Use of Ribonucleosides as Protecting Groups in Synthesis of Polynucleotides with Phosphorylated Terminals†

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ABSTRACT: Two new protected 5′-ribonucleotides, 2′,3′-*O*-bis(4,4′-dimethoxytrityl)uridine 5′-(4-chlorophenyl phosphate) and 2′,3′-*O*-(methoxymethylene)uridine 5′-(4-chlorophenyl phosphate), form the basis of a chemical procedure for phosphorylating the 5′-ends of DNA fragments synthesized by the phosphotriester approach. Condensation of either of these mononucleotide units with the free 5′-hydroxyl of an otherwise fully protected oligomer results in high-yield formation of a 5′–5′ triester linkage. Subsequently, the terminal ribonucleotide of the deprotected product rU5′-5′d(N-N_n-N) can be cleaved by periodate oxidation of its 2′,3′-*cis*-hydroxyl system followed by β-elimination, leaving its phosphate attached to the 5′-hydroxyl group of the oligodeoxyribonucleotide. This procedure together with a tactic employing a 2′,3′-*O*-acylribonucleotide residue at the 3′-terminus of the chain allows the synthesis of oligomers possessing monophosphate groups at either end or both ends. Furthermore, oligonucleotide intermediates possessing a 5′–5′-linked uridine terminal are shown to have a special application as acceptors in RNA ligase reactions, where the presence of the ribonucleoside cap on the 5′-phosphate limits ligation specifically to the 3′-ends of the oligomers. Removal of the uridine residues to expose free 5′-phosphates would then enable the products to participate as donors in further elongation reactions.

The phosphotriester method is the most widely used approach for chemical synthesis of defined-sequence DNA fragments in solution. The usual strategy in this system involves extension of the nascent DNA chain in the 3′- to 5′-direction, by joining the 5′-hydroxyl of an otherwise fully protected oligonucleotide to the 3′-phosphodiester function of a protected mononucleotide or oligonucleotide triester block. The product of the reaction is then a fully protected oligomer that may be further lengthened in the 5′-direction, after selective removal of the 5′-hydroxyl blocking group, by condensation with another incoming mono- or oligonucleotide 3′-phosphodiester. The cycle of 5′-deprotection followed by condensation is carried out as often as necessary to yield the desired sequence.

The essential feature of this type of chain extension in the 3′- to 5′-direction, namely, the repeated removal of a protecting group from the 5′-end of the growing molecule to expose a free 5′-hydroxyl, means that, once the final sequence has been obtained, full deprotection of the molecule also yields an oligonucleotide with a free 5′-hydroxyl group. For many biological and physicochemical purposes, however, it is desirable that synthetic oligonucleotides contain 5′-terminal monophosphates, and since enzymatic means of 5′-phosphorylation with polynucleotide kinase and ATP can be uneconomical, particularly in large-scale operations, effective chemical methods for adding a 5′-phosphate to the ends of oligodeoxyribonucleotides are of considerable value.

A number of chemical procedures already exist for achieving this objective (Neilson et al., 1975; van Boom et al., 1975; Reese & Yau, 1978; Sekine et al., 1979; Hsiung et al., 1980; Takaku et al., 1980; van der Marel et al., 1982). Nevertheless, our own requirements were more far-reaching, since we intended to test the 5′-phosphorylated molecules in a scheme for the stepwise synthesis of polynucleotides, using RNA ligase to join the respective units (Kaufman & Kallenbach, 1975; Walker et al., 1975; Sninsky et al., 1976; McCoy & Gumport, 1980). Our system demanded that a 5′-phosphate group attached to each acceptor unit be maintained in a protected state until after ligation between the acceptor and an appropriate donor. The phosphate would then be freed under mild conditions so that the extended molecule could itself serve as a donor in the next ligase reaction.

With these prerequisites in mind, we decided to examine the possibility of using a ribonucleoside in a dual role, for introducing phosphate and as a protecting group. The latter application was first proposed by Kathawala & Cramer (1967, 1968), who showed that the terminal phosphates of oligomers could be protected during phosphodiester syntheses by esterification with 2′,3′-*O*-(2,4-dimethoxybenzylidene)uridine through its 5′-hydroxyl. Subsequently, after acid hydrolysis of the benzylidene acetal function, the uridine residue could be removed from the phosphate by cleavage of its 2′,3′-*cis*-glycol system with periodate and β-elimination of the resulting dialdehyde.

Our newly developed methodology for 5′-phosphorylation employs 2′,3′-O-protected uridine 5′-(4-chlorophenyl phosphates) as monofunctional phosphorylating agents. Condensation of these reagents with the free 5′-hydroxyl of a suitably protected oligodeoxyribonucleotide results in high-yield formation of a 5′–5′ phosphotriester bond. The linkage is stable under all conditions encountered during synthesis and is converted to a 5′–5′ phosphodiester in the first step of the final deblocking process. Subsequently, after removal of the protecting groups from its 2′- and 3′-hydroxyls, the uridine residue can be detached by periodate oxidation and β-elimination, leaving a phosphate on the 5′-terminus of the oligomer.

Experimental Procedures

Materials. 5′-*O*-Acetyluridine (Fromageot et al., 1967), 2′,3′-*O*-(methoxymethylene)uridine (Griffin et al., 1967), TSNI[1] (Gough et al., 1979a), and TSNT (Jones et al., 1980)

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were prepared by published methods. $N^4,O^{2'},O^{3'}$-Tribenzoylcytidine was obtained from Sigma Chemical Co., St. Louis, MO. Spleen and snake venom phosphodiesterases and RNA ligase were purchased from P-L Biochemicals, Milwaukee, WI. Fully protected oligodeoxyribonucleotide blocks [$(MeO)_2$Tr]dibG^{o}dbzA^{o}dbzC^{o}(CNEt), [$(MeO)_2$Tr]dT^{o}-dT^{o}dbzC^{o}dbzA^{o}(CNEt), and [$(MeO)_2$Tr]dbzC^{o}dbzC^{o}-dbzA^{o}dbzA^{o}dbzC^{o}dbzC^{o}(CNEt) were synthesized from suitably protected mono- and dinucleotides by phosphotriester methods described previously (Gough et al., 1979a,b, 1982); the dinucleoside monophosphate [$(MeO_2$Tr]dbzA^{o}dT(Bz) was prepared by condensing the barium salt of [$(MeO)_2$Tr]-dbzA-(ClPh) with dT(Bz).

