Synthesis of 6′-Cyano-6′-deoxyhomoadenosine-6′-phosphonic Acid and Its Phosphoryl and Pyrophosphoryl Anhydrides and Studies of Their Interactions with Adenine Nucleotide Utilizing Enzymes[1]

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Abstract: Three adenine nucleotide analogs have been synthesized which have a $CH_2CH(CN)P$ grouping in place of the CH_2OP grouping in adenosine 5′-phosphate, 5′-diphosphate, and 5′-triphosphate (AMP, ADP, ATP), respectively. The synthesis involved conversion of 5′-*O*-tosyl-2′,3′-*O*-isopropylideneadenosine to an *N*,*N*-dibenzoyl derivative from which the corresponding 5′-iodo-5′-deoxy derivative was prepared. Condensation of the latter with diethyl cyanomethylphosphonate gave the diethyl ester of the 2′,3′-*O*-isopropylidene derivative of the desired analog of AMP as a mixture of mono- and di-*N*-benzoyl derivatives. Treatment of these in benzene solution with dry hydrogen bromide removed the two ethyl groups, and subsequent acidic and basic treatments removed the remaining blocking groups. The crystalline 6′-cyano-6′-deoxyhomoadenosine-6′-phosphonic acid so obtained was converted to the corresponding analogs of ADP and ATP by an established anion-exchange method for the conversion of nucleoside 5′-phosphates to phosphoanhydride derivatives. The known 6′-deoxyhomoadenosine-6′-phosphonic acid and its 6′-cyano derivative herein described were substrates of rabbit and pig AMP kinases (V_{max} 2 and 0.04%, respectively, that of AMP) and of rabbit AMP aminohydrolase (V_{max} 20 and 3%, respectively); they were competitive inhibitors of rabbit AMP kinase with enzyme–inhibitor dissociation constants (K_i) of 440 and 320 μM and of rabbit AMP aminohydrolase with K_i values of 58 and 19 μM, respectively. The 6′-cyano analogs of AMP, ADP, and ATP were all mixtures of approximately equal parts of the two possible 6′ epimers. With the pig and rabbit AMP kinases only one 6′ epimer of the AMP analog was a phosphoryl acceptor and only one epimer of the ATP analog was a phosphoryl donor; the donor and acceptor epimers were of opposite C-6′ configuration. One epimer of the ADP analog was a substrate of pyruvate kinase. The substrate and inhibitor properties of these nucleotide analogs show that the enzyme–adenine nucleotide complexes of the systems examined have room in the vicinity of the nucleotide O-5′ to accommodate a cyano group.

Adenosine 5′-phosphate (AMP) is the sole metabolic precursor of the AMP residue in adenosine 5′-triphosphate (ATP) and other adenine nucleotide coenzymes, and thus it indirectly plays an essential role in most areas of cell metabolism and is indispensable for cell multiplication. AMP and ATP are additionally important in metabolism by virtue of their involvement in allosteric regulation of enzyme activity.[4] In most, if not all, instances where AMP or ATP act as enzymic substrates or regulators, important interactions occur between the enzymes and the respective mono- or triphosphoryl moieties of these nucleotides. Such an interaction undoubtedly occurs when ATP is a substrate because the catalyzed reactions involve bond-breaking in the tripolyphosphoryl group of ATP; that it occurs also during enzyme regulation by AMP or ATP is evidenced by the dependence of the regulatory effect upon the presence of the correct number of nucleotide phosphoryl groups.[4] Likewise, the phosphoryl group of AMP can be presumed to interact with AMP kinase and 5′-nucleotidase because it participates in the reactions catalyzed by those enzymes; in other cases, such as with AMP aminohydrolase and adenylosuccinate lyase, interaction between the enzymes and the phosphoester portion of AMP is suggested by the almost total inability of these enzymes to catalyze transformation of the corresponding alcohol, adenosine.[5,6] That the phosphoester portions of other mononucleotides interact with enzymes[7] is indicated by evidence that inosine is neither a substrate nor a competitive inhibitor of inosine-5′-phosphate dehydrogenase[8] or of adenylosuccinate synthetase,[8] that guanine is neither substrate nor inhibitor of guanosine-5′-phosphate reductase,[9] and that xanthosine is not a substrate of xanthosine-5′-phosphate aminase.[10]

One promising approach to the study of the nature of the interactions between enzymes and phosphoryl groups of adenine nucleotides would be the use of adenine nucleotide analogs capable of binding selec-

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(2) This work was undertaken during the tenure of a Research Training Fellowship awarded by the International Agency for Research on Cancer.

(3) Cancer Research Unit, National Cancer Institute (Canada), Edmonton, Alberta, Canada, where preliminary aspects of the work were carried out.

(4) (a) D. E. Atkinson, *Annu. Rev. Biochem.*, **35**, 85 (1966); (b) E. R. Stadtman, *Advan. Enzymol.*, **28**, 41 (1966); (c) H. M. Kalckar, *et al.*, Ed., "The Role of Nucleotides for the Function and Conformation of Enzymes," Academic Press, New York, N. Y., 1969.

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(7) It might be held that the phosphoester moiety of a mononucleotide may not necessarily interact with the enzyme but instead could exert its effect by conferring on the nucleotide a conformation recognized by the enzyme. This possibility is weakened by evidence that the conformation of enzyme-bound adenosine resembles that of enzyme-bound AMP: A. Hampton, P. J. Harper, and T. Sasaki, *Biochemistry*, **11**, 4736, 4965 (1972).

(8) A. W. Nichol, A. Nomura, and A. Hampton, *Biochemistry*, **6**, 1008 (1967).

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tively to adenine nucleotide sites and thereafter bonding covalently to an amino acid residue in or near the phosphoryl interaction site. Earlier work concerned with the design of such reagents showed that simple replacement of an ionizable phosphate hydroxyl of inosine 5′-phosphate (IMP) by hydrogen apparently prevented binding of the molecule to the IMP site as evidenced by lack of substrate activity and lack of competitive inhibitory properties.[8] In addition, the more subtle replacement of a phosphate hydroxyl by sulfhydryl reduced affinity for the IMP site of IMP dehydrogenase[11] and of adenylosuccinate synthetase[12] as indicated by the dissociation constants of the resultant IMP analog. Since these findings indicated that modification of the phosphoryl group was undesirable in reagents of the type sought, an analog was synthesized which differed from AMP only by replacement of $C(O)OPO_3H_2$ for the $CH_2OPO_3H_2$ residue of AMP,[13] and this compound, though hydrolytically quite unstable, appears to react covalently at the AMP sites of some of the AMP utilizing enzymes studied. An approach to more stable and structurally less limited types of reagents was suggested by the enzymatic properties of 6′-deoxy-6′-homoadenosine-6′-phosphonic acid,[14] an isostere of AMP in which the $CH_2OPO_3H_2$ grouping of AMP is replaced by $CH_2CH_2PO_3H_2$. This is a substrate of AMP aminohydrolase[12] and AMP kinase (as reported herein) and, moreover, binds strongly to adenylosuccinate synthetase[12] and to AMP hydrolase (this communication); in addition, the corresponding isostere of IMP is a substrate of adenylosuccinate synthetase.[12] Considerable work with methylene isosteres of nucleoside polyphosphates has shown that substitution of the methylene group for a phosphoanhydride oxygen is compatible with maintenance of capacity to bind to the enzymic nucleotide site; for example, such isosteres are able to effectively inhibit polynucleotide phosphorylase[15] and protein synthesis,[16] to effect regulation of AMP aminohydrolase,[17] and to bind to the ATP site of phosphoenolpyruvate synthetase.[18] These findings suggested that 6′-substituted adenine nucleotide analogs containing a $CH_2CH(R)PO_3H_2$ system in place of the normal $CH_2OPO_3H_2$ system might be able to bond, through reactive 6′-substituents, to amino acid residues near phosphoryl interaction sites. To assess whether enzyme–adenine nucleotide complexes contain sufficient space to accommodate such 6′ substituents, we have studied methods for the synthesis of derivatives of 6′-deoxyhomoadenosine-6′-phosphonic acid bearing on C-6′ small substituents which could subsequently be converted, if required, into larger groups by methods expected to leave unaltered the remainder of the molecule. The present communication describes the synthesis of the 6′-cyano derivative, XIV, of 6′-deoxyhomoadenosine-6′-phosphonic acid and its conversion to the corresponding 6′-cyano-substituted ADP and ATP analogs XVIII and XIX. Substrate and inhibitor properties of these compounds with some AMP, ADP, and ATP utilizing enzymes are presented.

At the outset of the work the only reported nucleotide derivatives isosteric with normal nucleotides by virtue of substitution of $C–CH_2–P$ for the nucleotide C–O–P system were 6′-deoxyhomouridine and 6′-deoxyhomoadenosine-6′-phosphonic acids. These had been synthesized by conversion of the respective 2′,3′-*O*-isopropylidene nucleoside 5′-aldehydes into α,β-unsaturated nucleoside phosphonates with a Wittig reagent followed by reduction of the 5′,6′ double bond and removal of blocking groups.[14] The ethyl ester of 6′-deoxyhomoadenosine-6′-phosphonic acid had been prepared by condensation of a protected ribofuranose 5-aldehyde with tetraethylmethylene diphosphonate and attachment of the adenine moiety by subsequent reactions.[19] Clearly, neither of these routes is directly applicable to the preparation of 6′-substituted 6′-deoxyhomoadenosine-6′-phosphonates. In view of the readiness with which the methylene carbon of trialkylphosphonoacetates, $(RO)_2P(O)CH_2CO_2R$,can undergo alkylation,[20] the synthesis of 6′-substituted 6′-deoxyhomoadenosine-6′-phosphonates was approached instead through a study of reactions between various readily accessible adenosine derivatives bearing good leaving groups on C-5′ and phosphonate esters possessing an activated methylene adjacent to phosphorus. In the initial studies it was advantageous to utilize methyl 2,3-*O*-isopropylidene-5-deoxy-5-iodo-β-D-ribofuranoside[21] (I) as a structurally simplified model for the proposed types of adenosine reactants in the above scheme. The desired reaction of I with the sodio derivative of triethyl phosphonoacetate (IIa) did occur, but yields of the α-carboethoxy phosphonate IIIa did not exceed 19% under a wide variety of reaction conditions examined. However, when the carboethoxy group of IIa was replaced by the less bulky nitrile group, reaction between the resulting cyanomethylphosphonate IIb and I in dimethyl sulfoxide solution at 60° gave as much as 60% yield of the α-cyanophosphonate IIIb. The feasibility of extending the reaction from methyl ribofuranosides to adenine nucleosides was examined, initially, by converting 2′,3′-*O*-isopropylideneadenosine to its 5′-*O*-(*p*-nitrobenzenesulfonyl) derivative, because the corresponding derivatives of inosine[22] and methyl ribofuranoside[23] are highly reactive in displacement reactions at C-5′ and C-5, respectively. However, yields of the 5′-*O*-(*p*-nitrobenzenesulfonyl) derivative were low, and the compound was too unstable to serve as an intermediate since at room temperature it rapidly underwent quaternization at N-3 to give 2′,3′-*O*-isopropylidene-3,5′-cycloadenosine *p*-nitrobenzenesulfonate. Treatment of the significantly more stable 5′-

