Pyridoxal Phosphate. 5.[1] 2-Formylethynylphosphonic Acid and 2-Formylethylphosphonic Acid, Potent Inhibitors of Pyridoxal Phosphate Binding and Probes of Enzyme Topography

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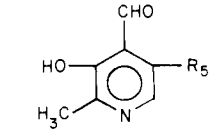
The title compounds (17 and 21) were prepared in good yield by synthesis of the phosphonate diester acetals (14 and 19), deesterification with chloro- or bromotrimethylsilane, hydrolysis of the acetal group, and formation of the characterized barium salts. The 3-carbon aldehydophosphonic acids (17 and 21) were potent inhibitors ($K_i = 2 \times 10^{-6}$) of pyridoxal phosphate (PPal) binding to apoaspartate aminotransferase (AAT) and are believed to span between and bind to the enzymic functional groups which bind the aldehyde and phosphate groups of PPal.

In previous papers in this series,[2] several types of phosphonic acid analogues of pyridoxal phosphate (PPal, 1) have been shown to inhibit the binding of PPal to its apoenzymes. The phosphonic acids most closely analogous to PPal and pyridoxol phosphate (PPol, 2), namely 3–6, in which the ester oxygen is replaced by a carbon unit, were found to bind to aspartate aminotransferase (AAT) almost as well as does PPal.[2] Other phosphonic acid analogues of PPal, such as 2-formylphenylphosphonic acids[3] (e.g., 7) and those[4] (e.g., 8) related to toxopyrimidine phosphate (9),[5] have also proved to be good inhibitors of the binding of PPal to AAT.[3,4]

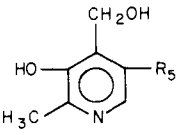
To extend these findings and to provide an additional basis for developing potential active-site-directed irreversible inhibitors[2,6] (or affinity labels[7]) of PPal-dependent enzymes, we sought the synthesis and biological evaluation of aliphatic, acyclic phosphonic acid derivatives related to PPal.

The specific goals for this study were (1) to simplify the structure necessary to obtain good inhibitor–apoenzyme binding and (2) to provide analogues that could be used to determine the distance and topography of the enzyme surface relating the binding points of the formyl and phosphoryl (phosphonyl) groups of PPal, the two groups[8] most important for binding PPal to its apoenzyme. (3) The target compounds were also expected to make available simple acyclic intermediates that could be used to prepare alicyclic analogues of PPal. The general formula for the target compounds is shown by 10.

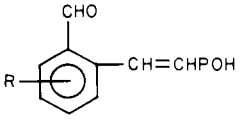
During the course of this work it was reported[9] that a series of aldehydo phosphates (11) was inhibitors of the holoenzyme of AAT. Inhibitory activity required the presence of the formyl (aldehydo) and the phosphate groups in the same molecule. Inhibition appeared due, at least in part, to the formation of an aldimine between the formyl group of the inhibitor and an amino group on the enzyme surface (proposed[9] to be an ϵ-aminolysyl group at the catalytic site). The inhibition was also dependent on the presence of a phosphoryl group in the aldehydo inhibitor. These observations on the holo-AAT, and the knowledge that phosphate anion[10–12] and that phosphate and sulfate monoesters[13–15] unrelated to PPal inhibited the recombination of PPal to AAT, strengthened our belief that simple acyclic phosphonic acid analogues would be good inhibitors of PPal-dependent enzymes.



1, $R_5 = CH_2OPO_3H_2$
3, $R_5 = CH_2CH_2PO_3H_2$
5, $R_5 = CH{=}CHPO_3H_2$



2, $R_5 = CH_2OPO_3H_2$
4, $R_5 = CH_2CH_2PO_3H_2$
6, $R_5 = CH{=}CHPO_3H_2$



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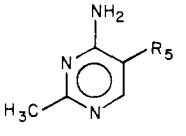


