Synthesis of Oligothymidylates and Nucleoside Cyclic Phosphates by Oxidation–Reduction Condensation

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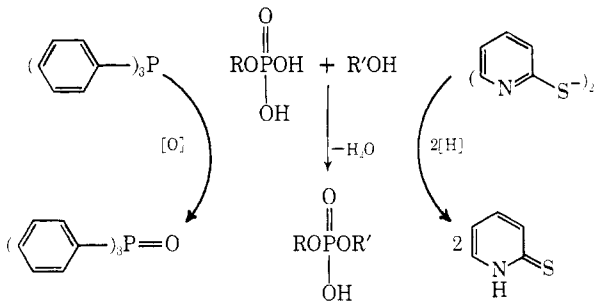
Abstract: Detailed studies on the synthesis of nucleotide derivatives by use of triphenylphosphine and 2,2′-dipyridyl disulfide (TPP–PDS) as coupling reagents are described. Active intermediates (I), phosphoroxyphosphonium salts, are formed immediately by the reactions of phosphates and the coupling reagents and are kept as stable salts in pyridine solution. By treatment of I with 5′-*O*-tritylthymidine, 2′,3′-*O*-isopropylideneuridine, and 3′-*O*-acetylthymidime 5′-phosphate, thymidylic (3′) acid, uridylic (5′) acid, and oligothymidylates (di, tri, and tetra) are obtained in 50–90% yields, accompanied by a very small amount of symmetrical pyrophosphates. Nucleoside 2′,3′- and 3′,5′-cyclic phosphates are also obtained in high yields by use of the present coupling reagents.

A general method for the specific synthesis of naturally occurring small molecules of nucleic acid, oligonucleotides having $C_{3'-5'}$ internucleotide linkage, has been established by the use of dehydrating reagents such as DCC and TPS[1] developed by Khorana and coworkers.

It has been reported in previous communications[2-4] that the use of triphenylphosphine and 2,2′-dipyridyl disulfide (TPP–PDS) as the coupling reagents led to the formation of diesters of phosphoric acid, phosphoramidates, and pyrophosphates in good yields. This paper describes the synthesis of oligonucleotides and nucleoside cyclic phosphates by the use of this phosphorylating reagent.

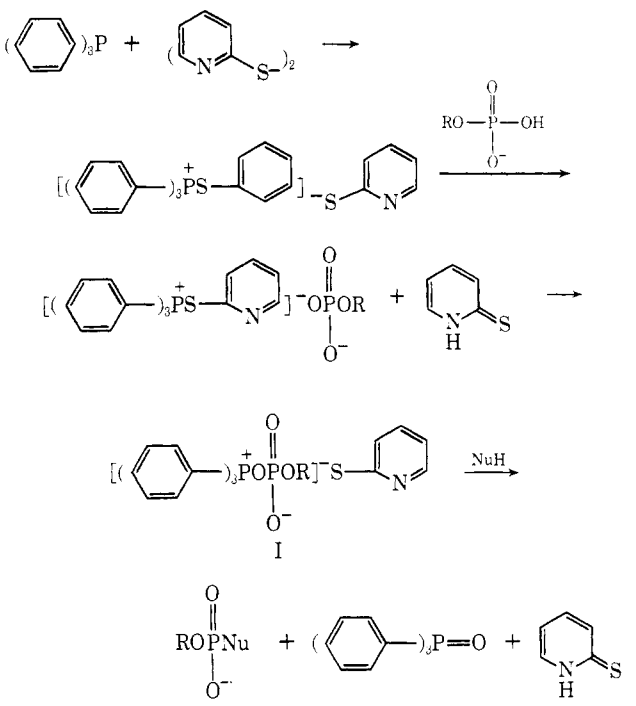
The dehydration between monoesters of phosphoric acid and nucleophilic components proceeds by eliminating one oxygen atom with triphenylphosphine (reduction) and two hydrogen atoms with 2,2′-dipyridyl disulfide (oxidation) to afford mixed esters of phosphoric acid, triphenylphosphine oxide, and 2 mol of pyrid-2-thione as seen in Scheme I.

