Volume 6 Number 4 April 1979 Nucleic Acids Research

Protected deoxyribonucleoside-3' aryl phosphodiesters as key intermediates in polynucleotide synthesis. *Construction of an icosanucleotide analogous to the sequence at the ends of Rous* sarcoma virus 35S RNA

G.R.Gough*, C.K.Singleton†, H.L.Weith† and P.T.Gilham*

Departments of Biological Sciences* and Biochemistry†, Purdue University, West Lafayette, IN 47907 USA

Received 5 February 1979

ABSTRACT

Several modifications have been incorporated into the phosphotriester strategy for chemical synthesis of oligodeoxyribonucleotides. These include high-yield methods for preparation and isolation of $O^{5'}$,N-protected deoxyribonucleoside-3' p-chlorophenyl phosphates which serve as key intermediates, and the elimination of some superfluous manipulation and purification steps commonly used in the process of synthesizing oligonucleotide blocks. In addition, two new arylsulfonyl nitroimidazole derivatives have been prepared and found to be highly effective agents for internucleotide bond formation. These techniques have been applied in construction of the icosamer d(G-C-C-A-T-T-T-T-A-C-C-A-T-T-C-A-C-C-A)-rC, equivalent to a ribonucleotide sequence located at both the 5' and 3' ends of Rous sarcoma virus 35S RNA.

INTRODUCTION

Recent studies on Rous sarcoma virus (RSV) 35S RNA have revealed the presence of -GCCAUUUUACCAUUCACCAC-poly(A) at the 3'-terminus[1], and an identical sequence of nucleotides, excluding the poly(A) tail, has been found at the 5'-terminus immediately adjacent to the cap.[2] The existence of a repeated terminal icosamer suggests a number of ways in which the linear viral RNA can direct synthesis of double-stranded circular proviral DNA. One plausible mechanism[1,2] involves the formation of a DNA intermediate containing a single copy of the icosamer. Participation of this sequence in a recombination event with host DNA could generate an integrated linear provirus possessing terminally repeated sequences, and subsequent transcription of the proviral gene by RNA polymerase would produce viral genomes identical with the original 35S RNA. In order to investigate this possibility, we decided to construct the ribonucleoside-terminated[3] nonadecadeoxyribonucleotide d(G-C-C-A-T-T-T-T-A-C-C-A-T-T-C-A-C-C-A)-rC corresponding to the repeated RNA segment of RSV, for use as a specific hybridization probe to determine the number of copies of this sequence present in circular proviral DNA, and for

studying the mechanism of provirus integration. In addition, we intended to employ the synthesis of the oligonucleotide for testing some modifications of the overall phosphotriester methodology which have been developed in this laboratory.

STRATEGY FOR SYNTHESIS OF d(G-C-C-A-T-T-T-T-A-C-C-A-T-T-C-A-C-C-A)-rC

Synthesis of the icosamer was simplified by the double occurrence of the sequence CCATT, which enabled the following strategy to be devised (Fig. 1). This scheme requires preparation of relatively large quantities of fully protected d(C-Ap) and d(T-Tp). From these, in conjunction with monomeric units, the subunit blocks 1, 2 and 3 could be assembled as shown. Sequential joining of these subunits, followed by addition of the 5'-terminal dG, would lead to the desired icosamer.

PREPARATION OF MONOMERS

The modified phosphotriester approach[4,5] constitutes the most successful methodology presently used for synthesis of oligodeoxyribonucleotides. By facilitating routine chemical preparation of moderately large quantities of oligonucleotides up to 20 units in length, it has led to such notable recent achievements as the synthesis, cloning, and expression in E. coli of a mammalian somatostatin gene.[6] The essential monomeric units in this system are the four protected deoxyribonucleoside-3' p-chlorophenyl 2-cyanoethyl phosphates, which are prepared from the corresponding blocked deoxyribonucleosides and serve, after removal of the 5'-OH protecting group, as 3'-terminal units in binary condensations. The phosphotriesters can be converted, by selective decyanoethylation[7], to nucleoside aryl phospho-

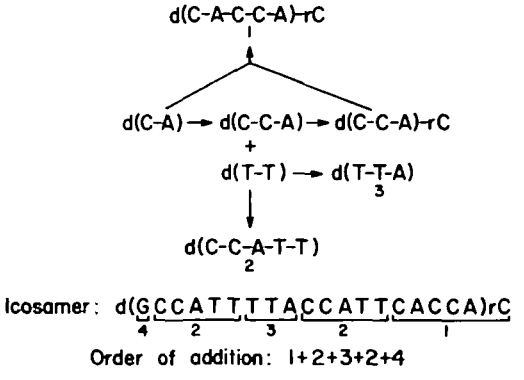

Figure 1. Scheme for synthesis of the icosamer. For the sake of clarity all protecting groups and terminal phosphates have been omitted.

diesters which are used to extend the growing oligonucleotide in the 3' → 5' direction. However, we have found that higher yields of the pure phosphodiesters (II) are obtained by preparing them directly as shown in Figure 2. II is separated from excess phosphorylating agent by precipitation as its barium salt, then converted by ion-exchange to the tetraethylammonium salt, which possesses more favorable solubility properties. The reactions can be carried out on a large scale and yields are in the range 92-97%. The fully protected phosphotriesters (III) are readily prepared from the corresponding phosphodiesters by reaction of II with cyanoethanol in the presence of the condensing agent 1-(mesitylenesulfonyl)-4-nitroimidazole. The triesters are purified by silica gel column chromatography and isolated in 90-97% yields.

