Whitlock of the University of Wisconsin (Madison) for enlightening discussions. This research was financially supported by the National Institutes of Health (Grant No. AI07795). The use of the CDC 1604 and 3600 computers and the UNIVAC 1108 computer at the University of Wisconsin Computing Center was made available through partial support of National Science Foundation and Wisconsin Alumni Research Foundation through the University Research Committee. One of us (R. M. S.) acknowledges the support of the National Institutes of Health through Graduate Research Fellowship No. 3-FO1-GM-39444-02.

Polynucleotides. VIII.[1] A New Method for the Synthesis of Protected Deoxyribooligonucleotides with 5′-Phosphate

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Abstract: Phosphoroanilidate was used to protect the 5′-terminal phosphate in the synthesis of derivatives of deoxyribooligonucleotides. Three deoxyribotrinucleotides containing acid labile N-benzoyldeoxyadenosine were synthesized. The synthetic step involved the condensation of thymidine 5′-phosphoranilidate and N-benzoyl-3′-O-acetyldeoxyadenosine 5′-phosphate using dicyclohexylcarbodiimide, and selective removal of the 3′-O-acetyl group to yield the protected dinucleotide (III, Scheme I) in a yield of 58%. For the synthesis of trinucleotides, III was condensed with N-benzoyl-3′-O-acetyldeoxyadenosine 5′-phosphate, N-anisoyl-3′-O-acetyldeoxycytidine 5′-phosphate, and N,3′-O-diisobutyryldeoxyguanosine 5′-phosphate. Treatment with isoamyl nitrite and subsequent removal of the 3′-O-acetyl group gave IVa, IVb, and IVc, respectively. Yields in the synthesis of the trinucleotides were 30–36%.

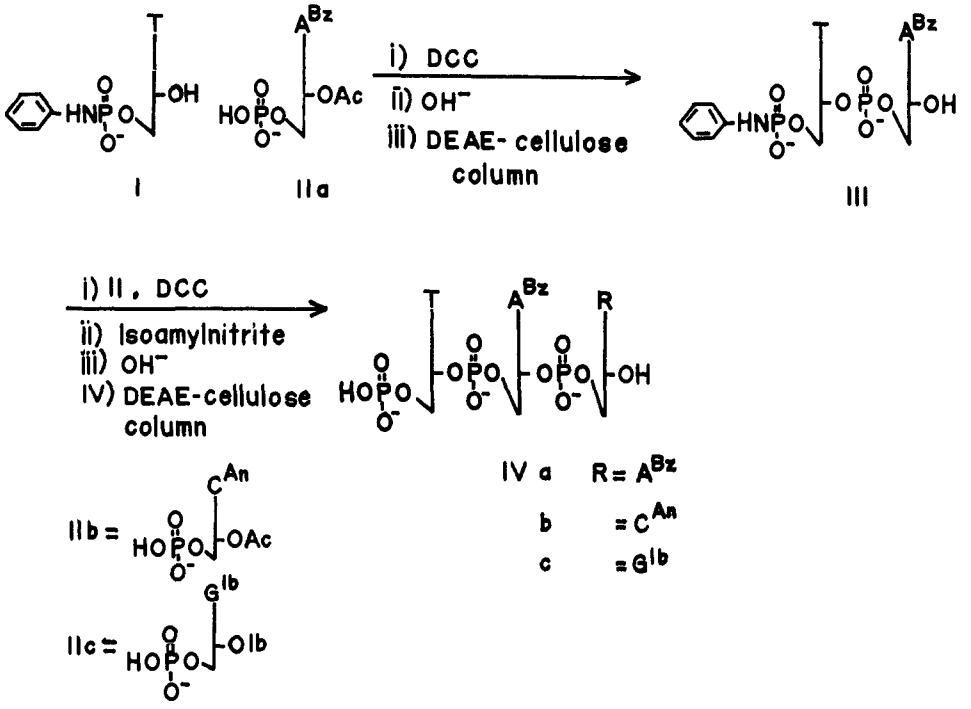
Nucleoside 5′-phosphates protected at the phosphate group have been used as key intermediates in the synthesis of deoxyribopolynucleotides.[2] β-Cyanoethyl ester is one of the most common protecting groups. Since it is cleaved by treatment with alkali,[3,4] reprotection of the 5′-phosphate is required after removal of the 3′-O-acetyl group.[5] Other useful protecting groups include the trichloroethyl ester, which is cleaved by reduction,[6] and the S-ethylphosphorothioates,[7] which are stable in alkali and are removed by mild oxidation. Blackburn described the synthesis of pTpTpT[8] on a phosphoramidate resin. The acid treatment[9] used to release the product, however, is not compatible with purine deoxyribonucleotides.