Scheme I



In this reaction, if the phosphoroxyphosphonium salt I[5a] shown in Scheme II exists as a comparatively stable intermediate, it is possible to prepare mixed esters of phosphoric acid, phosphoramidates, or asymmetrical

Scheme II

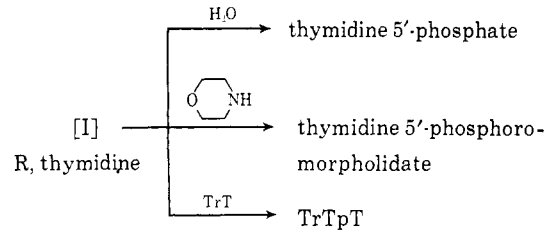


pyrophosphates by the further treatment of I with alcohols, amines, or phosphates, respectively, without the formation of by-products, such as symmetrical pyrophosphates. For example, when 3′-*O*-acetylthymidine 5′-phosphate was allowed to react with excess TPP–PDS in a small amount of pyridine,[5b] I was formed quite rapidly and could be kept as a stable salt in a highly concentrated solution as indicated by the following findings, though it was not isolated. First, symmetrical pyrophosphate was no longer produced even though the solution of I was further diluted with pyridine. Second, thymidine 5′-phosphate, a starting material, was recovered quantitatively by hydrolysis of the intermediate I at room temperature. Third, thymidine 5′-phosphoromorpholidate or 5′-*O*-tritylthymidylyl-(3′→5′)-3′-*O*-acetylthymidine ($TrTpT_{OAc}$) was produced by the addition of morpholine or 5′-*O*-tritylthymidine (TrT), respectively, to the solution of I (Scheme III).

Concerning its stability, tlc and paper electrophoresis showed no detectable decomposition after 1 day at

(1) R. Lohrman and H. G. Khorana, *J. Amer. Chem. Soc.*, **88**, 829 (1966).
(2) T. Mukaiyama and M. Hashimoto, *Bull. Chem. Soc. Jap.*, **44**, 196 (1971).
(3) T. Mukaiyama and M. Hashimoto, *ibid.*, **44**, 2284 (1971).
(4) T. Mukaiyama and M. Hashimoto, *Tetrahedron Lett.*, 2425 (1971).
(5) (a) H. Kaye and Lord Todd, *J. Chem. Soc.*, 1420 (1967). (b) When the same reaction was carried out in diluted pyridine solution, *sym*-pyrophosphate (P^1,P^2-dithymidyl pyrophosphate) was obtained as a main product.

Scheme III



room temperature. Further, after the solution of I had stood for 60 hr at room temperature, paper chromatography showed that 90% of thymidine 5′-phosphate was obtained along with 10% of symmetrical pyrophosphate after removal of the acetyl group by mild alkaline hydrolysis.

The reaction of 2′,3′-*O*-isopropylideneuridine with 2 mol equiv of β-cyanoethyl phosphate and 10 mol equiv of TPP–PDS in anhydrous pyridine afforded uridine 5′-phosphate in 90% yield after removal of the protecting groups in the usual manner. Similarly, thymidine 3′-phosphate[6] was obtained in 80% yield by treating 5′-*O*-tritylthymidine and 2 mol equiv of β-cyanoethyl phosphate with 10 mol equiv of TPP–PDS. These results indicate that the yields of nucleotides and uridine 5′- and thymidine 3′-phosphates increased remarkably in comparison with those obtained by the reaction between nucleoside and thioester[4] of phosphoric acid under similar conditions.

Next, it was established that $C_{3'-5'}$-linked deoxyribooligonucleotides were successfully synthesized by the present method. The reaction of 5′-*O*-tritylthymidine (0.6 mol) with 3′-*O*-acetylthymidine 5′-phosphate (0.5 mmol) and 5 mol equiv of TPP–PDS in 5 ml of anhydrous pyridine at room temperature for 8 hr afforded thymidylyl-(3′ → 5′)-thymidine[7] in 90% yield after removal of the protecting groups in the usual manner. Thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine[8] was obtained in 55% yield when 5′-*O*-tritylthymidylyl-(3′ → 5′)-thymidine and 2 mol equiv of 3′-*O*-acetylthymidine 5′-phosphate were allowed to react with 10 mol equiv of TPP–PDS under the same conditions. Further, TpTpTpT[9] was obtained in 50% yield from TrTpTpT and 3 mol equiv of 3′-*O*-acetylthymidine 5′-phosphate. The latter oligothymidylate, on degradation with spleen phosphodiesterase preparation, gave thymidine 3′-phosphate and thymidine in the expected ratio. A dinucleotide, 5′-*O*-phosphorylthymidylyl-(3′ → 5′)-thymidine,[10] was also obtained in 65% yield when β-cyanoethylthymidine 5′-phosphate and 3′-*O*-acetylthymidine 5′-phosphate were treated with 8 mol equiv of TPP–PDS at room temperature for 5 hr. On the other hand, in the case of β-cyanoethylthymidine 5′-phosphate[11] synthesis by the reaction with excess of β-cyanoethanol and thymidine 5′-phosphate, it was found that *sym*-pyrophosphate (P^1,P^2-dithymidylyl pyrophosphate) was always produced in about 20% yield as the by-product. For example, when thymidine 5′-phosphate (1 mmol) and β-cyanoethanol (30 mmol) were treated with 5 mol equiv of TPP–PDS in 2 ml of pyridine at room temperature, β-cyanoethylthymidine 5′-phosphate was obtained in 68% yield along with 32% yield of the *sym*-pyrophosphate (the ratio of β-cyanoethanol/pyridine = 1:1 v/v). But, the yield of the *sym*-pyrophosphate was reduced to 20% when 15 mol equiv of β-cyanoethanol and 3 ml of pyridine were used in the above experiment (β-cyanoethanol/pyridine = 1:3 v/v). The result shows that the dilution of the reaction mixture with an excess amount of β-cyanoethanol causes the velocity of the formation of I to slow down, which led to the production of the undesirable *sym*-pyrophosphate.