Thin-Layer Chromatography. Detritylation, decyanoethylation, and condensation reactions were examined for completeness by thin-layer chromatography on Eastman Chromagram sheets (silica gel with fluorescent indicator) with 2 or 5% methanol in chloroform (v/v) as solvent. Dimethoxytrityl-containing compounds were detected by exposing the dried plates to vapor from concentrated HCl.

Detritylation of Fully Protected Oligonucleotides. Dimethoxytrityl groups were removed from fully protected oligonucleotides by treatment at 0 °C with a 2% (w/v) solution of benzenesulfonic acid in chloroform–methanol (7:3 v/v); at least 20 μL of this reagent was used per μmol of mononucleotide residue in each oligomer. After 25 min, the reaction mixture was quenched with pyridine and diluted with chloroform (35 mL). The solution was first extracted with 5% $NaHCO_3$ (25 mL) containing 2-methyl-2-propanol (3 mL) and then with 10% NaCl (25 mL)–2-methyl-2-propanol (3 mL). The organic layer was dried (Na_2SO_4), and then the solvents were removed in vacuo below 35 °C by several coevaporations with anhydrous pyridine. The resulting oil was used without purification as the 5′-hydroxyl component in the subsequent condensation reaction.

Decyanoethylation of Fully Protected Oligonucleotides. Oligomers to be decyanoethylated were dried by repeated evaporations of pyridine below 35 °C and then treated in this solvent (2 mL) with anhydrous triethylamine (1 mL). After 6 h at 25 °C, excess alkylamine was removed by coevaporation with pyridine, leaving in each case a concentrated solution of an oligonucleotide 3′-phosphodiester ready for condensation.

Condensation Reactions. For each condensation, pyridine solutions of the phosphodiester and 5′-hydroxyl components were combined and rendered anhydrous by several additions and evaporations of dry pyridine at below 35 °C. The weight of pyridine ultimately remaining in the reaction mixture, determined by difference from the known amounts of reactants, was adjusted to make the concentration of the phosphodiester component at least 0.1 M. After treatment with the appropriate condensing agent for the specified time and subsequent hydrolysis, if noted, the reaction mixture was concentrated in vacuo until ca. 100 mg of pyridine remained. The residue was diluted with chloroform (10 mL) and chromatographed on silica gel as described below.

Silica Gel Chromatography of Oligonucleotides. The fully protected products of the condensation reactions were isolated on small (0.9 × 30 cm) columns of SilicAR CC-7 (Mallinckrodt) packed in chloroform and run at 3 °C. After application of the samples, the columns were washed with chloroform (100 mL); the oligonucleotide products were then eluted with the chloroform–methanol mixture specified for each case. The yields of purified products were measured by spectrophotometric determination of the dimethoxytrityl cation (Schaller et al., 1963).

Table I: HPLC Retention Volumes of Oligonucleotides

oligomer	retention vol (mL)[a]
rU5′-5′d(G-A-C-A-T)	55
d(pG-A-C-A-T)	99
d(G-A-C-A-T)	41
d(T-T-C-A)-rC	32
d(T-T-C-Ap)	56
2′,3′-[$(MeO)_2$Tr]$_2$rU5′-5′d(T-T-C-A)-rC	136
rU5′-5′d(T-T-C-A)-rC	50
d(pT-T-C-Ap)	102
2′,3′-[$(MeO)_2$Tr]$_2$rU5′-5′d(T-T-C-Ap)	200
rU5′-5′d(T-T-C-Ap)	77
rU5′-5′d(T-T-C-A)	32
d(pT-T-C-A)	48
d(T-T-C-A)	22
d(C-C-A-A-C-C)-rC	54[b]
d(C-C-A-A-C-Cp)	75[b]
rU5′-5′d(C-C-A-A-C-C)-rC	66[b]
d(pC-C-A-A-C-Cp)	108[b]
d(C-C-A-A-C-C)	39[b]

[a] Solvent system was 240 mL of aqueous 0.05 M KH_2PO_4 containing 15% (v/v) MeCN and a linear gradient of 0–0.3 M $(NH_4)_2SO_4$ at pH 6.0; flow rate was 2 mL/min. [b] Solvent system was 200 mL of aqueous 0.05 M KH_2PO_4 containing 15% (v/v) MeCN and a linear gradient of 0–0.5 M $(NH_4)_2SO_4$ at pH 6.0; flow rate was 2 mL/min.

Deprotection of Oligomers. Column-purified oligonucleotide triesters were deprotected by treatment with 1 M (tetramethylguanidinium)pyridine 2-aldoximate in dioxane–water (1:1 v/v) at 25 °C (Reese et al., 1978); 20 μL of this reagent was used per mg of fully protected oligomer. After 36 h, pyridine (0.05 mL/mg) and concentrated ammonia (0.5 mL/mg) were added to each, and the mixtures were allowed to stand for 3 days. Solvents were then removed in vacuo. For isolation of an oligomer with its dimethoxytrityl protection intact, the residue at this stage was dissolved in water, and a 2-μmol aliquot was applied to 45 cm of Whatman 3MM chromatography paper. The chromatogram was run in ethanol for 8 h to wash the pyridine aldoximate to the solvent front, then dried, and finally developed in 2-propanol–concentrated ammonia–water (6:1:3 v/v) for 18 h. The major ultraviolet-absorbing band was cut out and eluted with water. When molecules lacking dimethoxytrityl groups were required, the residues from the ammoniacal treatment were dissolved in acetic acid–water (4:1 v/v; 3 mL/mg of original protected oligomer) and allowed to stand for 50 min. The acid was then removed by coevaporation with water. The residues were chromatographed on paper as described above, except that for these separations the developing solvent used was 1-propanol–concentrated ammonia–water (55:10:35 v/v).

