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Syntheses and Characterizations of Adenosine 5′-(α,β-*N*-Methylimido)di and Triphosphates and Adenosine 5′-(β,γ-*N*-Methylimido)triphosphate—Comparisons with Their Nonmethylated Analogs

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N-Methylimidodiphosphate [PN(Me)P] has been synthesized for the first time by hydrolysis of *N*-methylimidodiphosphoryl tetrachloride. PN(Me)P was coupled with 5′-tosyladenosine to produce adenosine 5′-[α,β-*N*-methylimido]diphosphate [AMPN(Me)P] which in turn was treated with phosphocreatine in the creatine kinase reaction to generate adenosine 5′-[α,β-*N*-methylimido]triphosphate [AMPN(Me)PP]. Using the Michelson synthesis, PN(Me)P was coupled with adenosine 5′-monophosphate to produce adenylyl *N*-methylimidophosphate [AMP · PN(Me)P]. All three new purine nucleotide analogs were characterized by mass spectrometry and ^{31}P NMR spectroscopy. Their enzyme kinetic parameters (V_{max} and K_m values) were measured in the creatine kinase reaction and directly compared to similar kinetic parameters obtained for the corresponding nonmethylated analogs and ADP and ATP. AMPN(Me)P was shown to have a V_{max} value ca. 40% of that of ADP although its K_m value is ca. 100 times greater than that for ADP. AMPN(Me)PP was also a substrate, whereas AMP · PN(Me)P showed no detectable inhibitory properties.

INTRODUCTION

In 1971, Yount and co-workers (*1*) first reported the synthesis and inhibitory properties of adenylyl imidodiphosphate (AMP · PNP, **1**),[3] the β,γ-*N*–H analog of ATP. Since that time, AMP · PNP has been widely used in enzymological studies by virtue of its close structural similarity to ATP.

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[3] Abbreviations used: AMPPNP, adenylyl imidodiphosphate; AMPNP, adenosine 5′-[α,β-imido] diphosphate; AMPNPP, adenosine 5′-[α,β-imido]triphosphate; AMPCP, adenosine 5′-[α,β-methylene]diphosphate; AMPCPP, adenosine 5′-[α,β-methylene]triphosphate; PNP, imidodiphosphate; PN(Me)P, *N*-methylimidodiphosphate; AMPN(Me)P, adenosine 5′-[α,β-*N*-methylimido]diphosphate; AMPN(Me)PP, adenosine 5′-[α,β-*N*-methylimido]triphosphate; AMPPN(Me)P, adenosine 5′-[β,γ-*N*-methylimido]triphosphate or adenylyl *N*-methylimidodiphosphate; LSIMS, liquid secondary ion mass spectrometry; PEI, polyethylenimine; TEAB, triethylammonium bicarbonate; QAE, quaternary aminoethyl; *m*-CPBA, *meta*-chloroperoxybenzoic acid; DMF, dimethylformamide.

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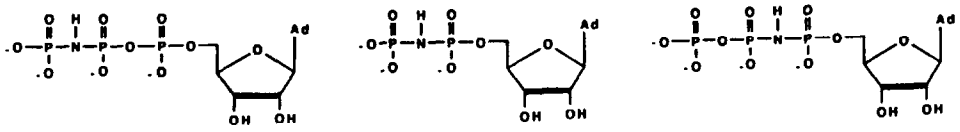
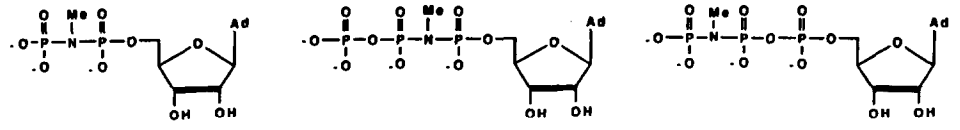


AMPPNP (1) AMPNP (2) AMPNPP (3)

(1)