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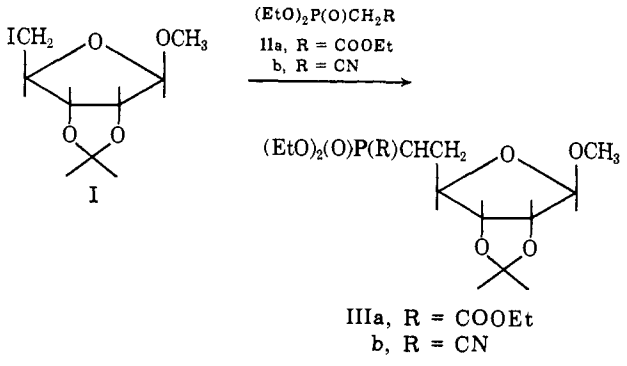
iodo-5′-deoxy-2′,3′-*O*-isopropylideneadenosine[24] with sodio diethyl cyanomethylphosphonate (IIb) under the conditions of the successful model reactions yielded almost exclusively the iodide of the same 3,5′-cycloadenosine. To enhance the stability of the nucleoside intermediate, 5′-*O*-tosyl-2′,3′-*O*-isopropylideneadenosine (IV)[25] was converted to its *N*,*N*-dibenzoyl[26] derivative V, because acylation of the adenine ring of 5′-halogeno-5′-deoxyadenosines hinders 3,5′-cyclization,[27] presumably by reduction in electron density at N-3, and because *N*-benzoyl groups on adenine residues tend to be more stable than acetyl or formyl groups.[28] Reaction of V with IIb required a higher temperature (90°) than reaction of the riboside I with IIb and produced predominantly the *N*-benzoyl 3,5′-cycloadenosine derivative, X. The *N*,*N*-dibenzoyl 5′-iodo-5′-deoxyadenosine derivative VI was obtained in 60% yield by treatment of V with sodium iodide. When VI was treated with the cyanomethylphosphonate IIb under the conditions established with the riboside I, diethyl *N*,*N*-dibenzoyl-6′-cyano-6′-deoxy-2′,3′-*O*-isopropylidenehomoadenosine-6′-phosphonate (VII)[29] and its *N*-monobenzoyl analog (VIII) were obtained as major components of the mixture together with lesser amounts of *N*-benzoyl-5′-iodo-5′-deoxy-2′,3′-*O*-isopropylideneadenosine (IX) and *N*-benzoyl-2′,3′-*O*-isopropylidene-3,5′-cycloadenosine (X) (Scheme I). The structure of compound X was corroborated by its identity with the product obtained by benzoylation of 2′,3′-*O*-isopropylidene-3,5′-cycloadenosine. When equimolar quantities of IIb and VI were employed in the reaction, VII and VIII were obtained in homogeneous form in 28 and 7% yields, respectively, whereas when IIb was present in tenfold molar excess over VI the yields of VII and VIII were 7 and 45%, respectively, and that of X was 11%.



IIIa, R = COOEt
b, R = CN

Treatment of VII or VIII with ethanolic ammonia produced the same debenzoylated product, XI, the structure of which was assigned from its elemental analysis and ultraviolet, infrared, and nmr spectra. That XI was a mixture of 6′ epimers was shown by the presence in the nmr spectrum of two signals each from the C-2 proton and the C-8 proton. The chemical shifts in both cases differed by 0.04–0.05 ppm and determination of the areas under the peaks showed that the ratio of epimers was 7:3. Treatment of compound XI with ammonium hydroxide readily cleaved one ethyl group, and subsequent acidic treatment removed the isopropylidene group in good yield to give monoethyl 6′-cyano-6′-deoxyhomoadenosine-6′-phosphonate (XIII). Compound XIII was most conveniently obtained directly from XI by simultaneous removal of the isopropylidene and one ethyl group with acetic acid. As in the case of other monoalkyl phosphonates,[30-32] the ethyl group of XIII was totally resistant to removal by further acidic or basic treatments. However, upon prolonged incubation with large amounts of snake venom phosphodiesterase XIII yielded the free phosphonic acid XIV. The poor substrate activity of XIII with this enzyme may be partly attributed to the presence in XIII of a C–CH_2–P system which in the case of the phenyl ester of UMP causes a fivefold decrease in reaction velocity;[14] in addition, the feeble substrate activity of XIII may be associated with α substitution on an alkyl phosphonate, since 2-hydroxyethyl 2′,3′-*O*-isopropylidene-5′-deoxyuridine-5′-phosphonate is also resistant to hydrolysis by venom phosphodiesterase.[32]

Enzymatic removal of the second ethyl group by a variety of commercially available phosphodiesterases was examined but proved to be impractical for preparative purposes, and further chemical approaches to the problem of obtaining the totally deblocked homonucleotide XIV were therefore studied. Facile transesterification of homonucleotide diphenyl esters has been achieved with sodium benzyloxide in dimethyl sulfoxide,[14] but the diethyl ester VIII was unaffected by such treatment. When VIII was treated with sodium benzyloxide in benzyl alcohol none of the desired dibenzyl ester was obtained and a phosphorus-free product of incompletely resolved structure resulted which from its uv, ir, and nmr spectra, and its chemical properties was found to contain a cyano group, a benzyl group, and a 2′,3′-*O*-isopropylidenehomoadenosine residue. Dialkyl esters of phosphonic acids have been successfully deesterified by treatment with trimethylchlorosilane and hydrolysis of the resulting silyl phosphonates,[33] but the diethyl esters VIII and XI were essentially unchanged by that procedure. However, when VII or VIII was treated in benzene solution with gaseous hydrogen bromide, as employed for the deesterification of diethyl 2′,3′-*O*-isopropylidene-5′-deoxyuridine-5′-phosphonate,[34] the ethyl groups and one of the two benzoyl groups were removed and partial removal of the isopropylidene group occurred. Removal of the isopropylidene group was completed by an aqueous acidic treatment to give a 42% yield of the *N*-benzoyl free phosphonic acid XVI and a 13% yield of its monoethyl ester XV which were identified by their electrophoretic mobility and ultra-

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(29) Systematic names for the compounds synthesized in this work are given in the Experimental Section.
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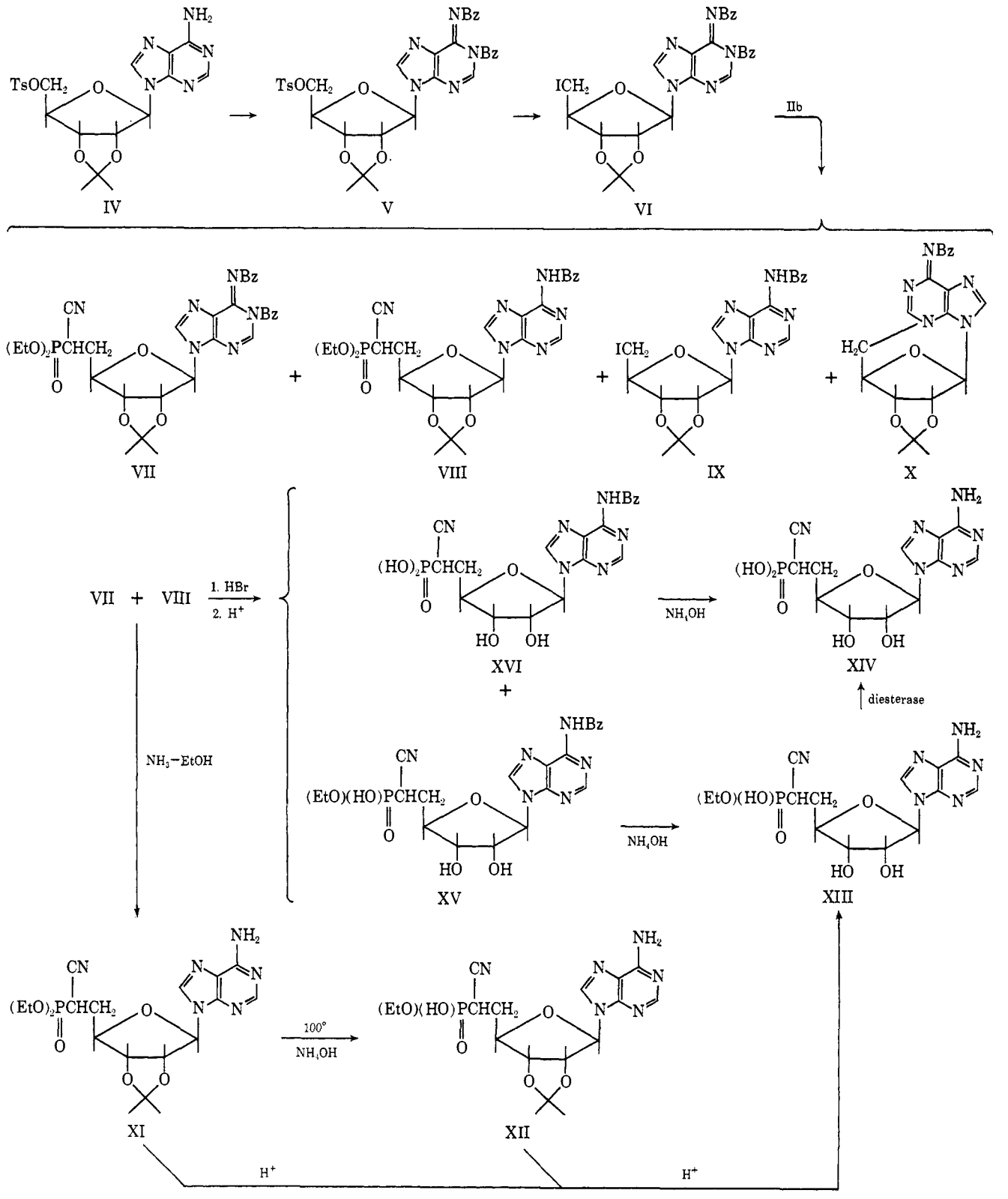
Scheme I



violet spectra. When the products resulting from the successive hydrogen bromide and aqueous acidic treatments were debenzoylated with ammonium hydroxide the principal products were 6′-cyano-6′-deoxyhomoadenosine-6′-phosphonic acid (XIV), adenine, and the monoethyl ester XIII, the latter being identical with the compound obtained by acidic treatment of either XI or XII.