In this paper we report the use of aromatic phosphoramidates as protecting groups for the 5′-phosphate group in the stepwise synthesis of deoxyribooligonucleotides. Aromatic amidates of protected ribonucleoside 3′-phosphate were previously shown to be stable in alkali and to be subject to selective removal with isoamyl nitrite.[10] A new general procedure for the synthesis of oligonucleotide blocks is outlined in Scheme I. Since the glycosyl bond of N-benzoyldeoxyadenosine is extremely labile under the condition in which those of N-benzoyladenosine and deoxyadenosine were stable,[11] the synthesis of trinucleotides containing N-benzoyldeoxyadenosine was chosen to test the stability of the glycosyl linkage during the treatment with isoamyl nitrite.

Thymidine 5′-phosphoranilidate (I) was prepared by a method similar to that described for the synthesis of adenosine 5′-phosphoro-*p*-anisidate.[12] Anilidate of the 5′-phosphate could be removed with isoamyl nitrite in a 1:1 mixture of acetic acid and pyridine or a 1:2 mixture of acetic acid and triethylamine. Although the latter mixture seems to give a slower rate, it might be safer to use a trialkylamine for acid labile deoxypurine nucleotides. Thymidine 5′-phosphoranilidate (I) and N-benzoyl-3′-O-acetyldeoxyadenosine 5′-phosphate (IIa) were allowed to react with dicyclohexylcarbodiimide (DCC). Triisopropylbenzenesulfonyl chloride (TPS) was also used as the condensing reagent in a preliminary experiment. After 4 days an aliquot was treated with ammonia. PhNHpTpA was found on paper chromatogram as almost the sole product (see Table I for R_f values of the protected and unprotected compound). The 3′-O-acetyl group was removed selectively by treatment with strong alkali to give III. The stability of the amidate in this treatment was checked by paper chromatography and paper elec-

(1) Paper VII: M. Ikehara, I. Tazawa, and T. Fukui, *Biochemistry*, **8**, 736 (1969).
(2) H. G. Khorana, *Pure Appl. Chem.*, **17**, 349 (1968).
(3) G. M. Tener, *J. Amer. Chem. Soc.*, **83**, 159 (1961).
(4) G. Weimann, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 3835 (1963).
(5) E. Ohtsuka and H. G. Khorana, *ibid.*, **89**, 2185 (1967).
(6) A. Franke, F. Eckstein, K. Scheit, and F. Cramer, *Chem. Ber.*, **101**, 944 (1968).
(7) A. F. Cook, M. J. Holman, and A. L. Nussbaum, *J. Amer. Chem. Soc.*, **91**, 6479 (1969).
(8) The abbreviation of nucleotides in this paper follows that described in *J. Biol. Chem.*, **241**, 527 (1966), and E. Ohtsuka, M. W. Moon, and H. G. Khorana, *J. Amer. Chem. Soc.*, **87**, 2956 (1965). Other abbreviations used were PhNHpTpA, 5′-phosphoranilidothymidilyl-(3′–5′)-deoxyadenosine; d-pG^{1b}-OIb, N,3′-O-diisobutyryldeoxyguanosine 5′-phosphate.
(9) G. M. Blackburn, M. J. Brown, and M. R. Harris, *J. Chem. Soc.*, 2438 (1967).
(10) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, **91**, 1537 (1969).
(11) M. W. Moon, S. Nishimura, and H. G. Khorana, *Biochemistry*, **5**, 937 (1966).
(12) J. G. Moffatt and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 649 (1961).