Since the P–N–P bridge in AMP · PNP is relatively inert to hydrolysis both in basic solution and in the presence of enzymes that catalyze either the hydrolysis or transfer of the γ-phosphoryl group of ATP, this compound has been a useful competitive inhibitor of both kinases and ATPases. In a few cases, P–N–P bonds can be cleaved by certain phosphoryl transfer enzymes (*1–3*). We have recently reported the syntheses of both adenosine 5′-(α,β-imido)diphosphate (AMPNP, **2**) and adenosine 5′-(α,β-imido)triphosphate (AMPNPP, **3**) (*4*). [An independent preliminary account of the synthesis and properties of **2** has also appeared recently (*5*).] Both **2** and **3** have been shown to be substrates for rabbit muscle creatine kinase (*4*). In addition, **2** is a substrate for pyruvate kinase, whereas **3** is a substrate for hexokinase. The properties of **2** and **3** are intriguing since the corresponding α,β-methylene analogs (AMPCP and AMPCPP) (*6, 7*) have had such a mixed record of success in binding productively to the ADP and ATP sites of enzymes (*8–18*). That the NH analogs are better accepted probably can be explained by the facts that (1) the bridging *N*–H still has a lone pair of electrons and can therefore act as either a hydrogen bonding acceptor or coordinate to an electrophilic metal ion and (2) crystal structures for imidodiphosphate (PNP) and pyrophosphate show that bond lengths and bond angles of the P–N–P and P–O–P linkages are nearly identical (*19*).

In this paper, we present the first syntheses of the corresponding *N*-methyl analogs, namely adenosine 5′-(α,β-*N*-methylimido)diphosphate (AMPN(Me)P, **4**), adenosine 5′-(α,β-*N*-methylimido)triphosphate (AMPN(Me)PP, **5**) and adenylyl *N*-methylimidodiphosphate (AMP · PN(Me)P, **6**). It is hoped that these new analogs can probe for bulk tolerance in ADP- and ATP-requiring enzymatic systems, especially among isoenzymes. They may also serve as competitive inhibitors.



AMPN(Me)P (4) AMPN(Me)PP (5) AMPPN(Me)P (6)

(2)

We also report their full characterizations using ^{31}P NMR, liquid secondary ion mass spectrometry (LSIMS) and pH-titration curves. Finally, some kinetic studies have been performed for **4**, **5**, and **6** in the rabbit muscle creatine kinase reaction. The results are compared with corresponding kinetic parameters for the

corresponding nonmethylated analogs **2** and **3** as well as those for ADP and ATP under the same reaction conditions.

MATERIALS AND METHODS

Adenosine 5′-monophosphoric acid (AMP), phosphocreatine, glucose, creatine kinase, glucose-6-phosphate dehydrogenase, pyruvate kinase, hexokinase, PEP, NADH, NADP, ADP, and ATP were all purchased from Sigma Chemical Co. Diethyl chlorothiophosphate, diethyl chlorophosphate, diphenyl chlorophosphate, 5′-tosyladenosine, and trimethylsilyl iodide were all products of Aldrich Chemical Co. Both AMPNP and AMPNPP were obtained as described previously (*4*). Pyridine was heated at reflux over NaOH pellets, distilled, and stored over molecular sieves (Aldrich, type 4A, 4–8 mesh). CH_2Cl_2 (Aldrich) was heated at reflux over CaH_2, distilled, and stored over molecular sieves. Dimethylformamide (Aldrich) was dried over molecular sieves for more than 2 weeks. Trioctylamine, tributylamine, and triethylamine (Aldrich) were all distilled prior to use and stored at 4–5°C. Triethylammonium bicarbonate solutions (1 M) were prepared by bubbling CO_2 through a sintered glass diffuser into a triethylamine solution until the pH reached 8.5. DEAE and QAE Sephadex A-25 (Pharmacia) were converted to their HCO_3^- form with 1 M solutions of $NaHCO_3$. Other chemicals were all obtained from either Aldrich or J. T. Baker and used directly.