8, $R_5 = CH{=}CHPO_3H_2$
9, $R_5 = CH_2OPO_3H_2$

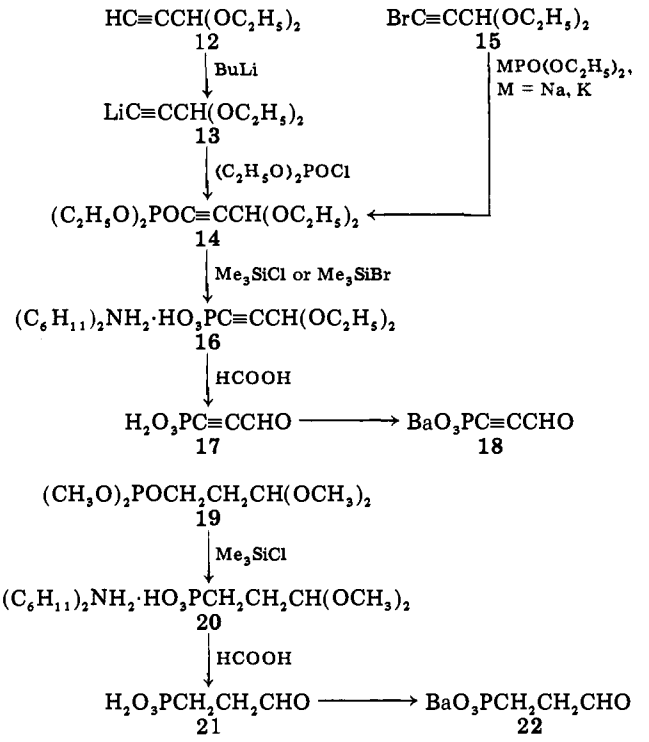
$OHC\text{-}(CH_x)_y\text{-}PO_3H_2$

10, $x = 0$, 1, or 2
$y = 2$, 3, or 4

$OHC\text{-}(CHOH)_n\text{-}CH_2OPO_3H_2$

11, $n = 0$, 1, or 2

Chart I



The synthesis of the subject compounds is outlined in Chart I. For the synthesis of the desired acetylenic phosphonic acid 17, the key unsaturated phosphonate intermediate, diethyl 3,3-diethoxy-1-propyn-1-ylphosphonate (14), was best prepared (in 70–80% yield) by reaction of 3,3-diethoxy-1-lithio-1-propyne (13) [prepared from 3,3-diethoxy-1-propyne (12)[16]] with diethyl phosphorochloridate at −65 °C. The product was easily purified by distillation. An alternative route, the reaction of 1-bromo-3,3-diethoxy-1-propyne (15)[17] with potassium or sodium diethyl phosphite according to published procedures,[18] gave only 30–40% yields of 14. Use of lithium diethyl phosphite in this procedure gave no yield of 14. The impurities from the use of sodium diethyl phosphite, which poisoned hydrogenation catalysts used to reduce 14