ARYLSULFONYL 4-NITROIMIDAZOLES AS CONDENSING AGENTS

The arylsulfonyl tetrazoles introduced by Stawinski et al.[8] are the most powerful condensing agents currently available for oligonucleotide synthesis. However, although they promote rapid high-yield formation of internucleotide bonds, these compounds are inherently unstable and must be freshly prepared at frequent intervals. Van Boom et al.[9] used triisopropyl-

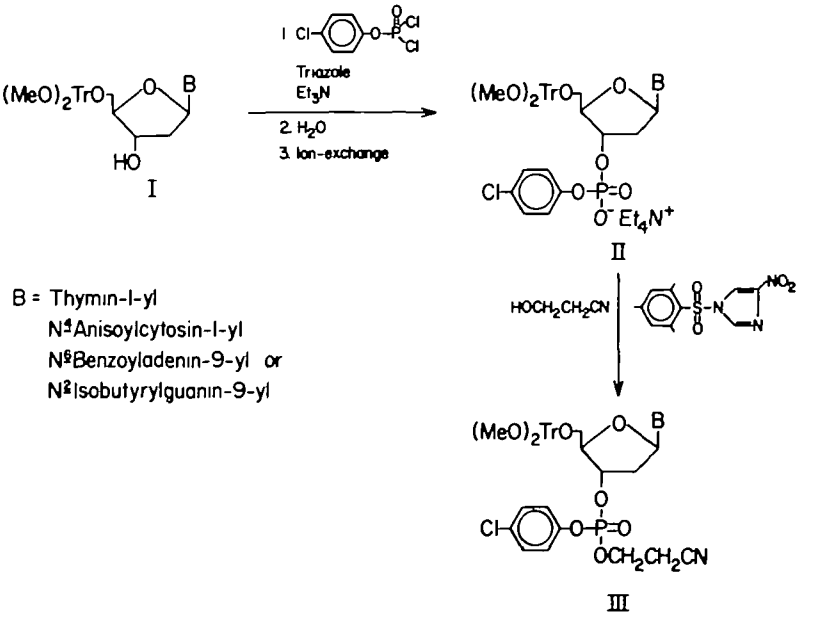

Figure 2. Synthesis of protected deoxyribonucleoside-3' aryl phosphodiesters and fully protected phosphotriesters.

benzenesulfonyl 4-nitroimidazole in phosphotriester synthesis of oligoribonucleotides, but this reagent requires extended reaction times (up to two days). In seeking other useful condensing agents we prepared mesitylenesulfonyl 4-nitroimidazole and p-toluenesulfonyl 4-nitroimidazole. The former compound was employed throughout the present work and we found that it is capable of joining even our longest oligonucleotide blocks in less than 16 hr. The use of p-toluenesulfonyl 4-nitroimidazole will be discussed in a future communication; preliminary studies involving the synthesis of model dinucleotides have indicated that this reagent gives improved yields of products, with less sulfonylation and less destruction of deoxyguanosine residues.

SIMPLIFICATION OF ISOLATION AND PURIFICATION PROCEDURES

Prior to each condensation reaction, it is necessary to detritylate a fully protected mono- or oligonucleotide in order to generate a 5'-hydroxyl component. In the present study this was carried out using benzenesulfonic acid in chloroform-methanol solution, as described by Stawinski et al.[8] However, after extraction of the acid, we departed from the published procedure. We found that isolation of the pure 5'-OH component from the resulting mixture is unnecessary, since free dimethoxytritanol does not interfere with internucleotide bond formation in the subsequent condensation and can be easily separated from the elongated oligonucleotide product when the reaction mixture is fractionated on a silica gel column. We have standardized this chromatographic step and, with the quantity of silica gel used (55 g), we are able to apply small scale reaction mixtures (< 0.2 mmol) directly to the columns after removal of all but ca. 250 µl of the solvent. Using these simplified procedures we prepared the required oligonucleotide blocks, including 1, 2, and 3, as outlined in Table 1.

DEPROTECTION AND ANALYSIS AT INTERMEDIATE STAGES OF SYNTHESIS OF THE ICOSAMER

Small amounts of the hexanucleotide block 1 (Table 1) were deprotected and analyzed as a check on the progress of the synthesis. Initially, two methods for removal of protecting groups were compared. In the first, 1 was treated at 70° for 3 hr with ammonia in aqueous pyridine. These conditions have been reported to effect cleavage of p-chlorophenyl groups from the internal phosphates and to remove the protecting groups from the exocyclic amino functions of the bases.[8] The second method involved treatment of the fully protected hexamer with an aqueous pyridine solution of tetrabutylammonium fluoride to remove the p-chlorophenyl groups[10], then with aqueous ammonia to deblock the $-NH_2$ and 3'-terminal -OH functions. In both cases, exposure to 80% acetic acid was used to remove the 5'-terminal dimethoxytrityl