Removal of Terminal Ribonucleoside Residues. Each sample (10–50 A_{260} units) of deprotected, ribonucleoside-terminated oligonucleotide in water (1 mL) was treated with 0.2 M $NaIO_4$ (0.24 mL) at 0 °C for 1 h. Excess periodate was then destroyed by adding 0.2 M L-methionine (0.36 mL). After a further 20 min at 0 °C, the mixture was treated with 1 mL of 1 M cyclohexylamine hydrochloride–0.25 M Hepes (pH 8 at 25 °C), immediately warmed to 45 °C, and incubated at this temperature for 1 h. The mixture was then streaked on Whatman 3MM paper (30 cm), and the chromatograms were developed in 1-propanol–concentrated ammonia–water

[1] Abbreviations: the symbol ^{o} denotes a 4-chlorophenyl phosphotriester linkage; TSNI, 1-(4-toluenesulfonyl)-4(5)-nitroimidazole; TSNT, 1-(4-toluenesulfonyl)-3-nitro-1,2,4-triazole; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PEI, poly(ethylenimine). Representations of mono- and oligonucleotides and their protected derivatives are consistent with the IUPAC–IUB Commission on Biochemical Nomenclature (1970) recommendations.

Table II: Products from Enzymatic Digestion of Oligonucleotides

oligomer	spleen phosphodiesterase products (molar ratios)	snake venom phosphodiesterase products (molar ratios)
d(pG-A-C-A-T)	none	pdG:pdA:pdC:pdT (1.0:2.0:1.0:1.0)
d(G-A-C-A-T)	dGp:dAp:dCp:dT (1.0:2.1:1.0:1.0)	dG + pdC:pdA:pdT (2.0:2.1:1.0)
d(T-T-C-Ap)	dTp:dCp:dAp (2.0:1.0:1.0)	none
d(pT-T-C-A)	none	pdT:pdC:pdA (2.0:1.0:1.0)
d(pT-T-C-Ap)	none	none
d(T-T-C-A)	dTp:dCp:dA (2.0:1.0:1.0)	dT:pdT:pdC:pdA (1.0:1.0:1.1:1.1)
d(C-C-A-A-C-Cp)	dCp:dAp (4.0:2.0)	none
d(pC-C-A-A-C-Cp)	none	none
d(C-C-A-A-C-C)	dCp:dAp:dC (2.9:2.0:1.0)	dC:pdC:pdA (1.1:3.0:2.0)

(55:10:35 v/v) for 18 h. To obtain the purified product, the oligonucleotide band was eluted with water.

High-Performance Liquid Chromatography. A small amount (ca. 0.6 A_{260} unit in 0.6 mL water) of each deprotected oligonucleotide was chromatographed on a column (0.46 × 15 cm) of cross-linked PEI on microparticulate silica (Lawson et al., 1983). Retention volumes of the various oligonucleotides and the solvent systems used are given in Table I.

Enzymatic Analysis of Oligonucleotides. Susceptible oligonucleotides were digested with the following enzymes for the purposes of characterization.

(1) Spleen Phosphodiesterase. A sample of oligomer (1.5 A_{260} units) dissolved in 1.25 mL of 0.01 M Mes (pH 6.5) was treated with spleen phosphodiesterase (0.25 unit) at 25 °C. The digestion was monitored spectrophotometrically at 260 nm, and when its absorbance stopped increasing (ca. 2 h), the solution was heated to 80 °C for 15 min. A portion (0.5 mL) of the mixture was chromatographed on the PEI column to verify complete degradation of the starting oligomer by the enzyme. The remainder was analyzed by a previously reported technique (Gough et al., 1982) to determine the base composition of the oligonucleotide. The measured ratios are shown in Table II.

(2) Snake Venom Phosphodiesterase. A small quantity (1.8 A_{260} units) of oligomer in 1.5 mL of 0.05 M Tris–HCl–0.05 M NaCl–0.08 M $MgCl_2$ (pH 8.9) was treated with venom phosphodiesterase (0.3 unit) at 25 °C. The reaction was monitored, and the products were analyzed as described above for the spleen enzyme.

(3) Calf Intestinal Alkaline Phosphatase. Unless otherwise noted, an oligomer to be dephosphorylated (ca. 10 A_{260} units) was dissolved in 0.5 mL of 0.1 M Tris-HCl (pH 8) and treated with alkaline phosphatase (0.5 unit) at 37 °C for 90 min. The mixture was then heated to 80 °C for 15 min and applied to 10 cm of Whatman 3MM paper. The chromatogram was developed in 1-propanol–concentrated ammonia–water (55:10:35 v/v) for 16 h. The band containing the dephosphorylated oligonucleotide was cut out and eluted with water.

Those oligonucleotides that by virtue of possessing 3′- or 5′-terminal phosphate groups were expected to be resistant to the action of venom or spleen phosphodiesterases, respectively, were treated in the following manner. A mixture of the oligonucleotide (0.6 A_{260} unit) and its terminally dephosphorylated equivalent (0.6 A_{260} unit) in 1 mL of the appropriate buffer was incubated with venom or spleen exonuclease for 20 min at 25 °C. The mixture was then heated to 80 °C for 15 min, diluted to twice its volume with water, and chromatographed on the PEI column. The elution profile was examined for complete disappearance of the nonphosphorylated molecule, while the peak area of the phosphorylated species was compared with that produced by a known quantity of control.

