Synthesis of Homoadenosine-6′-phosphonic Acid and Studies of Its Substrate and Inhibitor Properties with Adenosine Monophosphate Utilizing Enzymes†

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ABSTRACT: Hydroboration–oxidation of the diphenyl ester of the 5′,6′-unsaturated phosphonate isostere of 2′,3′-*O*-isopropylideneadenosine 5′-phosphate followed by removal of blocking groups furnished the title compound, 5′-deoxy-5′-(*C*-dihydroxyphosphinyl)hydroxymethyladenosine. That the position of substitution was α to phosphorus was established from the H-5′ and H-6′ signals at δ 2.28 and 4.12, respectively, in the proton magnetic resonance spectrum in D_2O of 2′,3′-*O*-isopropylidenehomoadenosine-6′-phosphonate. The homoadenosine-6′-phosphonate was comprised of equal amounts of its 6′ epimers; both epimers were substrates of muscle adenylate (adenosine monophosphate (AMP)) kinase but only one of the phosphorylphosphonates thereby produced was a substrate of muscle pyruvate kinase. With AMP kinase of pig muscle the Michaelis constant, K_m, of the epimeric mixture was 0.17 mM and the maximal velocity, V_{max} (micromoles per minute per milligram of protein), was 0.14, 0.15% of the V_{max} with AMP (V_{max} = 92.6, K_m = 0.19 mM). With rabbit AMP kinase, K_m was 0.71 mM and V_{max} was 0.20, 0.17% of the V_{max} with AMP (V_{max} = 119; K_m = 0.50 mM). With rabbit muscle AMP aminohydrolase both epimers were substrates (K_m = 4.0 and 0.025 mM; V_{max} = 1000 and 31, respectively, 91 and 2.8% of V_{max} for AMP); AMP values were K_m = 0.40 mM and V_{max} = 1100. The 6′ epimers produced an AMP-promoted inhibition of dephosphorylation of AMP by *Crotalus* venom 5′-nucleotidase and linear competitive inhibition of pig muscle AMP kinase and AMP aminohydrolase (K_i = 0.38 and 0.21 mM, respectively). The data indicate that the complexes of AMP with rabbit AMP aminohydrolase and with pig AMP kinase contain sufficient space in the region of O-5′ of AMP to accommodate a hydroxyl group.

The interactions which occur between the phospho ester portion [$-CH_2OP(O)(OH)_2$] of a purine mononucleotide and its enzymic substrate binding sites are frequently drastically reduced or eliminated by small steric or electronic modifications of the ionizable phosphate oxygens. Thus, previous studies with IMP analogs (Nichol *et al.*, 1967) showed that binding to the IMP site, as evidenced by substrate activity or competitive kinetics, was abolished when one phosphate hydroxyl was replaced by hydrogen or other small substituents. Furthermore, even replacement of one phosphate hydroxyl by sulfhydryl profoundly reduces substrate activity and/or binding in instances involving AMP (Murray and Atkinson, 1968), IMP (Hampton *et al.*, 1969; Hampton and Chu, 1970), and UMP and TMP (Eckstein and Sternbach, 1967). Useful information regarding these phospho ester binding sites should be obtainable by means of nucleotide analogs capable of acting as site-specific reagents. One reagent of this type is a carboxylic–phosphoric mixed anhydride isostere of AMP which differs from AMP only in that the $CH_2OPO_3H_2$ system of AMP is replaced by $C(O)OPO_3H_2$ (Hampton and Harper, 1971); this compound inactivitates some but not all of the AMP-utilizing enzymes tested, presumably by bonding covalently to AMP sites. An isostere of AMP, 6′-deoxyhomoadenosine-6′-phosphonic acid, in which the $CH_2OPO_3H_2$ system of AMP is replaced by $CH_2CH_2PO_3H_2$ (Jones and Moffatt, 1968), is a substrate of AMP aminohydrolase and AMP kinase (Hampton *et al.*, 1973) and binds to adenylosuccinate synthetase (Hampton and Chu, 1970), and the corresponding isostere of IMP is a substrate of adenylosuccinate synthetase (Hampton and Chu, 1970). These findings suggested that AMP analogs with suitable substituents attached to a methylene group α to phosphorus (*i.e.*, with a $CH_2CH(R)PO_3H_2$ system) might prove capable of derivatizing AMP-utilizing enzymes at or near their AMP phospho ester binding sites. To determine whether sufficient space is available in enzyme–AMP complexes to accommodate such α substituents, the synthesis of α-substituted phosphonate analogs of AMP has been undertaken. The present article examines several synthetic routes to the α-hydroxy phosphonate analog VIII (Scheme I) of AMP (homoadenosine-6′-phosphonic acid[1]) and describes a practicable method which furnishes VIII as a mixture of 6′ epimers. Kinetic evidence is presented for the substrate and inhibitor properties of the epimers of VIII with AMP aminohydrolase of rabbit muscle, 5′-nucleotidase of snake venom, and the AMP kinases of rabbit muscle and pig muscle.

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[1] Abbreviations and trivial names which are not listed in *Biochemistry* 5, 1445 (1966) are: homoadenosine-6′-phosphonic acid, 5′-deoxy-5′-(*C*-dihydroxyphosphinyl)hydroxymethyladenosine; DCC, *N,N′*-dicyclohexylcarbodiimide; Me_2SO, dimethyl sulfoxide; Me_4Si, tetramethylsilane; P-enolpyruvate, phosphoenolpyruvate; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide.