Table 1. Reaction Conditions for Synthesis of Oligonucleotide Blocks*

3' Aryl Phosphate Component (mmol)	5' Hydroxyl Component (mmol)	MSNI (mmol)	% MeOH in $CHCl_3$ for elution from silica gel	Product	Block No.	Yield
[$(MeO)_2$Tr]danC-(ClPh) (1.2)	dbzA^a(CNEt) (1.0)	3.0	2	[$(MeO)_2$Tr]danC^{a}dbzA^a(CNEt)		92%
[$(MeO)_2$Tr]danC-(ClPh) (0.6)	danC^{a}dbzA^a(CNEt) (0.5)	1.5	3	[$(MeO)_2$Tr]danC^{a}danC ^{a}dbzA^a(CNEt)		80%
[$(MeO)_2$Tr]danC^{a}danC ^{a}dbzA-(ClPh) (0.15)	rbzC(Bz)$_2$ (0.225)	0.5	2	[$(MeO)_2$Tr]danC^{a}danC ^{a}dbzA^{a}rbzC(Bz)$_2$		82%
[$(MeO)_2$Tr]danC ^{a}dbzA-(ClPh) (0.16)	danC^{a}danC^{a}dbzA ^{a}rbzC(Bz)$_2$ (0.123)	0.53	3	[$(MeO)_2$Tr]danC^{a}dbzA^{a}danC ^{a}danC^{a}dbzA^{a}rbzC(Bz)$_2$	1	89%
[$(MeO)_2$Tr]dT-(ClPh) (1.2)	dT^a(CNEt) (1.0)	3.0	2	[$(MeO)_2$Tr]dT^{a}dT^a(CNEt)		89%
[$(MeO)_2$Tr]dT ^{a}dT-(ClPh) (0.15)	dbzA^a(CNEt) (0.12)	0.375	3	[$(MeO)_2$Tr]dT^{a}dT ^{a}dbzA^a(CNEt)	3	84%
[$(MeO)_2$Tr]danC^{a}danC ^{a}dbzA-(ClPh) (0.46)	dT^{a}dT^a(CNEt) (0.4)	1.15	4	[$(MeO)_2$Tr]danC^{a}danC ^{a}dbzA^{a}dT^{a}dT^a(CNEt)	2	85%

*The p-chlorophenyl phosphotriester linkages are represented by the symbol ^{a} [22]

groups. Equivalent portions of each deblocking mixture were analyzed by high-resolution ion-exchange chromatography on Pellionex SAX. A significantly greater quantity of pure d(C-A-C-C-A)-rC was recovered from the second mixture, so the fluoride procedure was used for subsequent deprotections.

After each chain extension of block 1 (i.e. 2 + 1, 3 + (2 + 1) etc.) a small amount of the purified protected oligonucleotide was removed and deprotected. The resulting mixtures were fractionated on Dowex 1 ion-exchange resin. Small amounts of residual contaminants were then removed from the products, either by high-resolution chromatography on Pellionex SAX for analytical purposes, or preparatively by electrophoresis on a 20% polyacrylamide slab gel. The yields quoted for the 11-mer, 14-mer, 19-mer and 20-mer in Table 2 refer to material isolated from the Pellionex column. The sequences of these samples were confirmed by *mobility shift analysis*.[11]

As a postscript to the above, it has recently been shown[12] that oximate ion is capable of removing aryl protecting groups from the internal phosphotriester functions of short oligothymidylates, without significant cleavage of internucleotide bonds. This finding prompted us to use the novel reagent in a trial deblocking of the 19-mer. Pyridinealdoximate in aqueous

Table 2. Reactions Leading to the Intermediate Undecanucleotide, Tetradecanucleotide and Nonadecanucleotide, and the Final Icosanucleotide

3' Aryl Phosphate Component (mmol)*	5' Hydroxyl Component (mmol)*	MSNI (mmol)	Product (Yield)*	Deprotected Product (Yield)
Decyanoethylated 2 (0.025)	Detritylated 1 (0.0176)	0.21	Protected Undecamer (73%)	d(C-C-A-T-T-C-A-C-C-A)-rC (65%)
Decyanoethylated 3 (0.0197)	Detritylated Undecamer (0.0116)	0.21	Protected Tetradecamer (63%)	d(T-T-A-C-C-A-T-T-C-A-C-C-A)-rC (59%)
Decyanoethylated 2 (0.013)	Detritylated Tetradecamer (0.0069)	0.17	Protected Nonadecamer (64%)	d(C-C-A-T-T-T-T-A-C-C-A-T-T-C-A-C-C-A)-rC (40%)
I(MeO)$_2$Tr]dIbG-ClPh (0.0021)	Detritylated Nonadecamer (0.002)	0.043	Protected Icosamer (78%)	d(G-C-C-A-T-T-T-T-A-C-C-A-T-T-C-A-C-C-A)-rC (9%)

*These figures represent overestimates of the actual values, since they are based solely on weight and do not take into account the presence of minor amounts of contaminants unresolved by silica gel chromatography. The fully protected 19-mer and 20-mer, in particular, contained significant quantities of sulfonylated side products, and 4(5)-nitroimidazole derived from breakdown of the condensing agent. By the same token, the spectrophotometrically determined yields shown for the purified deprotected oligonucleotides are artificially low.

dioxane was indeed more effective than fluoride ion. It increased the yield of product to 50% and therefore appears to constitute the method of choice for