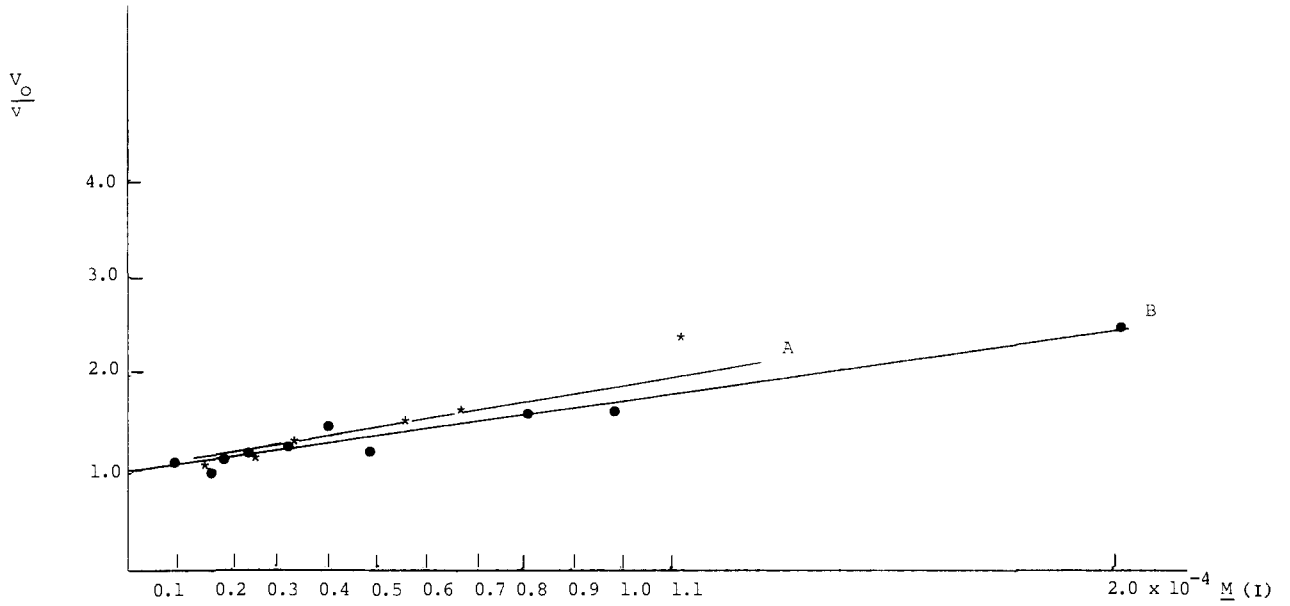


Figure 1. Lineweaver-Burke plots of enzyme kinetic data: A, dicyclohexylamine salt of 3,3-diethoxypropylphosphonic acid; B, dicyclohexylamine salt of 3,3-diethoxy-1-propyn-1-ylphosphonic acid.

(see subsequent papers), could not be easily removed by distillation; tedious TLC methods were necessary.

The deesterification of the phosphonate ester **14** was done by the Arbuzov-type dealkylation introduced by Rabinowitz[19] using chlorotrimethylsilane (Me_3SiCl). However, the method was dramatically improved by substituting the much more reactive bromotrimethylsilane (Me_3SiBr) for Me_3SiCl. Thus, simple mixing of **14** with Me_3SiBr at room temperature gave an exothermic reaction, which by NMR analysis appeared to yield the intermediate silyl ester in quantitative yield and which, after methanolysis, allowed isolation of the salt **16** in 79% yield. Use of the normal Me_3SiCl procedure to dealkylate **14** required heating of the reaction mixture in a steel bomb from 3 days at 100 °C and gave only 45% yield of salt **16**. The salt had no discrete melting point and was characterized by its spectral properties and by elemental analysis.

Hydrolysis of the acetal group was readily done with 97% formic acid at 50–60 °C and was complete within 15 min as determined by NMR analysis. The crude product appeared by NMR analysis to be only a mixture of dicyclohexylamine formate and **17**. The aldehydophosphonic acid (**17**) was most conveniently isolated as the insoluble barium salt. Attempts to isolate the pure dicyclohexylamine salt from the reaction mixture resulted in tars only.

The desired saturated phosphonic acid **21** was prepared by deesterification of the known[20] acetal phosphonate (**19**) using the Me_3SiCl method.[19] The time required was 2 days at reflux. The dicyclohexylamine salt of acid **20** was isolated in 83% yield. Formic acid hydrolysis of **20**, as done for **16**, gave **21**, isolated as the barium salt **22** in 45% yield.

Both barium salts **18** and **22** were characterized by their ir and NMR spectra and by their correct elemental analysis for C, H, P, and Ba.

Biological. The enzymatic evaluations were done with aldehydophosphonic acids **17** and **21**. It was found more convenient to prepare **17** and **21** in situ by formolysis of **16** and **20**, removal of the formic acid by lyophilization, and dilution of the residue to required concentrations. This was done because of the (apparent) quantitative facile formolysis of **16** and **20**, as shown by NMR analysis, and by the relatively tedious preparation of the barium salts. Enzymic study of the purified barium salts showed them to have the same inhibitory activity as the products from the formolysis route.

Table I. Inhibition of Recombination of Apoaspartate Aminotransferase and Pyridoxal Phosphate by 3-Carbon Phosphonic Acids

Compd	K_i,[a] M	$([I]/[S])_{0.5}$[b]
17	1.6×10^{-6}	6.5
21	2.2×10^{-6}	8.7
16[c]	$\sim 1 \times 10^{-4}$	~342
20[c]	$\sim 5 \times 10^{-5}$	~110

[a] The inhibitor constant calculated by the Lineweaver-Burke method using the conditions described in the Experimental Section. [b] The ratio of the concentration of the inhibitor to substrate (PPal) to give 50% inhibition using the conditions described in the Experimental Section. [c] Assay of these compounds gave inconsistent results for both K_i and $([I]/[S])_{0.5}$. Thus, data were selected which had in the case of the $([I]/[S])_{0.5}$ an intercept close to 1.0 and which were also internally consistent (high correlation coefficient). These data are thus recorded as approximate.