SCHEME I

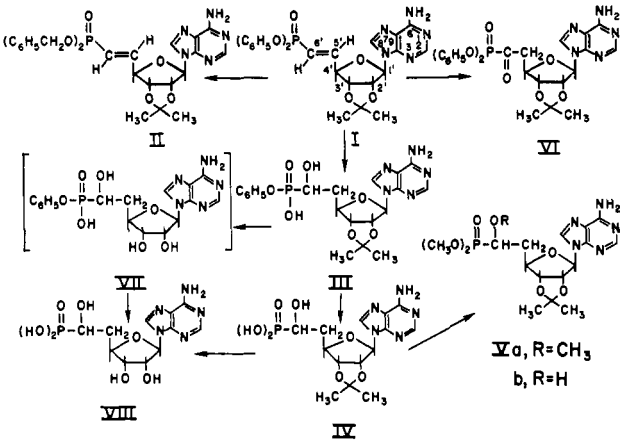

TABLE I: Silica Gel Chromatography.

Compound	R_F Values for Solvent A	B	C	D	Molisch Spray Color
2′,3′-*O*-Isopropylidene-adenosine	0.06	0.30	0.48	0.69	Purple
2′,3′-*O*-Isopropylidene-adenosine 5′-aldehyde	0.08	0.35	0.56		Gray
I (Scheme I)	0.16	0.48	0.68	0.80	Brown
II	0.08	0.37	0.65	0.78	Green
III	0.00		0.09	0.22	Green
IV			0.00	0.05	Green
Va	0.05	0.12	0.49	0.75	Green
Vb	0.02	0.08	0.42	0.69	Green

Materials and Methods

Enzyme Assays. Enzyme reactions were followed by measurement of the rate of change of absorbance at the stated wavelength in a Cary Model 15 spectrophotometer. Initial velocities were linear and proportional to primary enzyme concentration and were not increased by increasing the concentration of secondary enzymes used in coupled assays. Each substrate and inhibitor study employed at least four concentrations of substrate, and kinetic parameters were obtained from Lineweaver–Burke plots.

AMP aminohydrolase (Sigma, Grade IV, from rabbit muscle, 0.018 μg) was employed in 0.90 ml of a system (pH 6.5) 0.01 M in citrate and 0.005 M in KCl. The decrease in absorbance was measured at 265 nm where $\Delta\epsilon$ was calculated to be 6600.

5′-Nucleotidase (Sigma, from *Crotalus adamanteus* venom, 10 μg) was used in a coupled system with adenosine aminohydrolase (Sigma, type I, from intestinal mucosa, 100 μg) in 0.1 M Tris-HCl (pH 8.5). Measurement and calculation of $\Delta\epsilon$ were identical with those for AMP aminohydrolase.

In the case of AMP kinase, for substrate studies with homoadenosine-6′-phosphonate, the system contained lactic dehydrogenase (50 μg), pyruvate kinase (50 μg), and pig or rabbit AMP kinase (Boehringer) (10 μg) in 1 ml of 0.1 M Tris-Cl (pH 7.6) containing KCl (0.1 M), $MgSO_4$ (1 mM), ATP (0.28 mM), P-enolpyruvate (0.87 mM), and NADH (0.38 mM). Assays with AMP as substrate used 0.2 μg of AMP kinase, 4 μg of pyruvate kinase, and 4 μg of lactic dehydrogenase, the remaining conditions being identical with those employed with homoadenosine-6′-phosphonate. After the rate of absorbance decrease at 340 nm diminished to a constant value (30 min or less), the reactions were started by addition of AMP or homoadenosine-6′-phosphonate, and the absorbance decreases at 340 nm were followed.

Methods for Chemical Syntheses. Melting points (uncorrected) were determined by the capillary method. Elemental analyses were by Midwest Microlab, Ltd., Indianapolis, Ind. Infrared (ir) spectra were obtained with a Perkin-Elmer Model 137 spectrophotometer, ultraviolet (uv) spectra with a Cary Model 15 spectrophotometer, and nuclear magnetic resonance (nmr) spectra on Varian XL-100-15 and Jeolco MH 60 spectrometers. Chemical shifts are reported in parts per million from internal Me_4Si or from Me_4Si in a concentric capillary tube in the sample. Thin layer chromatography (tlc) was done on Merck F-254 silica gel plates in (A) ethyl acetate, (B) chloroform–methanol (19:1), (C) chloroform–methanol (9:1), (D) chloroform–methanol (6:4), and (E) ethyl acetate–ethanol (9:1). Silica gel column chromatography was performed with Merck silica gel (0.05–0.2 mm, 70–325 mesh). Chromatography was carried out on Whatman No. 1 paper and on 250-μ cellulose plates in (F) 2-propanol–NH_4OH–water (7:1:2, v/v), (G) 1-butanol–acetic acid–water (4:1:5, v/v, upper), (H) 1-butanol–acetic acid–water (5:2:3, v/v), and (I) 95% ethanol–1 M ammonium acetate (7:3, v/v, pH 7.5). The buffer for paper electrophoresis was 0.05 M triethylammonium bicarbonate (pH 7.5). (See Tables I and II.)

Results

Dibenzyl [9-(5′,6′-Dideoxy-2′,3′-O-isopropylidene-β-D-ribo-hex-5′-enofuranosyl)adenine]-6′-phosphonate (II). A solution of sodium benzoxide (0.04 mol) in benzyl alcohol (40 ml) was added to a solution of the α,β-unsaturated phosphonate I (Jones *et al.*, 1968; Jones and Moffatt, 1968) (5.035 g, 0.0094 mol) in benzyl alcohol (15 ml). After 0.5 hr (25°) ether (40 ml) and an excess of carbon dioxide were introduced. Volatiles were removed *in vacuo* from the filtered solution and the residue was dissolved in ethyl acetate (10 ml) and ether (20 ml) and precipitated with ligroin (bp 30–60°) (200 ml). Reprecipitation gave a yellowish gum (4.50 g, 85.0%). A portion (3.10 g) was chromatographed on silica gel (4.5 × 40 cm); elution with ether (2.5 l.), ethyl acetate (7 l.), and 9:1 ethyl acetate–ethanol (3 l.) successively removed benzyl al-

TABLE II: Paper Chromatography and Electrophoresis.