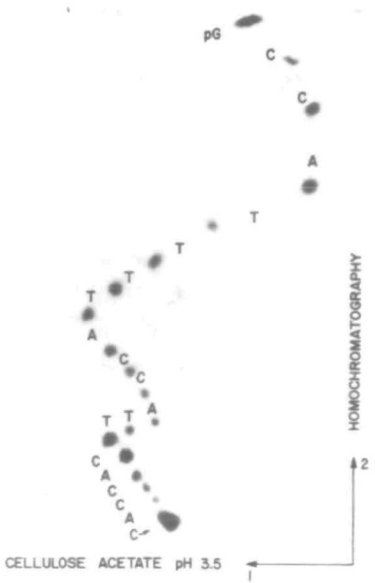

Figure 3. Autoradiogram showing two-dimensional fractionation of limited snake venom exonuclease digestion of 5'-terminally labeled icosamer. The spot corresponding to the mononucleotide was eluted from the thin layer and subjected to paper electrophoresis at pH 3.5. This material co-migrated with authentic pdG.

deprotection of longer oligodeoxyribonucleotide phosphotriesters.

CONCLUSION

The ease with which the icosamer was synthesized in the present study resulted from the following: 1) the ready preparation and use of protected nucleoside aryl phosphodiesters as the key synthetic units, 2) the use of arylsulfonyl nitroimidazole derivatives as condensing agents, and 3) elimination of some unnecessary intermediate purification procedures. Use of a terminal ribonucleoside in the synthesis should also be noted. We plan to exploit this residue as a universal protecting group for the ultimate 3' end of oligodeoxyribonucleotides, since it provides the option of generating either a 3'-terminal phosphate by means of periodate oxidation and β-elimination, or a free 3' hydroxyl by subsequent phosphatase treatment. Molecules possessing these termini are expected to be of considerable value in studies on RNA ligase mediated coupling of oligodeoxyribonucleotides, by analogy with systems of proven utility in oligoribonucleotide synthesis.[13,14]

EXPERIMENTAL

Materials

5'-O-Dimethoxytritylthymidine was prepared by the method of Schaller et al.[15] N^4-Anisoyldeoxycytidine[16], N^6-benzoyldeoxyadenosine[15], N^2-isobutyryl deoxyguanosine[17], and their respective 5'-O-dimethoxytrityl derivatives[15,17] were all synthesized by published procedures, as was p-chlorophenyl phosphorodichloridate.[18] Authentic samples of the fully protected nucleoside phosphotriesters III were purchased from Collaborative Research, Inc. The following materials were also obtained from commercial sources: 1,2,4-Triazole, 4(5)-nitroimidazole, benzenesulfonyl chloride, mesitylenesulfonyl chloride, 2-cyanoethanol, 2-pyridinealdoxime (Aldrich Chemical Co.), benzenesulfonic acid, p-toluenesulfonyl chloride, tetramethylguanidine (Eastman Co.), N^4,$O^{2'}$,$O^{3'}$-tribenzoylcytidine (Sigma Chemical Company), polynucleotide kinase (P-L Biochemicals) and [γ^{32}P]-ATP (ICN). Pyridine, 2,6-lutidine, dioxane, and triethylamine were distilled and stored over calcium hydride. 2-Cyanoethanol was distilled under reduced pressure and kept over molecular sieves (Type 4A). Tetra(n-butyl)ammonium fluoride was prepared by passing an aqueous solution of sodium fluoride (10 mmol) through a small column of Dowex 50W-X8 (tetrabutylammonium form). The eluate was evaporated

to dryness and the residue was dissolved in pyridine to give a total volume of 10 ml.

Thin-layer chromatography was carried out on Eastman Chromagram sheets (silica gel with fluorescent indicator). The completeness of condensation reactions was routinely monitored by TLC using 2% and 5% methanol in chloroform (v/v) as developing solvents. Trityl-containing bands were visualized by exposing the plates to vapors from concentrated hydrochloric acid.

Silica gel chromatography was carried out at 3° on columns (2.4 x 30 cm) packed with 54.6 g of SilicAR CC-7 Special (Mallinckrodt) in chloroform. Unless otherwise specified, after application of the mixture to be fractionated the column was eluted with chloroform (300 ml) followed by chloroform containing methanol (500 ml). The relevant proportions by volume of methanol in the second eluant are given in Table 1 and in the text.

Ion-exchange chromatography

Quantities of deprotected oligonucleotides up to 50 A_{260} units were chromatographed on the Dowex resin AG1-X2 (-400 mesh, Bio-Rad Laboratories). The column (0.4 x 100 cm) was eluted under pressure at 20-25 ml/hr with a 400 ml linear gradient of 0.3-1.0 M NH_4Cl (pH 8) in 40% ethanol.[19] Improved resolution of smaller amounts of oligonucleotides (ca. 5 A_{260} units) was obtained on AS Pellionex SAX (Whatman, Inc.).[19] For example, a column (0.4 x 50 cm) of this material easily separates the 19-mer from the 20-mer when eluted with 400 ml of 0.2-0.8 M NH_4Cl (pH 8) in 40% ethanol. Purified products obtained from either of the above systems were desalted by concentrating the appropriate pooled fractions to small volumes and passing them through a column (1 x 60 cm) of Sephadex G-10 (Sigma Chemical Co.) using 20% ethanol as the eluting solvent.

