. The protected decamer which was estimated to

Table III: Mobilities of Protected Mono- and Oligodeoxyribonucleotides on Silica Gel High-Pressure LC and TLC

compd	LC[a] retention time (min)	R_f (silica gel TLC)[b] 5% M/C[c]	10% M/C	15% M/C	20% M/C
monomers					
d-[(MeO)$_2$Tr]TpCE	8.9	0.09	0.56, 0.52		
d-[(MeO)$_2$Tr]bzCpCE	11.8	0.12	0.55		0.72
d-[(MeO)$_2$Tr]bzApCE	7.5	0.33	0.57		
d-[(MeO)$_2$Tr]ibuGpCE	11.2, 12.0	0.11, 0.07	0.53, 0.47		0.71, 0.68
d-TpCE	8.6	0.04	0.39		0.51
d-bzCpCE	8.2	0.05	0.32		0.61
d-bzApCE	7.7	0.18	0.35		
dimers					
d-[(MeO)$_2$Tr]TpTpCE	9.5	0.20, 0.16	0.37, 0.34		0.65
d-[(MeO)$_2$Tr]bzCpbzCpCE	11.8	0.29	0.55		0.70
d-[(MeO)$_2$Tr]bzApbzApCE	10.2	0.22	0.60	0.65	
d-[(MeO)$_2$Tr]ibuGpibuGOAc	15.2	0.06, 0.04	0.32, 0.28		0.60
d-[(MeO)$_2$Tr]ibuGpbzCpCE	10.1, 10.7	0.08, 0.06	0.49, 0.42		0.72
d-bzApbzApCE	12.3	0.08	0.37	0.53	
d-ibuGpibuGOAc	17.3	0.00	0.14, 0.10		0.51, 0.45
d-ibuGpbzCpCE	12.9	0.00	0.22	0.43	
oligomers					
d-[(MeO)$_2$Tr]bzCpbzCpbzApbzApCE	11.4	0.02	0.50	0.64	
d-[(MeO)$_2$Tr]TpTpibuGpibuGOAc	20.9		0.10		0.42
d-TpTpibuGpibuGOAc	17.1	0.00	0.10	0.27	
d-[(MeO)$_2$Tr]bzCpbzCpbzApbzApibuGpbzCpCE	15.1	0.00	0.28	0.51	
d-[(MeO)$_2$Tr]bzCpbzCpbzApbzApibuGpbzCpTpTpibuGpibuGOAc	18.2	0.00	0.23	0.47	

[a] High-pressure LC: HC Pellosil (2.1 mm × 1 m); 0–20% methanol in chloroform; 1 mL/min; 40-mL total volume. [b] TLC: EM silica gel 60 F_{254}, 0.2 mm thick. [c] M, methanol; C, chloroform.

be 65% pure based on high-pressure LC analysis was contaminated with unreacted tetra- and hexanucleotide. Therefore, the yield of the protected decamer given in Table II is estimated by taking into account the presence of these contaminants. A small amount of the decamer was further purified by preparative high-pressure silica gel column chromatography to obtain a pure sample for UV spectral analysis.

The fully protected mono- and oligonucleotides were characterized by ultraviolet spectral analysis. A computer program was written which calculates and displays the UV spectrum of protected oligodeoxyribonucleotides of any base sequence (L.S. Kan et al., unpublished results). This program assumes a simple additive contribution from each chromophore of the oligomer to the overall spectrum. This situation should prevail in 95% ethanol solution where base–base interactions are minimized. Table IV presents the UV spectral data of these compounds and a comparison of the calculated and observed ratios of extinction coefficients at selected wavelengths.

For further characterization, the protecting groups were removed from small amounts of each of the intermediate oligonucleotides. The dinucleotides were treated sequentially with 75% concentrated ammonium hydroxide in pyridine, followed by 80% aqueous acetic acid. Examination of the reaction mixture by high-pressure LC on a Pellionex column showed that each dimer was at least 95% pure. The protected tetra- and hexanucleotides were first treated with tetra-*n*-butylammonium fluoride in aqueous tetrahydrofuran–pyridine solution (Itakura et al., 1975; Ogilvie & Beaucage, 1979) to selectively remove the *p*-chlorophenyl phosphate protecting groups. After separation of the fluoride ions from the partially deprotected oligomer by DEAE-cellulose chromatography, the remaining protecting groups were removed by using the standard base and acid deblocking sequence. This procedure gave pure d-TpTpGpG which amounted to 84% of the total A_{254} units applied to the high-pressure LC column. The remaining UV-absorbing material appeared to consist of smaller mono- and oligonucleotides. The other tetramer was obtained as a mixture of d-CpCpApAp and d-CpCpApAp in a ratio of 2.66:1 which comprised 93% of the total A_{254} applied to the column. The hexamer was obtained as a mixture of d-CpCpApApGpCp and d-CpCpApApGpCp in a ratio of 1.56:1 which comprised 89% of the total UV-absorbing material.