Compound	R_F Values for Solvent F	G	H	I	Electrophoretic Mobility (pH 7.5), M_{AMP}
I (Scheme I)	0.89		0.89	0.92	
III	0.75	0.63	0.73	0.75	0.42
IV	0.25	0.47	0.59	0.33	0.85
VII	0.55		0.49	0.61	0.38
VIII	0.05	0.11	0.29	0.14	0.89
Adenosine 5′-phosphate	0.13	0.09	0.23	0.10	1.00

cohol, compound I, and compound II. Homogeneous II (solvents B, C, and E) (2.84 g; 78% yield) was obtained as a resinous powder upon removal of solvents: mp 70–100°; ir 3.05, 3.20 (NH_2), 8.01, 8.30 (P=O), 9.45 (P—O—C), 10.20 (trans C=C), 13.61, and 14.35 μ (phenyl); uv λ_{max}^{EtOH} 261 nm (ϵ 15,950); proton magnetic resonance (pmr) ($CDCl_3$, external Me_4Si, 100 MHz) δ 8.91 (s, 1, H-8), 8.65 (s, 1, H-2), 7.12 (br s, 2, NH_2 exchangeable D_2O), 6.87 (d, 1, $J_{1',2'} <$ 0.5 Hz, H-1'), 5.80 (d of d, 1, $J_{2',3'}$ = 5.5 Hz, H-2'), 5.52 (m, 2, H-3', H-4'), 7.70 (m, 1, H-5'), 6.12 (d of d of d, 1, $J_{6',P}$ = 22.0 Hz, $J_{5',6'}$ = 18.0 Hz, $J_{4',6'}$ = 1.5 Hz, H-6'), 5.55 (d, 4, J_{POCH} = 10.5 Hz, benzyl CH_2), 7.94 (s, 10, benzyl phenyl), and 2.06 and 1.95 (two s, 6, CMe_2).

5'-Deoxy-5'-(C-hydroxyphenoxyphosphinyl)hydroxymethyl-2',3'-O-isopropylideneadenosine (III). To a stirred solution of compound I (2.42 g, 4.51 mmol) in dry tetrahydrofuran (30 ml) at 5°, 1 M borane in tetrahydrofuran (7 ml) was added dropwise over 2 min, under N_2. The solution was stirred for 1 hr after which additional borane in tetrahydrofuran (13 ml) was added and the solution was stored at 25° for 18 hr.

The ir spectrum (tetrahydrofuran) within 20 min of BH_3 addition showed alkylborane formation by the appearance of bands at 3.95 (R_2BH), 4.30 and 4.40 (RBH_2), and 4.82 μ (RBH_2 and R_2BH) (Brown, 1962, p 178ff) and by the disappearance of the bands at 6.10 and 10.65 μ due to the 5',6' C=C. The reaction mixture was cooled and tetrahydrofuran–water (1:1) (18 ml) was added. After effervescence ceased, 30% H_2O_2 (27 ml) and 2 M NaOH (54 ml) were added simultaneously. The suspension was stirred at 0–25° for 5 hr, volatiles were removed *in vacuo*, the residue was dissolved in aqueous 30% methanol (300 ml), and the pH was adjusted to 5.0 by the addition of Dowex 50 (H^+) resin (200–400 mesh) at 2°. The resin was filtered off and washed with aqueous 30% methanol. The methanol was evaporated at 15 mm and the aqueous solution was extracted with ether (4 × 250 ml) and evaporated to dryness *in vacuo*. The residue was coevaporated three times with absolute methanol (100 ml) after which the sodium salt of III was obtained as a yellowish powder by lyophilization of its aqueous solution. The yield, determined spectrophotometrically, was 71%; the preparation showed one major spot on chromatography in systems F, G, and H and on electrophoresis. A solution of a portion of this salt (1.57 mmol) in aqueous 5% methanol was chromatographed on a DEAE-cellulose (HCO_3^-) column (3 × 40 cm) with a linear gradient of water (2 l.) to 0.4 M aqueous triethylammonium bicarbonate (2 l.). Evaporation of appropriate fractions gave the triethylammonium salt as a colorless powder (84% recovery). The material was chromatographically and electrophoretically homogeneous: ir 3.03, 3.17 (NH_2), 8.28, 8.44 (P=O), 9.41 (P–O–C), 13.10, and 13.80 μ (phenyl); uv λ_{max}^{MeOH} 260 (ϵ 14,700), 259 (pH 2), and 260.5 nm (pH 12); pmr (carried out on the free acid form, obtained by treatment of the sodium salt in methanol with Dowex 50 (H^-), and purified in solvent system D) (Me_2SO-d_6, external Me_4Si, 100 MHz) δ 8.73 (s, 1, H-8), 8.58 (s, 1, H-2), 7.30 (br s, 2, NH_2), 6.48 (d, 1, $J_{1',2'}$ = 3.0 Hz, H-1'), 5.85 (d of d, 1, $J_{2',3'}$ = 6.0 Hz, H-2'), 5.20 (d of d, 1, $J_{3',4'}$ = 3.0 Hz, H-3'), 4.48 (m, 1, H-4'), 2.20 (m, 2, H-5'), 3.50 (br m, 1, H-6'), 7.51 (m, 5, phenyl), and 1.92 and 1.70 (two s, 6, CMe_2); ^{31}P nmr (ammonium salt, D_2O, external H_3PO_4, 40.5 MHz) δ − 8.72 (m, J_{PCH} = 24.0 Hz, collapses to s upon heteronuclear decoupling).