The results of the enzyme inhibition studies are presented in Figures 1 and 2 and the K_i and $[(I)/(S)]_{0.5}$ values obtained for **16**, **17**, **20**, and **21** are summarized in Table I. Compounds **16**, **17**, **20**, and **21** were all competitive inhibitors of the PPal binding to apo-AAT. The aldehydophosphonic acids, **17** and **21**, were much more potent inhibitors ($K_i = 1.6\text{–}2.2 \times 10^{-6}$ M) than the acetal phosphonic acids **16** and **20** ($K_i = 10^{-4}\text{–}10^{-5}$ M). Studies independent of the enzymic analysis showed that the acetal groups were stable under the conditions of enzymic evaluation, thus showing that the results obtained were due to the acetal group and not products of partial hydrolysis. Incubation of the inhibited enzyme with PPal caused a progressive recovery of enzyme activity with time.

The ability of the aldehydophosphonic acids **17** and **21** to quench protein fluorescence of AAT, in a manner similar to the known[21] quenching by PPal, was also tested. Neither **17** nor **21** at concentrations as high as 1.4×10^{-4} M caused any quenching of fluorescence. Pyridoxal phosphate in concentrations as low as 1.6×10^{-6} M caused significant quenching of protein fluorescence.

Discussion

This study has revealed the inhibitor constants for **17**

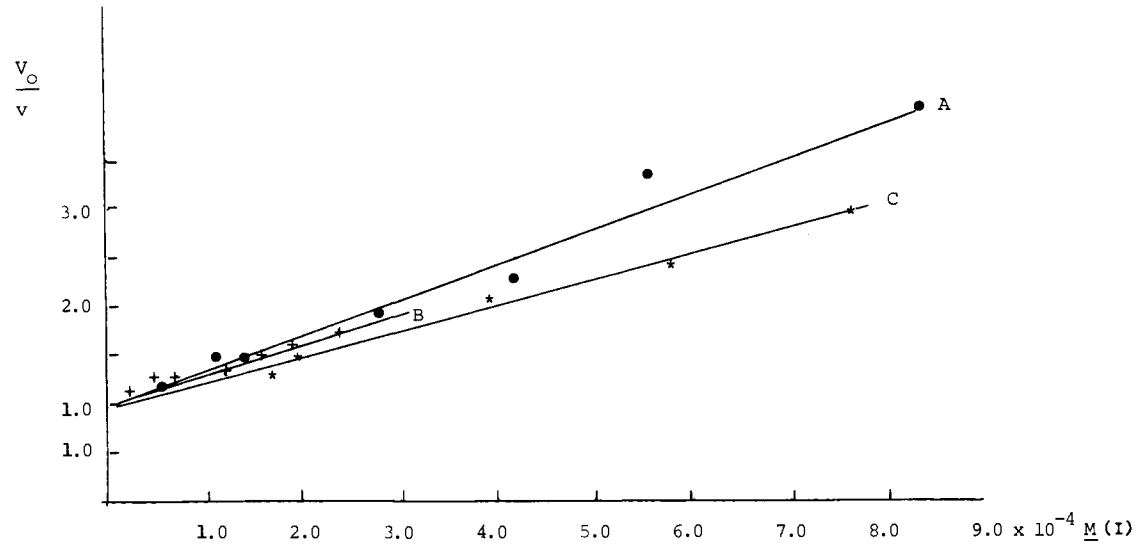


Figure 2. Lineweaver-Burke plots of enzyme kinetic data: A, 2-formylethynylphosphonic acid derived by formolysis; B, the purified barium salt of 2-formylethynylphosphonic acid; C, 2-formylethylphosphonic acid derived by formolysis.

and **21** to be similar to the K_i reported for phosphate anion vs. PPal in apo-AAT ($K_i = 0.215 \times 10^{-6}$ M)[22,23] and to be similar in magnitude to the K_m' determined for PPal in this enzyme ($K_m' = 0.6 \times 10^{-6}$ M). The present study with **16**, **17**, **20**, and **21** has also shown the aldehyde group of **17** and **21** to be important for good inhibition. The inhibitor constants for **16** and **20** were much poorer than those observed for **17** and **21**.

The lack of protein fluorescence quenching by **17** and **21** may be interpreted in one of three ways: (1) the compounds are binding to apo-AAT in a different manner from that of PPal; (2) the compounds are unable to induce an apo → holoenzyme transformation that appears to occur with PPal and its closely related analogues[24,25] when they are bound to PPal-dependent apoenzymes (assuming that this is related to protein fluorescence quenching); (3) the compounds do not possess the same ability to quench protein fluorescence as do the more aromatic analogues of PPal. The present authors believe that the latter interpretation ("three") is the more likely for **17** and **21**.