Scheme I



trophoresis. The yield of III obtained by chromatography on a column of DEAE–cellulose was 58%.

For the synthesis of d-pTpA^{Bz}pA^{Bz} (IVa), the protected dinucleotide (III) was condensed with d-pA^{Bz}-OAc (IIa) using DCC. The amidate group of the 5′-phosphate was removed by treatment with isoamyl nitrite in a buffer of acetic acid and triethylamine and the 3′-O-acetyl group was removed with strong alkali. The product IVa was isolated on a column of DEAE–cellulose. The elution pattern and identification of peaks are shown in Figure 1. Peak V contained the pure trinucleotide IVa (36%). The purity was checked by paper chromatography and paper electrophoresis before and after removal of benzoyl group. The unprotected trinucleotide, d-pTpApA, was completely degraded with purified snake venom phosphodiesterase to give pT and d-pA in the ratio of 1.0:2.0.

Other trinucleotides, d-pTpA^{Bz}pC^{An} (IVb) and d-pTpA^{Bz}pG^{Ib} (IVc) were synthesized by condensing the pyridinium salt of III with d-pC^{An}-OAc (IIb) and d-pG^{Ib}-OIb (IIc), respectively. The products were isolated as above in yields of 31 and 30%, respectively. The spectral properties and R_f values are given in Table I.

Table I

Compd	Spectral properties λ_{max}, nm	Spectral properties λ_{min}, nm	Paper chromatography, R_f in solvent A	B	C	D	Paper electrophoresis, relative mobility pH 7.5	pH 3.5
pT			0.17	0.33	0.49	0.48	1.00	1.00
d-pA			0.13	0.23	0.32	0.35		
d-pC					0.65	0.38		
d-pG				0.12	0.39	0.28		
d-pTpA	261	231				0.30		
d-pTpApA	259	231			0.06	0.26	1.02	
d-pTpTpC	262	235			0.11	0.23	1.09	
d-pTpApG	257, 265 (sh)	228			0.08	0.15	1.09	
d-PhNHpT	230, 269	249	0.65	0.75		0.86	0.73	
d-PhNHpTpA	234, 262	226, 245	0.40			0.78	0.81	
d-PhNHpTpApA	234 (sh), 259	242	0.17				0.84	
d-PhNHpTpApC	235 (sh), 262	245				0.58	0.88	
d-PhNHpTpApG						0.37	0.89	
d-pTpA^{Bz}	278	240		0.30			0.96	1.02
d-pTA^{Bz}pA^{Bz}	280	240		0.27			0.96	1.14
d-pTpA^{Bz}pC^{An}	281, 300 (sh)	241		0.28			0.98	
d-pTpA^{Bz}pG^{Ib}	263, 274 (sh)	238		0.32			1.10	
d-PhNHpTpA^{Bz}				0.69				

The use of aromatic phosphoramidates for protection of the terminal 5′-phosphate and their removal with isoamyl nitrite thus proved to be suitable for the synthesis of N-protected deoxyribooligonucleotides, which could be used in subsequent condensation reactions. The yield of condensation involving the protected dinucleotide and mononucleotides should be improved by using triisopropylbenzenesulfonyl chloride.[13] Side

(13) T. M. Jacob and H. G. Khorana, *J. Amer. Chem. Soc.*, 86, 1630 (1964).

reactions such as the formation of pyrophosphates and further activations of secondary phosphate anions would have to be studied in detail if this method is to be used for the synthesis of larger polynucleotides.

Application of this method to the synthesis of protected oligonucleotides on a polymer support is under investigation.