Barium 2′,3′-O-Bis(4,4′-dimethoxytrityl)uridine 5′-(4-Chlorophenyl phosphate). 5′-*O*-Acetyluridine (286 mg, 1 mmol) was dried by repeated additions and evaporations of anhydrous pyridine and then treated in 3 mL of dry pyridine with 4,4′-dimethoxytrityl chloride (1 g, 3 mmol). After 36 h at 55 °C, the reaction mixture was cooled to 25 °C and treated with ethanol (2 mL) for 15 min. Ethyl acetate (150 mL) was added, and the solution was extracted once with 5% $NaHCO_3$ (50 mL) and twice with water (50 mL). The ethyl acetate layer was evaporated to a thick oil, which was dissolved in dioxane–ethanol (1:1 v/v, 6 mL) and treated with 1.5 mL of 20% aqueous tetraethylammonium hydroxide. After 1 h at 25 °C, the mixture was diluted with chloroform (100 mL) and extracted with 10% NaCl (3 × 50 mL). The organic phase was evaporated to a gum, which was redissolved in chloroform (10 mL) and applied to a column (2.5 × 30 cm) of SilicAR CC-7 at 3 °C. The column was washed with chloroform (300 mL); elution with chloroform–methanol (98:2 v/v) then gave pure 2′,3′-*O*-bis(4,4′-dimethoxytrityl)uridine (0.64 mmol, 64%). For analytical purposes, a small portion (ca. 8 mg) of the tritylnucleoside was dissolved in 80% acetic acid (5 mL). After 16 h at 25 °C, the acid was removed by evaporation and coevaporation with water (2 × 20 mL). The residue was dissolved in warm ethanol (2 mL) and added to chloroform (30 mL), which was then washed with water (3 × 20 mL) to extract uridine. The aqueous extracts were combined, evaporated to dryness, and redissolved in 500 mL of water. This solution was found by spectrophotometric measurement to contain 9.4 μmol of uridine (ϵ_{262} = 10000). The chloroform layer was also evaporated to dryness, and the residue was dissolved in 10 mL of EtOH. Assay of this solution by the method of Schaller et al. (1963) showed that the original chloroform contained 19.5 μmol of dimethoxytrityl groups [based on ϵ_{498} = 74500 in ethanol–70% $HClO_4$ (1:1 v/v) for the dimethoxytrityl cation].

For phosphorylation of the nucleoside, 1,2,4-triazole (208 mg, 3 mmol) was dissolved in warm, dry dioxane (5 mL), and the solution was cooled to 25 °C. Triethylamine (0.42 mL, 3 mmol) and then 4-chlorophenyl phosphorodichloridate (0.245 mL, 1.5 mmol) were added with vigorous stirring. After 45 min at 25 °C, the mixture was passed through a coarse glass sinter to remove precipitated triethylammonium chloride. The filtrate was added directly, with rapid mixing, to a solution of the nucleoside (0.5 mmol, dried by coevaporation of pyridine) in anhydrous 2,6-lutidine (1 mL). The mixture was allowed to stand for 2 h at 25 °C and then added dropwise with stirring to water (25 mL). After 1 h, this solution was poured into stirred, ice-cold aqueous $BaCl_2 \cdot 2H_2O$ (1 g in 100 mL of water). The resulting suspension was warmed slowly with constant stirring to 30 °C, producing a filterable solid. The mixture was then cooled to 3 °C and stirred for 1 h. The white, flocculent barium salt of (ClPh)-rU[$MeO)_2$Tr]$_2$ was collected on a coarse sinter, washed with cold water (250 mL), and dried in vacuo over P_2O_5 to constant weight (500 mg, 85%). After being washed with diethyl ether to remove a trace of contaminating dimethoxytritanol, the nucleotide was

chromatographically homogeneous [TLC in chloroform–methanol (85:15 v/v)]. It was used in subsequent reactions without further purification. For analysis, a sample (200 mg) was dissolved in dioxane (2 mL) and poured into cold 1% barium chloride solution (200 mL). The resulting suspension was warmed, filtered, washed, and dried as before. Anal. Calcd. for $C_{57}H_{51}ClN_2O_{13}PBa_{0.5}\cdot 4H_2O$: C, 58.06; H, 5.04; N, 2.38; P, 2.63. Found: C, 58.35; H, 5.20; N, 2.57; P, 2.75. To confirm the location of the aryl phosphate group on the nucleoside, the barium salt (ca. 2 mg) was first dissolved in 80% acetic acid (6 mL). After 50 min at 25 °C, the acid was removed by evaporation, and the detritylated nucleotide (ClPh)-rU was isolated by preparative paper chromatography in 1-propanol–concentrated ammonia–water (7:1:2 v/v). A portion of this material was then treated with snake venom phosphodiesterase under the standard conditions described above. Analysis of the reaction mixture by paper chromatography (Whatman No. 1, using the above solvent) showed complete conversion of (ClPh)-rU to uridine 5′-phosphate.

As an alternative preparation of (ClPh)-rU[$(MeO)_2$Tr]$_2$, 2′,3′-*O*-bis(dimethoxytrityl)uridine (0.3 mmol) was dissolved in dry pyridine (1 mL) and mixed with 1,2,4-triazole (1.2 mmol). 4-Chlorophenyl phosphorodichloridate (0.6 mmol) was added with stirring to the solution, and after 5 min at 25 °C the reaction was treated with dioxane–water (2:3 v/v, 15 mL) and allowed to stand for 30 min. The product was isolated as described above. It was chromatographically indistinguishable from the previous preparation of nucleotide barium salt and gave an equally satisfactory elemental analysis.

2′,3′-O-(Methoxymethylene)uridine 5′-(4-Chlorophenyl 2-cyanoethyl phosphate). 2′,3′-*O*-(Methoxymethylene)uridine (1.19 g, 4.17 mmol) in 2,6-lutidine (8.8 mL) was treated with a dioxane solution of 4-chlorophenyl phosphoroditriazolide (12.5 mmol), prepared as described above. After 1 h at 25 °C, 2-cyanoethanol (2.92 mL, 42 mmol) was added, and the mixture was allowed to stand for a further 4 h. It was then diluted with ethyl acetate (100 mL), and the resulting solution was extracted with 0.1 M triethylammonium bicarbonate (pH 8, 3 × 60 mL) and 10% sodium chloride (60 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to an oil, which was dissolved in chloroform and applied to a column (2.5 × 30 cm) of SilicAR CC-7. The column was washed with chloroform (250 mL), followed by chloroform–methanol (99:1 v/v). The uridine phosphotriester (1.79 g) was obtained as a hygroscopic foam in 81% yield. Anal. Calcd. for $C_{20}H_{21}ClN_3O_{10}P\cdot H_2O$: C, 43.85; H, 4.23; N, 7.67; P, 5.65. Found: C, 43.92; H, 4.35; N, 7.78; P, 5.40. This material could be converted to the corresponding phosphodiester by the decyanoethylation method described above.