The results presented in this paper are significant in that simple acyclic molecules (**17** and **21**) are demonstrated to be excellent inhibitors of PPal recombination with apoenzymes and that they are similar in effectiveness to most reported aromatic analogues bearing close resemblance to PPal.[26,27]

Published findings on 3-glyceraldehyde phosphate[28] indicate that this inhibitor of AAT can bind to apo-AAT via its aldehyde group through Schiff base formation with an amino group in the active site. No inhibitor constants for glyceraldehyde phosphate vs. PPal were given. The present results (good inhibitor constants for **17** and **21** and the apparent importance of the aldehyde group for good inhibition) would appear to suggest that a similar process may be occurring with **17** and **21**.

Cycloaddition reactions of diethyl 2-formylethynylphosphonate derived from the intermediate **14** have been studied and are being reported elsewhere.[29]

Experimental Section

Infrared spectra were recorded on a Perkin-Elmer grating spectrophotometer. The proton NMR spectra were obtained on a Varian T-60 spectrophotometer using tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as internal standards. All chemical shifts are reported in parts per million. The fluorescence measurements were made with a Perkin-Elmer Model MPF-2A fluorescence spectrophotometer equipped with a constant temperature unit. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, Tenn.

Diethyl 3,3-Diethoxy-1-propyn-1-ylphosphonate (14). A. A solution of 21 g (0.164 mol) of 1,1-diethoxy-2-propyne (**12**)[16] in 100 ml of THF was cooled to −70 °C and stirred rapidly and 104 ml of BuLi (1.6 M in hexane) was added dropwise at −60 °C. Stirring was continued and 29.24 g (0.17 mol) of $(EtO)_2POCl$ was added dropwise at −60 °C. After the mixture had been stirred 8 h at −65 °C, 50 ml of H_2O was added and the aqueous phase extracted with Et_2O (3 × 50 ml). The Et_2O extracts were dried over $MgSO_4$ and filtered and the Et_2O was evaporated under reduced pressure. Distillation yielded pure **14**: 30.5 g (75%); bp 113–117 °C (0.1–0.15 Torr); ir (neat) 4.5 (C≡C), 7.85 (PO), 8.95, 9.45, 9.70, 10.12 μ (POC, COC); NMR (CCl_4) δ 1.23 (t, J = 7 Hz, CH_3), 1.38 (t, J = 7 Hz, CH_3), 3.68 (AB q, J = 7 Hz, CH_2O), 4.15 (2 q, J = 7, 9 Hz, CH_2OP), 5.30 [d, J = 2.8 Hz, $CH(OR)_2$]. Anal. ($C_{11}H_{21}O_5P$) C, H, P.

B. A solution of $NaPO(OC_2H_5)_2$ was prepared by reacting 20.7 g (0.15 mol) of $HPO(OC_2H_5)_2$ with 0.155 mol of NaH in 200 ml of THF. This was cooled to −75 °C and stirred rapidly while 30.05 g (0.15 mol) of **15**[17] in 20 ml of THF was added dropwise. The reaction mixture was stirred 16 h at −70 °C, allowed to warm to room temperature, and centrifuged to remove precipitated NaBr. The solid was dissolved in 40 ml of H_2O and extracted with Et_2O (2 × 20 ml). The organic phases were combined, dried over $MgSO_4$, filtered, and evaporated under reduced pressure to yield an oil. Distillation of this oil gave 17 g (64%) of **14**: bp 119–130 °C (0.13 Torr). Redistillation of the product gave 14 g of **14**: bp 105–108.5 °C (0.05 Torr). TLC of this material indicated the presence of small amounts of impurity which could not be removed by distillation. An analytical sample was prepared by preparative TLC using silica PF-254 and hexane–acetone (9/1) as the developing solvent. In this way 1.35 g of material spotted on eight plates of 1-mm thickness yielded 0.84 g (62% recovery) of eluted **14**: bp 80 °C (0.005 Torr).

C. A THF solution of 0.05 M $KPO(OC_2H_5)_2$, prepared by dissolving 2.0 g of potassium in 6.9 g (0.05 mol) of $HPO(OC_2H_5)_2$ and 100 ml of THF at room temperature, was cooled to −70 °C. A solution of 10.35 g (0.05 mol) of **15** in 12 ml of THF was added dropwise to the stirred solution of $KPO(OC_2H_5)_2$ at −70 °C. Work-up was identical with that of the sodium salt. Distillation yielded 4.4 g (30%) of **14**: bp 100–110 °C (0.03–0.04 Torr). The NMR and ir spectra were identical with those obtained in the previous synthesis.