Next, the synthesis of nucleoside 2′,3′-[12–14] and 3′,5′-[15,16] cyclic phosphate by the oxidation–reduction condensation was studied. It was established that ribonucleoside 2′,3′-cyclic phosphates were obtained quantitatively by treating nucleoside 2′- (or 3′-) phosphates with triphenylphosphine and 2,2′-dipyridyl disulfide in hexamethylphosphorus triamide (HMPA) or methanol–water. The results are summarized in Table I.

Table I. Ribonucleoside 2′,3′-Cyclic Phosphates Syuthesis

Ribonucleoside	Solvent	Condition[a]	$\lambda_{max}^{7.0}$	$10^3\epsilon_{max}$
Adenosine	HMPA	rt, 3 hr	259	14.6
Uridine	HMPA	rt, 3 hr	262	10.0
Cytidine	HMPA	70°, 40 min	271	9.1
Guanosine	HMPA	70°, 40 min	254	12.95
Riboflavin (4′,5′)	HMPA	rt, 1 hr	264 (370, 442)	
Adenosine	CH_3OH–H_2O (1:1)	rt, 5 min	259	14.6

[a] rt = room temperature.

In the case of nucleoside 3′,5′-cyclic phosphates, undesirable pyrophosphates were formed when the reactions were carried out under the same conditions as mentioned above. However, cyclization to the nucleoside 3′,5′-cyclic phosphates was the major course when nucleoside 5′-phosphates were allowed to react with TPP–PDS under high dilution conditions at temperatures ranging from 70 to 120°. When a mixture of adenosine 5′-phosphate and 5 mol equiv of TPP–PDS was refluxed in anhydrous pyridine for 3 hr, adenosine 3′,5′-cyclic phosphate[17] was obtained in 86% yield. In a similar manner, uridine and thymidine 3′,5′-cyclic phosphates were obtained in 80 and 70% yields, respectively.

Since the solubility of the 4-morpholine *N*,*N*′-di-

(6) G. M. Tener, *J. Amer. Chem. Soc.*, **83**, 159 (1961).
(7) (a) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958); (b) G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962).
(8) (a) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3828 (1963); (b) T. M. Jacob and H. G. Khorana, *ibid.*, **86**, 1630 (1964).
(9) (a) G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962); (b) R. L. Letsinger and K. K. Ogilvie, *ibid.*, **91**, 3350 (1969).
(10) H. Kössel, M. W. Moon, and H. G. Khorana, *ibid.*, **89**, 2148 (1967).
(11) E. Ohtsuka, M. W. Moon, and H. G. Khorana, *ibid.*, **87**, 2956 (1965).
(12) A. M. Michelson, *J. Chem. Soc.*, 3655 (1959).
(13) T. Tanaka, *Yakugaku Zasshi*, **78**, 627 (1958).
(14) M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Amer. Chem. Soc.*, **80**, 6204 (1958).
(15) M. Smith, G. I. Drummond, and H. G. Khorana, *ibid.*, **83**, 698 (1961).
(16) G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *ibid.*, **80**, 6224 (1958).
(17) When HMPA was used as solvent, the formation of nucleoside 3′,5′-cyclic phosphates took place rapidly at 70° and gave the corresponding cyclic phosphates accompanied by undesirable by-product, nucleoside *N*,*N*-dimethylphosphoramidate. The phosphoramidate was produced from the reaction of nucleotide and HMPA, and the amount of the phosphoramidate increases as the reaction temperature rises or with addition of base.

cyclohexylcarboxyamidinium salts of cytidine and guanosine 5′-phosphates was increased by the addition of triphenylphosphine, cytidine 3′,5′-cyclic phosphate was successfully obtained in 56% yield from cytidine 5′-phosphate without the protection of the amino group of cytidine. Guanosine 3′,5′-cyclic phosphate was also obtained in 85% yield by the use of lyophilized powder of the 4-morpholine *N*,*N*′-dicyclohexylcarboxyamidinium salt of guanosine 5′-phosphate. Nearly the same result was obtained when *N*-benzoylguanosine 5′-phosphate, easily soluble in pyridine, was used in place of the salt of guanosine 5′-phosphate in the above experiment. All of the compounds described above were always identified with the corresponding authentic samples.