Preparation of 2′,3′-[(MeO)$_2$Tr]$_2$rU5′ºdibGºdbzAº-dbzCº(CNEt) (**1**). The fully protected trinucleotide block [$(MeO)_2$Tr]dibGºdbzAºdbzCº(CNEt) (50 μmol) was detritylated and combined with the barium salt of (ClPh)-rU-[$(MeO)_2$Tr]$_2$ (100 μmol). The anhydrous solution in pyridine (350 mg) was treated with TSNI (220 μmol) and allowed to react for 24 h. Pyridine (1 mL) was then added, followed by water (0.2 mL). After 1 h, the reaction mixture was worked up and chromatographed on silica gel as described above. Elution with chloroform–methanol (97:3 v/v) yielded **1** (37 μmol, 75%).

Preparation of 2′,3′-[(MeO)$_2$Tr]$_2$rU5′ºdibGºdbzAº-dbzCºdbzAºdT(Bz) (**2**). Compound **1** (34 μmol) was decyanoethylated, and the dimer [$(MeO)_2$Tr]dbzAºdT(Bz) (26 μmol) was detritylated. An anhydrous mixture of the two products in pyridine (250 mg) was treated with TSNI (150 mg) for 20 h. The resulting hexamer **2** was eluted from a silica gel column with chloroform–methanol (98:2 v/v) in 85% yield.

Preparation of d(pG-A-C-A-T). A sample (2 μmol) of **2** was fully deprotected to give 82 A_{260} units of rU5′-5′d(G-A-C-A-T). A portion of this material (45 A_{260} units) was oxidized and subjected to β-elimination yielding, after purification, 38 A_{260} units of d(pG-A-C-A-T). A total of 16 A_{260} units of this molecule was treated with calf intestinal alkaline phosphatase; preparative paper chromatography yielded 15 A_{260} units of the product d(G-A-C-A-T).

[(MeO)$_2$Tr]dTºdTºdbzCºdbzAºrbzC(Bz)$_2$ (**3**). The cyanoethyl group was removed from 63 μmol of the fully protected tetranucleotide block [$(MeO)_2$Tr]dTºdTºdbzCºdbzAº-(CNEt), and the resulting 3′-phosphodiester was combined with N^4,$O^{2'}$,$O^{3'}$-tribenzoylcytidine (120 μmol) in pyridine (300 mg). The anhydrous mixture was treated with TSNI (240 μmol) for 18 h and then purified by silica gel chromatography with chloroform-methanol (98:2 v/v) to yield 56.5 μmol (90%) of ribonucleoside-terminated pentamer **3**.

d(T-T-C-Ap). A sample (1 μmol) of **3** was deprotected, and the product d(T-T-C-A)-rC was isolated by preparative paper chromatography (33 A_{260} units). The cytidine residue was removed from 10 A_{260} units of this material by oxidation and β-elimination to yield 6.3 A_{260} units of d(T-T-C-Ap).

2′,3′-[(MeO)$_2$Tr]$_2$rU5′ºdTºdTºdbzCºdbzAºrbzC(Bz)$_2$ (**4**). A portion (46 μmol) of **3** was detritylated and combined with 90 μmol of the barium salt of (ClPh)-rU[$(MeO)_2$Tr]$_2$ in pyridine (350 mg). The mixture was treated with TSNI (60 mg, 200 μmol) for 22 h. Column chromatography using chloroform–methanol (97:3 v/v) yielded **4** (41 μmol, 89%).

Conversion of **4** *into d(pT-T-C-Ap).* A sample of **4** (2 μmol) was fully deprotected, yielding 70 A_{260} units of rU5′-5′d(T-T-C-A)-rC. Fifty A_{260} units of this intermediate was oxidized and subjected to β-elimination, giving 32 A_{260} units of d(pT-T-C-Ap) after purification.

Conversion of **4** *into d(pT-T-C-A).* Partial deprotection of **4** (2 μmol) gave 79 A_{260} units of 2′,3′-[$(MeO)_2$Tr]$_2$rU5′-5′d-(T-T-C-A)-rC. One-third of this material, after oxidation/β-elimination and paper chromatographic purification (Whatman 3MM paper, 1-propanol–concentrated ammonia–water, 55:10:35 v/v), gave 19 A_{260} units of 2′,3′-[$(MeO)_2$Tr]$_2$rU5′-5′d(T-T-C-Ap), which was then detritylated. The resulting rU5′-5′d(T-T-C-Ap), after removal of acid by coevaporation with water, was dephosphorylated in 0.25 mL of 0.1 M Tris·HCl (pH 8) by using *Escherichia coli* alkaline phosphatase (0.4 unit). After incubation at 37 °C for 2 h, the mixture was concentrated to ca. 100 μL and treated with 2 N NaOH (35 μL) for 15 min. The solution was neutralized with glacial acetic acid (10 μL), and 17 A_{260} units of the product rU5′-5′d(T-T-C-A) was isolated by preparative paper chromatography. Periodate oxidation and β-elimination then gave 13 A_{260} units of d(pT-T-C-A), after purification.

A separate sample of the intermediate rU5′-5′d(T-T-C-Ap) was prepared and purified by paper chromatography for use in the RNA ligase experiments described below. The oligonucleotides d(pT-T-C-A) and d(pT-T-C-Ap) were also produced, in experiments analogous to those above, by using the mononucleotide 2′,3′-*O*-(methoxymethylene)uridine 5′-(4-chlorophenyl phosphate) as an alternative phosphorylating agent.