High-performance liquid chromatography was performed on a Beckman 110A instrument using an ODS column (Partisil M9 10/50 ODS-3, Whatman) with a linear gradient in solvent B (0.1 M TEAB/EtOH) of 0.25%/min as eluent (*20*). The chromatographic fractions were detected either by uv at 275 nm or by use of polyethylenimine-cellulose thin-layer chromatography (PEI-cellulose TLC) (*21*). The total amount of phosphorus in pooled fractions was measured by the acid-labile phosphate method (*22*). ^{31}P NMR spectra were recorded either at 40.5 MHz using a Varian XL-100 Spectrometer or at 79.5 MHz using a Nicolet NTCFT-1180 spectrometer. Broadband proton decoupling was routinely employed. A sweep-width of 4000 Hz, a probe temperature of 22°C, and broadband ^1H decoupling were used in ^{31}P NMR measurements, and chemical shifts were determined relative to 85% H_3PO_4 with positive shifts being downfield of the reference. ^1H NMR spectra were taken at 80 MHz on a Varian FT-80 spectrometer. High-resolution LSIMS spectra were recorded at the University of California (San Francisco) on a Kratos MS-50 mass spectrometer (negative ion probe) with ATP as reference. pH-titration curves were generated and analyzed as described previously (*23*, *24*).

ENZYME KINETIC STUDIES

Enzyme assays were performed and computer analyzed essentially as described by Cook *et al.* (*25*). In the forward reaction (ATP consumption) the assay solutions (pH 9.0, 25°C) contained glycine (0.2 M), $Mg(OAc)_2$ (12 mM), KOAc (0.2 M),

creatine (66 mM), PEP (2.8 mM), NADH (0.23 mM), lactic dehydrogenase (80 U/ml), pyruvate kinase (65 U/ml), creatine kinase (23 U/ml), and varying concentrations of ATP (or ATP analogs). In the reverse reaction (ATP formation, pH 7.0, 25°C) the assay solutions contained glycine (0.2 M), $Mg(OAc)_2$ (12 mM), glucose (71 mM), phosphocreatine (29.5 mM), NADP (0.94 mM), hexokinase (30 U/ml), glucose-6-phosphate dehydrogenase (33 U/ml), creatine kinase (12 U/ml), and varying concentrations of ADP (or ADP analogs). Initial rates were measured by the change of ultraviolet absorbance at 340 nm.

Progress in the creatine kinase reactions could also be followed semi-quantitatively using the PEI-cellulose TLC method of Rowley and Kenyon (*21*).

SYNTHESES

Preparation of Tetraethyl N-Methyl PNP

(*a*) *Diethyl N-methylphosphorothioylamidate.* Monomethylamine (19.19 g, 0.6179 mol) was bubbled through a glass frit into 250 ml of CH_2Cl_2 at 0°C. This solution was transferred to a 500-ml round bottom flask equipped with a magnetic stir bar and an addition funnel. Diethyl chlorothiophosphate (58.27 g, 0.3089 mol), dissolved initially in 50 ml of CH_2Cl_2, was added dropwise with stirring at 0°C. Stirring was continued for 1 h at 0°C and then overnight at room temperature. Next, precipitated monomethylammonium chloride was removed by filtration, and the solvent was removed under reduced pressure to give an oil (58.3 g). The product was purified by vacuum distillation at 68–70°C (0.1 Torr) and weighed 55.23 g (97.6% of the theoretical amount).

Anal. Calcd for $C_5H_{14}NO_2PS$: C, 32.77; H, 7.7; N, 7.65; P, 16.91; S, 17.50. Found: C, 32.95; H, 7.68; N, 7.58; P, 16.94; S, 17.38.