In order to determine more precisely the purity of the hexanucleotide, a small sample of d-[(MeO)$_2$Tr]-bzCpbzCpbzApbzApibuGpbzCpCE was first treated with pyridine-2-aldoximate (Reese et al., 1978) to remove the *p*-chlorophenyl phosphate groups, followed by sequential treatment with ammonium hydroxide and 80% acetic acid. Under these conditions, d-CpCpApApGpCp comprised 94% of the total UV-absorbing material.

Small samples of the protected decamer were deblocked by two of the procedures described above, and the reaction products were analyzed by high-pressure LC. Treatment with fluoride, followed by sequential treatment with base and acid, gave the decamer as ~33% of the total UV-absorbing material. The best results were obtained by using the pyridine-2-aldoximate treatment to remove the phosphate protecting groups. This procedure gave the decamer peak as 61% of the total nucleotidic UV-absorbing material in the reaction mixture. The bulk of the protected decamer was deblocked by using this procedure.

The deprotected oligonucleotides were purified by preparative high-pressure LC on Pellionex AL WAX or on Partisil ODS-2 columns. The oligomers were freed of ammonium acetate, which was used as a buffer for both columns, by absorption of the oligomer to DEAE-cellulose, followed by elution with the volatile buffer, ammonium bicarbonate. Alternatively, the oligomers were precipitated from 95% ethanol in which ammonium acetate is completely soluble. The recovery of oligomers was between 70 and 90%. By use of the above pyridine-2-aldoximate deblocking procedure, followed by preparative Pellionex chromatography and desalting on DEAE-cellulose, 16.5 μmol of the decamer was obtained, which represents a 21% isolated yield. The UV spectral properties and high-pressure LC mobilities of the oligonucleotides are presented in Table V. The chromatographic profiles of d-CpCpApApGpCpTpTpGpG on Pellionex and

Table IV: Ultraviolet Spectral Characteristics of Protected Mono- and Oligodeoxyribonucleotides[a]

compd	λ_{max} (nm)	λ_{min} (nm)	$\epsilon_{306}/\epsilon_{240}$ calcd	obsd	$\epsilon_{280}/\epsilon_{240}$ calcd	obsd	$\epsilon_{260}/\epsilon_{240}$ calcd	obsd	$\epsilon_{260}/\epsilon_{280}$ calcd	obsd
d-[(MeO)$_2$Tr]TpCE	232		0	0	0.44	0.42	0.55	0.63	1.24	1.47
	267	254								
d-[(MeO)$_2$Tr]bzCpCE	236	229	0.36	0.35	0.43	0.40	0.82	0.86	1.92	2.2
	260	250								
	305	290								
d-[(MeO)$_2$Tr]bzApCE	235	232	0.11	0.12	0.80	0.76	0.49	0.48	0.61	0.64
	280	257								
d-[(MeO)$_2$Tr]ibuGpCe	236		0.14	0.16	0.62	0.59	0.79	0.76	1.27	1.28
	252 (sh)									
	259 (sh)									
	275	229								
	281 (sh)	270								
d-TpCE	265	234	0.03	0.02	1.50	1.55	2.39	2.70	1.60	1.74
d-bzCpCE	265	232	0.81	0.97	0.69	0.85	1.78	1.98	2.58	2.56
	304	285								
d-bzApCE	233 (sh)		0.31	0.25	1.85	1.85	1.07	1.09	0.58	0.59
	280	246								
d-[(MeO)$_2$Tr]TpTpCE	235	231	0.00	0.00	0.62	0.55	0.86	0.89	1.39	1.63
	265	248								
d-[(MeO)$_2$Tr]bzCpbzCpCE	239	231	0.49	0.47	0.50	0.54	1.09	1.16	2.19	2.14
	262	245								
	304	290								
d-[(MeO)$_2$Tr]bzApbzApCE	233	231	0.17	0.22	1.09	1.09	0.65	0.65	0.60	0.61
	280	255								
d-[(MeO)$_2$Tr]ibuGpibuGOAc	239		0.26	0.06	0.84	0.80	1.12	1.00	1.33	1.25
	255	233								
	261 (sh)	246								
	278	273								
d-[(MeO)$_2$Tr]ibuGpbzCpCE	239		0.38	0.39	0.65	0.71	1.14	1.17	1.76	1.64
	262	230								
	300 (sh)	244								
d-bzApbzApCE	235 (sh)		0.31	0.32	1.85	1.74	1.07	1.03	0.58	0.59
	280	247								
d-ibuGpibuGOAc	258	233	0.56	0.43	1.42	1.47	1.99	1.98	1.40	1.35
	280	273								
d-ibuGpbzCpCE	260		0.71	0.63	0.99	1.04	1.87	1.85	1.89	1.79
	285 (sh)	233								
d-[(MeO)$_2$Tr]bzCpbzCpbzApbzApCE	263		0.43	0.43	0.95	0.93	1.09	1.07	1.15	1.14
	235	246								
d-[(MeO)$_2$Tr]TpTpibuGpibuGOAc	238									
	263	232	0.24	0.20	0.96	0.90	1.34	1.18	1.34	1.31
	275 (sh)	245								
d-TpTpibuGpibuGOAc	262		0.39	0.33	1.44	1.27	2.11	1.84	1.47	1.47
	275 (sh)	233								
d-[(MeO)$_2$Tr]bzCpbzCpbzApbzApibuGpbzCpCE	262	247	0.51	0.46	0.97	0.95	1.30	1.25	1.32	1.32
	238	232								
d-[(MeO)$_2$Tr]bzCpbzCpbzApbzApibuGpbzCpTpTpibuGpibu-GOAc	262	240	0.48	0.44	1.08	0.99	1.49	1.28	1.38	1.29