The organoborane intermediate from I was treated with the Jones reagent (Bowers *et al.*, 1953) and the mixture subjected to silica gel chromatography to give in 5% yield diphenyl [9-(5'-deoxy-2',3'-*O*-isopropylidene-β-D-*ribo*-hexofuran-6-ulosyl)adenine]-6'-phosphonate (VI): pmr ($CDCl_3$, internal, Me_4Si, 100 MHz) δ 8.37 (s, 1, H-8), 7.94 (s, 1, H-2), 6.29 (br s, 2, NH_2), 6.10 (d, 1, $J_{1',2'}$ = 2.0 Hz, H-1'), 5.61 (d of d, 1, $J_{2',3'}$ = 6.0 Hz, H-2'), 5.02 (d of d, 1, $J_{3',4'}$ = 4.0 Hz, H-3'), 4.34 (m, 1, H-4'), 2.24 (m, 2, H-5'), 7.29 (m, 10, phenyl), and 1.64 and 1.41 (two s, 6, CMe_2); ir 5.64 (C=O), *cf.* 5.68 μ (C=O) for $(C_6H_5O)_2P(O)C(O)CH_3$ (Ogata and Tomioka, 1970).

5'-Deoxy-5'-(C-dihydroxyphosphinyl)hydroxymethyl-2',3'-O-isopropylideneadenosine (IV). A solution of the sodium salt of III (0.870 mmol) in 0.1 M Tris buffer (5 ml), 0.3 M magnesium acetate (5 ml), and water (5.5 ml) was brought to pH 8.8 by addition of solid Tris. Crude phosphodiesterase from *Crotalus atrox* (type IV, Sigma Chemical Co.) (45 mg) was added and the solution was shaken overnight at 37°. Cellulose tlc (solvents F and H) and paper electrophoresis showed only one component. The solution was heated at 90° for 5 min, filtered, and adjusted to pH 5.1 with Dowex 50 (H^+) resin (3 ml). The resin was washed with aqueous 10% methanol. The filtrate and washings were evaporated to *ca.* 50 ml and passed through a Dowex 50 (K^+) column (1.5 × 6.0 cm), which was washed with water. The combined eluates, which contained IV in 91% yield, were chromatographed on Whatman No. 3MM paper in solvent G to separate compound IV (R_F 0.5) from a minor component (R_F 0.4). Compound IV was eluted with aqueous 50% methanol (71.5% recovery), and obtained as a colorless powder which was dried over P_2O_5, 0.01 mm, at 78.5°; ir (KBr pellet) 3.02, 3.15 (NH_2), 8.30, 8.42 (P=O), and 9.50 μ (P–O–C); uv $\lambda_{max}^{H_2O}$ 257 (pH 2) (ϵ 14,600) and 260 nm (pH 6) (ϵ 15,170); pmr (D_2O, external Me_4Si, 100 MHz) δ 8.70 (s, 1, H-8), 8.63 (s, 1, H-2), 6.58 (d, 1, $J_{1',2'}$ = 2.0 Hz, H-1'), 5.88 (d of d, 1, $J_{2',3'}$ = 6.0 Hz, H-2'), 5.42 (d of d, 1, $J_{3',4'}$ = 3.0 Hz, H-3'), 4.62 (m, 1, H-4'), 2.28 (m, 2, H-5'), 4.12 (br m, 1, H-6'), 2.11 and 1.88 (two s, 6, CMe_2), and 1-butanol Me at 1.70.

Anal. Calcd for $C_{14}H_{19}N_5O_7PK \cdot 0.25\ C_4H_{10}O$: C, 39.3; H, 4.7; N, 15.3; P, 6.8. Found: C, 38.6; H, 4.9; N, 14.8; P, 6.5.

Methylation of IV. The barium salt of IV (2.05 mmol) was prepared from a solution (pH 8) of the sodium salt of IV and an excess of barium acetate by precipitation with acetone. A solution of this salt in 225 ml of aqueous 10% methanol was adjusted to pH 5.9 by the addition of Dowex 50 (H^+) (200–400 mesh) resin, and then applied to a Dowex 50 (H^+) column (1.7 × 6 cm) at 5°. The column was washed with 1 l. of H_2O. The eluates, which contained OD units equivalent to 1.79 mmol of nucleotides, were lyophilized. The colorless powder, which contained VIII, adenine, and IV, was suspended at 0° in 65 ml of absolute methanol. Diazomethane (0.7 g in 31 ml of ether) was added over 10 min. After 15 min at 15°, methanol (50 ml) followed by 1.4 g of diazomethane in 62 ml of ether was added. The mixture was stirred overnight at 25° and evaporated to dryness. The residual gum was dissolved in chloroform and chromatographed on silica gel (3 × 88 cm) by elution with chloroform (2 l.), followed by linear gradients of chloroform (2. l) to chloroform–methanol (1:1) (2 l.) and 1:1 chloroform–methanol (1 l.) to 1:2 chloroform–methanol (1 l.). Fractions (15 ml each) 201–249 contained Va (17.3% of the OD units methylated), 250–270 contained Vb (5.5%), and 271–287 contained adenine (17.5%). Evaporation of solvents gave Va as a yellowish gum containing polymethylene: ir 8.25 (P=O) and 9.50 μ (P–O–C); uv λ_{max}^{MeOH} 259 nm; pmr ($CDCl_3$, external, Me_4Si, 100 MHz) δ 8.87 (s, 1, H-8), 8.55 (s, 1, H-2), 7.68 (br s, 2, NH_2, exchanges with D_2O), 6.60 (d, 1, $J_{1',2'}$ = 2.5 Hz, H-1'), 6.02 (d of d, 1, $J_{2',3'}$ = 6.5 Hz,

TABLE III: Substrate Properties of Homoadenosine-6′-phosphonic Acid (VIII) with AMP Aminohydrolase and AMP Kinase.