Experimental Section

General Methods and Materials. Reagent grade pyridine was distilled after treating with *p*-toluenesulfonyl choride and dried over calcium hydride for several weeks. HMPA and DMF were distilled and dried over molecular sieves (Linde molecular sieves, 4A). For condensations, solutions were rendered anhydrous by repeated evaporation of added pyridine to pyridine solutions of the compounds using the vacuum from an oil pump and a liquid nitrogen trap. Paper chromatography was performed using the descending technique on Toyo Roshi No. 51 except where noted otherwise. The solvent systems used for paper chromatography were: isopropyl alcohol–concentrated ammonia–water (7:1:2) (solvent I); *n*-butyl alcohol–acetic acid–water (5:2:3) (solvent II); ethyl alcohol–0.5 *M* ammonium acetate, pH 3.8 (5:2) (solvent III); isopropyl alcohol–concentrated ammonia–0.1 *M* boric acid (7:1:2) (solvent IV); and saturated aqueous ammonium sulfate–1 *M* sodium acetate–isopropyl alcohol (80:18:2) (solvent V). Paper electrophoresis was carried out on Toyo Roshi (No. 51A, 15 × 60 cm) impregnated with the solvents described below at 1200 V for 1.5 hr, using an apparatus similar to that described by Markham and Smith:[18] buffer I, 0.05 *M* potassium phosphate (pH 8.0); buffer II, 0.05 *M* potassium phosphate (pH 6.0). The R_f values and the paper electrophoretic mobility of different compounds are listed in Table II. The trityl-containing compounds were made visible on paper chromatograms after spraying the chromatograms with the perchloric acid spray and warming with a hot dryer.

3′-*O*-Acetylthymidine 5′-phosphate was prepared by acetylation of thymidine 5′-phosphate with acetic anhydride in pyridine. 5′-*O*-Tritylthymidine was prepared by treatment of thymidine with trityl chloride in pyridine.

2,2′-Dipyridyl disulfide was prepared by the procedure given in the literature.[19,20] Triphenylphosphine was obtained from a commercial source and purified by recrystallization.

Phosphorus-containing compounds were detected by molybdate–perchloric acid spray.

Thymidylyl-(3′ → 5′)-thymidine. To a solution of 5′-*O*-tritylthymidine (0.6 mmol) and 3′-*O*-acetylthymidine 5′-phosphate (0.5 mmol) in dry pyridine (5 ml) TPP–PDS (2.5 mmol) was added and the mixture was kept standing for 8 hr at room temperature. To this reaction medium water (2ml) was added and the aqueous pyridine mixture was kept standing at room temperature for 2 hr with stirring. After removal of the solvent *in vacuo*, the residue was dissolved in 1:1 methanol–concentrated ammonia and kept standing overnight. The solvent was removed *in vacuo*. After addition of 80% acetic acid (10 ml), the solution was heated at 100° for 10 min. Evaporation was repeated several times to remove acetic acid completely after addition of water. The residue was dissolved in water (10 ml) and the insoluble tritanol and triphenylphosphine oxide were removed by filtration. The filtrate was evaporated to a small bulk and chromatographed in solvent system I. The R_f values of the bands obtained were 0.75 (pyrid-2-thione, thymidine), 0.48 (thymidylyl-(3′ → 5′)-thymidine), and 0.16 (thymidine 5′-phosphate). After elution of the main band (R_f 0.48) with water, the yield of thymidylyl-(3′ → 5′)-thymidine was estimated to be 90% as based on the nucleotide [λ_{max} 267 mμ (ε 18,500)]. Symmetrical pyrophosphate (P^1,P^2-dithymidylyl pyrophosphate) was not produced at all.

Thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine.[21] An anhydrous mixture of pyridinium 5′-*O*-tritylthymidyl-(3′ → 5′)-thymidine (0.1 mmol) and thymidine 5′-phosphate (0.2 mmol) in dry pyridine (2 ml) was treated with TPP–PDS (1 mmol) for 8 hr at room temperature in the dark. Work-up of the reaction mixture as described above gave thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine (55%), which was estimated spectrophotometrically (solvent I) after elution of the spot (R_f 0.23) from the paper chromatograms with water [λ_{max} 267 mμ (ε 25,400].[22] Another main spot was thymidine 5′-phosphate.

Thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine. The reaction of TrTpTpT (0.1 mmol) with pyridinium 3′-*O*-acetylthymidine 5′-phosphate (0.3 mmol) and TPP–PDS (393–330 mg, 1.5 mmol) in anhydrous pyridine (1 ml) for 8 hr at room temperature afforded TrTpTpTpT after removal of the acetyl group by mild alkaline hydrolysis. The reaction mixture was applied to four sheets of Whatman 3MM chromatographic paper and chromatographed in solvent system I. The R_f values of the bands obtained were 0.75 (pyrid-2-thione), 0.40 (not investigated), 0.35 (TrTpTpTpT), and 0.16 (thymidine 5′-phosphate). The main band (R_f 0.35) was eluted with 1:1 water–methanol and the eluate was evaporated *in vacuo*. Exactly one-half of TrTpTpTpT was dissolved in 80% acetic acid (5 ml) and heated at 100° for 10 min. Acetic acid was evaporated off *in vacuo* after repeated addition of water. The product was applied to two sheets of Whatman 3MM chromatographic paper and chromatographed in solvent system I. The band (R_f 0.17) was cut out and eluted with water and spectrophotometric analysis of the eluate showed the yield of the desired tetranucleoside triphosphate to be 50% [λ_{max} 266 mμ (ε 34,000)].[23]

Enzymic Hydrolysis of TpTpTpT. To a solution of thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine (NH_4 salt, 10 mg) in 0.25 *M* sodium succinate buffer (pH 6.5, 0.4

Table II. Paper Chromatography and Paper Electrophoresis of Different Compounds

Compound	R_f's of different compounds I	II	III	Electrophoretic mobility[a] pH 6.0 (II)	pH 8.0 (I)
TrT	0.85				
TrTpT	0.70				0.28
TrTpTpT	0.50	0.41			0.51
TrTpTpTpT	0.35				0.60
T	0.66	0.65			
TpT	0.48	0.34			0.52
TpTpT	0.23	0.18			0.62
TpTpTpT	0.18	0.08			0.75
pT	0.16	0.25		1.00	1.00
C_EpT	0.55	0.43			0.56
pTpT	0.08	0.19		1.20	1.00
TppT	0.28			1.20	0.75
pTOAc					0.95
Cyclic Phosphates					
2′,3′-AMP	0.45	0.28	0.62	0.88	0.56
2′,3′-CMP	0.32				0.56
2′,3′-GMP	0.24				0.50
2′,3′-UMP	0.35				0.65
4′,5′-FMN	0.50	0.31			0.53
3′,5′-AMP	0.46	0.27	0.60	0.87	0.56
3′,5′-CMP	0.30	0.25	0.38		0.60
3′,5′-GMP	0.25	0.15	0.20		0.50
3′,5′-UMP	0.36	0.21	0.25		0.58
3′,5′-TMP	0.43				0.61

[a] The mobilities under these columns for the compounds derived from different nucleotides are relative to those of the parent nucleoside 5′-phosphate.

(18) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).
(19) R. A. Jones and A. R. Katrizky, *J. Chem. Soc.*, 3610 (1958).
(20) H. Kubota and T. Akita, *Yakugaku Zasshi*, **81**, 511 (1961).
(21) This was identical with an authentic sample which was prepared by the procedure of Khorana[8] in comparison of paper chromatography and paper electrophoresis.
(22) R. L. Letsinger, M. H. Caruthers, and D. M. Jerina, *Biochemistry*, **6**, 1379 (1967).
(23) T. M. Jacob and H. G. Khorana, *J. Amer. Chem. Soc.*, **87**, 368 (1965).

ml) spleen diesterase solution (0.1 ml) was added. The mixture was incubated at 37° for 5 hr. The mixed solution of uranyl acetate and perchloric acid was added to the mixture. The total mixture was evaporated in small bulk and applied to a sheet of Whatman 3MM chromatographic paper. Development with solvent system I gave two bands: a nucleoside, thymidine (R_f 0.66), a nucleotide, thymidylic (3′) acid (R_f 0.15). The bands were eluted with water and spectrophotometric analysis at 267 mμ gave the value 3 for the molar ratio of thymidylic (3′) acid to thymidine.

5′-*O*-Phosphorylthymidylyl-(3′ → 5′)-thymidine. The reaction of pyridinium 3′-*O*-acetylthymidine 5′-phosphate (0.5 mmol) with pyridinium β-cyanoethylthymidine 5′-phosphate (0.5 mmol) and TPP–PDS (1.048–0.880 g, 4 mmol) in anhydrous pyridine (5 ml) for 5 hr at room temperature afforded C_EpTpT_{OAc}. After removal of the protecting groups in the usual manner, the reaction mixture was applied to four sheets of Whatman 3MM chromatographic paper and chromatographed in solvent system I. The R_f values of the bands obtained were 0.16 (thymidine 5′-phosphate), 0.08 (pTpT), and 0.03 (unidentified 5%). After elution of the main band (R_f 0.08) with water, spectrophotometric analysis of the eluate showed the yield of the desired pTpT to be 65% as based on thymidine 5′-phosphate [λ_{max} 266 mμ (ε 19,200)]. Thymidine 5′-phosphate was recovered in 30% yield.