[a] Spectra were measured in 95% ethanol.

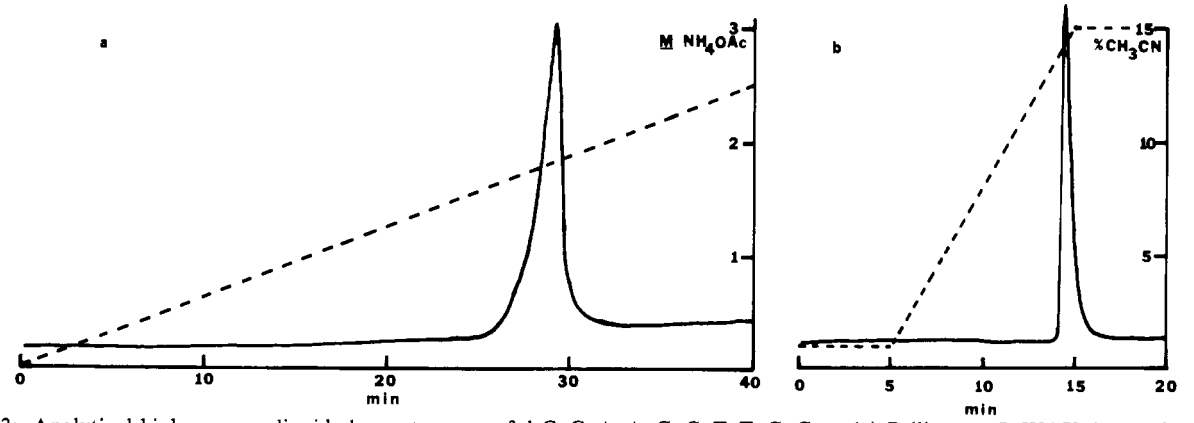

FIGURE 3: Analytical high-pressure liquid chromatograms of d-CpCpApApGpCpTpTpGpG on (a) Pellionex AL WAX, ion-exchange and (b) ODS-2, reversed-phase columns. The specific column parameters are given under Experimental Section.

ODS-2 high-pressure LC columns are shown in Figure 3.

The oligomers were further characterized by digestion with snake venom phosphodiesterase. The oligomers terminating in 3′-phosphoryl groups were first dephosphorylated by treatment with bacterial alkaline phosphatase, followed by purification on a DEAE-cellulose column. Each oligomer was

Table V: UV Spectral and High-Pressure LC Chromatographic Characteristics of Oligodeoxyribonucleotides[a]

oligomer	λ_{max} (nm)	λ_{min} (nm)	$\epsilon_{260}/\epsilon_{280}$	retention time (min) ODS-2[b]	retention time (min) Pellionex AL WAX[c]
d-CpCpApAp	260	230	2.06	1.8	18.3
d-CpCpApA	260	228	2.09	4.6	10.1
d-CpCpApApGpCp	257	227	1.71	2.3	26.6
d-CpCpApApGpC	258	229	1.50	4.8	17.0
d-TpTpGpG	257 270 (sh)	229	1.58	5.9	12.8
d-CpCpApApGpCpTpTpGpG	257	228	1.71	10.0	29.3
dpA				1.6	7.2

[a] Measured in H_2O at pH 7. [b] 7–11% acetonitrile in 0.10 M ammonium acetate, pH 5.8; 2.5 mL/min; 50-ml total volume. [c] 0.001–2.5 M ammonium acetate in 60% ethanol, pH 6.5; 1.0 mL/min; 40-mL total volume.