Compd	K_m (mM)	V_{max} (μmol per min per mg of Protein)	V_{max} Rel to AMP (%)
	AMP Aminohydrolase		
AMP	0.40	1100	100
VIII	4.0	1000	91
	0.025	31	2.8
	AMP Kinase (Rabbit Muscle)		
AMP	0.50	119	100
VIII	0.71	0.20	0.17
	AMP Kinase (Pig Muscle)		
AMP	0.19	92.6	100
VIII	0.17	0.14	0.15

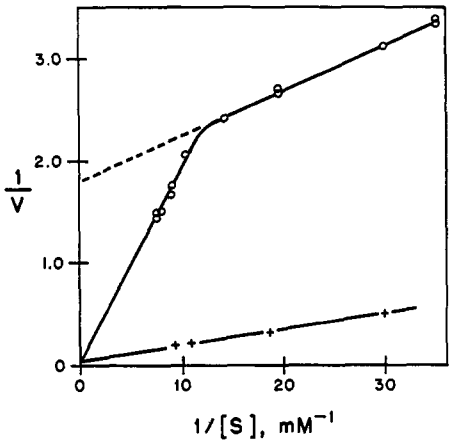

FIGURE 1: Deamination of AMP (+) and homoadenosine-6′-phosphonate (O) by AMP aminohydrolase of rabbit muscle; V represents nanomoles of substrate converted per minute.

H-2′), 5.49 (d of d, 1, $J_{3',4'}$ = 3.5 Hz, H-3′), 4.75 (d of d of d, 1, $J_{4',5'}$ = 7.0 Hz, $J_{4',6'}$ = 3.0 Hz, H-4′), 2.50 (m, 2, H-5′), 3.56 (d of d of t, 1, $J_{5',6'}$ = 11.0 Hz, $J_{6',P}$ = 24.0 Hz, H-6′), 4.19 (d, 6, J_{POCH} = 11.0 Hz, PO $(OMe)_2$), 4.17 (s, 3, OMe), 2.13 and 1.91 (two s, 6, CMe_2), and 1.80 (m, 2, polymethylene); signals 2.50, 3.56, and 4.19 collapsed to sharper m, d of d, and s, respectively, upon heteronuclear decoupling; mass spectrum (50 eV) *m/e* (relative intensity) 443 (0.5), 428 (9.5), 414 (17), 279 (39), 221 (24), 164 (100), and 136 (47).

Anal. Calcd for $C_{17}H_{26}N_5O_7P\cdot-(CH_2)_{x=1}-$: C, 47.3, H, 6.2. Found: C, 47.4, H, 6.3.

The compound in fractions 250–270 was concluded to be Vb from its tlc behavior and nmr spectrum.

5′-Deoxy-5′-(C-dihydroxyphosphinyl)hydroxymethyladenosine (VIII). A solution at pH 2.5 of III (sodium salt) (0.24 mmol) in water (50 ml), methanol (15 ml), and acetic acid was heated at 90° for 1 hr. The solution was evaporated *in vacuo* and the residue was dissolved in 0.1 M Tris buffer, pH 8.8 (4 ml), 0.3 M magnesium acetate (4 ml), and water (5 ml); solid Tris (to restore the pH to 8.8) and phosphodiesterase from *Crotalus atrox* (28 mg) were added, and the mixture was kept for 2 hr at 37°, then heated at 90° for 10 min, filtered, and adjusted at 2° to pH 4.5 by the addition of Dowex 50 (H^+). The filtered solution was adjusted to pH 8.5 with LiOH and barium acetate (1.280 mmol) was added. The suspension was centrifuged and 30 ml of ethanol was added to the supernatant. The precipitated barium salt of VIII (74% yield from III) was homogeneous upon paper electrophoresis and contained VIII as the major component by chromatography in systems H and I. A portion (0.16 mmol) was chromatographed on a Dowex 1 (Cl^-) (200–400 mesh) column (2 × 38 cm) using a linear gradient from 0.001 (1.5 l.) to 0.008 N HCl (1.5 l.) at 5°. Fractions containing VIII were neutralized with triethylamine and evaporated *in vacuo*. The residue was washed with ethanol to remove triethylamine hydrochloride, dissolved in water, and then passed through Dowex 50W (K^+) to give, after evaporation, the potassium salt of VIII as a white solid (37% recovery), which was homogeneous upon paper electrophoresis and chromatography in solvents F, G, H, and I: ir 3.05, 3.15 (NH_2), 8.25 (P=O), and 9.30 μ (P–O–C); uv $\lambda_{max}^{H_2O}$ 257 (pH 2) and 259 nm (pH 6).

When compound IV was heated in aqueous acetic acid (pH 2.1) at 90° for 1.2 hr, the product was chromatographically (solvents F and H) and electrophoretically indistinguishable from VIII obtained from VII. The reaction mixture contained 13% of IV, 72% of VIII, and 14% of adenine.