1-Mesitylenesulfonyl-4-nitroimidazole (MSNI)

Mesitylenesulfonyl chloride (9.75 g, 44.6 mmol) was added to a stirred suspension of finely ground 4(5)-nitroimidazole (5.04 g, 44.6 mmol) in anhydrous dioxane (120 ml). The mixture was treated with triethylamine (6.24 ml, 44.6 mmol) and stirring was continued at 25° for 24 hr with exclusion of moisture. Insoluble triethylamine hydrochloride was removed by filtration and the yellow filtrate was evaporated in vacuo, leaving a crystalline residue which was dissolved in hot benzene. The benzene solution, after filtration and cooling, was treated with petroleum ether and the resulting crystalline product was collected, yielding 11.8 g of pale yellow needles, mp 125-135°. Recrystallization from benzene-cyclohexane gave 10.5 g (80%) of white needles, mp 133-135°. The analytical sample was crystallized

from benzene: mp 135-136°. Anal. Calcd. for $C_{12}H_{13}N_3O_4S$: C, 48.80; H, 4.44; N, 14.23. Found: C, 48.84; H, 4.26; N, 14.40.

1-(p-Toluenesulfonyl)-4-nitroimidazole (TSNI) was prepared from p-toluenesulfonyl chloride as described above for MSNI. Recrystallization from chloroform-benzene gave white needles, mp 167-169°, (72% yield). Anal. Calcd. for $C_{10}H_9N_3O_4S$: C, 44.94; H, 3.39; N, 15.72. Found: C, 44.98; H, 3.49; N, 15.50.

General Procedure for Synthesis of Protected Deoxyribonucleoside-3' p-Chlorophenyl Phosphates (II)

For 1 mmol: Triazole (415 mg, 6 mmol) was dissolved with stirring in warm anhydrous dioxane (10 ml). The solution was cooled to 25° and treated with triethylamine (0.84 ml, 6 mmol) followed by p-chlorophenyl phosphorodichloridate (0.492 ml, 3 mmol). The thick slurry was stirred for 45 min, then mixed with a solution of the protected nucleoside I (1 mmol) in anhydrous 2,6-lutidine (2.1 ml). Stirring was continued for 6 hr at 25°. In the case of the deoxyguanosine derivative the phosphorylating agent was filtered to remove triethylamine hydrochloride before addition to the protected nucleoside, and the reaction was allowed to proceed for 2 hr. The resulting mixture was added dropwise with stirring to water (50 ml). After standing for 1 hr, the clear solution was poured into vigorously stirred aqueous barium chloride (2 g of $BaCl_2 \cdot 2H_2O$ in 200 ml water) at 3°. The mixture was gently warmed to ca. 35° in a water bath without allowing the flocculent barium salt to settle. Upon formation of a filterable solid, the suspension was again cooled to 3°, stirred for 1 hr, then filtered through a coarse sinter under gravity flow. The collected precipitate was washed thoroughly with water (400 ml) and dried in vacuo over P_2O_5.

For conversion to the tetraethylammonium salt, the barium salt was dissolved in 30 ml of dioxane-water (2:1), or 70 ml of dioxane-water (1:1) in the case of the deoxyguanosine derivative, and passed through a column of Dowex 50W-X8 (100-200 mesh, Et_4N^+ form, 1.3 x 7 cm) followed by aqueous dioxane (20 ml) and water (50 ml). The eluate was adjusted to pH 7 with tetraethylammonium hydroxide solution if necessary, then evaporated to ca. 10 ml and immediately lyophilized. Tetraethylammonium 5'-O-dimethoxytrityl-thymidine-3' p-chlorophenyl phosphate (95% yield): Anal. Calcd. for $C_{45}H_{55}ClN_3O_{10}P \cdot 2.5H_2O$: C, 59.43; H, 6.65; N, 4.62; P, 3.41. Found: C, 59.22; H, 6.90; N, 4.31; P, 3.46. Tetraethylammonium 5'-O-dimethoxytrityl-N^6-benzoyldeoxyadenosine-3' p-chlorophenyl phosphate (97% yield): Anal. Calcd. for $C_{52}H_{58}ClN_6O_9P \cdot 3H_2O$: C, 60.55; H, 6.25; N, 8.15; P, 3.00. Found: C,

60.55; H, 6.32; N, 8.24; P, 3.12. Tetraethylammonium 5'-O-dimethoxytrityl-N^4-anisoyldeoxycytidine-3' p-chlorophenyl phosphate (92% yield): Anal. Calcd. for $C_{52}H_{60}ClN_4O_{11}P \cdot 3H_2O$: C, 60.20; H, 6.41; N, 5.40; P, 2.99. Found: C, 60.25; H, 6.41; N, 5.53, P, 3.10. Tetraethylammonium 5'-O-dimethoxytrityl-N^2-isobutyryldeoxyguanosine-3' p-chlorophenyl phosphate (96% yield): Anal. Calcd. for $C_{49}H_{60}ClN_6O_{10}P \cdot 4H_2O$: C, 57.05; H, 6.64; N, 8.15; P, 3.00; Found: C, 57.20; H, 6.58; N, 8.05; P, 3.02.