Experimental Section

General Methods. Paper chromatography was performed by the descending technique using Toyoroshi No. 51A paper. The solvent systems used were: solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); solvent B, ethyl alcohol–1 *M* ammonium acetate, pH 7.5 (7:3, v/v); solvent C, saturated ammonium sulfate–water–isopropyl alcohol (79:19:2, v/v); solvent D, *n*-propyl alcohol–concentrated ammonia–water, (55:10:35, v/v). The R_f values of different compounds are given in Table I. Paper electrophoresis was performed at 900 V/40 cm. Triethylammonium bicarbonate (0.05 *M*, pH 7.5) and ammonium formate (0.05 *M*, pH 3.5) were used.

Pyridine was treated with *p*-toluenesulfonyl chloride, fractionally distilled, and then refluxed over potassium hydroxide, decanted, and redistilled. It was stored over Molecular Sieves (4 A) (Linde Co.). All reaction mixtures for condensation reactions were made anhydrous by repeated evaporation of added pyridine; the last three times the flask was opened in a drybox in which an anhydrous atmosphere was maintained by phosphorus pentoxide.

Pyridinium N-benzoyl-3′-O-acetyldeoxyadenosine 5′-phosphate (IIa) and N-anisoyl-3′-O-acetyldeoxycytidine 5′-phosphate (IIb) were synthesized as described previously.[14] Pyridinium N,3′-O-diisobutyryldeoxyguanosine 5′-phosphate[2] was prepared according to the unpublished method by Büchi, Weber, and Khorana using isobutyric anhydride (40 equiv) in pyridine.

The molar extinction values at neutral pH at 280 nm for the nucleotides are as described in ref 15. The molar extinction value of d-pG^{Ac} was used for that of d-pG.[1b] The abbreviation OD_{260} refers to the extinction at 260 nm of a nucleotidic solution in a 1-ml volume using a 1-cm light-path quartz cell.

Phosphorus analysis was done by Allen's method.[16]

For removal of the N- and O-acyl protecting groups the compounds were treated with a large excess of concentrated ammonia for 20 hr at 25° or 4 hr at 55°. The selective removal of the 3′-O-acetyl group was performed using 1 *N* sodium hydroxide at 0° for 2 min. The anilidate group was removed with isoamyl nitrite (40–100-fold excess) in a mixture of acetic acid and triethylamine (2:1, v/v) for 4 hr at 20°.

Purified venom phosphodiesterase was purchased from Worthington Biochemical Corp. and further purified by cation exchange chromatography.[17] This purification is not always necessary. Oligonucleotides (*ca.* 3 OD_{260} units) were digested with 10 μg of the enzyme in 0.2 *M* ammonium carbonate (100 μl) for 4.5 hr at 37°.

Thymidine 5′-Phosphoranilidate (I). This compound was prepared by essentially the same method used for the synthesis of adenosine 5′-phosphoro-*p*-anisidate. The reaction could be carried out at room temperature for 3 days with a larger excess of aniline. N,N′-Dicyclohexyl-N″-phenylguanidinium thymidine 5′-phosphoranilidate was crystallized from 50% aqueous pyridine saturated with ether (mp 163–164°). *Anal.* Calcd for $C_{35}H_{50}N_6O_7P$: P, 4.4%. Found: P, 4.3%.