D. A solution containing 0.05 mol of $LiPO(OC_2H_5)_2$ was prepared by reacting 31 ml of BuLi (1.6 M in hexane) with 6.85 g (0.05 mol) of $HPO(OC_2H_5)_2$ in 40 ml of THF at −50 °C. The solution was cooled to −70 °C and 10.2 g of **15** was added dropwise with rapid stirring. The reaction mixture was kept at −70 °C for 12 h and an additional 12 h at room temperature. Water (100 ml) was added and the H_2O phase extracted with Et_2O (3 × 40

ml). The ether extracts were dried over $MgSO_4$ and concentrated to yield 14 g of residue. Distillation of the syrup yielded two fractions: bp 38–55 °C (0.02 Torr) and bp 105–116 °C (0.025 Torr). NMR spectroscopy of both fractions failed to reveal the presence of the desired products and, therefore, the fractions were not investigated further.

Dicyclohexylamine Salt of 3,3-Diethoxy-1-propynylphosphonate (16). A. A solution of 1.0 g of **14** in 10 ml of Me_3SiCl was heated in a stainless steel Parr bomb at 100 °C for 3 days. Evaporation of the excess Me_3SiCl yielded a residue which was freed of all traces of Me_3SiCl by repeated evaporation of benzene. The silyl ester groups were hydrolyzed by dissolving the residue in EtOH, film evaporating, and repeating the process several times. The phosphonic acid thus obtained was dissolved in 10 ml of Et_2O and a solution of 0.672 g of dicyclohexylamine in 10 ml of Et_2O was added. The resultant 0.67 g of precipitate (45% crude yield) was filtered, redissolved in hot EtOH, treated with charcoal, and filtered, and the filtrate was cooled to yield a white crystalline salt. An analytical sample was prepared by recrystallization from EtOH: mp ~180 °C with gradual decomposition; ir (KBr) 3.36–4.4 (CH, NH, OH), 4.5 (C≡C), 8.53, 9.15, 9.45, 9.65, 9.90, 10.10, 10.70 μ (PO, CO); NMR ($CDCl_3$) δ 1.18 (t, J = 7 Hz, CH_3), 1.05–2.23 (hump, dicyclohexylamine protons), 3.58 (AB q, J = 7 Hz, CH_2O), 5.21 [d, J = 2.5 Hz, $CH(OR)_2$]. Anal. ($C_{19}H_{36}NO_5P$) C, H, N, P.

B. At room temperature, 54.0 g (0.353 mol) of Me_3SiBr was added cautiously to 39.3 g (0.15 mol) of **14**, care being taken to exclude all moisture from the reaction mixture. An exothermic reaction occurred which lasted ~15 min after which time the reaction mixture was heated at reflux for 45 min and cooled and the volatiles were removed in vacuo at room temperature. The residue was poured into MeOH solution of dicyclohexylamine (36.3 g in 350 ml of MeOH). The MeOH was evaporated, 200 ml of MeOH again added to the residue, and the process repeated a number of times. The resultant residue was dissolved in 250 ml of hot ethanol and crystallized to give 22.2 g (38%) of a first crop of **16** and 24 g (41%) of additional crops. The ir and NMR spectra of the products were the same as for **16** obtained by the Me_3SiCl cleavage of **14**, described in **A**.

Barium 2-Formylethynylphosphonate (18). A solution of 0.38 g (1 mmol) of **16** in 15 ml of 97% HCOOH was heated at 50 °C for 15 min. The solution was frozen and lyophilized under high vacuum. The residue was dissolved in 3 ml of H_2O and 0.3 g (1.2 mmol) of barium acetate was added. The solution was centrifuged to remove insoluble matter and 2 vol of ethanol was added to precipitate **18**. The salt was isolated by centrifugation, washed successively with EtOH and Et_2O, and dried to give **18** in 100% crude yield. This material was reprecipitated a number of times from H_2O with EtOH to give analytically pure **18** in 54% yield: ir (KBr) 6.02, 8.75, 9.18, 10.1 μ; NMR (D_2O) δ 9.3 (s, CHO). Anal. ($C_3HBaO_4P \cdot 0.5H_2O \cdot 0.5C_2H_5OH$) C, H, Ba, P; solvate: calcd, 11.88; found, 5.03.