Conversion of Protected Deoxyribonucleoside-3' p-Chlorophenyl Phosphates (II) to Fully Protected Triesters (III)

The tetraethylammonium salt of the protected nucleoside-3' aryl phosphate (II, 1 mmol) was rendered anhydrous by several additions and evaporations of dry pyridine. After the final evaporation ca. 2 g pyridine was allowed to remain and the resulting solution was treated with cyanoethanol (0.35 ml, 5 mmol) and MSNI (885 mg, 3 mmol) for 16 hr at 25°. The mixture was then added to ethyl acetate (100 ml) and extracted with 0.1 M aqueous triethylammonium bicarbonate solution (3 x 60 ml) followed by 10% aqueous NaCl (60 ml). The ethyl acetate layer was dried (Na_2SO_4) and evaporated to a thick oil which was dissolved in chloroform (3 ml) and chromatographed on silica gel. Elution of the column with 600 ml of $CHCl_3$, followed by 600 ml of $CHCl_3$-MeOH (99:1 or, in the case of the deoxyguanosine derivative, 98:2) yielded fully protected phosphotriester III (90-97%). The thin-layer chromatographic mobility of each of the deoxyribonucleoside phosphotriesters prepared as described above was identical with that of its commercially available counterpart.

Detritylation and Decyanoethylation of Fully Protected Phosphotriester Intermediates

Detritylation was effected by treatment with 2% benzenesulfonic acid in chloroform-methanol (7:3) at 0° for 25 min. As a general rule, 10 ml of this solution were used for each mmol of protected intermediate. However quantities smaller than 0.3 mmol were in all cases deprotected with 3 ml of the reagent. After the trityl cleavage was complete, the mixture was neutralized with 5% $NaHCO_3$ and transferred into chloroform (100 ml). The chloroform layer was washed with 5% $NaHCO_3$ (25 ml) and water (25 ml), then dried (Na_2SO_4). The glassy residue remaining after removal of chloroform was used as the 5'-hydroxyl component in the subsequent condensation reaction without further purification.

For all quantities less than 1 mmol, selective decyanoethylation was carried out by treating an anhydrous solution of the fully protected triester

intermediate in pyridine (2 ml) with dry triethylamine (1 ml) for 6 hr at 25°. Following removal of most of the solvent *in vacuo* and several evaporations of dry pyridine, the residue was employed as the 3' aryl phosphodiester component in the subsequent reaction.

Synthesis of the Fully Protected Dinucleotide $[(MeO)_2Tr]danC^{Ac}dbzA^{Ac}(CNEt)$

5'-*O*-Dimethoxytrityl-N^6-benzoyldeoxyadenosine-3' *p*-chlorophenyl 2-cyanoethyl phosphate ($[(MeO)_2Tr]dbzA^{Ac}(CNEt)$, 1 mmol) was detritylated as described above and combined with the tetraethylammonium salt of 5'-*O*-dimethoxytrityl-N^4 anisoyldeoxycytidine-3' *p*-chlorophenyl phosphate ($[(MeO)_2Tr]danC$-(ClPh), 1.2 mmol). After several additions and evaporations of pyridine, the last of which left 2 ml of residual solvent, the mixture was treated with MSNI (3 mmol) for 16 hr at 25°. The resulting suspension was then added to ethyl acetate (100 ml) and the organic phase was washed with 0.1 M triethylammonium bicarbonate (3 x 60 ml) and 10% aqueous NaCl (60 ml). After standing over Na_2SO_4, the ethyl acetate solution was evaporated to dryness and the residue was chromatographed on silica gel. The fully protected oligonucleotide blocks $[(MeO)_2Tr]danC^{Ac}danC^{Ac}dbzA^{Ac}(CNEt)$, $[(MeO)_2Tr]dT^{Ac}dT^{Ac}(CNEt)$, and $[(MeO)_2Tr]danC^{Ac}danC^{Ac}dbzA^{Ac}dT^{Ac}dT^{Ac}(CNEt)$ were prepared and worked up similarly. The reaction conditions for the synthesis and isolation of these compounds are summarized in Table 1.

Synthesis of the Ribonucleoside Terminated Block $[(MeO)_2Tr]danC^{Ac}dbzA^{Ac}danC^{Ac}danC^{Ac}dbzA^{Ac}rbzC(Bz)_2$ (1, Figure 1)

(a) $[(MeO)_2Tr]danC^{Ac}danC^{Ac}dbzA^{Ac}(CNEt)$ (295 mg, 0.15 mmol) was decyanoethylated as described above. The resulting 3'-phosphodiester was mixed with N^4,$O^{2'}$,$O^{3'}$-tribenzoylcytidine (125 mg, 0.225 mmol), co-evaporated with pyridine and treated in *ca.* 0.5 ml anhydrous pyridine with MSNI (150 mg, 0.5 mmol) for 16 hr. Water (10 µl) was added and the mixture was evaporated *in vacuo* until *ca.* 250 mg pyridine remained. The resulting oil was dissolved in chloroform (1.5 ml) and chromatographed on silica gel. The column was eluted with 300 ml of $CHCl_3$ and 500 ml of $CHCl_3$-MeOH (97:3). The desired oligonucleotide was incompletely separated from the faster running protected nucleoside $rbzC(Bz)_2$. The mixture (439 mg) was rechromatographed; elution with 1000 ml of $CHCl_3$-MeOH (99:1) followed by 500 ml of $CHCl_3$-MeOH (98:2) yielded the pure tetranucleoside triphosphate (302 mg, 82%).