d-PhNHpTpA^{Bz} (III). (i) Using DCC. Pyridinium thymidine 5′-phosphoranilidate (I, 0.16 mmol) and pyridinium d-pA^{Bz}-OAc (IIa, 0.16 mmol) were allowed to react with DCC (1.6 mmol) in the presence of pyridinium Dowex 50-X2 (0.2 g) in pyridine (2 ml) at 20° for 6 days. The reaction could be checked by treating an aliquot with ammonia and chromatographed in solvent A. Water (2 ml) was added to the reaction mixture and DCC was extracted with *n*-hexane. The aqueous pyridine solution was kept at room temperature for 12 hr and filtered to remove insoluble materials. The solution was treated with 2 *N* sodium hydroxide (4 ml) in an ice bath for 2 min. Sodium ions were removed by pyridinium Dowex 50-X2 (20 ml in wet volume) and the diluted solution was applied to a column (2.2 × 30 cm) of DEAE-cellulose (bicarbonate). A linear gradient of triethylammonium bicarbonate in 10% ethyl alcohol was used for elution at 4°, 2 l. of 0.005 *M* of salt being in the mixing chamber and an equal volume of 0.15 *M* of salt in the reservoir. The protected dinucleotide (III) was eluted at about 0.07 *M* of the salt. The yield of III was 2280 OD_{280} units (0.093 mmol, 58%). The spectral properties in water of the product are shown in Table I.

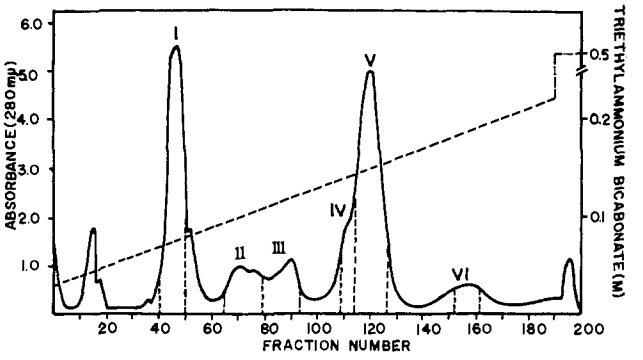


Figure 1. Chromatography of the products obtained in the synthesis of d-pTpA^{Bz}pA^{Bz} on a column of DEAE-cellulose (bicarbonate, 2.2 × 30 cm). Elution was carried out using a linear gradient of triethylammonium bicarbonate in 10% ethyl alcohol (2 l. of 0.03 *M* in the mixing vessel and an equal volume of 0.25 *M* salt in the reservoir). Fractions of 18 ml were collected at 4°. Peak I contained d-pA^{Bz}. The main component of peak II was a derivative of the trinucleotide. Peak III was a mixture of three compounds. Peak IV contained mainly d-pTpA^{Bz}pA^{Bz} and peak V contained pure d-pTpA^{Bz}pA^{Bz}. Peak VI was a higher compound.

(ii) Using TPS. The guanidinium thymidine 5′-phosphoranilidate (0.1 mmol) and IIa (0.1 mmol) were made anhydrous as described in general method. The anhydrous pyridine solution (1 ml) was treated with TPS (0.3 mmol) at 10° for 12 hr. Aqueous pyridine (50%, 1 ml) and triethylamine (0.6 mmol) were added with cooling. The extent of the reaction, estimated by paper chromatography in solvent D, was 89%.

d-PtpA^{Bz}pA^{Bz} (IVa). Triethylammonium salt of II (1780 OD_{280} units, 0.072 mmol) and pyridinium d-pA^{Bz}-OAc (0.16 mmol) were dissolved in 50% aqueous pyridine (3 ml) and passed through a column (1 × 10 cm) of pyridinium Dowex 50-X2. The effluent and washings were rendered anhydrous and treated with DCC (1.6 mmol) in pyridine (2 ml) at 20° for 4 days. Aqueous pyridine treatment was given for 12 hr and after removal of DCC as above the solution was made anhydrous by evaporation of pyridine. The nucleotides were precipitated in ether from their anhydrous pyridine solution. The precipitate was collected by centrifugation and treated with isoamyl nitrite (0.5 ml), triethylamine (0.25 ml), acetic acid (0.5 ml), and DMF (0.5 ml) at 20° for 4 hr. Most of the volatile materials were removed *in vacuo* and the residue was precipitated with a mixture of ether and *n*-hexane. The precipitate was dissolved in 50% aqueous pyridine and treated with 2 *N* sodium hydroxide as described in the preparation of compound III. The diluted solution was applied to a column of DEAE-cellulose (bicarbonate). The elution pattern and the identification of peaks are shown in Figure 1. Peak V contained the pure trinucleotide, d-pTpA^{Bz}pA^{Bz} (980 OD_{280} units, 0.026 mmol). The yield was 36%, $\epsilon_{(P)}$ at 280 nm being found to be 12.5×10^3. The spectral properties in water and R_f values of the product are shown in Table I. The fully unprotected nucleotide d-pTpApA (*ca.* 3 OD_{260} units) was digested with purified snake venom phosphodiesterase and the products were analyzed by paper chromatography in solvent C. pT (0.081 μmol) and d-pA (0.16 μmol) were the sole products.