(*b*) *Tetraethyl phosphorothioyl N-methylphosphoramidate.* Dimethoxyethane (ethylene glycol, dimethyl ether) was heated at reflux and distilled over $LiAlH_4$ prior to use. NaH (4.32 g, 90.1 mmol, as a 50% oil dispersion) was activated by washing with hexane under a dry N_2 atmosphere, then quickly transferred to a 300 ml round bottom flask, and covered with 100 ml of dry dimethoxyethane. A magnetic stir bar was added and an addition funnel and $CaCl_2$ drying tube were attached after the apparatus was purged with dry N_2. Diethyl *N*-methylphosphorothioylamidate (15.00 g, 81.8 mmol), dissolved initially in 25 ml of dry dimethoxyethane, was added dropwise using the addition funnel with stirring at 0°C. Stirring was continued for 30 min at 0°C and then for 1½ h at room temperature. Next, diethyl chlorophosphate (14.11 g, 81.8 mmol), dissolved initially in 25 ml of dry dimethoxyethane, was added dropwise using the addition funnel with stirring at 0°C. After 15 min at 0°C, stirring was continued at room temperature for 48 h. The reaction mixture was then filtered to remove NaCl, and the solvent was removed under reduced pressure. The product was purified by fractional vacuum distillation, bp 106–109°C (0.15 Torr) [literature (*26*) bp 120°C, 1.0 Torr] and weighed 17.53 g (67% of the theoretical yield).

Anal. Calcd for $C_9H_{23}NO_5P_2S$: C, 33.85; H, 7.26; N, 4.39; P, 19.40; S. 10.04. Found: C, 34.01; H, 7.24; N, 4.35; P, 19.30; S, 10.13.

(*c*) *Tetraethyl phosphoroyl-N-methylphosphoramidate*. A solution of *m*-chloroperoxybenzoic acid (11.0 g, 63.7 mmol) in 120 ml of CH_2Cl_2 was added dropwise with stirring to tetraethyl phosphorothioyl-*N*-methylphosphoramidate (5.00 g, 15.7 mmol) in a 250-ml round bottom flask. A $CaCl_2$ drying tube was then attached and stirring was continued for 30 min. The reaction mixture was filtered and then washed with 80 ml of 10% sodium sulfite followed by 2 × 30 ml of saturated $NaHCO_3$. The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The resulting residue was filtered to give a colorless oil which weighed 5.6 g. The product was purified by vacuum distillation at 109–110°C (0.25 Torr) and weighed 2.54 g (53.4% of the theoretical amount). ^1H NMR ($CDCl_3$): δ 1.35 (12H, t, J_{HH} = 7.0 Hz), 2.95 (3H, J_{PH} = 9.7 Hz), 4.16 (10H, m).

Anal. Calcd for $C_9H_{23}NO_6P_2$: C, 35.65; H, 7.64; N, 4.62; P, 20.43. Found: C, 36.02; H, 7.49; N, 4.39; P, 20.16.

Preparation of PN(Me)P

(*a*) *N-Methylimidodiphosphoryl tetrachloride* (*8*). CH_2Cl_2 was dried by heating at reflux over CaH_2. Dichlorophosphoryl trichloroiminophosphorane (*27*) (15.00 g, 55.7 mmol) was dissolved in 50 ml of dry CH_2Cl_2 in a 200-ml round bottom flask equipped with a magnetic stir bar, addition funnel, and $CaCl_2$ drying tube. The reaction vessel was cooled to ca. −20°C using a dry ice/acetone bath. Next, absolute methanol (1.785 g, 55.7 mmol), dissolved initially in 25 ml of dry CH_2Cl_2, was added dropwise with stirring using the addition funnel. Stirring was continued at −20°C for 30 min and then at room temperature for 2 h. The solvent was removed under reduced pressure, and the last traces of HCl were removed by application of high vacuum (0.1 Torr) at room temperature. The resulting oil was then heated at 60–75°C in an oil bath for 4 h, while being protected from moisture with a $CaCl_2$ drying tube. The product was purified by vacuum distillation at 72–73°C (0.01 Torr) [literature (*28*) bp 95–98°C, 0.3 Torr]. This material rapidly solidified to give a white glassy solid, mp 49–50°C [literature (*28*) mp 50–51°C] which weighed 8.78 g (59.5% of the theoretical amount). ^1H NMR $CDCl_3$: δ 3.38 (t, J_{PN} = 13.6 Hz).