AMP Aminohydrolase Studies. Compound VIII gave a complex rate curve (Figure 1). At concentrations above 90 μM the plot gave values of K_m = 4 mM and V_{max} = 1000 μmol per min per mg of protein. At concentrations below this a sharp inflection in the plot occurred to give a series of points which, when extrapolated, gave K_m = 25 μM and V_{max} = 31 μmol per min per mg of protein. When the aminohydrolase-catalyzed transformation of VIII was allowed to proceed to completion, the total change in absorbance at 265 nm was the same, within experimental error of 5%, as that produced from the same initial level of AMP, thus confirming that both 6′ epimers of VIII were substrates. The kinetic constants of AMP (Table III) were similar to those calculated from the results of Smiley *et al.* (1967) (V_{max} = 1380 μmol per min per mg of protein; K_m = 500 μM.) Compound VIII produced competitive inhibition at levels of 53.6 and 107 μM; the replot of inhibitor concentration *vs.* slope was linear and gave K_i = 210 μM.

5′-Nucleotidase Studies. With the coupled assay system employed, AMP gave values of K_m = 100 μM and V_{max} = 1.14 μmol per min per mg of protein. Compound VIII was examined as an inhibitor of AMP hydrolysis at levels of 50 and 100 μM (Figure 2). At AMP levels near 25 μM, small (*ca.* 10%) inhibition was noticeable, but as AMP levels increased, the inhibition increased markedly, to the extent that 70% inhibition was obtained with 95 μM AMP and 100 μM of compound VIII.

AMP Kinase Studies. The K_m and V_{max} values of AMP and VIII are given in Table III. K_m values of 0.26 and 0.50 mM for AMP and the rabbit enzyme have been reported previously (Noda, 1958; Callaghan, 1959). Compound VIII, tested at levels of 27, 67, and 201 μM, produced competitive inhibition; the replot of inhibitor level against slope was linear and gave K_i = 380 μM.

An assay mixture containing 10 μg of rabbit AMP kinase was allowed to stand for 15 min after which the rate of decrease in absorbance at 340 nm had reached a slow constant value (0.00866 per hr). Sufficient AMP was then added to give an initial concentration of 73 μM. In duplicate experiments

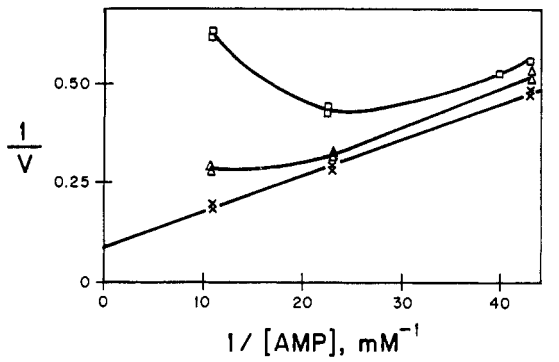

FIGURE 2: Inhibition by 50 μM (△) and 100 μM (□) homoadenosine-6′-phosphonate of the action of 5′-nucleotidase on AMP; V, nanomoles of AMP dephosphorylated per minute.

the decrease in absorbance at 340 nm was 0.870 and 0.906, respectively (calculated for 100% conversion, 0.898, based on ϵ 6220 for NADH), during 6 min, after which time the base-line rate was resumed. The procedure was repeated with a 72 μM solution of VIII and 10 μg of enzyme, whereupon after 8 hr the total decrease in absorbance at 340 nm was 0.74 and the base-line rate had been resumed. After subtracting the base-line decrease in absorbance (0.07) during the 8-hr period, the change due to AMP kinase action on VIII was 0.670, or 74.6% of the expected change if each molecule of VIII had given rise to two molecules of NAD^+ as in the case of AMP. In an identical experiment with 20 μg of the pig muscle kinase, the absorbance decrease produced by VIII was 74.5% of the maximum theoretical amount.

Discussion

Synthesis of Homoadenosine-6′-phosphonic acid. Prior to these studies the only examples of nucleoside phosphonates isosteric with nucleoside 5′-phosphates by virtue of replacement of the C–O–P system by C–CH_2–P were 6′-deoxyhomouridine- and 6′-deoxyhomoadenosine-6′-phosphonic acids (Jones and Moffatt, 1968). These compounds were obtained by condensation of the respective 2′,3′-*O*-isopropylidene nucleoside 5′-aldehydes with the Wittig reagent diphenyl triphenylphosphoranylidenemethylphosphonate (Jones *et al.*, 1968) to give α,β-unsaturated nucleoside phosphonates (*e.g.*, compound I) followed by reduction of the olefinic linkages and removal of protecting groups. In addition, the monoethyl ester of 6′-deoxyhomoadenosine-6′-phosphonic acid has been obtained *via* reaction of a suitably blocked ribofuranose 5′-aldehyde with tetraethyl methylenediphosphonate followed by attachment of the adenine residue by means of a further series of reactions (Montgomery and Hewson, 1969). The reported accessibility of the α,β-unsaturated phosphonate I prompted us to examine the feasibility of obtaining the α-hydroxy phosphonate VIII by addition of the elements of water across the α,β double bond. The phenyl groups of I resisted removal by catalytic hydrogenolysis, and to enable hydrogenolytic deblocking to be employed in final steps of the projected synthesis, I was treated with sodium benzoxide in benzyl alcohol, a procedure which gave the dibenzyl phosphonate II in high yield.