Thymidine 3′-Phosphate. The reaction of 5′-*O*-tritylthymidine (1 mmol) with β-cyanoethyl phosphate (2 mmol) and TPP–PDS (10 mmol) in pyridine (10 ml) for 1 day at room temperature afforded thymidine 3′-phosphate after removal of the protecting groups in the usual manner and it was isolated as the barium salt after successive treatment with barium hydroxide. The product was dried by washing with ethanol, followed by acetone, and then ether and finally by removing traces of solvent *in vacuo;* the yield of vacuum-dried material was 358 mg which analyzed as the trihydrate (70% yield) [λ_{max} 267 mμ (ε 9600)]. Uridine 5′-phosphate was obtained in 90% yield by the reaction of 2′,3′-*O*-isopropylideneuridine (1 mmol) with β-cyanoethyl phosphate (2 mmol) and TPP–PDS (10 mmol) in pyridine (10 ml) for 1 day at room temperature after removal of the protecting groups in the usual manner [λ_{max} 260 mμ (ε 10,000)], R_f 0.15 (solvent I).

β-Cyanoethylthymidine 5′-Phosphate. A mixture of pyridinium thymidine 5′-phosphate (2 mmol), hydroacrylonitrile (2.5 ml, 36 mmol), pyridine (7.5 ml), and TPP–PDS (2.62–2.20 g, 10 mmol) was allowed to stand at room temperature for 6 hr. Paper electrophoresis at this stage showed disappearance of thymidylic acid. Water (20 ml) was added and the solvent was evaporated *in vacuo.* The residue was dissolved in water (40 ml) and the insoluble triphenylphosphine oxide was filtered off. The filtrate and washings were concentrated to 5 ml and this solution was applied on top of a DEAE-cellulose (carbonate) column (60 × 3 cm). Elution was carried out at 4° using a linear gradient of triethylammonium bicarbonate (2 l. of water in the mixing vessel and 2 l. of 0.1 *M* triethylammonium bicarbonate in the reservoir), fractions of about 20 ml being collected every 15 min. Fractions from 31 to 52 contained the desired product. The combined eluate was treated with Dowex 50 pyridinium resin and concentrated by evaporation in the presence of pyridine. The desired β-cyanoethylthymidine 5′-phosphate was obtained in 70% yield as based on the nucleotide [λ_{max} 266 mμ (ε 9600)]. The product was homogeneous by paper chromatography (solvent I, R_f 0.55) as well as on paper electrophoresis. After hydrolysis in alkali, it gave a single product identical with thymidine 5′-phosphate.

Cyclic Phosphates. Ribonucleoside 2′,3′-Cyclic Phosphates. Adenosine 2′,3′-Cyclic Phosphate. The suspension of adenosine 2′-(3′-) phosphate (0.1 mmol) (triethylammonium salt) with 3 mol equiv of TPP–PDS in HMPA (1 ml) was stirred at room temperature. After 3 hr, the solution became clear and at this stage paper electrophoresis showed only one spot. Then the solution was evaporated to dryness *in vacuo.* The residue was suspended in a mixture of water (100 ml) and dichloromethane (100 ml). The aqueous layer was concentrated to a small volume and applied to a sheet of Whatman 3MM chromatographic paper and chromatographed with solvent system I. After elution of the band (R_f 0.45) with water, spectrophotometric analysis of the eluate showed the yield of the 2′,3′-cyclic phosphate to be quantitative.

In cases where 1:1 water–methanol was used in place of HMPA, the cyclization reaction was completed within only 5 min. Uridine, cytidine, guanosine (2′,3′-), and riboflavin (4′,5′-) cyclic phosphates were obtained quantitatively under the same conditions as mentioned above.

Adenosine 3′,5′-Cyclic Phosphate. Adenosine 5′-phosphate (1.0 mmol of the free acid) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine (293 mg, 1.0 mmol) were dissolved in pyridine (25 ml) containing water (5 ml) and the solution was evaporated to dryness. After removal of water by coevaporation with pyridine, the nucleotide salt was dissolved in boiling pyridine (150 ml). The solution of TPP–PDS (5 mmol) in pyridine (50 ml) was added rapidly to the boiling solution. The reaction mixture was heated under reflux for 3 hr and then water (250 ml) was added. After the mixture had been kept standing for 2 hr at room temperature, it was evaporated to dryness. The resulting product was dissolved in a mixture of water (150 ml) and dichloromethane (150 ml). The aqueous layer was concentrated to a small volume and applied to four sheets of Toyo Roshi No. 51 chromatographic paper and chromatographed with solvent system I. After elution of the band (R_f 0.46) with water, the solution was evaporated to dryness. The resulting light yellow semisolid product was dissolved in 50% ethanol (4 ml); 1 *N* hydrochloric acid was added to bring the solution to pH 2.0. After cooling to −15°, the solid was collected, washed with ethyl alcohol, and dried *in vacuo* to yield cyclic phosphate as the monohydrate (296 mg, 85%): ultraviolet adsorption 258 mμ (ε 14,650) at pH 7.0; ratio of adenosine (determined spectrophotometrically) to phosphate, 1:1.1 (required 1:1).