Conversion of d(pT-T-C-Ap), d(T-T-C-Ap), and d(pT-T-C-A) into d(T-T-C-A). The oligonucleotide d(pT-T-C-Ap) (15 A_{260} units) was treated with calf intestinal alkaline phosphatase to give, after paper chromatographic purification, 13.5 A_{260} units of d(T-T-C-A). Tetramers d(pT-T-C-A) and

d(T-T-C-Ap) (6 and 3 A_{260} units, respectively) were dephosphorylated similarly. The oligonucleotide products of all three phosphatase reactions were indistinguishable on the basis of their paper chromatographic and HPLC mobilities.

Preparation of [$(MeO)_2Tr$]*dbzC*ºdbzCºdbzAºdbzAºdbzCºdbzCºrbzC(Bz)$_2$ (**5**) *and* 2′,3′-[$(MeO)_2Tr$]$_2$*rU5′*º5′*dbzC*ºdbzCºdbzAºdbzAºdbzCºdbzCºrbzC(Bz)$_2$ (**6**). The fully protected triester block [$(MeO)_2$Tr]dbzCºdbzCºdbzAºdbzAºdbzCºdbzCº(CNEt) (37 μmol) was decyanoethylated and allowed to react in anhydrous pyridine (250 mg) for 1 h with 75 μmol of rbzC(Bz)$_2$ and TSNT (168 μmol) as the condensing agent. The mixture was then treated with pyridine–water (10:1 v/v, 1 mL). After a further hour, it was added to chloroform (25 mL), and the solution was extracted with 5% $NaHCO_3$ (25 mL)–2-methyl-2-propanol (3 mL), followed by 10% NaCl (25 mL)–2-methyl-2-propanol (3 mL). The organic layer was dried (Na_2SO_4), filtered, evaporated, and coevaporated several times with pyridine. The residue in pyridine (100 mg) was diluted with chloroform (10 mL) and chromatographed on silica gel with chloroform–methanol (98:2 v/v) to give **5** (33 μmol, 89%). Detritylated **5** (26 μmol) was then combined with 52 μmol of the barium salt of (ClPh)-rU[$(MeO)_2Tr]_2$ in pyridine (250 mg), and the anhydrous solution was treated with TSNT (170 μmol). After 1 h, the mixture was worked up and chromatographed as described above to give an 88% yield of **6**.

d(C-C-A-A-C-Cp) and d(pC-C-A-A-C-Cp). Oligonucleotide triesters **5** and **6** were deprotected, oxidized, and subjected to β-elimination, giving d(C-C-A-A-C-Cp) and d(pC-C-A-A-C-Cp), respectively, in good overall yields. Portions of these hexamers were dephosphorylated with alkaline phosphatase to give the common product d(C-C-A-A-C-C).

Ligation of rU5′-5′d(T-T-C-A)-rC to d([^{32}*P*]*pC-C-A-T-T-C-A-C-C-Ap*). Incubation mixtures (10 μL) consisted of 50 mM Hepes (pH 7.6), 0.5 μg of bovine serum albumin, 20 mM dithiothreitol, 60 μM ATP, 0.01 M $MnCl_2$, and 6 μM T4 RNA ligase, with the concentration of the labeled donor molecule d([^{32}P]pC-C-A-T-T-C-A-C-C-Ap) (Lawson et al., 1983) at 60 μM. Each solution was treated with one of the three potential acceptors rU5′-5′d(T-T-C-A)-rC, d(T-T-C-A)-rC, and rU5′-5′d(T-T-C-Ap) at a concentration of 120 μM and kept at 20 °C for 16 h. The mixtures were then analyzed by electrophoresis on 20% polyacrylamide gels, and the quantity of ligated species present in each case was determined by scintillation counting. The yields of ligated products were 21, 20, and 0%, respectively.

Results and Discussion

Nucleotides designed to serve as phosphate carriers were synthesized by phosphorylating the 5′-positions of two appropriately protected uridine derivatives. To obtain the first of these, 5′-*O*-acetyluridine (Fromageot et al., 1967) was converted into 2′,3′-*O*-bis(dimethoxytrityl)uridine by tritylation and subsequent deacylation. The ditrityl derivative was then phosphorylated with a mixture of 1,2,4-triazole and 4-chlorophenyl phosphorodichloridate and isolated by the general method originally developed by Gough et al. (1979a,b) for preparation of protected deoxyribonucleoside 3′-(aryl phosphates): hydrolysis followed by precipitation with dilute $BaCl_2$ gave an 85% yield of the barium salt of the diester 2′,3′-*O*-bis(4,4′-dimethoxytrityl)uridine 5′-(4-chlorophenyl phosphate), (ClPh)-rU[$(MeO)_2Tr]_2$. The same phosphodiester was also produced more rapidly with a modified phosphorylation technique recently introduced by Sung & Narang (1982).

For synthesis of the second nucleotide, 2′,3′-*O*-(methoxymethylene)uridine (Griffin et al., 1967) was similarly phosphorylated at its 5′-position. However, in this case, because the barium salt of the phosphodiester product proved to be soluble in water, it was necessary to adopt a less straightforward isolation procedure. The diester was converted to the corresponding triester by addition of an excess of 2-cyanoethanol to the phosphorylation mixture, following the method of Itakura et al. (1974). The resulting 2′,3′-*O*-(methoxymethylene)uridine 5′-(4-chlorophenyl 2-cyanoethyl phosphate) was obtained in 81% yield by adsorption chromatography. This material could be reconverted by treatment with triethylamine to the phosphodiester (ClPh)-rU>CH(OMe), which was then ready for condensation with the 5′-hydroxyl group of an oligonucleotide.