Table VI: Enzymatic Hydrolysis of Oligodeoxyribonucleotides with Snake Venom Phosphodiesterase

oligomer	obsd products[a]	base ratio calcd	base ratio found
d-CpCpApA	dC	1.00	1.06
	dpC	1.00	1.02
	dpA	2.00	2.00
d-TpTpGpG	dT	1.00	1.00
	dpT	1.00	0.97
	dpG	2.00	1.86
d-CpCpApApGpC	dC	1.00	1.04
	dpC	2.00	2.00
	dpA	2.00	1.96
	dpG	1.00	1.26
d-CpCpApApGpCpTpTpGpG	dC	1.00	1.00
	dpC	2.00	1.95
	dpA	2.00	1.98
	dpT	2.00	1.98
	dpG	3.00	3.01

[a] The products were separated by reversed-phase high-pressure LC on a Partisil 10/25 ODS-2 column. The column was eluted with 0.10 M ammonium acetate (pH 5.8) containing 1.0% acetonitrile (12.5 mL), followed by a linear gradient of 1.0–15% acetonitrile (25 mL) at flow rate of 2.5 mL/min.

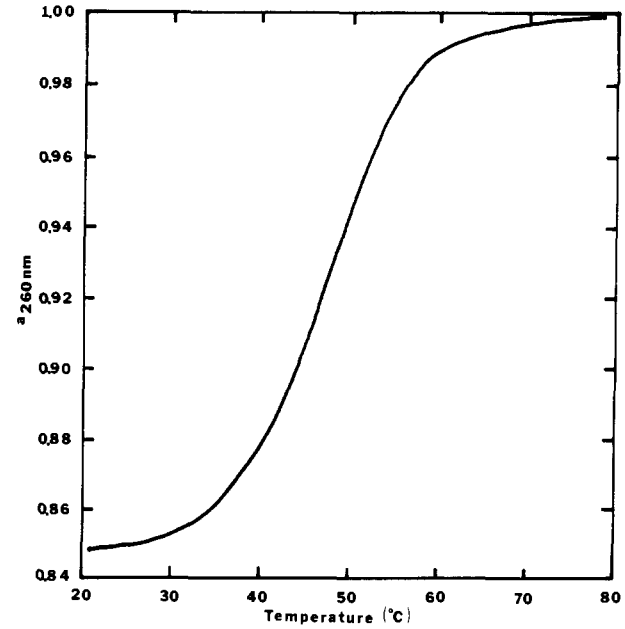

FIGURE 4: UV melting profile of d-CpCpApApGpCpTpTpGpG at a strand concentration of 13 μM in 0.10 M sodium chloride, 0.01 M sodium phosphate, and 0.10 mM ethylenediaminetetraacetate at pH 7.0.

completely digested to its constituent nucleoside and mononucleotides. The digests were analyzed by reverse-phase ODS-2 high-pressure LC. The products of each digestion and the observed base ratios are given in Table VI. The decamer was also completely digested with spleen phosphodiesterase and gave dG plus the four deoxyribonucleoside 3′-phosphates in the expected ratio.

The decamer contains nucleotide sequences which are recognized by *Alu*I (d-ApGpCpT–) and by *Hin*dIII and *Hsu*I (d-ApApGpCpTpT–) restriction endonucleases. The decamer was completely hydrolyzed by *Alu*I and gave the two pentanucleotides, d-CpCpApApG and d-pCpTpTpGpG, in the the expected 1:1 ratio. No cleavage of the decamer was observed when treated with *Hin*dIII at either 0, 22, or 37 °C.

For physical studies by NMR, the decamer was freed of paramagnetic metal ions by passage through a Chelex column. The decamer was then repeatedly lyophilized from D_2O. In making up samples for physical studies, we took extreme precautions to avoid any biological contamination of the sample. This is particularly important for solutions containing phosphate buffer. The water and buffers used to prepare the NMR samples were autoclaved before use. The buffer solutions were filtered through 0.45-μm Millipore filters and were stored at −20 °C. When these precautions were followed, no degradation of the oligonucleotides was observed even after 24 h of incubation at 37 °C.