On treatment with 1 *N* hydrochloric acid at 100° for 1 hr, adenosine 3′,5′-cyclic phosphate yields adenine (74%), adenosine 3′,5′-cyclic phosphate (21%), and adenosine monophosphate (5%).

In alkaline hydrolysis (0.1 *M* barium hydroxide at 100° for 40 min) adenosine 3′,5′-cyclic phosphate yields two products, adenosine 3′-phosphate (81%) and adenosine 5′-phosphate (19%). The hydrolysis products were characterized by paper chromatography in solvents I, IV, and V and estimated spectrophotometrically.

Adenosine 3′,5′-Cyclic Phosphate from P^1,P^2-Diadenosine Pyrophosphate. Adenosine 5′-phosphate (0.25 mmol) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine (74 mg, 0.25 mmol) were dissolved in pyridine (50 ml) under anhydrous conditions at 100°; TPP–PDS (131–110 mg, 0.5 mmol) was added and the solution was kept at 100° for 20 min. The solution was cooled to 0°, whereupon it set to a gel. Chromatographic examination of the product in solvent I showed P^1,P^2-diadenosine 5′-pyrophosphate to be the only ultraviolet absorbing product. The gel was liquefied by warming and the resultant viscous solution was run dropwise into a solution of TPP–PDS (262–220 mg, 1 mmol) in pyridine (25 ml) under reflux over a period of 1 hr and continued heating under reflux for an additional 1 hr. Paper chromatography and paper electrophoresis showed adenosine 3′,5′-cyclic phosphate to be the main product with a trace amount of by-product.

Thymidine 3′,5′-Cyclic Phosphate. Thymidine 5′-phosphate (0.5 mmol, pyridinium salt) was dissolved in anhydrous pyridine (30 ml) and TPP–PDS (655–550 mg, 2.5 mmol) was added to the solution. The resulting solution was stirred for 30 min at room temperature and then heated at 100° for 2 hr. After the mixture was concentrated to small amounts it was applied to Toyo Roshi No. 51 chromatographic paper and chromatographed with solvent system I. The R_f values of the bands obtained were 0.15 (thymidine 5′-phosphate, 18%), 0.29 (P^1,P^2-dithymidylyl pyrophosphate, 12%), and 0.43 (thymidine 3′-,5′-cyclic phosphate, 70%); λ_{max} 265 mμ (ε 9600) at pH 7.0.

Uridine 3′,5′-Cyclic Phosphate. The procedure used in the preparation of adenosine 3′,5′-cyclic phosphate was used. Chromatographic examination of the product in solvent system I showed uridine 3′,5′-cyclic phosphate (R_f 0.35) 80% [λ_{max} 260 mμ (ε 10,000)] at pH 7.0 and P^1,P^2-diuridine pyrophosphate (R_f 0.17) 20%.

Cytidine 3′,5′-Cyclic Phosphate. The procedure used in the preparation of adenosine 3′,5′-cyclic phosphate was used except that solvent volume was doubled and the reaction time lengthened to 5 hr. After separation of the product using chromatographic paper (Toyo Roshi No. 51, solvent system I), spectrophotometric analysis of the eluate showed the yield of cytidine 3′,5′-cyclic phosphate to be 56% [λ_{max} 272 mμ (ε 9340)] at pH 7.0. Undissolved cytidine 5′-phosphate was recovered in 40% yield.

Guanosine 3′,5′-Cyclic Phosphate. The lyophilized powder of the 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium salt of guanosine 5′-phosphate (0.5 mmol) was suspended in pyridine (200 ml) and TPP–PDS (1.31–1.10 g, 5 mmol) was added to the solution. The resulting solution was heated under reflux for 5 hr and then the solution was evaporated to dryness. The resulting products were dissolved in a mixture of water (150 ml) and dichloromethane (150 ml). After the aqueous layer was concentrated to a small volume, separation of the product was done with the same method as described above. The spectrophotometric analysis showed the yield of the desired guanosine 3′,5′-cyclic phosphate

Table III. Formation of Adenosine 5′-*N*,*N*-Dimethylphosphoroamidate

Base	Conditions Temp, °C	Time, hr	AMP, %	Amidate, %	
	rt	24	91.5	8.5	
	100	1	85.5	6.2	8.3 not identified
	150	1	12.5	25.0	18.7 adenine 43.8 ADP ?
Pyridine	rt	24	76.0	10.0	14.0 not identified
Triethylamine	rt	24	71.0	29.0	
4-Morpholine[a]	rt	24	70.0	30.0	

[a] 4-Morpholine *N*,*N*′-dicyclohexylcarboxamidine.

to be 85% [λ_{max} 254 mμ (ε 12,950)] at pH 7.0. Another product was guanosine 5′-phosphate (15%).