The free phosphonic acid XIV was obtained in crystalline form following purification by charcoal adsorption and anion-exchange chromatography. The overall yield of homogeneous XIV from VII or VIII was 35% and the product was indistinguishable from that obtained by phosphodiesterase treatment of XIII (Scheme I). As expected, the ultraviolet spectral properties of XIV were the same as those of adenosine 5′-phosphate (AMP) and the infrared and proton magnetic resonance spectra were entirely consistent with the

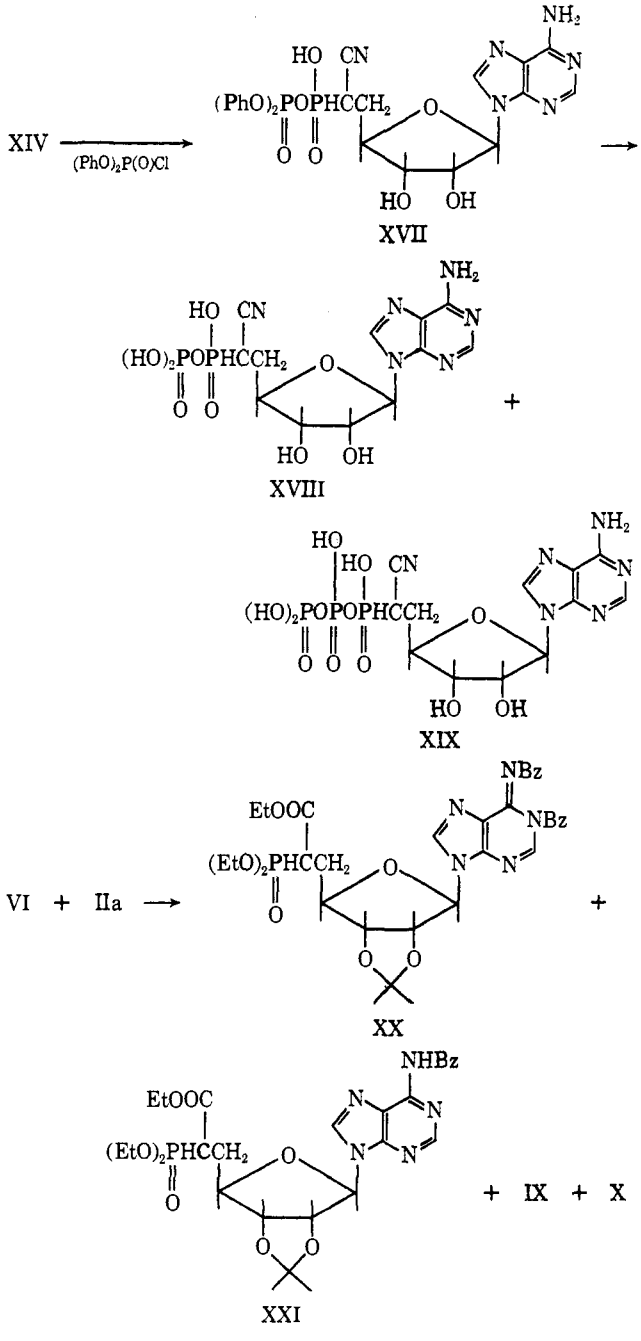
structure expected from the mode of synthesis. The similar behavior of XIV and AMP on ion exchange and paper chromatograms and on paper electrophoretograms is shown in Table I.

Table I. Paper Chromatography and Electrophoresis

Compd	Electrophoretic mobility[a] pH 7.5	pH 4.0	R_f values in system H	J	K	M
AMP	1.0	1.0	0.54	0.82	0.14	
XIV	1.0	1.1	0.50	0.80	0.14	0.52
ADP	1.2	1.7	0.34	0.70		
XVIII	1.2	1.7	0.28	0.69	0.09	0.34
ATP	1.6	1.9	0.04	0.32		
XIX	1.6	1.8	0.04	0.33	0.06	0.25

[a] Values are relative to those of AMP.

Scheme II



The anion-exchange method of Michelson[35] for the conversion of 5′-nucleotides to phosphoanhydride derivatives was successfully utilized for the conversion of the AMP isostere XIV to XIX, the corresponding 6′-substituted isostere of ATP. Thus the tri-*n*-butylammonium salt of XIV was treated with diphenyl phosphorochloridate and the resultant diphenyl phosphonylphosphate, XVII, was reacted *in situ* with tributylammonium pyrophosphate. The products were separated by anion-exchange chromatography on diethylaminoethyl-cellulose in the bicarbonate form from which the sodium salt of XIX was obtained in 52% yield; in addition, a small amount of the phosphorylphosphonate XVIII was obtained, in analogy to the formation of small amounts of ADP during the conversion of AMP to ATP by this procedure[25] (Scheme II). Compounds XVIII and XIX possessed the same ultraviolet spectra as ADP and ATP, and after further purification by paper chromatography were homogeneous on paper chromatograms and thin layer anion-exchange chromatograms in a number of solvents and on electrophoresis at pH 4 and 7.5 (Table I) with properties closely similar to those of ADP and ATP, respectively.

In view of the successful condensation of the blocked 5′-iodo-5′-deoxynucleoside VI with diethyl cyanomethylphosphonate it was of interest to examine the reaction of VI with triethyl phosphonoacetate (IIa). A number of trials were made using conditions similar to those established in the model reactions with the 5-iodo-5-deoxyriboside I, but in the best instance the yield of the desired *N*,*N*-dibenzoyl nucleoside (XX) was only 6% and of its N-monobenzoyl counterpart (XXI) only 2%, the principal components of the mixture being *N*-benzoyl-3,5′-cycloadenosine (X; 25%), monodebenzoylated VI (IX; 19%), and VI itself (28% recovery).

Studies with Adenine Nucleotide Utilizing Enzymes. 6′-Cyano-6′-deoxyhomoadenosine-6′-phosphonic acid was a substrate of rabbit muscle AMP aminohydrolase; a double reciprocal plot of substrate concentration against reaction velocity (illustrated in Figure 1 with AMP as substrate) was linear, and the K_m (Michaelis constant) and V_{max} (maximal velocity) values obtained therefrom are listed in Table II together with values for 6′-deoxyhomoadenosine-6′-phosphonic acid similarly obtained. When the deamination of XIV was allowed to proceed to completion (Figure 2), the total decrease in absorbance at 265 nm was the same as given by an identical initial level of AMP, showing that both of the two 6′ epimers of XIV are substrates. The reaction rate for one isomer was considerably less than for the other, and the position of the inflection in the rate plot (Figure 2) shows that the two epimers were present in approximately equal proportions.

To confirm that the product of deamination of XIV was an IMP analog, adenylate deaminase (100 μg) was added to a solution of XIV (3 mg) in a mixture of 0.9 ml of 0.01 *M* citrate buffer (pH 6.5) and 0.5 ml of 1 *M* KCl. After 14 hr at 37°, the mixture showed no further decrease in optical density at 265 nm, and was accordingly heated in boiling water for 5 min and centrifuged to remove coagulated protein. The supernate possessed an absorption maximum at 248.5 nm, as expected for an inosine derivative at pH 6.5, and with

(35) A. M. Michelson, *Biochim. Biophys. Acta*, 91, 1 (1964).

Table II. Kinetic Constants of Homonucleotide Analogs as Substrates and Inhibitors of AMP Utilizing Enzymes

Enzyme	Compd	K_m, mM	V_{max}, rel %	K_i, μM
5′-Nucleotidase	AMP	0.027	100.00	
	XIV			110[e]
AMP aminohydrolase	AMP	1.00	100.00[b]	
	6′-DeoxyhomoAMP[a]	1.70	20.00	58[f]
	XIV	0.50	3.00	19[f]
Adenylate kinase (rabbit muscle)	AMP	0.50	100.00[c]	500[g]
	6′-DeoxyhomoAMP	0.17	2.28	440[f]
	XIV	0.027	0.039	320[f]
Adenylate kinase (pig muscle)	AMP	0.19	100.00[d]	
	6′-DeoxyhomoAMP	0.07	1.96	
	XIV	0.017	0.033	

[a] 5′-Deoxy-5′-(dihydroxyphosphinyl)methyladenosine (6′-deoxyhomoadenosine-6′-phosphonic acid). [b] V_{max} for AMP was 1210 μmol min^{-1} mg^{-1} of protein. [c] V_{max} for AMP was 121 μmol min^{-1} mg^{-1} of protein. [d] V_{max} for AMP was 88 μmol min^{-1} mg^{-1} of protein. [e] Noncompetitive inhibition. [f] Competitive inhibition. [g] L. Noda, *Enzymes*, **6**, 146 (1962).