Anal. Calcd for $C_1H_3NO_2P_2Cl_4$: C, 4.54; H, 1.14; N, 5.29; P, 23.39. Found: C, 4.40; H, 1.16; N, 5.14; P, 23.18.

(*b*) *N-Methylimidodiphosphate, tributylammonium salt* (*PN(Me)P*. *N*-Methylimidodiphosphoryl tetrachloride (0.53 g, 2 mmol) was treated with 1 N NaOH (19.0 ml, 19.0 mmol) added in small amounts in an ice bath for 1 h. The reaction was then warmed to room temperature with stirring until all of the white solid had dissolved. This sample was purified by anion-exchange chromatography at 2–4°C on QAE Sephadex A-25 (HCO_3^-) using a 3-liter linear gradient of 0.1–0.7 M triethylammonium bicarbonate, pH 8.5, as eluent. Fractions were assayed for acid-labile phosphate according to the procedure of Ames (*22*). PN(Me)P is eluted from the column by 0.36 M triethylammonium bicarbonate under these conditions. The appropriate fractions were pooled, 1 ml of tributylamine was added, and the resulting solution was concentrated to a syrup on a rotary evaporator using a vacuum pump and a dry ice/ethanol trap with a bath temperature below 25°C. An

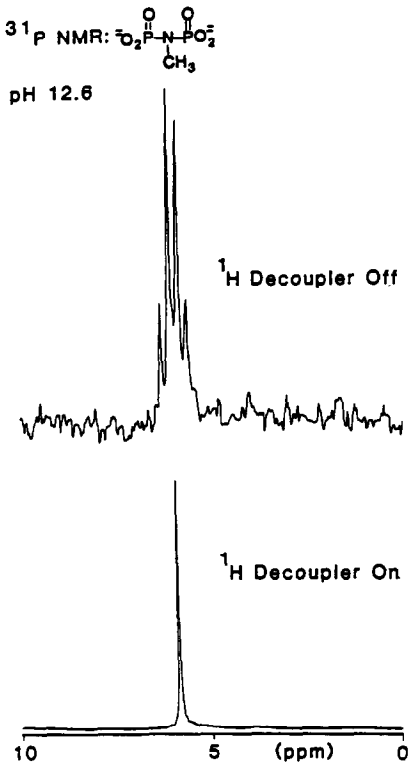


FIG. 1. ^{31}P NMR spectra of PN(Me)P at pH 12.6. Above, proton decoupler off. Below, proton decoupler on. H_3PO_4 (85%) was used as an external standard.

additional 1 ml of tributylamine was added, and the syrup was stripped of residual water and triethylammonium bicarbonate by repeated evaporation of 25-ml aliquots of absolute methanol under reduced pressure. The amount of $PN(CH_3)P$ was determined by the Ames method (*22*) (1.6 mmol, 80% yield). ^{31}P NMR (20% D_2O, pH 12.6, proton decoupled): δ 5.83 (s); (proton coupled) (quartet, J_{PH} = 9.4 Hz) (see Fig. 1).

Preparation of Tributylammonium Salt of Adenylyl Diphenylphosphate

AMP · H_2O (2 mmol, free acid) was dissolved in several milliliters of absolute methanol containing 2 mmol of trioctylamine (0.0706 g) with stirring under reflux. The methanol was removed under reduced pressure with a bath temperature of 25–30°C and the residue dried by repeated addition and evaporation of three 10-ml portions of dry dimethylformamide. The residue was again dissolved in dimethylformamide (10 ml); 0.6 ml of diphenyl chlorophosphate (3.0 mmol) and 0.94 ml of tributylamine (4.0 mmol) were added, and the solution was allowed to stand at room temperature for 2 h while being protected from moisture. Solvents were removed under reduced pressure and dry ether (100 ml) was added to the residue

with swirling. The flask was cooled in an ice bath for 20 min and the ether decanted. The remaining ether was removed under reduced pressure and the product stored in a stoppered flask at −20°C until used.