At early stages of this reaction (1.5 hr), paper electrophoresis showed three spots: guanosine 5′-phosphate (25%), P^1,P^2-diguanosine pyrophosphate (36%), and guanosine 3′,5′-cyclic phosphate (38%).

In cases where *N*-benzoylguanosine 5′-phosphate was used in place of guanosine 5′-phosphate, the procedure used in the preparation of adenosine 3′,5′-cyclic phosphate was applied. After the *N*-benzoyl group was removed by treatment with 95% ethyl alcohol and concentrated ammonia (1:2 v/v) and the liquid heated in a sealed tube at 100° for 2 hr, guanosine 3′,5′-cyclic phosphate was obtained in 85% yield.

Adenosine 5′-*N*,*N*-Dimethylphosphoroamidate. The amidate was obtained by treating adenosine 5′-phosphate with excess HMPA at temperatures ranging from 20 to 150°. The amount of amidate increases as the reaction temperature rises or with addition of bases. The results are summarized in Table III. This amidate was stable in neutral solution but in acidic medium (pH 1) it decomposed to adenosine 5′-phosphate (about 70% in 1 day): λ_{max} 258 mμ (ε 15,400); R_f 0.66 (solvent I), electrophoretic mobility relative to AMP 0.40 (buffer I).

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Solvolysis of Adenine Nucleosides. I. Effects of Sugars and Adenine Substituents on Acid Solvolyses

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Abstract: The acidic solvolyses of 2′,3′-dideoxy-2′,3′-didehydroadenosine > 2′-deoxyadenosine > 9-β-D-psicofuranosyladenine ≫ 3′-deoxyadenosine > 8-bromoadenosine > adenine arabinoside ~ 2-chloroadenosine ~ N^6-methyladenosine ≳ adenosine ~ 2-methyladenosine > 1-methyladenosine ~ N^6-dimethyladenosine ≳ adenine xyloside > 8-methoxyadenosine ~ 2′*C*-methyladenosine result in the respective sugar and stable adenine moiety except in the case of 1-methyladenosine where the resultant 1-methyladenine is more slowly transformed into 5-aminoimidazole-4-*N*′-methylcarboxamidine. The typical ranking of relative activities are given above for 80° in 0.10 *M* HCl. Studies have been conducted at various acid and buffer concentrations, and at various temperatures for many of these compounds. The facts that only specific acid catalyzed solvolyses of the protonated and nonprotonated species were observed and that there was no maximum in solvolysis rate in the low pH region supported the argument against a Schiff base intermediate subsequent to ethereal oxygen attack. The probability of an A-1 mechanism for solvolyses of diprotonated adenine nucleosides with protons on the nitrogens in the 1 and 7 positions is favored by the fact that the entropies of activation, $\Delta S^{\pm}$, are close to zero. Although the inductive effect of the 2′ hydroxyl on the sugar moiety of adenosine inhibits acid solvolysis, a less significant increase in reactivity is introduced by the substitution of a hydrogen for the 3′ hydroxyl. The effects of substituents on the pyrimidine ring of the adenine moiety lead to only minor effects in reactivity whereas substitution of a bromine or methoxyl group on C-8 of the imidazole portion has a more pronounced effect consistent with the appropriate order for inductive effects aiding protonation. This and other evidence is consistent with the presumption that hydrogen ion attack of the protonated purine nucleoside to form a solvolyzing dication by an A-1 mechanism is on the imidazole moiety and most probably at the 7 position rather than on the ethereal oxygen. The fact that the 1-methyladenosine cation solvolyses in acid at about the same rate as the adenosine cation strongly suggests that it is the 1-protonated form of the latter that reacts with a second proton to result in a solvolyzing dication.

A mechanistic explanation of the acid solvolysis of the nucleosides to a base and a sugar has been proposed based on the analogous solvolysis of the simpler glycosylamines.[1] The essential steps were considered to be the protonation of the sugar ring oxygen, sugar ring opening, followed by water attack on the Schiff base to yield the heterocyclic base and sugar.[2-4] If the heterocyclic base could be protonated elsewhere, the ease of proton transfer to the sugar ring oxygen was considered to be an important factor. However, the validity of this mechanism for nucleoside solvolysis, based on the analogy to the acid solvolysis of the simple glycosylamines, has been seriously questioned.[2,5,6]

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