The first test of the 5′-phosphorylation system involved the fully protected triester block [$(MeO)_2$Tr]dibGºdbzAºdbzCº(CNEt). This trimer was detritylated to free its 5′-hydroxyl group and was then allowed to react with the barium salt of (ClPh)-rU[$(MeO)_2Tr]_2$ in the presence of the condensing agent TSNI. The product 2′,3′-[$(MeO)_2Tr]_2$rU5′º5′dibGºdbzAºdbzCº(CNEt) (**1**) was in turn extended at its 3′-terminus. The reaction of decyanoethylated **1** with the protected dinucleotide dbzAºdT(Bz) in the presence of TSNI produced the fully protected hexanucleotide 2′,3′-[$(MeO)_2Tr]_2$rU5′º5′dibGºdbzAºdbzCºdbzAºdT(Bz) (**2**) in 85% yield after purification. Removal of the phosphate-, base-, and hydroxyl-protecting groups from this oligomer under standard conditions gave the product rU5′-5′d(G-A-C-A-T), which was readily converted to the pentanucleotide d(pG-A-C-A-T) by periodate oxidation and β-elimination. In this case, as in those to be described below, oligonucleotide conversions were first verified by observing changes in retention volumes on HPLC (Table I). The products were then characterized by analysis of the nucleosides and nucleotides formed by subjecting the oligomers to enzymatic degradation (Table II).

The barium salt of (ClPh)-rU[$(MeO)_2Tr]_2$ consequently provides a convenient means of introducing a 5′-phosphate group into an oligodeoxyribonucleotide at the phosphotriester level. After removal of protecting groups, the ribonucleoside residue is quantitatively detached in a simple oxidation/β-elimination step, a process that has been used previously in the synthesis of oligonucleotides with 3′-terminal phosphates (Sninsky et al., 1976; Ulrich et al., 1983) and in sequencing of RNA by stepwise degradation (Keith & Gilham, 1974). The conditions used for removal of terminal ribonucleosides are mild: the entire reaction sequence is complete in 2.5 h and is carried out over a pH range of 4.5–8.0. The end result of these manipulations is the transfer of phosphate from uridine to the 5′-terminus of an oligodeoxyribonucleotide; the procedure generates molecules that can serve as substrate components in internucleotide-joining reactions catalyzed by DNA ligase and provides an economically attractive alternative to enzymatic phosphorylation methods when large amounts of synthetic oligomers containing 5′-monophosphates are required, as, for example, in the commercial preparation of "linker" or "adaptor" molecules or in the production of high-purity oligomers for physical studies.

A useful feature of this new method of chemical phosphorylation is that it can be combined with a similar, previously reported procedure for generation of 3′-monophosphates (Gough et al., 1979a; Ulrich et al., 1983) to allow the synthesis of oligonucleotides containing any combination of terminal monophosphates. This strategy was demonstrated by using a tetranucleotide block as a starting point. The various transformations of this material are diagrammed in Figure 1, which shows how, from the single fully protected tetramer

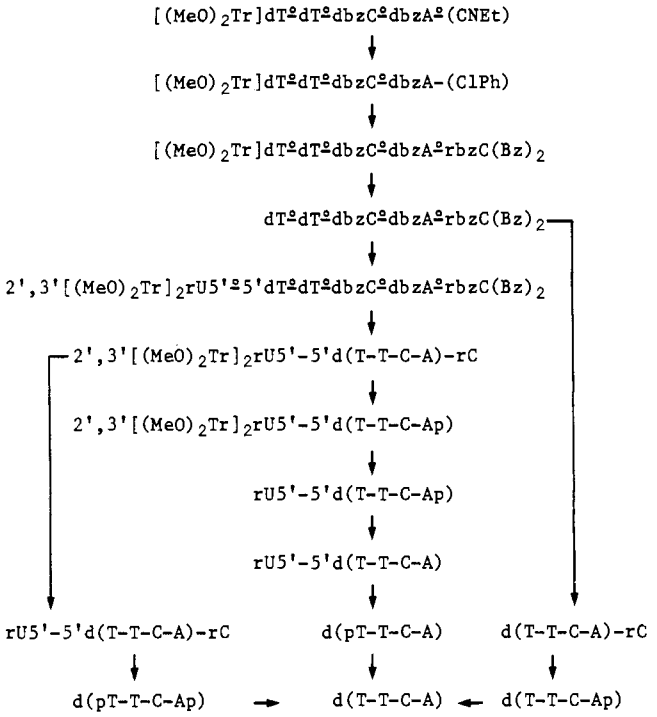

FIGURE 1: Scheme for oligonucleotide conversions.

$[(MeO)_2Tr]dT^sdT^sdbzC^sdbzA^s(CNEt)$, it is possible to produce the three terminally phosphorylated species d(T-T-C-Ap), d(pT-T-C-A), and d(pT-T-C-Ap) by methods that should be generally applicable to any deoxyribonucleotide sequence. The utility of molecules with the structure d(pN-N_n-N) as substrates for DNA ligase has been mentioned above. Molecules of the type d(pN-N_n-Np) are useful as donor substrates in the joining of oligodeoxyribonucleotides by RNA ligase (McCoy & Gumport, 1980); in these reactions, the 3'-phosphate prevents the oligomer from functioning as an acceptor. Furthermore, oligonucleotides with the structure d(N-N_n-Np) can be phosphorylated on their 5'-ends with polynucleotide kinase and may prove useful in situations requiring 5'-^{32}P-labeled molecules.