d-pTpA^{Bz}pC^{An} (IVb). The pyridinium salt of III (0.069 mmol) and pyridinium d-pC^{An}-OAc (0.14 mmol) were allowed to react with DCC (1.4 mmol) in pyridine (1 ml) and DMF (1 ml) in the presence of pyridinium Dowex 50-X2 (0.1 g) using the same condition as above. The product was isolated by DEAE-cellulose column chromatography as described in Figure 1a. It was eluted at a salt concentration of 0.15 *M*. The yield was 765 OD_{280} units, 0.022 mmol, 31%, assuming $\epsilon_{(P)} = 12{,}000$ at 280 nm.

d-pTpA^{Bz}pG^{Ib} (IVc). Pyridinium salt of III (0.07 mmol) and pyridinium d-pG^{Ib}-OIb (IIc) (0.15 mmol) were condensed using DCC (1.5 mmol) as above. IVc was eluted at a salt concentration

(14) H. Schaller and H. G. Khorana, *J. Amer. Chem. Soc.*, **85**, 3828 (1963).
(15) H. Kössel, H. Büchi, and H. G. Khorana, *ibid.*, **89**, 2185 (1967).
(16) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).
(17) E. Keller, *Biochem. Biophys. Res. Commun.*, **17**, 412 (1964).

of 0.16 *M* in DEAE-cellulose chromatography using the same condition described in Figure 1. The yield was 640 OD_{270} units, 30%, assuming $\epsilon_{(P)}$ = 10,400 at 280 nm. The unprotected trinucleotide pTpApG (*ca.* 3 OD_{260} units) was degraded with purified snake venom phosphodiesterase to give pT (0.066 μmol), d-pA (0.068 μmol), and d-pG (0.064 μmol) in paper chromatography (solvent C). The spectral properties and R_f values of the trinucleotide derivatives are given in Table I.

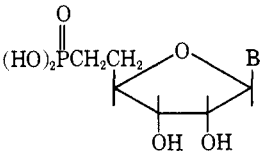
Communications to the Editor

Synthesis of Isosteric Phosphonate Analogs of Some Biologically Important Phosphodiesters

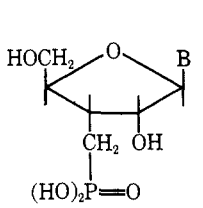
Sir:

Recently we have developed syntheses of isosteric phosphonate analogs of both nucleoside 5′-phosphates (**1**)[1] and nucleoside 3′-phosphates (**2**)[2] in which the ester oxygen is replaced by a methylene group. Conversion of these compounds into analogs of natural phosphodiesters containing specific chemically and enzymatically stable bonds provides powerful tools for studying the mechanism of enzyme and hormone action. Such syntheses are described in this communication.

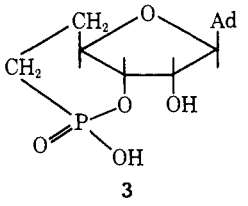
The intramolecular, high-dilution cyclization[3] of **1a** using dicyclohexylcarbodiimide (DCC) in hot pyridine readily gave the 3′,6′-cyclic phosphonate **3** as the crystalline free acid in 90% yield without need for chromatography: mp >220° dec; λ_{max} 259 mμ (ε 14,900).[4] The cyclization of **1a** to **3** was much more facile than that of adenosine 5′-phosphate and could be carried out in concentrated solution, and even in aqueous pyridine. Thus, reaction of the tributylammonium salt of **1a** with 4 equiv of DCC in refluxing pyridine–water (95:5) gave 89% of crystalline **3**.