The melting profile of the decamer measured in 0.13 M sodium chloride–phosphate buffer at a strand concentration of 13 μM is shown in Figure 4, while the CD spectra of the decamer at 19 °C and 80 °C are shown in Figure 5. Under the conditions of these experiments, the melting temperature of the decamer duplex is 47 °C. The proton NMR spectra of the base (including H_2, H_5, H_6, H_8, and CH_3) and the $H_{1'}$ proton resonances at high (90 °C) and low (20 °C) temperature are shown in Figure 6. The tentative assignments of the proton resonances at 90 °C were made by a variety of techniques, including (1) incremental sequence analysis of component oligodeoxyribonucleotides (Borer et al., 1975), (2) measurements of T_1 relaxation times and coupling constants, and (3) homodecoupling experiments. A detailed description of the assignment procedures will be given in subsequent publications.

Discussion

The goal of this study was to prepare a self-complementary decadeoxyribonucleotide, d-CpCpApApGpCpTpTpGpG, in sufficient purity and quantity for extensive physical studies by nuclear magnetic resonance spectroscopy. For the synthesis, we chose the modified phosphotriester approach originally described by Narang and co-workers (Itakura et al., 1975) and since elaborated upon by Narang and others (Katagiri et al., 1975; Stawinski et al., 1977; Sood & Narang, 1977; Hirose et al., 1978; Crea et al., 1979; Hsiung et al., 1979). In our hands we encountered a number of difficulties which

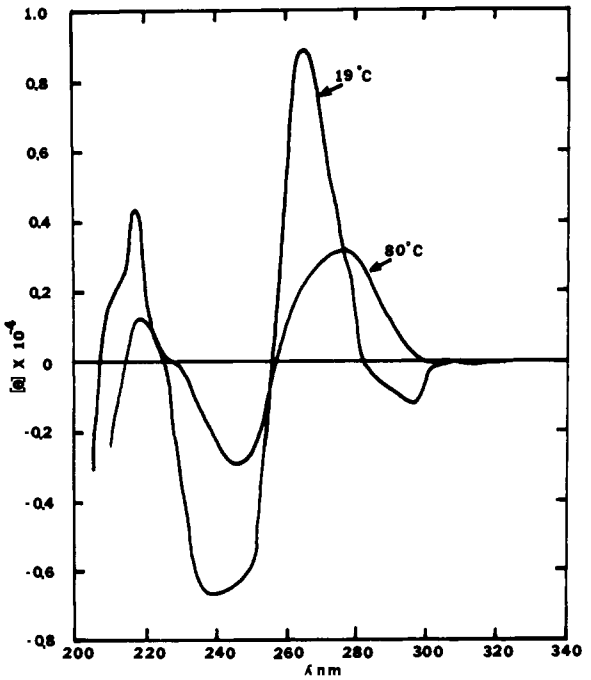

FIGURE 5: Effect of temperature on the CD spectrum of 13 μM d-CpCpApApGpGpTpTpGpG in 0.10 M sodium chloride, 0.01 M sodium phosphate, and 0.10 mM ethylenediaminetetraacetate at pH 7.0.

were not adequately described in the existing literature and which required modifications of the reported procedures.

The general synthetic scheme shown in Figure 1 was used to prepare the decamer. This scheme was chosen for the following reasons. First, since G-rich oligonucleotides are usually the most difficult to prepare, the protected tetramer d-TpTpibuGpibuGOAc was chosen as the limiting oligomer in the synthesis. Second, this approach involves detritylation of only one oligomer longer than a dimer, that is, d-[$(MeO)_2$Tr]TpTpibuGpibuGOAc. We have found that longer reaction times, which increase the possibility of depurination, were required to detritylate oligomers of increasing chain length. On the other hand, the rate of removal of the cyanoethyl group from 3′-terminal *p*-chlorophenyl β-cyanoethyl phosphoryl groups apparently is not affected by the chain length of the oligomer. Finally, the synthetic scheme provides intermediates which can be used to prepare oligonucleotides required for the assignment of the decamer ^{1}H NMR resonances by the incremental assignment technique (Borer et al., 1975).