Attempts were made to effect Markovnikov hydration of the alkenyl systems of I and II by means of the oxymercuration–demercuration procedure of Brown and Geoghegan (1967), but both I and II were unaffected by treatment with mercuric acetate in aqueous tetrahydrofuran. This accords with the inertness toward mercuric acetate of a sugar derivative with a vinyl system even less hindered than those of I and II (Rosenthal and Sprinzl, 1971). The hydroboration–oxidation procedure for anti-Markovnikov hydration of olefins (Zweifel and Brown, 1963) was then investigated. The olefinic linkage of the dibenzyl ester II readily underwent hydroboration with borane in tetrahydrofuran. Attempts were made to oxidize the product with alkaline hydrogen peroxide, the Jones reagent (Brown and Garg, 1961), or CrO_3 in aqueous pyridine (Cornforth *et al.*, 1962), but silica gel chromatography and paper electrophoresis indicated little or no reaction even after 1 day. Aqueous potassium permanganate converted *ca.* 25% of it to a mixture of products of which the major was adenine. No oxidation procedure yielded products which were anionic at neutral pH.

Hydroboration of the diphenyl ester I also occurred readily, but in this case oxidation of the organoborane with alkaline hydrogen peroxide gave a 70% yield of a single product which was concluded to be the monophenyl α-hydroxy phosphonate III on the basis of its ultraviolet, infrared, and proton magnetic resonance spectra. Assignment of the chemical shifts of H-5′ and H-6′ was made from the reported values for methyl 5-deoxy-2,3-*O*-isopropylidene-β-D-allofuranoside (Ryan *et al.*, 1964; Montgomery and Hewson, 1964) and the similar values found in this laboratory for unambiguously synthesized 6′-cyano-6′-deoxyhomoadenosine-6′-phosphonate (Sasaki and Hampton, 1972). The phenyl group of compound III was readily removed by snake venom phosphodiesterase to furnish the 2′,3′-*O*-isopropylidene derivative IV of the free phosphonic acid. The pmr evidence that III and IV are α- as opposed to β-substituted phosphonates was corroborated when Jones oxidation of the organoborane from I gave the α-keto phosphonate VI, although the yield in a single trial was not high. A signal from the 6′-hydroxyl of III or IV was absent from their pmr spectra due to solvent exchange, and to obtain direct evidence for the presence of this 6′-hydroxyl, the methylation of IV was undertaken. When IV (as the free acid or its barium salt) was treated with boron trifluoride in methanol no methylation occurred, but treatment of the free acid with diazomethane in ether–methanol gave the dimethyl α-hydroxy phosphonate Vb and the dimethyl α-methoxy phosphonate Va in a 1:3 ratio. The presence in Va of the 6′-methoxyl group was established from its pmr spectrum. The structure of Va was confirmed by its high resolution mass spectrum in which the molecular ion was present in 0.5% relative abundance; the major fragmentation path gave 9-formyladenine + H (100%), which is usually an abundant ion in adenine nucleoside spectra (Shaw *et al.*, 1970), together with the remaining portion of the molecule, $C_{11}H_{20}O_8P$ (39%).

Treatment of the nucleotide III with aqueous acid gave phenyl homoadenosine-6′-phosphonate VII, which upon treatment with snake venom phosphodiesterase furnished homoadenosine-6′-phosphonate VIII, in 70% overall yield. Homoadenosine-6′-phosphonate was also obtained in high yield by acidic treatment of IV.

The organoborane obtained from I possessed infrared bands in the 3.95–4.82-μ range characteristic of mono- and dialkylboranes. The formation of a trialkylborane is unlikely from steric considerations (Brown, 1962, p 205). That oxidation of these organoboranes furnished an α-hydroxyphosphonate in high yield shows that the boron is bonded to C-6′ of I. This result accords with evidence that electron-withdrawing substituents (in this case the diphenylphosphono

group) attached to olefins tend to direct the entering boron onto the carbon atom nearest to that substituent (Brown and Keblys, 1964). The inertness toward alkaline peroxide of the organoborane from the dibenzyl ester II may be related to the finding that at the pH employed II itself was stable for at least three times the time needed for complete oxidation of the diphenylorganoborane, whereas the diphenyl ester I was hydrolyzed to the corresponding monophenyl ester in a fraction of the time required for liberation of III from its organoborane precursor. This suggests that initial hydrolytic removal of one phenyl group may be necessary for subsequent insertion of oxygen into the carbon–boron bond of the organoborane formed from I.

Enzyme Studies. Both of the 6′ epimers of VIII were substrates of AMP aminohydrolase from rabbit muscle. The V_{max} value of the more active epimer is essentially the same as that of AMP itself and five times greater than that of the AMP analog in which the 6′ hydroxyl of VIII is replaced by hydrogen (Hampton *et al.*, 1973). It can be speculated that this enhanced substrate activity might result from an ability of the 6′ oxygen of the more active epimer of VIII to perform some function of O-5′ of AMP in the catalytic process. That the V_{max} value of the less active epimer is eightfold less than that of its 6′-deoxy analog could indicate an insufficiency of space in the enzyme–substrate complex to accommodate O-6′ of that epimer, since data on the structural requirements for substrate activity with this enzyme, discussed previously (Hampton *et al.*, 1972), strongly indicate that the purine ring, the sugar ring, and the phosphate segment of AMP all interact simultaneously with the enzyme at some stage of the catalytic process. At that time, the permissible range of the 5′,6′ torsion angle would hence be very restricted and the 6′-hydroxyl groups of the two epimers would of necessity occupy different positions in the enzyme–substrate complex.

Inhibition of 5′-nucleotidase action by VIII was strongly enhanced by AMP (Figure 2). The AMP-promoted inhibition apparently involves the 6′ hydroxyl of VIII because replacement of this hydroxyl by a cyano group (Sasaki and Hampton, 1972) abolishes the effect. This suggests that the 6′-O of VIII may be able to mimic some function of the 5′-O of AMP in the enzyme–AMP interaction, as was speculated in the case of AMP aminohydrolase.