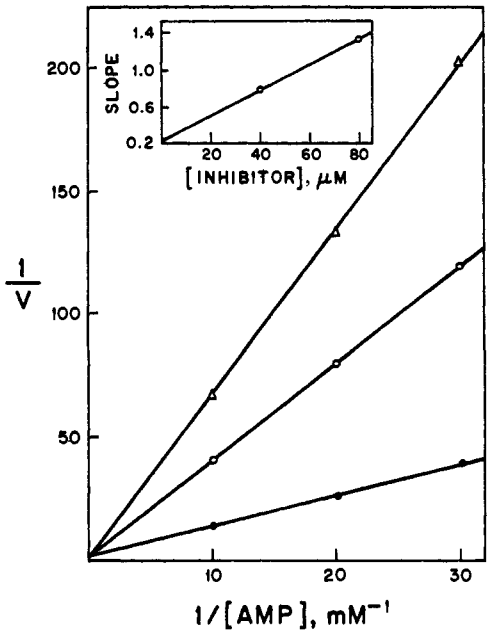


Figure 1. Inhibition of AMP aminohydrolase by XIV. Initial velocity is expressed as change in optical density at 265 nm/min. XIV concentrations were: 0 (●), 40.7 μM (○), and 81.4 μM (△).

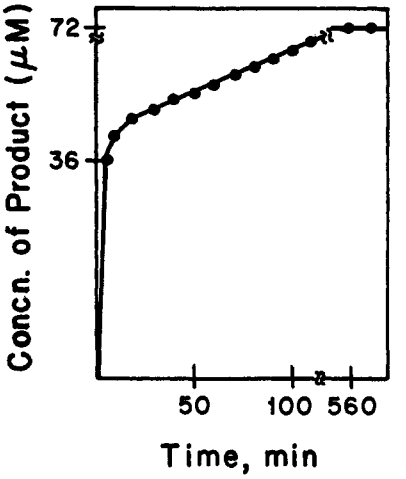


Figure 2. Deamination of XIV by AMP aminohydrolase. The mixture contained 5 μg of enzyme in 0.9 ml of 10 mM citrate buffer (pH 6.5) which contained 25 mM KCl and was initially 72 μM in respect to XIV.

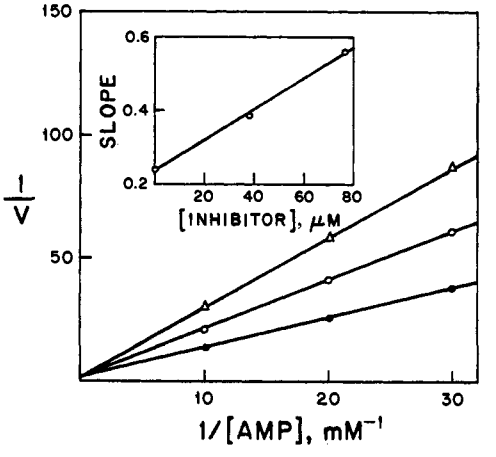


Figure 3. Inhibition of AMP aminohydrolase by 6′-deoxyhomoadenosine-6′-phosphonic acid. Velocity is expressed as in Figure 1. Inhibitor concentrations were: 0 (●), 38.2 μM (○), and 76.4 μM (△).

paper chromatography in saturated ammonium sulfate–0.1 M potassium phosphate buffer (pH 7.2)–2-propanol (79:19:2, v/v), it gave a single ultraviolet-absorbing spot with the same R_f (0.53; XIV, 0.28) as IMP.

Figure 1 shows that XIV is a linear competitive inhibitor of the deamination of AMP by AMP aminohydrolase. Figure 3 shows that 6′-deoxyhomoadenosine-6′-phosphonic acid exerts the same type of inhibitory action. The enzyme–inhibitor dissociation constants (K_i value) are listed in Table II.

Both XIV and 6′-deoxyhomoadenosine-6′-phosphonic acid were substrates of rabbit and pig muscle AMP kinase; the kinetic constants were obtained by double reciprocal plots and are given in Table II. The two AMP analogs were linear competitive inhibitors of the conversion by the rabbit muscle enzyme of AMP to ADP (Figures 4 and 5 and Table II). Figure 6 shows the time course of the production of NAD in the assay of AMP kinase coupled with pyruvate kinase and lactate dehydrogenase when either compound XIV is substituted for AMP or compound XIX is substituted for ATP as a substrate of AMP kinase. Figure 6 shows also the rate of production of NAD when compound XVIII was substituted for ADP as a substrate of pyruvate kinase.

6′-Cyano-6′-deoxyhomoadenosine-6′-phosphonate was a linear noncompetitive inhibitor of the dephos-

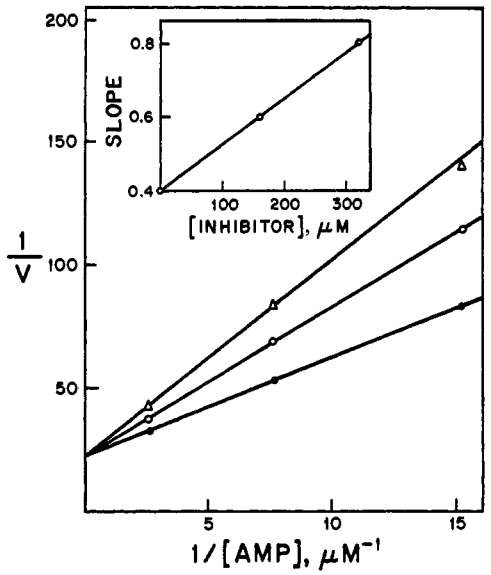


Figure 4. Inhibition of rabbit AMP kinase by XIV. Initial velocity is expressed as change in optical density at 340 nm/min. XIV levels were: 0 (●), 160 μM (○), and 320 μM (△). *Inset*: secondary plot of slopes against XIV concentration.

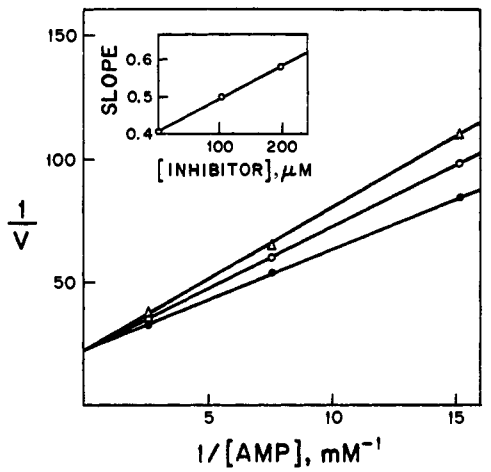


Figure 5. Inhibition of rabbit AMP kinase by 6′-deoxyhomoadenosine-6′-phosphonic acid. Units of velocity are as in Figure 4. Inhibitor concentrations were: 0 (●), 100 μM (○), and 200 μM (△). *Inset*: secondary plot of slopes against inhibitor level.

phorylation of AMP by the 5′-nucleotidase of snake venom (Figure 7, Table II).

Discussion

Both 6′ epimers of 6′-cyano-6′-deoxyhomoadenosine (XIV) were substrates of the AMP aminohydrolase, and the position of the sharp inflexion which occurred during the course of the deamination of XIV (Figure 2) revealed that the two epimers were present in essentially equal proportion. In confirmation of this, it was found that only 50% of the ADP analog XVIII, which was synthesized from XIV by reactions unable to affect the configuration at C-6′, served as a substrate of the pyruvate kinase (Figure 4).

In the present studies the action of AMP kinase, which mediates the phosphoryl transfer AMP + ATP ⇆ 2ADP, was measured in a coupled system containing pyruvate kinase for which ADP and phosphoenolpyruvic acid are substrates and give rise to pyruvic

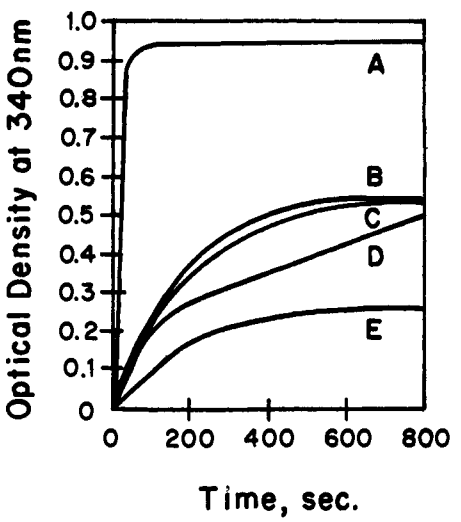


Figure 6. Substrate properties of XIV, XVIII, and XIX with AMP kinase and pyruvate kinase in a system coupled with lactate dehydrogenase. Reaction mixtures were as described in the Experimental Section except that they contained altered quantities of adenylate kinase (10 μg), pyruvate kinase (100 μg), and lactic dehydrogenase (100 μg). The substrates employed were as follows: curve A, 0.08 mM AMP and 0.28 mM ATP; curve B, 0.08 mM XVIII (AMP, ATP, and AMP kinase omitted); curve C, 0.08 mM XIX and 0.28 mM AMP; curve D, 0.08 mM 6′-deoxyhomoadenosine-6′-phosphonic acid and 0.28 mM ATP; curve E, 0.08 mM XIV and 0.28 mM ATP.