Dicyclohexylamine Salt of 3,3-Dimethoxypropylphosphonate (20). A solution of 25.9 g (0.12 mol) of **19** and 200 ml of Me_3SiCl was refluxed 63 h. Excess Me_3SiCl was evaporated and the residue was worked up in the same way as for **16** to give 26.4 g (60%) of **20**. A second crop of 10.3 g (23%) was obtained by addition of ether. An analytical sample was crystallized from dimethoxyethane and then from ethyl acetate–ethanol (3/1): ir (KBr) 3.4–4.24 (CH, NH, POH), 8.38, 8.73, 8.80, 9.40, 9.7, 10.02, 10.34 μ (CO, PO); NMR ($CDCl_3$) δ 0.8–2.4 (methylene hump), 3.33 (s, CH_3O), 4.41 [t, J = ~5 Hz, $CH(OR)_2$]. Anal. ($C_{13}H_{36}NO_5P$) C, H, N, P.

Barium 2-Formylethylphosphonate (22). The compound was prepared from **20** in the same way as **18** in 45% yield: ir (KBr) 5.9, 6.28, 7.34, 8.7, 9.5, 10.25 μ; NMR (D_2O) δ 1.83, 3.0 (poorly resolved set of multiplets, $-CH_2CH_2-$), 9.8 (CHO). Anal. ($C_3H_5BaO_4P \cdot 0.5H_2O$) C, H, Ba, P.

Biological. Aspartate Aminotransferase. The enzyme[30] was obtained from pig heart ventricules by using published isolation procedures.[31,32] Minced beef heart ventricules (100 lb) were extracted with 20 l. of 0.05 M glutarate buffer, pH 6.0, containing 0.005 M EDTA. The resultant homogenate was heat denatured in the manner described by Jenkins[31] and the enzyme isolated by fractional precipitation with $(NH_4)_2SO_4$ [enzyme precipitated at 460 g/l. of $(NH_4)_2SO_4$]. The AAT was further purified by column chromatography on hydroxyapatite[31] and on CM-Sephadex C-50.[32] The α-subform of AAT thus isolated was shown to be pure by starch gel electrophoresis.[32]

Apo-AAT was obtained using previously published procedures.[2] The resolved enzyme (apo-AAT) was subjected to a $NaBH_4$ reduction so that any unresolved AAT could be reduced to its inactive form (enzyme–PPal Schiff base reduced to a secondary amine).

PPal saturation curves of this apo-AAT gave $0.60 \pm 0.1 \times 10^{-6}$ M as the concentration of PPal required for $0.5V_{max}$ and this was used as the K_m for the enzyme.

Assay Procedure. The enzyme kinetic studies were done using the following procedure.[2] A solution composed of 0.5 ml of diluted apo-AAT (0.01 mg/ml), 0.1 ml of PPal (1×10^{-5} M), and 0.1 ml of inhibitor (at the appropriate concentrations) was incubated 40 min at 0 °C. This enzyme solution was then diluted with 2 ml of a 37 °C phosphate–α-ketoglutarate (α-KG) buffer (0.009 M in α-KG in 0.135 M phosphate buffer, pH 7.4) and the resultant solution warmed ~15 s in a 37 °C water bath. Addition of 0.1 ml of aspartate (0.6 M) solution started the enzyme reaction which was followed at 257 nm using a Gilford 2400 spectrophotometer equipped with a wavelength programmer and a reference compensator. Water was used as a blank. The initial reaction velocity was obtained from the slope of the trace obtained during 1–2 min of the enzyme reaction. Each run used to determine the $([I]/[S])_{0.5}$ and K_i values included a PPal control to give the V_{max}, a PPal control for the V_0, and at least four concentrations of inhibitor. Duplicate assays of each concentration were run. Calculations were done using a linear regression analysis program written for a Hewlett-Packard 9820 (Model 20) calculator for the plot V_0/v vs. [I] from which the $[I]/[S]_{0.5}$ and K_i values were obtained.

Protein Fluorescence Quenching Experiments. All measurements were performed at an excitation wavelength of 280 nm and an emission wavelength of 340 nm and the solutions were all in 0.1 M Tris–acetate buffer, pH 7.4. The fluorimetric titration procedure used to titrate apo-AAT and to measure protein fluorescence quenching was similar to that used by Churchich.[21] Fluorimetric titration of active apo-AAT with an inhibitor or cofactor was carried out by adding the inhibitor or cofactor solution to apo-AAT (usually 0.2 mg/ml) at 20% increments until the molar ratio of active sites to cofactor or inhibitor was 1:1. The titration was continued by further addition of cofactor or inhibitor until significant decreases in protein fluorescence ceased. The total volume change introduced by the addition of the inhibitor or cofactor was made to be less than 2%. The effect of this dilution on protein fluorescence was considered negligible and, therefore, no corrections were made for dilution. All fluorescence measurements were made at 25 °C and for each increment of inhibitor or cofactor an incubation period of 15 min was used to ensure complete reaction.