The primary requirement for physical studies of oligonucleotides is that the oligomer be highly pure. This consideration and the requirement of relatively large quantities of material for NMR studies necessitate that care be taken at every step of the synthesis to purify each mono- or oligonucleotide intermediate. The key intermediates required for the synthesis are the protected mononucleotides, d-[$(MeO)_2$Tr]NpCE (see Figure 2). These monomers were prepared in from 60 to 80% yield by using a modification of a synthetic procedure described by Agarwal & Riftina (1978). This procedure avoids using large excesses of hydroacrylonitrile which can contaminate the mononucleotide even after silica gel column chromatography. Such contamination gives rise to side products in subsequent condensation reactions. The resulting d-[(MeO_2)Tr]NpCE was carefully purified by silica gel column chromatography and was isolated by precipitation from hexane. We have found that protected mono- and oligonucleotides isolated in this way are stable indefinitely when

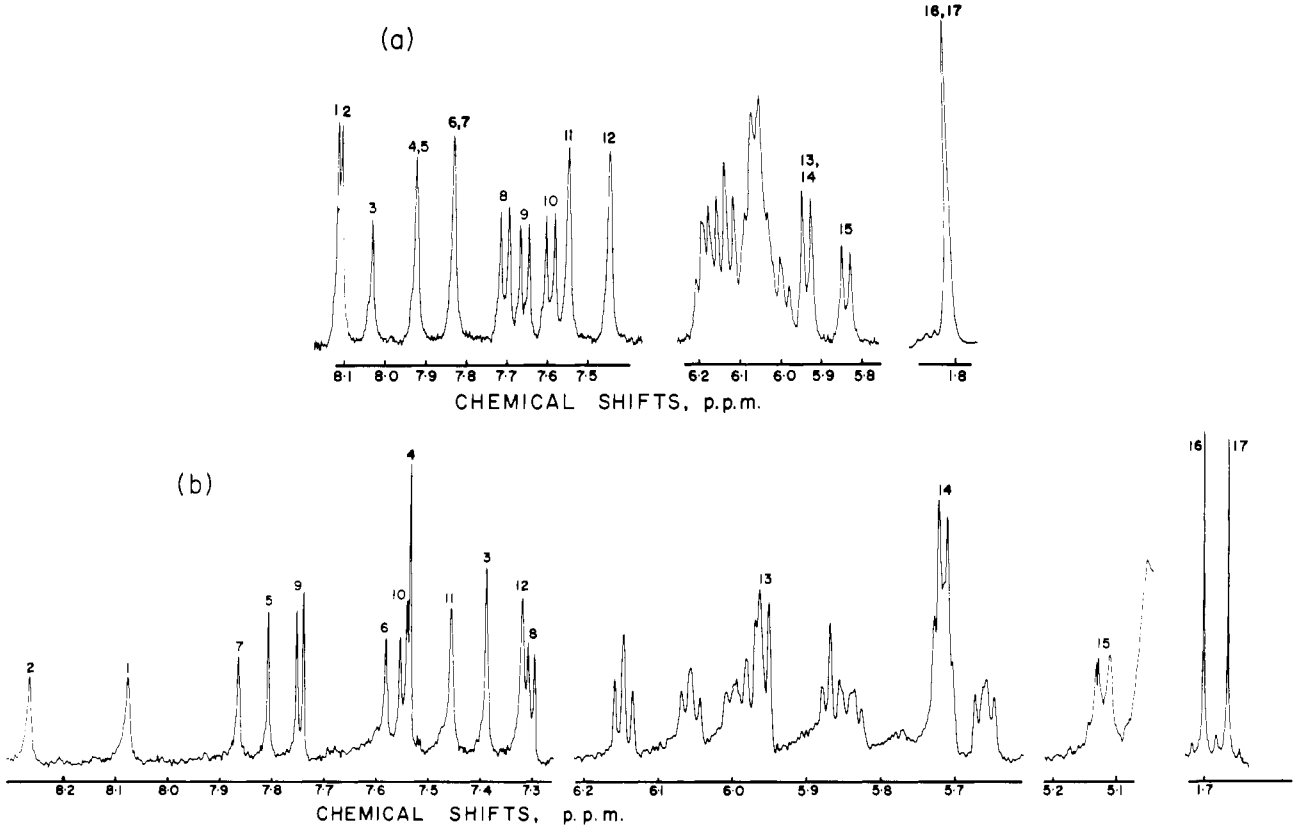

FIGURE 6: ^{1}H NMR spectra of 6.3 mM d-$C^1pC^2pA^3pA^4pG^5pC^6pT^7pT^8pG^9pG^{10}$ in 1×10^{-4} M EDTA and 0.01 M phosphate buffer, pH 7.0: (a) 360-MHz spectrum at 90 °C; (b) 600-MHz spectrum at 20 °C. The tentative assignments of the base proton resonances are as follows: 1, A^4-H_8; 2, A^3-H_8; 3, A^4-H_2; 4, A^3-H_2; 5, G^{10}-H_8; 6, G^5-H_8; 7, G^9-H_8; 8, C^6-H_6; 9, C^1-H_6; 10, C^2-H_6; 11, T^7-H_6; 12, T^8-H_6; 13, C^1-H_5; 14, C^2-H_5; 15, C^6-H_5; 16, T^7-CH_3; 17, T^8-CH_3.

stored in a desiccator at 0 °C. Thus, it is possible to stock large amounts of these materials for future use. Recently, Gilham and co-workers (Gough et al., 1979a,b) have described an alternative approach which involves the isolation of protected deoxynucleoside 3′-(*p*-chlorophenyl phosphates) as their barium salts.