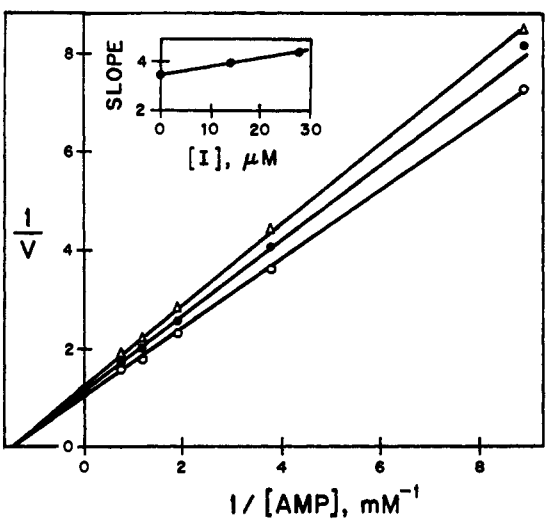


Figure 7. Inhibition of 5′-nucleotidase by XIV. Initial velocity is expressed as change in optical density at 265 nm/1000 sec. XIV concentrations were: 0 μM (●), 14 μM (○), and 28 μM (△). *Inset*: secondary plot of slopes against XIV concentration.

acid. Under the conditions used, essentially all the AMP was eventually converted to ADP and this in turn gave rise to an equivalent amount of pyruvate. In an AMP kinase mediated reaction which was allowed to go to completion (Figure 6), the AMP analog XIV produced only 25% as much pyruvate as did AMP itself, revealing that only one 6′ epimer of XIV was a substrate of AMP kinase and gave rise to an ADP analog which showed no substrate activity toward the relatively large amounts of pyruvate kinase employed in the assay. On the other hand, when the ATP analog XIX was substituted for ATP in the AMP kinase system, it produced 50% as much pyruvate as

did ATP itself, thus showing that only one 6′ epimer of XIX was serving as a phosphoryl donor with AMP kinase and was thereby giving rise to an ADP analog which was a substrate of the pyruvate kinase. These findings demonstrate that the configuration at C-6′ of the epimer of XIV which serves as a phosphoryl acceptor with AMP kinase is opposite to the configuration at C-6′ of the epimer of XIX which acts as a phosphoryl donor with the enzyme. This stereospecificity was shown by the AMP kinase from pig muscle as well as that from rabbit muscle. A further observation (Table II) is that the V_{max} values of XIV and of 6′-deoxyhomoadenosine-6′-phosphonic acid relative to those of AMP are the same, within experimental error, for the pig and rabbit AMP kinases, thus serving as a delicate indicator of close similarities in the catalytic sites and mechanisms of the two enzymes.

With AMP deaminase the above two AMP analogs were respectively 100- and 10-fold more effective as substrates than they were with the AMP kinases. A similar finding was obtained with an analog differing from XIV by replacement of the 6′-cyano group by a hydroxyl group,[36] and the relatively poor substrate activity with the kinases is possibly associated with the greater proximity of the 6′ substituent to the site of the catalyzed reaction.

Both 6′-deoxyhomoadenosine-6′-phosphonic acid and its 6′-cyano derivative XIV were linear competitive inhibitors of the transformation of AMP by AMP kinase and AMP aminohydrolase. Since both analogs are substrates of these enzymes, it is reasonable to conclude that the enzyme–inhibitor dissociation constants (K_i values) are an index of the affinity of the analogs for the respective enzymic AMP binding sites. If predominantly one 6′ epimer of XIV were involved in the binding, the actual K_i value for XIV could be as little as one-half the value listed. In any event, for AMP aminohydrolase the K_i value of XIV is at least three times less than that of 6′-deoxyhomoadenosine-6′-phosphonic acid, indicating enhanced binding, and for rabbit AMP kinase binding of XIV is slightly stronger than binding of either 6-deoxyhomoadenosine-6′-phosphonic acid or AMP.

The present studies have included six enzyme systems[37] for which either AMP, ADP, or ATP are substrates, and for each system it was possible to introduce a small substituent in the vicinity of O-5′ of the adenine nucleotides without preventing binding to the enzyme site as judged by persistence of substrate activity. Furthermore, in the two AMP utilizing systems studied, the α-cyano group has been found to slightly increase rather than to decrease affinity for the AMP site and parallel studies[36] in this laboratory have shown than an α-hydroxy group, likewise, does not significantly decrease affinity for the AMP site of the same enzymes. 6′-Deoxy-6′-substituted adenine homonucleotides hence appear to hold some promise of providing useful substrate analogs with which to investigate phosphate interaction sites and other properties of enzymes for which adenine nucleotides are substrates.

(36) A preliminary account of the synthesis and enzymic properties of this compound has been presented: F. Perini, P. J. Harper, and A. Hampton, Abstracts, Division of Biological Chemistry, 164th National Meeting of the American Chemical Society, New York, N. Y., Sept 1972, No. 231; A. Hampton, F. Perini, and P. J. Harper, *Biochemistry*, **12**, 1730 (1973).

(37) The systems were: pig and rabbit AMP kinases and rabbit AMP aminohydrolase (substrate, AMP); pyruvate kinase (substrate, ADP); pig and rabbit AMP kinases (substrate, ATP).

Experimental Section

Materials and Methods. Dimethyl sulfoxide was distilled at 2 mm from calcium hydride. Diethyl ethoxycarbonylmethylphosphonate and diethyl cyanomethylphosphonate (Aldrich Chemical Co., Milwaukee, Wis.) were dried with Linde 3A Molecular Sieves and distilled. Sodium hydride (50% in mineral oil) was supplied by Metal Hydrides, Inc. Darco 60 charcoal (Matheson Coleman and Bell) was used for adsorbing nucleosides and nucleotides and was activated prior to use by the method of Lipkin.[38] Petroleum ether employed in purifications boiled at 30–60°. Thin-layer chromatograms were run on Merck F-254 silica gel plates in (A) chloroform–ethyl acetate–ethanol (70:30:1); (B) chloroform–ethanol (8:2); (C) chloroform–ethanol (19:1); (D) cyclohexane–ethyl acetate–ethanol (8:1:1); (E) ethanol–benzene (2:8); (F) chloroform–ethanol (9:1); and on Baker-flex poly(ethylenimine)-cellulose in (H) 1.0 *M* LiCl; (J) 2.0 *M* HCOOH–2.0 *M* LiCl (1:1). Paper chromatography was carried out by the descending technique on Whatman 1 paper in the following systems: (K) 2-propanol–concentrated ammonium hydroxide–water (7:1:2); (L) 1-butanol–glacial acetic acid–water (5:2:3); (M) isobutyric acid–1 *M* ammonium hydroxide (10:6). Electrophoresis was performed on Whatman 1 or 3 MM paper at 57–80 V/cm for 30–60 min at pH 7.5 (0.05 *M* triethylammonium bicarbonate), and 57 V/cm for 30 min in pH 4.0 (0.05 *M* acetate) buffer. Mobility values (M_{AMP}) are relative to those of adenosine 5′-phosphate. Spots on chromatograms were detected by their ultraviolet absorption and by spraying silica gel chromatograms with the Molisch reagent. Melting points (uncorrected) were determined by the capillary method. Ultraviolet spectra were determined with a Cary Model 15 spectrophotometer. Infrared spectra were determined in KBr disks with a Perkin-Elmer spectrophotometer, Model 137, and pmr spectra were obtained with a Varian XL-100-15 instrument. Evaporations were carried out *in vacuo* at bath temperatures below 30° unless stated otherwise. Elemental analyses were made by Atlantic Microlabs, Atlanta, Ga., and Midwest Microlab, Ltd., Indianapolis, Ind.

Methyl 5-Deoxy-5-(diethoxyphosphinyl)cyanomethyl-2,3-*O*-isopropylidene-β-D-ribofuranoside (IIIb). Diethyl cyanomethylphosphonate (IIb, 291 mg, 1.7 mmol) was added dropwise at 5° to a slurry of sodium hydride (36 mg, 1.5 mmol) (previously washed several times with anhydrous light petroleum) in 5 ml of dry DMSO. The mixture was stirred for 1 hr at room temperature, by which time gas evolution had ceased. A solution of methyl-2,3-*O*-isopropylidene-5-deoxy-5-iodo-β-D-ribofuranoside[21] (I, 320 mg, 1.0 mmol) in 5 ml of dry DMSO was added dropwise with stirring over 30 min to the above solution of sodio diethyl cyanomethylphosphonate maintained at 50–60°. The mixture was stirred for a further 30 min at 50–60°, then cooled to room temperature. All the foregoing operations were performed in a dry nitrogen atmosphere. The mixture was acidified at 0° to pH 5 with 10% aqueous acetic acid. The solution was extracted with chloroform (3 × 10 ml) and the combined extracts were washed with water and dried (magnesium sulfate). The chloroform was removed *in vacuo* to give a brown syrup (390 mg). A sample was subjected to preparative tlc on silica gel (system A). The band corresponding to IIIb (R_f 0.78; R_f of I 0.92) was eluted with chloroform and the extract was filtered through Celite and evaporated to a syrup which gave a negative Beilstein halogen test and a positive Molisch test: yield, 210 mg (57%); ir (cm^{-1}) 2250 (C≡N); 1235 (P=O); 1157, 1050, and 1020 (P—O—Et).

Anal. Calcd for $C_{15}H_{26}NO_7P$: C, 49.58; H, 7.16; N, 3.85; P, 9.54. Found: C, 50.54; H, 7.51; N, 3.89; P, 9.25.

When *N*,*N*-dimethylformamide was used as solvent the reaction proceeded more slowly and gave a lower yield. No reaction occurred with tetrahydrofuran as solvent.

Methyl 5-Deoxy-5-(diethoxyphosphinyl)carboethoxymethyl-2,3-*O*-isopropylidene-β-D-ribofuranoside (IIIa). The reaction was carried out as described above, with 36 mg of sodium hydride, 380 mg of triethyl phosphonoacetate (IIa), and 320 mg of the iodo sugar (I) in DMSO, at 60–70° for 5 hr, to give 61 mg (15%) of a

(38) D. Lipkin, P. T. Talbert, and M. Cohn, *J. Amer. Chem. Soc.*, **76**, 2871 (1954).

pale yellow syrup which had R_f 0.81 in system A (positive Molisch test), showed infrared absorption (cm^{-1}) at 1720 (C=O), 1245 (P=O), and 1159 (P—O—Et) and gave a negative Beilstein test. Reactions carried out in *N,N*-dimethylformamide or in hexamethylphosphoramidate gave no higher yields. No reaction was detected when dioxane, benzene, or tetrahydrofuran were used as solvent.

Anal. Calcd for $C_{17}H_{31}O_9P$: P, 7.56. Found: P, 6.92.