Preparation of the Triethylammonium Salt of AMP · PN(Me)P

The tributylammonium salt of PN(Me)P (2.4 mmol) was dissolved in 10 ml of DMF and 30 ml of pyridine. Tributylammonium adenylyl diphenylphosphate (2.0 mmol), dissolved in 10 ml of DMF, was added to this solution dropwise over a 15-min period with stirring. The reaction mixture was allowed to stand at room temperature for 30 min with stirring and then solvents were removed under reduced pressure as before. The residue was extracted with cold ether (70 ml), and the ether was decanted after standing in an ice bath for 20 min. The remaining ether was removed under pressure, and the residue was dissolved in 100 ml of cold water containing 0.8 ml of concentrated ammonium hydroxide. The cloudy solution was extracted with 100 ml of ether, and the clear water layer was applied to a 2.5 × 70-cm column of DEAE Sephadex (A-25 HCO_3^-) at 0–20°C with a 2400-ml linear gradient eluent of 0 to 0.6 M TEAB. The fractions were detected by their uv absorbance at 275 nm, and AMP · PN(Me)P was localized by PEI-cellulose TLC. The fractions with the same R_f value as ATP were pooled and evaporated to dryness under reduced pressure at a bath temperature below 20°C. The dry residue was purified again using HPLC (ODS column). The fraction containing AMP · PN(Me)P was dried as before, and the crude product, dissolved in small amounts of cold water, was again passed through a 1 × 50-cm column of DEAE Sephadex A-25 with a 1200-ml linear gradient of 0 to 0.6 M TEAB as eluent. The procedure was the same as in the primary separation, and the appropriate fractions were collected and lyophilized to dryness. The triethylammonium salt of AMP · PN(Me)P, a white fluffy powder, was obtained (140 mg, 0.15 mmol, 7.6% yield). ^{31}P NMR (D_2O, ^1H broadband decoupling, pH 11.58) δ 2.85 (d, J_{PP} = 23 Hz, γ-P), −6.46 (app.t, J = 23Hz, β-P), −11.04 (d, J_{PP} = 23 Hz, α-P). LSIMS (accurate mass measurement, M-1, free acid): calcd for $C_{11}H_{18}N_6O_{12}P_3$: 519.0196; found: 519.0176.

Preparation of the Triethylammonium Salt of AMP · N(Me)P

The tris(tributylammonium) salt of PN(Me)P (522 mg, 0.7 mmol) was dissolved in 1.0 ml of CH_3CN, and 211 mg (0.5 mmol) of 5′-tosyladenosine was added. The mixture was allowed to react for 36 h under N_2 at room temperature, and then the CH_3CN was removed under vacuum. The residue was dissolved in 40 ml of cold H_2O containing 0.3 ml concd aq. NH_3. This solution was extracted with 3 × 40 ml diethyl ether. The clear aqueous layer was loaded onto a DEAE Sephadex A-25 column (HCO_3^-, 3 × 30 cm) and eluted with a 2.5-liter linear gradient of 0–0.6 M triethylammonium bicarbonate buffer (pH 8.5). The separation was followed by analysis using PEI-cellulose TLC, the fractions with the same R_f value as ADP were pooled, and the resulting solution was lyophilized to dryness. The triethylammonium salt of AMP · N(Me)P (59 mg, 0.08 mmol, 16% yield) was obtained. It gave a single spot with R_f value = 0.5 on PEI-cellulose TLC using 0.5 M triethyl-

ammonium bicarbonate buffer as eluent. ^{31}P NMR (D_2O, pD = 11.6, ^1H broadband decoupler off): δ 6.95 [s(broad), α-P]; 3.38 (q, J = 8.5 Hz, β-P). (^1H broadband decoupling on): δ 6.95 (d, J = 18 Hz, α-P); 3.4 (d, J = 18 Hz, β-P). Accurately mass measured parent ion [LSIMS, negative ion probe, free acid mass, M-1]: calculated for $C_{11}H_{17}N_6O_9P_2$: 439.0531; found: 439.0548.