With the pig and rabbit adenylate kinases both 6′ epimers of VIII were substrates but neither was more than 5% as effective as even the less active of them was with AMP aminohydrolase (Table III), possibly because in the case of these kinases the 6′-hydroxyl group of VIII is much closer to the site of the catalyzed reaction, and the steric and electronic requirements for substrate activity are more exacting in that area of the enzyme–substrate complex. These requirements were shown in the similar case of pyruvate kinase by its ability to utilize as substrate only one of the epimers of VIII after their phosphorylation by AMP kinase. This property enabled us to determine that the 6′ epimers of VIII were present in a 1:1 ratio through measurement of the amount of NAD^+ formed from VIII in the coupled assay of AMP kinase with pyruvate kinase and lactic dehydrogenase. It can be noted from Table III that the V_{max} of VIII relative to that of AMP is the same, within experimental error, for the rabbit and the pig AMP kinases, suggesting that those portions of the two kinases which are adjacent to the CH_2–O–P group of AMP in the kinase–AMP complex may possess many structural features in common.

Compound VIII was a linear competitive inhibitor of phosphorylation of AMP by pig muscle AMP kinase, and the fact that both 6′ epimers are substrates makes it probable that the enzyme–inhibitor dissociation constant derived from these inhibition studies is a measure of the affinity of VIII for the AMP site. It is possible that this dissociation constant (0.38 mM) corresponds to binding of mainly one 6′ epimer of VIII, in which case the true value would be approximately one-half, since the epimers are present in equal proportion. On the assumption that for pig AMP kinase the K_m of AMP (0.19 mM) is equal to the enzyme–substrate dissociation constant of AMP as it is with rabbit AMP kinase (Noda, 1962), it would follow that introduction of a hydroxyl group α to the phosphorus of AMP *via* C-6′ of VIII has not greatly affected affinity for the AMP site of pig AMP kinase.

The substrate and inhibitor properties of VIII with AMP aminohydrolase were qualitatively the same as with the AMP kinase and showed that the dissociation constant for binding to the AMP site was 210 μM if both epimers were binding to an equal extent, or 105 μM if one epimeric species was binding preferentially. When the 6′ hydroxyl of VIII is replaced by a hydrogen atom the dissociation constant is 58 μM (Hampton *et al.*, 1973), indicating that there is sufficient space in the O-5′ region of the aminohydrolase–AMP complex to accommodate the 6′ hydroxyl of at least one of the 6′ epimers of VIII.

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The α-Chymotrypsin-Catalyzed Hydrolysis of *N*-Acetyl-L-tryptophan *p*-Nitrophenyl Ester in Dimethyl Sulfoxide at Subzero Temperatures†

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ABSTRACT: The effect of subzero temperatures and aqueous Me_2SO solutions (liquid state) on the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan *p*-nitrophenyl ester has been investigated. As the Me_2SO concentration increases, the value of k_{cat} decreases proportionately to the decrease in water concentration; however, the value of K_m increases exponentially. The effect on K_m is attributed to the less polar Me_2SO binding more strongly than water to the substrate binding site. The pK_a for k_{cat} (deacylation) is unaffected by the presence of 65% aqueous Me_2SO. A Van't Hoff plot of k_{cat} using 65% Me_2SO was linear over the temperature range 0 to $-45°$. Below this temperature turnover occurred extremely slowly; since acylation was still very rapid the acyl-enzyme could be accumulated and was isolated by gel filtration. The intrinsic spectral properties of the enzyme (ultraviolet, circular dichroism, and fluorescence) were examined as a function of Me_2SO concentration in order to detect any Me_2SO-induced structural changes. No structural effects were observed, although solvent effects were noted. Similarly the effect of temperature (0 to $-80°$) on the intrinsic fluorescence of α-chymotrypsin in 65% Me_2SO did not indicate any structural changes. We conclude that the pathway of the enzyme-catalyzed reaction in 65% aqueous Me_2SO at subzero temperatures is essentially unchanged from that in the absence of Me_2SO and at ambient temperatures.

The rapidity of enzyme-catalyzed reactions of specific substrates has been a major problem in studying the dynamic processes occurring during the catalysis. In an attempt to overcome this problem rapid reaction techniques, *e.g.*, stopped-flow and temperature-jump spectrophotometry, have been used with a considerable degree of success with respect to determining kinetic parameters. However, in order to propose a detailed mechanism one must know the nature of the various intermediates and transition states on the reaction pathway. Some success has been achieved in this direction by the use of nonspecific substrates and substrate analogs. However the fact that intermediates such as acyl-enzymes can be isolated using nonspecific substrates may indicate that they are not on the productive pathway for good (specific) substrates.

We have begun a comprehensive study of the use of subzero temperatures (and fluid solutions) as a means of obtaining information about the nature of intermediates in the catalysis of specific substrates. This approach is predicated on the following basis. The dramatic decrease in reaction rates, and the enhanced difference in rates for different enthalpies of activation which occur with decreases in temperature of the order of 100° could allow the accumulation of intermediates in enzyme-catalyzed reactions. The "trapped" intermediate could then be studied by a variety of chemical and physical techniques to provide information concerning its structure (especially enzyme–substrate interactions), as well as yielding kinetic and thermodynamic data. We are initially applying the technique to some well studied enzymes, such as α-chymotrypsin, so that we may compare data obtained at the low temperatures with that obtained by other means.

In order to preclude problems arising from rate-limiting diffusion, fluid solvent systems are required. A few aqueous–organic solvent systems are known which are fluid to temperatures in the range of $-100°$, and which may be suitable for the contemplated experiments. Previous studies have shown that some enzymes are stable in high concentrations of potentially useful aqueous–organic solvents, *e.g.*, trypsin

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