***N,N*-Dibenzoyl-5′-*O*-tosyl-2′,3′-*O*-isopropylideneadenosine (V).** Benzoyl chloride (30 ml; freshly distilled) was added dropwise with stirring during 30 min to a solution of 2′,3′-*O*-isopropylidene-5′-*O*-tosyl-5′-deoxyadenosine[25] (IV, 10 g) in dry pyridine (150 ml; dried over CaH_2) at 5°. The mixture was stirred at 5° for 50 hr when it slowly turned pink and a white precipitate separated. The mixture was evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in chloroform (200 ml), and the solution was washed with cold water, followed by 10% sodium bicarbonate solution, and then water and dried over sodium sulfate. Tlc (system B) of the solution on silica gel showed one major spot of R_f 0.80 with a trace amount of slowly traveling contaminant (R_f 0.43; R_f of IV was 0.17) corresponding to *N*-monobenzoyl-2′,3′-*O*-isopropylidene-3,5′-cycloadenosine. The chloroform extract was concentrated *in vacuo* to *ca.* 50 ml and added slowly to 250 ml of petroleum ether. The cream-colored precipitate was filtered and washed with petroleum ether, yield 12.5 g (88%), mp 97–100° dec. The melting point was raised to 100–102° dec by a second precipitation (74%) from an ethereal solution by addition of petroleum ether. A sample was purified for analysis by preparative tlc in system B; in ethanol it showed λ_{max} 247 nm (ϵ 39,600) and 272 (20,600; shoulder). It exhibited an amide band at 1695 cm^{-1} and covalent sulfonate at 1170 cm^{-1} and no absorption at 1020 cm^{-1} (ionic sulfonate).

Anal. Calcd for $C_{34}H_{31}N_5O_8S$: C, 60.98; H, 4.63; N, 10.46. Found: C, 61.09; H, 4.78; N, 10.28.

***N,N*-Dibenzoyl-5′-iodo-5′-deoxy-2′,3′-*O*-isopropylideneadenosine (VI).** The 5′-tosylate V (10 g) was dissolved in 500 ml of dry acetone (Spectroquality grade; Matheson Coleman and Bell). Sodium iodide (20 g, dried over P_2O_5 at 100° for 10 hr) was added and the mixture was heated under gentle reflux for 5 hr when tlc on silica gel (system C) revealed no starting material. The mixture was chilled and filtered and the solution was evaporated to dryness *in vacuo*. The residue was dissolved in benzene (200 ml), insoluble material was removed by filtration, and the filtrate was washed with 10% sodium sulfite solution and then with water and dried over magnesium sulfate. The benzene extract was concentrated *in vacuo* to *ca.* 30 ml and added slowly to 150 ml of light petroleum to give a cream-colored solid, 8.3 g (88%), mp 95°–100° dec. With tlc on silica gel (system C), it gave one main spot (R_f 0.72, R_f of V 0.55) and an unidentified product of R_f 0.17. A solution of the product in a minimum volume of chloroform was applied to a column of 70 g of silica gel and the column was eluted with ethyl ether. The fractions containing material of R_f 0.85 (silica gel plate developed with ether) were evaporated and the product was precipitated from benzene by addition of light petroleum. *N,N*-Dibenzoyl-5′-iodo-5′-deoxy-2′,3′-*O*-isopropylideneadenosine (VI) could not be obtained in crystalline form and the amorphous powder (6.1 g, 58%) melted at 105–107° dec; in ethanol it showed absorption maxima at 249 nm (ϵ 39,600) and 272 sh (20,500); ir amide absorption at 1695 (broad doublet), and no absorption at 1170 and 1020 cm^{-1} (due to covalent or ionic sulfonate).

Anal. Calcd for $C_{27}H_{24}N_5O_5I$: C, 51.84; H, 3.84; N, 11.20; I, 20.32. Found: C, 51.96; H, 3.91; N, 11.38; I, 20.23.

***N,N*-Dibenzoyl-5′-deoxy-5′-(diethoxyphosphinyl)cyanomethyl-2′,3′-*O*-isopropylideneadenosine (VII).** Diethyl cyanomethylphosphonate (IIb, 389 mg, 2.2 mmol) was added dropwise with occasional cooling to a stirred suspension of sodium hydride (48 mg, 2 mmol) (previously washed two times with anhydrous light petroleum) in 10 ml of dry DMSO. The mixture was stirred for 1 hr at room temperature (when gas evolution ceased) and filtered. The filtrate was added dropwise to a stirred solution of VI (1.25 g, 2 mmol) in 10 ml of dry DMSO at 50–60°. After addition was complete (*ca.* 30 min), the mixture was stirred for an additional 30 min at 50–60°. All operations were carried out under dry nitrogen in apparatus which was oven-dried and assembled hot under nitrogen. The reaction mixture was acidified to pH 5 at 0° with 10% aqueous acetic acid. The solution was extracted with chloroform (3 × 10 ml) and the combined chloroform extracts were washed with water and then dried over magnesium sulfate. Tlc (sysem D) of the extract revealed four major ultraviolet-absorbing components of R_f 0.52 (VI), R_f 0.34 (*N*-monobenzoyl-2′,3′-*O*-isopropylidene-5′-iodo-5′-deoxyadenosine, IX), R_f 0.28 (VII and *N*-monobenzoyl-2′,3′-*O*-isopropylidene-3,5′-cycloadenosine, X), R_f 0.17 (VIII), and R_f 0.02 (unidentified material). Tlc with threefold repeated development in system D separated VII and X which had R_f values 0.49 and 0.46, respectively. The red chloroform extracts were evaporated to *ca.* 5 ml and applied to a column (3 × 10 cm) of Merck silica gel 7734, which was eluted with solvent D, and fractions of 10–13 ml were collected. Tlc showed that compound VII was eluted in fractions 75–124 together with a portion of X. These fractions were evaporated *in vacuo* and rechromatographed as described above. Fractions containing VII were evaporated to a semisolid; this was precipitated from chloroform by addition of light petroleum to give 377 mg (28%) of a white amorphous powder, mp 105–110° dec. It showed absorption maxima (in ethanol) at 249 nm (ϵ 39,500) and 272 sh (20,700); ir absorption occurred at 2232 cm^{-1} (C≡N), 1692 (C=O), 1245 (P=O), 1159 (P-O-Et).

Anal. Calcd for $C_{33}H_{35}N_6O_8P$: C, 58.75; H, 5.19; N, 12.46; P, 4.59. Found: C, 58.85; H, 5.48; N, 12.45; P, 4.33.

The component of R_f 0.34 was concluded to be IX on the basis of its R_f value, ultraviolet absorption (λ_{max}^{EtOH} 280 nm), and positive Beilstein test. In addition, this compound (*ca.* 5 mg) was refluxed in dioxane (*ca.* 3 ml) for 2 hr after which tlc in system D showed one major spot (R_f 0.25; R_f of starting material 0.33) which showed $\lambda_{max}^{EtOH \text{ and pH } 11}$ 266 and 308 nm, $\lambda_{max}^{pH\ 2}$ 253 and 300 nm identical with that of authentic monobenzoyl-2′,3′-*O*-isopropylidene-3,5′-cycloadenosine (X).

***N*-Benzoyl-5′-deoxy-5′-(diethoxyphosphinyl)cyanomethyl-2′,3′-*O*-isopropylideneadenosine (VIII).** Fractions 270–299 from the foregoing column chromatography were evaporated to dryness. Precipitation by the same procedure as described for VII gave 80 mg (7%) of product VIII which melted with decomposition at 81–86°; in ethanol it showed an absorption maximum at 279 nm (18,600); ir absorption was at 1692 (C=O), 2232 (C≡N), 1253 (P=O), 709 cm^{-1} (phenyl).

Anal. Calcd for $C_{26}H_{31}N_6O_7P$: C, 54.73; H, 5.44; N, 14.73; P, 5.44. Found: C, 54.70; H, 5.52; N, 14.49; P, 5.37.

Compound VIII was secured in higher yield by a modified reaction in which 38.9 g (0.22 mol) of the phosphonate IIb, 4.32 g (0.18 mol) of sodium hydride, and 12.5 g (0.02 mol) of VI were maintained at 70–75° for 1 hr; tlc (system D) showed that the spot corresponding to compound VI had disappeared. Chromatography as described above yielded 0.9 g (7%) of product VII and 5.1 g (45%) of product VIII after precipitation of these compounds from chloroform by addition of light petroleum.

***N*-Benzoyl-2′,3′-*O*-isopropylidene-3,5′-cycloadenosine (X).** A mixture of 2′,3′-*O*-isopropylidene-3,5′-cycloadenosine tosylate[24] (4.61 g, 10 mmol), benzoic anhydride (4.52 g, 20 mmol), and 100 ml of pyridine was stirred magnetically at 40–50° overnight. The resulting yellow solution was concentrated to *ca.* 5 ml and poured into 200 ml of water. The mixture was stirred at room temperature for 1 hr, and the white precipitate was filtered, washed with 150 ml of ether, and air dried. Crystallization from hot aqueous 90% alcohol gave 1.31 g (32%) of fine needles, mp 245–246°, uv λ_{max}^{MeOH} 266 nm (ϵ 12,500) and 308 (12,600).

Anal. Calcd for $C_{20}H_{19}N_5O_4 \cdot H_2O$: C, 58.39; H, 5.10; N, 17.03. Found: C, 58.30; H, 4.88; N, 16.98.

Attempted Transbenzylation of Compound VIII. (A) Sodium benzyloxide (70 mg of sodium in 10 ml of benzyl alcohol) was added to a solution of VIII (570 mg) in dry benzyl alcohol (10 ml). Silica gel tlc (system E) indicated that compound VIII (R_f 0.50) was converted within 3 hr at 25° to one major uv-absorbing product (R_f 0.41) that gave a positive reaction to Molisch spray reagent. The reaction mixture was acidified to pH 6.0 with aqueous acetic acid and extracted with chloroform (30 ml), and the extract was washed with water and dried over magnesium sulfate. The solution was concentrated to a small volume (*ca.* 3 ml) and light petroleum added to precipitate a white amorphous solid (0.405 g): mp 83° dec; uv max (pH 2, pH 7 or pH 12 in 50% aqueous ethanol), 263 nm; ir 2250 cm^{-1} (C≡N) 745, 685 cm^{-1} (phenyl), no absorption at 1700–1750 cm^{-1} (C=O); nmr (DMSO-d_6, external standard, TMS) δ 8.68 (s, 1, H-8), 8.56 (s, 1, H-2), 7.75 (m, 7, $C_6H_5CH_2$), 7.28 (s, 1, NH), 6.58 (d, 1, H-1′), 5.94 (m, 1, H-2′), 5.40 (m, 1, H-3′), 4.74 (m, 1, H-4′), 3.66 (broad s, 2, H-6′), 3.19 (m, 2, H-5′), 1.89 and 1.68 (s, 3, isopropyl methyls).