Condensation reactions were carried out in anhydrous pyridine using mesitylenesulfonyl tetrazolide (Stawinski et al., 1977) as the condensing agent. Although the reactions appeared to be quite "clean" and appeared to go to greater than 80% completion when checked by silica gel TLC, examination by silica gel high-pressure LC clearly showed a number of components other than the desired product. These side products included unreacted starting materials, materials which appeared to be the 5′-O-sulfonylated oligomer and other unidentified compounds. These contaminants have mobilities similar to those of the desired oligomer on silica gel TLC and thus are difficult to detect by this method. Since the oligomers are composed of a mixture of diastereoisomers, the spot on the TLC slide is usually elongated and in some cases may consist of a series of spots of similar mobility. On silica gel high-pressure LC, the diastereoisomers often consist of a series of overlapping peaks and thus the product peak may have a number of shoulders. The broadening of the product peak becomes more serious as the chain length, and hence the number of diastereoisomers, increases. This property is a drawback of the phosphotriester method, since it makes evaluation of the purity of longer protected oligomers difficult.

The presence of side products in the condensation reactions was confirmed by preparative silica gel column chromatography. The columns were carefully eluted with increasing percentages of methanol in chloroform. Fractions, which were collected on a fraction collector, were monitored by silica gel TLC. We found that when the reaction mixture was processed in this way, side products could be readily distinguished from the desired product by silica gel TLC even though they had mobilities similar to those of the product. The oligonucleotide bearing the 3′-(*p*-chlorophenyl phosphoryl) group, which is employed in excess, remained absorbed to the silica gel column, except in the case of the protected decamer. In general, we found that purification of the protected mono- and oligonucleotides by this method could be accomplished in 4 h. Chromatography was always carried out in such a manner that the material was not allowed to remain on the column over 8 h.

Using these procedures, it was possible to obtain large quantities of the protected mono- and oligonucleotides up to the hexanucleotide in 95% purity or greater. In the case of the protected decanucleotide, considerable difficulty was encountered in separating the starting materials from the decamer. Thus, the protected decamer was obtained with an estimated purity of 65%. Preliminary studies demonstrated that the protected decamer could be further purified by silica gel high-pressure LC. However, since this compound was the end point of the synthesis and since the contaminating oligonucleotides could be separated from the desired decamer after removal of the protecting groups, further large-scale purification by high-pressure LC was not carried out.

The intermediate protected oligonucleotides were characterized by UV spectral analysis and by analysis of the products obtained after removal of the protecting groups. The UV spectra of the protected oligomers in ethanol solution were compared with computer-calculated spectra. In all cases, the agreement between the observed and calculated spectra was good.

Removal of the blocking groups from the protected oligonucleotides was carried out using three different procedures. The standard sequential treatment with concentrated ammonium hydroxide–pyridine solution, followed by treatment with 80% acetic acid, was found to be satisfactory for protected monomers and dinucleotides. For longer oligomers, this procedure, which results in some cleavage of the internucleotide linkages, is less satisfactory since increasing amounts of shorter oligonucleotides are produced as the chain length increases (Adamiak et al., 1977). We found that pretreatment of the oligonucleotides with fluoride ion (Itakura et al., 1975; Ogilvie & Beaucage, 1979) in aqueous tetrahydrofuran–pyridine solution, which partially removes the *p*-chlorophenyl phosphate protecting groups, led to increased yields of the desired oligomer. However, internucleotide bond cleavage again becomes a serious problem for oligomers with chain lengths greater than tetramer. We also found that pretreatment with F^- cleaves the terminal *p*-chlorophenyl group of those oligomers having 3′-*p*-chlorophenyl β-cyanoethyl phosphotriester groups and thus yields oligomers which terminate in a 3′-(β-cyanoethyl phosphoryl) group. Subsequent treatment with ammonium hydroxide removes the cyanoethyl group, giving an oligonucleotide terminating with a 3′-phosphate. Thus, these oligomers, which are intermediates in the overall synthetic scheme, may themselves be used for various physical and biochemical studies.