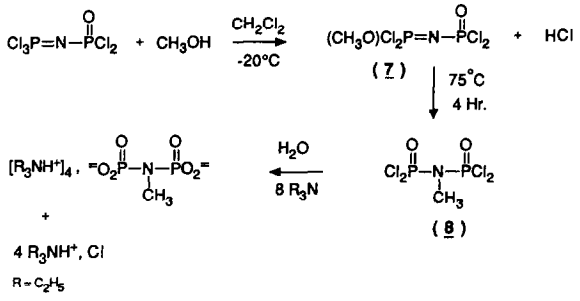
Preparation of the Triethylammonium Salt of Adenosine 5'-(N-Methylimido)triphosphate [AMP · N(Me)PP]

The triethylammonium salt of AMP · N(Me)P (37 mg, 0.05 mmol) and phosphocreatine (400 mg, 1.6 mmol) were dissolved in 15 ml of buffer solution (0.2 M glycine, 12 mM $Mg(OAc)_2$, pH 9), and then creatine kinase (6 mg) in 0.5 ml of Hepes buffer (pH 7.6) was added. The solution was kept at room temperature for 36 h and then purified as described above using chromatography on the DEAE Sephadex A-25 column. Fractions were monitored using PEI-cellulose TLC, and those with the same R_f value as ATP were pooled and lyophilized to dryness to give 18 mg of the triethylammonium salt of AMP · N(Me)PP (40% yield). It gave a single spot with an R_f value = 0.2 on PEI-cellulose TLC using 0.5 M triethylammonium bicarbonate buffer as eluent. ^{31}P NMR (D_2O, pD = 12.6, ^1H broadband decoupling off): δ 4.5 (m, broad, α-P); −5.8 (d, J = 21 Hz, γ-P); −7.3 (m, broad, β-P). (^1H broadband decoupling on): δ 4.6 (d, J = 21 Hz, α-P); −5.9 (d, J = 22 Hz, γ-P); −7.3(app.t, J = 21 Hz, β-P). Accurately mass measured parent ion (LSIMS, negative ion probe, free acid mass, M-1): calcd: 519.0196; found: 519.0187.

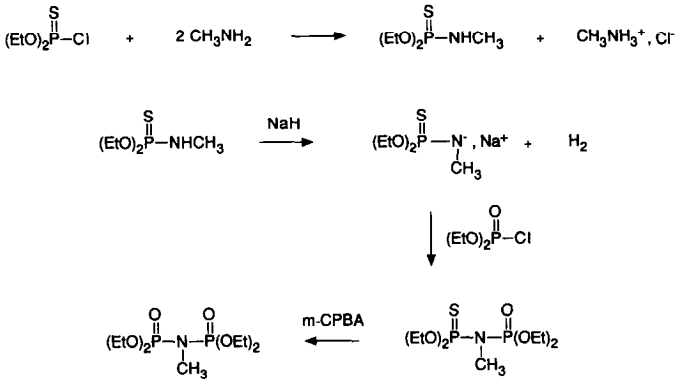
RESULTS AND DISCUSSION

Synthesis and Characterization of PN(Me)P

The synthesis of PN(Me)P begins with dichlorophosphoryltrichloroiminophosphorane (*27*) as shown in Scheme I. The first two steps were performed according to Riesel *et al.* (*28*). In the first step methanol attacks in an addition–elimination (Michael tye) reaction at the iminophosphorane position to expel chloride ion. In the second step, intermediate **7** is rearranged to the more thermodynamically



SCHEME I



SCHEME II

stable *N*-methylimidodiphosphoryl tetrachloride **(8).** Hydrolysis of **8** in the presence of 1 N NaOH has produced PN(Me)P for the first time. This last step parallels our earlier synthesis of imidodiphosphate (PNP) (*24*).

The ^{31}P NMR spectra of PN(Me)P at pH 12.6 with and without ^{1}H decoupling are shown in Fig. 1. The chemical shift is 5.8 ppm from 85% H_3PO_4 (external standard), and the ^{31}P–^{1}H coupling constant to the CH_3 group is 9.4 Hz.