Anal. Found: C, 65.78; H, 5.66; N, 20.78; P, none.

Hydrogenation of the compound (8 mg) in 50% aqueous ethanol at 40–45° for 14 hr with Adam's catalyst (10 mg) gave a product, $\lambda_{max}^{pH\ 3}$ 257 nm, $\lambda_{max}^{pH\ 13}$ 259 nm, which upon electrophoresis at pH 6.8 migrated at 53% the rate of AMP, but toward the cathode.

(B) Sodium benzyloxide (9.2 mg of sodium in 0.8 ml of benzyl alcohol) was added to a solution of VIII (57 mg) in 3 ml of DMSO. Silica gel tlc showed that no reaction had occurred after 3 days at room temperature.

5′-Deoxy-5′-(diethoxyphosphinyl)cyanomethyl-2′,3′-*O*-isopropylideneadenosine (XI). A solution of 1.36 g of VIII in ethanol (50 ml, previously saturated at 0° with ammonia) was stored at room temperature for 2 days in a sealed pressure bottle. The solvent was removed *in vacuo* and the residual yellow syrup was extracted with 30 ml of chloroform and the solution was concentrated *in vacuo* to *ca.* 3 ml; addition of light petroleum precipitated crude XI as a pale yellowish powder; yield 906 mg (82%). On tlc (silica gel, system F), the product showed a main spot of R_f 0.58 (R_f of VIII 0.96) together with minor spots of R_f 0.96 (compound VIII) and 0.62. The product was purified by preparative tlc in system F. The ir spectrum of the resulting powder (mp 80–85°, dec) showed no absorption at *ca.* 1720 cm^{-1} (due to amide C=O) but did show bands at 2250 cm^{-1} (C≡N) and 1236 cm^{-1} (P=O); λ_{max}^{EtOH} 258 nm; nmr ($CDCl_3$, internal standard, TMS) δ 8.29 and 8.25 (d, 1, H-8), 7.90 and 7.85 (d, 1, H-2), 6.82 (s, 2, NH_2), 6.10 (d, 1, J = 2 Hz, H-1′), 5.58 (q, 1, J = 2 and 6 Hz, H-2′), 5.08 (q, 1, J = 3 and 6 Hz, H-3′), 4.55 (q, 1, J = 3 and 8 Hz, H-4′), 4.13 (m, 4, CH_2 of C_2H_5), 3.41 (d of q, 1, J = 3, 12, and 24 Hz, H-6′), 2.38 (broad m, 2, H-5′), 1.61 and 1.39 (s, 3, isopropylidene methyl), and 1.28 (m, 6, CH_3 of C_2H_5).

Anal. Calcd for $C_{19}H_{27}N_6O_6P \cdot 0.1CHCl_3$: C, 47.97; H, 5.64; N, 17.63; P, 6.48. Found: C, 48.03; H, 5.63; N, 17.24; P, 6.39.

The compound was formed also from VII under the same conditions.

5′-Deoxy-5′-(ethoxyhydroxyphosphinyl)cyanomethyl-2′,3′-*O*-isopropylideneadenosine (XII). XI (50 mg) was treated with 15 ml of concentrated NH_4OH at 100° for 20 hr in a sealed tube, after which the reaction mixture contained material of R_f 0.76 in system K as the main product (87%) together with small amounts of XI (R_f 0.96). The R_f 0.76 material had M_{AMP} 0.43 on paper electrophoresis at pH 7.5 and had $\lambda_{max}^{H_2O}$ 259 nm. After removal of volatiles *in vacuo*, the residue was used for the subsequent deacetonization without further purification. A sample purified by paper chromatography in system K was homogeneous also in system L (R_f 0.78) and showed nmr signals in DMSO-d_6 (concentric capillary of TMS, as external standard) at δ 8.63 (s, 1, H-8), 8.47 (s, 1, H-2), 7.55 (s, 2, NH_2), 6.45 (d, 1, J = 2 Hz, H-1′), 5.79 (q, 1, J = 2 and 6 Hz, H-2′), 5.29 (q, 1, J = 3 and 6 Hz, H-3′), 4.58 (q, 1, J = 3 and 8 Hz, H-4′), 3.84 (m, 2, CH_2 of C_2H_5), 3.29 (m, 1, H-6′), 2.35 (m, 2, H-5′), 1.86 and 1.65 (s, 3, isopropylidene methyls), and 1.44 (m, 3, CH_3 of C_2H_5).

5′-Deoxy-5′-(ethoxyhydroxyphosphinyl)cyanomethyladenosine (XIII). The preparation of XII was heated at 100° in 10 ml of aqueous acetic acid (pH 2.5) for 1.5 hr. The main component of the reaction mixture had R_f 0.62 (system K), R_f 0.63 (system L), and M_{AMP} 0.45 on paper electrophoresis at pH 7.5. After purification by paper electrophoresis the product was homogeneous in solvents K and L, $\lambda_{max}^{H_2O}$ 258, $\lambda_{min}^{H_2O}$ 226 nm. The yield, determined spectrophotometrically, was 67%.

Removal of Ethyl Groups from VIII by Treatment with Hydrogen Bromide. Compound VIII (120 mg) was dissolved in dry benzene (50 ml) and anhydrous hydrogen bromide gas was passed into the solution at 20° for 17 hr. A white precipitate formed immediately. Solvent was removed by slow passage of dry nitrogen gas overnight, and water (10 ml) was added to the resulting pale yellowish powder. The pH was adjusted to 2.5 with NaOH and the solution was heated at 100° for 1.5 hr, then evaporated to dryness. Charcoal (Darco G-60) was added to a stirred aqueous solution (adjusted to pH 3) until the optical density at 280 nm (1-cm cell) of the solution fell to 0.07. The charcoal was collected by filtration, washed with water, and eluted with a 5% solution of ammonium hydroxide in 50% aqueous ethanol until the optical density at 280 nm of the eluate became negligible. Volatiles were removed *in vacuo* to give 67 mg of material which was chromatographed in system K on Whatman 3 MM paper. Bands corresponding to the monobenzoyl cyanohomoadenosinephosphonic acid (XVI, R_f 0.60; M_{AMP} 0.82 at pH 7.5), its monoethyl ester (XV, R_f 0.78; M_{AMP} 0.40) and monobenzoyladenine (M_{AMP} 0.13) were present. The yields, determined spectrophotometrically after elution, assuming that ϵ_{max} at 280 nm is the same (18,600) as found for *N*-benzoyladenosine, were 42% of XVI and 13% of XV. The ultraviolet absorption maxima of XV and XVI ($\lambda_{max}^{H_2O}$ 279, shoulders at 232 and 250, $\lambda_{min}^{H_2O}$ 243 nm) were very similar to those reported previously for *N*-benzoyladenosine.[26] Compound XV (8 mg) obtained after paper electrophoretic purification was dried over P_2O_5 at 78° and retreated with HBr–benzene (5 ml) as described above. Paper electrophoresis at pH 7.5 showed that *ca.* 18% of this compound was converted to XVI.

5′-Deoxy-5′-(dihydroxyphosphinyl)cyanomethyladenosine (XIV).

(A) **From VIII.** VIII (2.3 g) was dissolved in dry benzene (500 ml) and dry hydrogen bromide gas passed through the solution at 20° with mechanical stirring. After 17 hr volatiles were removed by bubbling through dry nitrogen gas at 25°; water and methanol were added to the residual pale yellowish powder and the pH was adjusted to 2.5 with NaOH. The solution was heated at 100° for 1.5 hr. Volatiles were removed *in vacuo* and the residual red gum was dissolved in 10 ml of dioxane. Concentrated ammonium hydroxide (10 ml) was added, and after the turbid mixture had clarified, additional concentrated ammonium hydroxide (30 ml) was added and the turbid solution was stirred at 5° for 3 days. The ammonium hydroxide was removed by evaporation. A solution of the residue in 15 ml of water was adjusted to pH 3.5 with acetic acid, and Darco G-60 was added. The suspension was stirred for 15 min at room temperature, and the charcoal was removed by filtration. The process was repeated until the solution contained less than 3% of the original ultraviolet-absorbing material. The charcoal was washed with water to remove acetic acid, then the uv-absorbing material was extracted at room temperature for 30 min with *ca.* 150 ml of 50% aqueous ethanol containing 5% ammonium hydroxide and filtered. The filtrate was evaporated to dryness followed by two evaporations with 30-ml portions of water. The residue, which contained 28,600 $OD_{260\ nm}$ units, was dissolved in 3 ml of water and applied to a column (3 × 30 cm) of Dowex 1 anion exchange resin (200–400 mesh, 8% cross linkage, chloride form). The column was washed with water (1.7 l.) which removed adenine (1300 OD_{260} units). Elution with 0.002 *N* HCl (250 ml) afforded an unidentified compound (600 OD_{260} units; $\lambda_{max}^{pH\ 2}$ 268 nm). Elution with 0.004 *N* HCl (530 ml) removed the monoethyl ester XIII (4800 OD_{260} units), which was identical with the product obtained from XII. Elution with 0.006 *N* HCl removed the chromatographically and electrophoretically homogeneous cyanohomoadenosinephosphonic acid XIV (21,200 OD_{260} units; 35% yield from VIII). The eluate was concentrated *in vacuo* to *ca.* 3 ml at 18°, ethanol (4 ml) was added, and the mixture was allowed to stand at 2° when it formed prisms: mp 146–149° (decomposition with gas evolution); $\lambda_{max}^{pH\ 2}$ 257 nm (ε 14,900), $\lambda_{max}^{pH\ 12}$ 259 (15,200); ir (KBr) 2250 (C≡N), 1235 (P=O), 3240 (OH), 3080 (NH), 1610 and 1572 cm^{-1} (C=C and C=N of the purine ring); nmr (D_2O, external standard, concentric capillary of TMS) δ 8.63 (s, 1, H-8), 8.37 (s, 1, H-2), 6.37 (d, 1, J = 5 Hz, H-1′), 5.07 (H-2′, partial overlap of HDO and H-2′ bands caused slight uncertainty in location of the H-2′ signal), 4.73 (broad s, 2, H-3′ and H-4′), 3.35 (broad m, 1, H-6′), and 2.71 (m, 2, H-5′).