1a, B = adenine
b, B = uracil



2a, B = adenine
b, B = uracil



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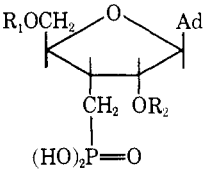
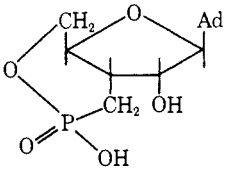
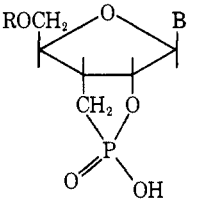
Cyclization of **2a** to the branched-chain cyclic phosphonate **5** requires prior protection of the 2′-hydroxyl group. To this end **2a** was converted in 70% yield into its 5′-*O*-monomethoxytrityl derivative (**4a**) as described for adenosine 3′-phosphate.[5] Subsequent reaction of **4a** with acetic anhydride in the presence of 30 molar equiv of tetraethylammonium acetate in pyridine gave the desired 2′-*O*-acetate **4b** as the major product together with variable amounts of the 2′-cyclic ester[6]



4a, R_1 = MeOTr; R_2 = H
b, R_1 = MeOTr; R_2 = Ac



5



6a, B = Ad; R = H
b, B = Ad; R = MeOTr
c, B = Ur; R = H

6b. The mixture was sequentially treated with 80% acetic acid to remove the methoxytrityl group, with DCC in pyridine to effect intramolecular cyclization to the 5′-hydroxyl group, and then deacetylated with ammonium hydroxide giving a mixture of the 3′,5′-cyclic phosphonate **5** and the 2′,3′-cyclic phosphonate **6a**. Completely selective hydrolysis of the five-membered cyclic compound **6a**[7] was achieved with 0.5 *N* hydrochloric acid at 22° for 2 hr and pure **5** was isolated by ion-exchange chromatography. Subsequent acidification gave **5** as the crystalline free acid in 10% overall yield from **2a**: mp >220° dec; λ_{max} 258 mμ (ε 14,400). Biological studies on **3** and **5** which are phosphonate analogs of adenosine 3′,5′-cyclic phosphate[8] will be described separately.

In order to obtain substrates with which to study the mechanism of enzyme action (*e.g.*, RNase) we have also prepared nucleoside 2′,3′-cyclic phosphonates (**6a**, **6c**) and the isomeric phosphonate analogs (**7** and **8**) of

(1) G. H. Jones and J. G. Moffatt, *J. Amer. Chem. Soc.*, **90**, 5337 (1968).
(2) H. P. Albrecht, G. H. Jones, and J. G. Moffatt, *ibid.*, **92**, 5511 (1970).
(3) M. Smith, G. I. Drummond, and H. G. Khorana, *ibid.*, **83**, 698 (1961).
(4) All purified products gave satisfactory analytical values and 100-MHz nmr spectra.
(5) Y. Lapidot and H. G. Khorana, *J. Amer. Chem. Soc.*, **85**, 3857 (1963).
(6) Using 10 equiv of tetraethylammonium acetate, which gives quantitative 2′-*O*-acetylation of the corresponding phosphate derivative,[5] the predominant product was the cyclic phosphonate **6b**.
(7) *Cf.* the relative stabilities of five- and six-membered cyclic phosphates: H. G. Khorana, G. M. Tener, R. S. Wright, and J. G. Moffatt, *J. Amer. Chem. Soc.*, **79**, 430 (1957).
(8) R. W. Butcher, G. A. Robison, J. G. Hardman, and E. W. Sutherland, *Advan. Enzyme Regul.*, **6**, 357 (1968).