All the phosphorylation reactions of oligonucleotides mentioned so far have employed TSNI as the condensing agent. Although this reagent gives high yields (75–90%) of fully protected 5'-phosphorylated oligonucleotides, it is rather slow acting, requiring 18–20 h for complete internucleotide bond formation. Use of the more rapid condensing agent TSNT was demonstrated in the phosphorylation of protected d(C-C-A-A-C-C)-rC (**5**). The oligomer was detritylated and mixed with (ClPh)-rU$[(MeO)_2Tr]_2$ in the presence of TSNT. After 1 h, the reaction was judged to be complete by thin-layer chromatography, and the product, **6**, was purified by silica gel chromatography and isolated in 88% yield. This material was deprotected and subjected to periodate oxidation and β-elimination to produce d(pC-C-A-A-C-Cp). A potential difficulty arising from the use of TSNT in this phosphorylation procedure is that the reagent causes significant modification of the uracil moieties by formation of a nitrotriazole derivative (Reese & Ubasawa, 1980). However, such modification was not expected to be a problem in this methodology for two reasons. First, the nitrotriazole derivative reverts to uracil during the standard deprotection of the oligonucleotide with oximate (Reese & Ubasawa, 1980). Second, any type of modification of the base moiety that might persist through the oximate deprotection step should not affect the removal of the ribonucleoside residue from the final oligonucleotide by periodate oxidation and β-elimination. In fact, in the experiment described, modification by TSNT during the synthesis of **6** was not detectable by thin-layer chromatography, nor did it interfere with any of the steps leading to the final 5'-phosphorylated product d(pC-C-A-A-C-Cp).

Apart from their role in introducing phosphate groups as described above, "inverted" uridine residues have an added function, that of protecting the 5'-terminal phosphate groups of oligonucleotides during RNA ligase reactions. Oligodeoxyribonucleotides bearing 3'-terminal ribonucleosides (Gough et al., 1979a, 1980), which have been tested as acceptor substrates for RNA ligase (G. B. Kelly, C. K. Singleton, and H. L. Weith, unpublished experiments), were used with the model donor d([^{32}-P]pC-C-A-T-T-C-A-C-C-Ap) to demonstrate this application. A series of RNA ligase reactions were carried out, and the products were analyzed by gel electrophoresis. When rU5'-5'd(T-T-C-A)-rC was employed as the acceptor, a single ligated product, with a mobility corresponding to a chain length of 16, was obtained in 20% yield. An analogous product was formed in the same yield with d(T-T-C-A)-rC as the acceptor substrate under identical conditions, while no ligation occurred when rU5'-5'd(T-T-C-Ap) was used. These experiments indicate that (i) an inverted uridine at the 5'-terminus of an acceptor oligomer does not affect its ability to engage in ligation at its 3'-terminus with the donor species and (ii) the 5'-inverted uridine residue does not itself behave as an acceptor 3'-terminus in the RNA ligase reaction. The ribonucleoside may therefore be used to protect a 5'-phosphate group on an acceptor species undergoing ligation; its subsequent removal would permit the extended molecule itself to function as a donor in ligation to another acceptor.

Although, in the experiments described above, the inertness of the uridine cap in RNA ligase reactions was demonstrated with hybrid acceptors (oligodeoxyribonucleotides with 3'-ribonucleoside terminals), we anticipate that this method of protecting the 5'-phosphate of an acceptor will also be useful in the stepwise enzymatic joining of "pure" RNA or DNA oligomers. Moreover, the outcome of the RNA ligase experiments raises the possibility that the inverted uridine could be used in a similar manner during other enzymatic manipulations of oligo- or polynucleotides. Hence, the ability to protect and subsequently expose 5'-terminal monophosphates at will, under conditions that do not affect the chemical integrity of nucleic acids, may have some significant applications in genetic engineering.

Finally, we note that our method for phosphorylating the 5'-termini of DNA oligonucleotides should be applicable to chemically synthesized oligoribonucleotides as well, if their 3'-terminal *cis*-diol functions are suitably protected against periodate oxidation while the uridine residues at the 5'-ends are being removed; the general concept could also be adapted for use in solid-phase systems for oligonucleotide synthesis employing either phosphate or phosphite triester chemistries.

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Base Protonation Facilitates B–Z Interconversions of Poly(dG-dC)·Poly(dG-dC)†

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ABSTRACT: Comparative studies on the salt titration and the related kinetics for poly(dG-dC)·poly(dG-dC) in pH 7.0 and 3.8 solutions clearly suggest that base protonation facilitates the kinetics of B–Z interconversion although the midpoint for such a transition in acidic solution (2.0–2.1 M NaCl) is only slightly lower than that of neutral pH. The rates for the salt-induced B to Z and the reverse actinomycin D induced Z to B transitions in pH 3.8 solutions are at least 1 order of magnitude faster than the corresponding pH 7.0 counterparts. The lowering of the B–Z transition barrier is most likely the consequence of duplex destabilization due to protonation as indicated by a striking decrease (~40 °C) in melting temperature upon H^+ binding in low salt. The thermal denaturation curve for poly(dG-dC)·poly(dG-dC) in a pH 3.8, 2.6 M NaCl solution indicates an extremely cooperative melting at 60.5 °C for protonated Z DNA, which is immediately followed by aggregate formation and subsequent hydrolysis to nucleotides at higher temperatures. The corresponding protonated B-form poly(dG-dC)·poly(dG-dC) in 1 M NaCl solution exhibits a melting temperature about 15 °C higher, suggesting further duplex destabilization upon Z formation.

Continuous acidification of a DNA solution of medium salt concentration can result in acid denaturation at pH below 3 (Gulland et al., 1947; Cox & Peacocke, 1957; Sturtevant et al., 1958). However, in a moderately acidic solution (pH 3–5) the DNA exists in a partially base-protonated duplex state (Cavalieri & Rosenberg, 1957; Peacocke & Preston, 1958; Geiduschek, 1958) that exhibits a dramatic decrease (~40 °C) in melting temperature from the corresponding neutral counterpart (Zimmer et al., 1968; Courtois et al., 1968; Chen, 1983a). This protonated duplex greatly enhances binding to some polycyclic aromatic hydrocarbons (Chen, 1984) and causes a striking sign reversal for the induced circular dichroism (CD) of DNA-bound pyrene (Chen, 1983a). This interesting sign inversion has been shown to result from changes in base sequence specificity of pyrene from preference for dA-dT (and/or dT-dA) in neutral DNA (Chen, 1983b) to an affinity for all purine–pyrimidine sequences, especially dG-dC (and/or dC-dG), in protonated DNA (Chen, 1983a).

The exact nature of this protonated duplex state, however, is still unclear. There is controversy as to which base of the DNA is the first to be protonated. On the basis of the fact

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