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References and Notes

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Design of Substrate-Site-Directed Inhibitors of Adenylate Kinase and Hexokinase. Effect of Substrate Substituents on Affinity for the Adenine Nucleotide Sites

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Nineteen derivatives of adenosine 5′-phosphate (AMP) bearing acylaminomethyl, acetoxy, or alkylaminomethyl substituents on the phosphate–ribose bridge (5′ and O-5′ positions) of AMP together with 2′,3′-*O*-ethylidene, 2′,3′-*O*-isopropylidene, and 2′,3′-di-*O*-acetyl derivatives of AMP have been synthesized. Their substrate and/or competitive inhibitor properties with pig and rabbit muscle AMP kinases indicate that all the substituents except 2′,3′-*O*-ethylidene with the pig enzyme permitted binding of AMP at its enzymic site. Determination of enzyme–inhibitor dissociation constants showed that several compounds with substituents on the ribose–phosphate bridge bind as well or better than AMP. The affinity is ascribed in part to interaction between substituents and a lipophilic region of the enzymes adjacent to the ribose–phosphate bridge in the enzyme–AMP complexes. The enzyme–inhibitor dissociation constants reveal a structural dissimilarity between the pig and rabbit enzymes in the vicinity of the lipophilic region. The substrate and inhibitor properties of eight ATP derivatives gave evidence that affinity of ATP for its substrate site on the AMP kinases is compatible with acetyl- or chloroacetylaminomethyl groups at the phosphate–ribose bridge or with 2′,3′-*O*-ethylidene or isopropylidene residues. The yeast hexokinase–ATP complex tolerated an acetylaminomethyl group at C-5′ or a benzoylaminomethyl group adjacent to O-5′. The present findings regarding substituent tolerance could be used in the design of adenine nucleotide site-directed irreversible inhibitors.

Adenylate kinase mediates reversible phosphate transfer from ATP to AMP to produce ADP and plays an important role in the energy economy of living systems.[1] To investigate the possibility of designing substrate-site-directed exo-site reagents with their potential to act as species-selective irreversible enzyme inhibitors,[2] we have introduced substituents at various positions of AMP and ATP and examined the effect on formation of enzyme–substrate complexes as indicated by substrate and inhibitor properties of the AMP and ATP derivatives. Studies with small substituents showed that binding to the AMP site of the kinase still occurs when a hydroxyl or cyano group[3,4] is inserted α to phosphorus (equivalent to O–5′ of AMP) in the phosphonate isostere of AMP (**1**, R^1 = H; R^2 = PO_3H_2) or when a methyl group is attached to C-5′ of AMP.[5] Substitution on the exocyclic N^6 of the adenine ring appears to prevent binding of AMP to its site because 1,N^6-etheno-AMP is not a substrate[6] and N^6-benzoyl-AMP is not a substrate and is a noncompetitive inhibitor with respect to AMP.[7] Formation of the ATP–enzyme complex was not prevented by introduction of a cyano group near O-5′.[4] This paper describes the substrate and inhibitor characteristics of derivatives of AMP and ATP with substituents on O-2′ and O-3′ (structure **4**) or on the phosphate–ribose bridge (5′ and O-5′ positions) (structures **1–3**).

Chemical Syntheses. The syntheses of 5′-deoxy-5′-(*C*-acetoxyphosphonomethyl)adenosine (**1a**) and of the 5′-deoxy-5′-[*C*-(acylaminomethyl)phosphonomethyl]-adenosines **1c,d,f,g,h** were reported previously.[8] The benzamidomethyl-substituted phosphonate analogue of AMP, **1e**, was obtained in 40% yield as its purified disodium salt by treatment of 5′-deoxy-5′-[*C*-(aminomethyl)phosphonomethyl]adenosine (**1b**)[8] with 2 equiv each of benzoic acid and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.[9] The AMP analogues **1c** and **1e** were converted to the corresponding ATP analogues **1i** and **1j** by the general procedure of Michelson[10] for the conversion