Longer oligomers were deprotected by using a three-step procedure. The *p*-chlorophenyl groups were first removed by treatment with pyridine-2-aldoximate (Reese et al., 1978). This reagent gave no apparent internucleotide bond cleavage in the case of the protected hexanucleotide, although smaller oligomers were noted after treatment of the protected decamer. The remaining protecting groups were then removed by sequential base and acid treatment.

The deblocked oligonucleotides were purified by preparative high-pressure LC on a reverse-phase Partisil ODS-2 column or on a Pellionex AL WAX ion-exchange column. The reverse-phase column which apparently separates oligonucleotides on the basis of both base composition and chain length (Fritz et al., 1978; McFarland & Borer, 1979) was found to be satisfactory for the purification of the tetra- and hexanucleotides. In general, a separation involving at least 800 A_{254} units of reaction mixture could be accomplished in 60 min. The recovery of oligonucleotides from the column was at least 75% of the total material applied.

The decanucleotide was purified by high-pressure LC of the deblocked reaction mixture on Pellionex AL WAX using a linear gradient of ammonium acetate buffer in 60% ethanol (Leutzinger et al., 1978). Up to 1100 A_{254} units of reaction mixture could be applied to the column, which was developed in 90 min. Recovery of material applied to the column appeared to be 90% or greater.

The decamer appeared as a single UV-absorbing peak when analyzed by high-pressure LC (Figure 3). The decamer and the other oligonucleotide intermediates were further characterized by base ratio analysis using snake venom phosphodiesterase and spleen phosphodiesterase. In each case the expected deoxyribonucleosides and deoxyribonucleoside monophosphates were obtained in the correct ratios. The decamer was readily hydrolyzed by the restriction endonuclease, *Alu*I, which recognizes the internal nucleotide sequence d-ApGpCpT. Although the decamer contains the sequence d-ApApGpCpTpT– which is recognized by *Hin*dIII restriction endonuclease, the decamer was not hydrolyzed by this enzyme. Similar behavior was observed by Scheller et al. (1977), who

found that d-pCpCpApApGpCpTpTpGpG was not a substrate for *Hsu*I which also recognizes the sequence d-ApApGpCpTpT-.

A total of 16.5 μmol (1318 A_{257} units) of the decadeoxyribonucleotide was obtained in this initial preparation. This quantity of material is sufficient for all the anticipated ^1H NMR studies. For ^{13}C NMR studies any additional material required may be prepared from the remaining protected hexanucleotide. The total time devoted to the preparation of this oligomer was equivalent to 7 man months, starting from the nucleosides. Most of this time was spent in purifying and characterizing the products of the various synthetic reactions. The final purification and characterization of the decamer was greatly aided by the use of analytical and preparative high-pressure LC. It can be expected that further improvement in synthetic techniques and preparative high-pressure LC systems will shorten the time required to make oligonucleotides for physical studies.

Duplex formation was confirmed by examining the effect of temperature on the UV, CD, and ^1H NMR spectra of the decamer (Figures 4, 5, and 6). Under the conditions of the UV and CD experiments, the melting temperature of the decamer duplex is 47 °C. The shape of the CD spectrum of the decamer at low temperature is qualitatively similar to that of DNA, suggesting that the duplex has a B-type geometry.

The high-temperature ^1H NMR spectrum of the single-stranded decamer shows that all the base proton resonances are well separated with the exception of the two T-CH_3 resonances which have identical chemical shift values. The tentative assignments of these resonances are given in Figure 6a. The absence of extraneous resonances confirms the purity of the decamer.

Plots of the base proton chemical shifts vs. temperature give sigmoid-shaped curves. This behavior is consistent with a transition from a single-stranded to a duplex form as the temperature of the decamer is lowered. Under the conditions of the NMR experiment, the T_m estimated from these plots is ~62 °C. The base proton chemical shifts tend to level off or to shift to low field in very small increments at temperatures below 50 °C. Although the base proton resonances of the decamer duplex were not well resolved at 360 MHz, they are well separated at 600 MHz (Figure 6b). The tentative assignments of the proton resonances in the duplex were achieved by carefully tracing the chemical shift vs. temperature data from high to low temperature and were confirmed by measurements of T_1 and coupling constants and by homonuclear decoupling experiments. The high quality of the ^1H NMR spectra of d-CpCpApApGpCpTpTpGpG in both the single-stranded and duplex forms and the characteristic shifts of each of the base proton signals can be used to analyze the conformation of this model DNA helix. The results of these analyses will be reported in future publications.

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