(b) $[(MeO)_2Tr]danC^{Ac}danC^{Ac}dbzA^{Ac}rbzC(Bz)_2$ (302 mg, 0.123 mmol) was detritylated and combined with decyanoethylated $[(MeO)_2Tr]danC^{Ac}dbzA^{Ac}(CNEt)$ (229 mg, 0.16 mmol) in anhydrous pyridine (*ca.* 0.4 ml). MSNI (160 mg, 0.53 mmol) was added

and the reaction mixture was allowed to stand for 16 hr. Following addition of water (10 µl) and removal of half of the pyridine, the residue was chromatographed directly on silica gel. The column was eluted with 300 ml of $CHCl_3$ then with 500 ml of $CHCl_3$-MeOH (97:3). Pure 1 (383 mg) was obtained in 89% yield.

Deprotection and Purification of 1

Two samples of 1 were separately deprotected as follows:

(i) Five mg of 1 were dissolved in pyridine-conc. NH_4OH-H_2O (1:1:1, 9 ml) and heated in a pressure bottle at 70° for 3 hr. The solvents were removed *in vacuo* and the residue was treated with 80% aqueous acetic acid (3 ml) for 20 min at 25°. After several evaporations of water the deprotected material was taken up in 40% aqueous ethanol (5 ml) and 0.2 ml of this solution was chromatographed on Pellionex SAX. The major oligonucleotide peak contained 1.83 A_{260} units (59%) of the hexamer d(C-A-C-C-A)-rC.

(ii) Five mg of 1 were dissolved in a pyridine solution of tetrabutylammonium fluoride (1 M, 0.1 ml) containing water (20 µl), and allowed to stand for 2 days at 25°. Pyridine (0.3 ml) and concentrated NH_4OH (3 ml) were then added. After 3 more days the solution was evaporated to dryness and the residue was treated with 80% acetic acid as described above. Further treatment was also as described in (i), but in this case 2.46 A_{260} units (80%) of the hexamer were obtained from the Pellionex column. Procedure (ii) was used for subsequent deblockings.

Syntheses of the Undecamer, Tetradecamer, Nonadecamer and Icosamer

Table 2 summarizes the reaction conditions used to prepare these oligonucleotides. In every case, after a 16 hr treatment with MSNI in pyridine (< 250 µl), the reaction mixtures were chromatographed directly on silica gel. The fully protected 11-mer and 14-mer were each obtained from a standard column eluted with 300 ml of $CHCl_3$ followed by 500 ml of $CHCl_3$-MeOH (97:3) The 19-mer was obtained by elution with 300 ml of $CHCl_3$ followed by 400 ml of $CHCl_3$-MeOH (97:3) and 300 ml of $CHCl_3$-MeOH (95:5). The small-scale reaction mixture which produced the 20-mer was chromatographed on a 0.9 x 30 cm column eluted with 75 ml of $CHCl_3$ and 200 ml of $CHCl_3$-MeOH (95:5). A sample (3 mg) of each fully protected oligonucleotide product was deprotected with tetrabutylammonium fluoride, ammonia and acetic acid, then purified by Dowex 1 and Pellionex SAX column chromatography. The yields obtained are shown in the last column of Table 2.

Deprotection of the 19-mer with Oximate Ion

Fully protected 19-mer (3 mg) was treated with N^1,N^1,N^2,N^2-tetramethyl-

guanidinium pyridine-2-carboxaldoximate (60 µl of a 1 M solution in 1:1 dioxane-water) at 25°. Tetramethylguanidine (1 µl) was added after 16 hr and the deblocking was allowed to proceed for a total of 48 hr. Thereafter, treatment with ammonia and acetic acid was as described for the fluoride deprotections. Following ion-exchange chromatography, a 50% yield (26.4 A_{260} units) of the 19-mer was obtained.

Preparative Isolation of Deprotected Products

Initial fractionation of deblocked oligonucleotides was accomplished by anion-exchange column chromatography. Subsequent purification involved polyacrylamide gel electrophoresis. A 20% (w/v) denaturing gel (0.33 x 20 x 40 cm) with a single slot (0.33 x 15 cm) was used. Up to 30 A_{260} units in 200 µl of buffer could be loaded on this gel. Electrophoresis was carried out in 0.05 M Tris-borate, pH 8.3, 0.001 M EDTA, for 5-6 hours at 1000 volts and 35-45 milliamps. Mobilities of the icosamer and nonadecamer relative to the bromophenol blue dye marker were 0.65 and 0.67, respectively, giving a separation of bands of 0.5-0.7 cm. The oligonucleotide bands were located by fluorescence quenching using a UV transilluminator and fluorescent indicator TLC plates. The oligonucleotides were eluted from the gel by the crush and soak method.[20] Desalting was done on a Biogel P2 column (200-400 mesh, 1 x 30 cm) using 0.01 M triethylammonium bicarbonate, pH 8, as elution buffer. These procedures consistently gave better than 85% recovery of the oligonucleotides and final purity greater than 95%.