Anal. (material dried at 78°, 0.02 mm). Calcd for $C_{12}H_{15}N_6O_6P \cdot 2H_2O$: C, 35.46; H, 4.67; N, 20.68; P, 7.63. Found: C, 35.29; H, 4.37; N, 19.89; P, 7.39.

(B) **From XIII.** Compound XIII (30 $OD_{260\ nm}$ units) in 0.4 ml of water was incubated for 3 days at 37° with 0.1 ml of 0.3 *M* magnesium acetate, 0.1 ml of 0.1 *M* Tris–HCl buffer (pH 8.8), and enzyme solution which contained 10 mg of crude snake venom phosphodiesterase of *Crotalus atrox* (type IV, from Sigma Chemical Co.). Paper electrophoresis and spectrophotometric assay of the eluted bands showed that 46% of XIII had been converted to the free phosphonic acid XIV. That the product was XIV was confirmed by paper chromatography in solvent systems K and L (R_f values 0.14 and 0.23, respectively).

5′-Deoxy-5′-(hydroxypyrophosphoroxyphosphinyl)cyanomethyladenosine (XIX). Reagent grade tetrasodium pyrophosphate·10 H_2O (1 mmol, 446 mg) was dissolved in water and passed through a column of Dowex 50-W resin (15 ml) in the pyridinium form. The resin was washed with water (30 ml) and the total effluent evaporated *in vacuo* to a volume of *ca.* 1 ml. Pyridine (30 ml) was added followed by tri-*n*-butylamine (1 ml, 4.2 mmol). The resulting homogeneous solution was evaporated to a syrup and rendered anhydrous by four evaporations with 2-ml portions of pyridine (dried by distillation from, and stored over, calcium hydride). The residue was dissolved in 1 ml of dry pyridine.

The nucleotide XIV (81 mg, 0.2 mmol) was dissolved in pyridine (2 ml) and tri-*n*-butylamine (0.6 mmol) added. Solvent was removed under reduced pressure and traces of moisture were removed from the residue by dissolution in DMF (2 ml) followed by evaporation to dryness at 22° under reduced pressure. To a solution of the residual tri-*n*-butylammonium salt of XIV in dry dioxane (0.5 ml) and dry DMF (0.05 ml) was added diphenyl phosphorochloridate (0.06 ml) and tri-*n*-butylamine (0.07 ml) and the solution kept at

room temperature for 3 hr under anhydrous conditions. Solvents were removed under reduced pressure, and diethyl ether was added to the residue to precipitate the mixed anhydride XVII. The mixture was kept at 0° for 1 hr and the ethyl ether was removed by decantation. Dioxane (0.2 ml) was added to the precipitated material and the solution concentrated to a syrup under reduced pressure. A solution of di(tri-*n*-butylammonium) pyrophosphate (0.4 mmol) in pyridine (0.15 ml) was added and the mixture was shaken and kept at room temperature for 45 min. Pyridine was removed under reduced pressure and anhydrous ethyl ether was added. The precipitate was dissolved in water and the solution applied to a column (2 × 20 cm) of DEAE-cellulose in the bicarbonate form. Elution was carried out with a linear gradient of water (1 l.) and 0.4 *M* triethylammonium bicarbonate (1 l., pH 7.5) and 10-ml fractions were collected. The starting material (XIV; 23% recovery) was eluted in fractions 63–98. The phosphonyl-phosphate XVIII (210 OD_{260} units; 7% yield) and the phosphonyl-pyrophosphate XIX (1560 OD_{260} units; 52% yield) were eluted as symmetrical peaks in the elution diagram in fractions 99–109 (0.18 *M* salt) and 111–158 (0.25 *M* salt), respectively. Fractions 111–158 were evaporated at 20° (12 mm), and the residue was dried by addition and evaporation of 15 ml of ethanol. This was followed by two evaporations of 0.1 ml of triethylamine in 5 ml of 50% ethanol, and one of 10 ml of ethanol. A solution of the residue in 3 ml of dry methanol was mixed with 2 ml of 1 *M* NaI in dry acetone, and the sodium salt was precipitated by addition of 10 ml of acetone. The salt was washed with acetone (four 20-ml portions) and dried at 20° (2 mm). The residue was freed from residual XVIII by ascending chromatography on acid-washed Whatman 3 MM paper with repeated development in isobutyric acid–1 *M* NH_4OH (100:60). The compound (XIX) obtained by lyophilization of the aqueous eluate had $\lambda_{max}^{H_2O}$ 258 nm and was homogeneous on paper chromatography, electrophoresis, and PEI-cellulose chromatography with properties similar to those of ATP (Table I).

***N*,*N*-Dibenzoyl-5′-deoxy-5′-(diethoxyphosphinyl)carboethoxymethyl-2′,3′-*O*-isopropylideneadenosine (XX).** The reaction was carried out as described for the preparation of VII, utilizing 36 mg (1.5 mmol) of sodium hydride, 403 mg (1.8 mmol) of triethyl phosphonoacetate (IIa), and 625 mg (1 mmol) of VI in 15 ml of DMSO, at 65–75° for 2 hr. Tlc of the chloroform extract with five successive developments in system D showed the presence of six or more ultraviolet-absorbing components which were quantitated spectrophotometrically and had respectively R_f 0.92 (VI) (28% yield), 0.73 (*N*-monobenzoyl-2′,3′-*O*-isopropylidene-5′-iodo-5′-deoxyadenosine, IX) (19% yield), 0.51 (the dibenzoyl phosphonate XX) (6% yield), 0.46 (monobenzoyl-2′,3′-*O*-isopropylidene-3,5′-cycloadenosine, X) (25% yield), 0.29 (monobenzoyl analog XXI of XX) (2% yield), and 0.01 (unidentified product).

A sample was purified by preparative tlc on silica gel as described above. Material of R_f 0.51 was eluted with chloroform and the extract was filtered through Celite and evaporated to a solid which afforded a white amorphous powder (37 mg, 5.1%) from chloroform solution after addition of petroleum ether. This product showed λ_{max}^{EtOH} 247 nm (ε 39,000) and 272 (20,200) and mp 92–99° dec. It exhibited ir absorption at 1720 (C=O) and at 1250 cm^{-1} (P=O).

Anal. Calcd for $C_{35}H_{40}N_5O_{10}P$: C, 58.25; H, 5.54; N, 9.70. Found: C, 57.85; H, 5.93; N, 10.21.

The component of the reaction mixture which had R_f 0.29 was concluded to be XXI because of its relatively low R_f value compared to that of XX and because it had a negative Beilstein halogen test and an absorption maximum in EtOH of 280 nm indicative of a mono-*N*-benzoyladenosine.

Enzyme Kinetic Studies. All assays were carried out by measuring the rate of change of optical density (OD) at a suitable wavelength in a Cary Model 15 spectrophotometer using 1-cm cells containing a final volume of 1 ml at 20°. In all systems the initial velocity with AMP or XIV as substrate was linear and proportional to the concentration of primary enzyme and independent of the concentration of secondary enzymes used in coupled assays. Michaelis, inhibition constants, and maximal velocity (V_{max}) values were determined graphically by the method of Lineweaver and Burk.[39]

Adenylate deaminase (Sigma, grade IV, from rabbit muscle) activity was measured by following the decrease in absorbance at 265 nm in a system containing 1 ml of 0.01 *M* citrate buffer (pH 6.5) and 25 m*M* KCl on addition of 0.03 and 0.93 μg (for AMP and XIV, respectively) of AMP deaminase which was diluted into 1 *M* KCl prior to use. The decrease in OD was measured at 265 nm where Δε for the conversion was 6600.

5′-Nucleotidase activity (Sigma, grade II, *Crotalus adamanteus* venom) was measured by following the decrease in absorbance at 265 nm in a coupled assay with adenosine deaminase (20 μg), 5′-nucleotidase (1.8 μg), and AMP and XIV in 1 ml of 0.1 *M* Tris hydrochloride (pH 8.5). Measurement and calculation of Δε were identical with AMP deaminase.

Each adenylate kinase (Boehringer, pig, and rabbit muscles) was studied in 1 ml of 0.1 *M* Tris hydrochloride (pH 7.6) containing $MgSO_4$ (1 m*M*), KCl (0.1 *M*), PEP cyclohexylammonium salt (0.87 m*M*), ATP (0.28 m*M*), NADH sodium salt (0.38 m*M*), adenylate kinase (0.43 μg for AMP and 40 μg for XIV), pyruvate kinase (Boehringer, 4 μg for AMP and 400 μg for XIV), and lactic dehydrogenase (Sigma, 4 μg for AMP and 400 μg for XIV). After an initial period of 30 min during which the OD change at 340 nm reached a constant value, the reaction was started by the addition of either AMP or XIV.

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(39) H. Lineweaver and D. J. Burk, *J. Amer. Chem. Soc.*, **56**, 658 (1934).

Communications to the Editor

Direct Formation of the Steroid Nucleus by a Nonenzymic Biogenetic-Like Cyclization.[1] Preparation of the Cyclization Substrate

Sir:

The aim of the present work was to synthesize the trienynol **1** and to study its cyclization. This substrate incorporates a cyclohexenol moiety of a type known[2] to initiate facile stereoselective cyclization so as to produce an A/B cis ring fusion. At the same time the substrate contains the terminal methylacetylenic residue which has been shown[3] to yield the C/D trans 6/5 ring system, so that in the event of complete cyclization the product would be the tetracyclic substance **2**. Finally, the substrate **1** has the advantage over previous systems that have been examined in that it has an asymmetric center at C-5 (steroid numbering) rendering it susceptible to obtention in its enantiomeric forms which

(1) For the previous paper in this series see W. S. Johnson, M. B. Gravestock, R. J. Parry, and D. A. Okorie, *J. Amer. Chem. Soc.*, **94**, 8604 (1972).

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