Sequence Analysis of Deblocked Products.

The 5'-labelling of the products was carried out using polynucleotide kinase and [γ^{32}P]-ATP (specific activity > 2000 Ci/mmol). Two to twenty pmol of sample and an equivalent amount of [γ^{32}P]-ATP were dissolved in 10 µl of 0.05 M glycine, 0.01 M $MgCl_2$, 5 mM dithiothreitol, pH 9.5, containing 1-2 units of kinase. Reactions were run in drawn-out capillaries for 30 min at 37°. Two dimensional fractionation of the labelled products was accomplished by electrophoresis at pH 3.5 on cellulose acetate, followed by homochromatography on DEAE-cellulose (1:7.5) thin-layers using a 4% 30 min hydrolyzed homomix.[21] Partial snake venom phosphodiesterase digestions were carried out on isolated spots from the thin layer plate. Samples were taken up in 7 µl of VPDE-mix (0.1 mg VPDE/ml of 0.05 M Tris HCl, 0.5 mM $MgCl_2$, pH 8.9 buffer solution) and incubated at 37°. Aliquots were removed at 0, 20, 40, 60, 90, 120, and 150 min and frozen on dry ice. The recombined aliquots were fractionated as described above, and autoradiography was used to visualize the partially digested products.

ACKNOWLEDGEMENTS

The work was supported by NIH Grants GM 19395, GM 11518, CA 23332 and a grant from The American Cancer Society. CKS is supported by NIH training grant 5T32GM07076, and HLW by NIH Research Career Development Award CA 00447. This is paper No. 7504 from the Purdue University Agricultural Experiment Station.

REFERENCES

1. Schwartz, D. E., Zamecnik, P. C., and Weith, H. L. (1977) Proc. Natl. Acad. Sci. USA 74, 994-998.
2. Haseltine, W. A., Maxam, A., and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 989-993.
3. The inclusion of a 3'-terminal ribonucleoside was considered desirable so that the icosamer could serve as a removable primer for DNA sequence analysis by template copying. In addition, the ribonucleoside residue is a versatile protecting group for the 3' end of oligodeoxyribonucleotides, as noted in the text.
4. Catlin, T. C. and Cramer, F. (1973) J. Org. Chem. 38, 245-250.
5. Itakura, K., Bahl, C. P., Katagiri, N., Michniewicz, J. J., Wightman, R. H., and Narang, S. A. (1973) Can. J. Chem. 51, 3649-3651.
6. Itakura, K., Hirose, T., Crea, R., Riggs, A. D., Heyneker, H. L., Bolivar, F., and Boyer, H. W. (1977) Science 198, 1056-1063.
7. Adamiak, R. W., Barciszewska, M. Z., Biala, E., Grzeskowiak, K., Kierzek, R., Kraszewski, A., Markiewicz, W. T., and Wiewiorowski, M. (1976) Nucleic Acids Res. 3, 3397-3408; Sood, A. K. and Narang, S. A. (1977) Nucleic Acids Res. 4, 2757-2765.
8. Stawinski, J., Hozumi, T., Narang, S. A., Bahl, C. P., and Wu, R. (1977) Nucleic Acids Res. 4, 353-371.
9. van Boom, J. H., Burgers, P. M. J., van der Marel, G., Verdegaal, C.H.M., and Wille, G. (1977) Nucleic Acids Res. 4, 1047-1063.
10. van Boom, J. H. and Burgers, P. M. J. (1978) Rec. Trav. Chim. Pays-Bas 97, 73-80.
11. Donelson, J. E., Barrell, B. G., Weith, H. L., Kössel, H., and Schott, H. (1975) Eur. J. Biochem. 58, 383-395.
12. Reese, C. B., Titmas, R. C., and Yau, L. (1978) Tetrahedron Lett. 2727-2730.
13. Sninsky, J. J., Last, J. A., and Gilham, P. T. (1976) Nucleic Acid Res. 3, 3157-3166.
14. Uhlenbeck, O. C. and Cameron, V. (1977) Nucleic Acid Res. 4, 85-89.
15. Schaller, H., Weimann, G., Lerch, B., and Khorana, H. G. (1963) J. Amer. Chem. Soc. 85, 3821-3827.
16. Cashion, P., Notman, H., Sathe, G., Cadger, T., and Tranquilla, T. (1977) J. Chromatog. 136, 159-161.
17. Büchi, H. and Khorana, H. G. (1972) J. Mol. Biol. 72, 251-288.
18. Maguire, M. H. and Shaw, G. (1953) J. Chem. Soc. 1479-1482.
19. Asteriadis, G. T., Armbruster, M. A., and Gilham, P. T. (1976) Anal. Biochem. 70, 64-74.
20. Maxam, A. M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-564.
21. Barrell, B. G. (1971) in Procedures in Nucleic Acid Research, Cantoni, G. L. and Davies, D. R., Eds., Vol. 2, pp. 751-779. Harper and Row, N.Y.
22. We advocate the use of the space saving symbol ^{R}, suitably defined with respect to phosphate protecting group R, to denote a 3'-5' phosphotriester linkage between nucleosides. This abbreviation has no prior connotation, is typographically convenient, and is simply derived from the established usage: Np(R)N → N-()N → N^{R}N.