In the course of attempting to prepare PN(Me)P by other routes, we carried out the successful synthesis of the tetraethyl ester as shown in Scheme II. Although this synthesis proceeded in good overall yields, using trimethylsilyl iodide we never were able to succeed in removing the ethyl ester groups without also cleaving the *N*-methyl group. If other, milder ester removal conditions can be found in the future, the synthetic route in Scheme II could become a general one for other *N*-substituted derivatives of PNP.

Synthesis and Characterization of AMP · PN(Me)P

AMP · N(Me)P was prepared for the first time from PN(Me)P and AMP by the Michelson procedure (*29*). It proved to be very difficult to purify, and the final purification required three chromatographic steps. The ^{31}P NMR spectrum rather closely resembles that for ATP itself, although, of course, the chemical shifts for the individual phosphorus resonances are different. Its pH titration behavior using ^{31}P NMR is discussed below.

Synthesis and Characterization of AMPN(Me)P and AMPN(Me)PP

AMPN(Me)P was prepared in the same manner as AMPNP (*4*) using the general methodology of Davisson *et al.* (*30*), starting with commercially available 5′-tosyladenosine. A $J_{^{31}P-^{31}P}$ value of 8.5 Hz was found. Interestingly, no $J_{^{31}P-^{31}P}$ was observable for the corresponding nonmethylated AMPNP (*4*). AMPN(Me)P was found to be a respectable substrate for rabbit muscle creatine kinase (Table 1), and this permitted its rather facile conversion to AMPN(Me)PP. As with AMP ·

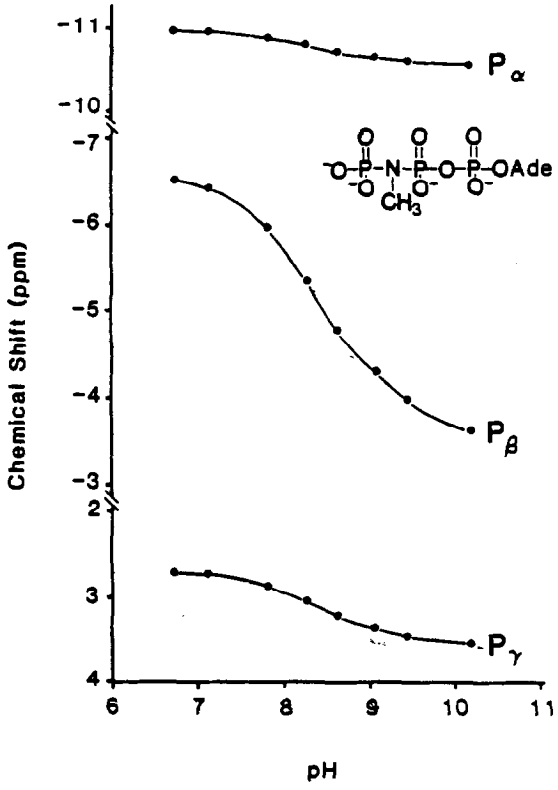


FIG. 2. ^{31}P NMR titration curves for the α-, β-, and γ-phosphoryl groups of AMPPN(Me)P.

PN(Me)P, its ^{31}P NMR spectrum showed large $J_{^{31}P-^{31}P}$ coupling constants (22 Hz), similar to those of ATP.

pH-Titration Curves Using ^{31}P NMR Spectroscopy

Of particular interest were the pH-titration curves for the α-, β-, and γ-phosphorus resonances of AMP · PN(Me)P. Earlier, we and others had studied (*24*, *31*, *32*) the similar pH-titration curves for AMP · PNP. For AMP · PNP, the β-phorphorus shows by far the greatest shift of the three upon titration in the physiological pH range, even though we established quite clearly by pH titrations using ^{17}O NMR that only the oxygens of the γ-phosphorus are protonated in the pH range 5–12. Thus the observed ^{31}P NMR shift of the β-phosphorus is an indirect effect and evidently has nothing to do with direct protonation of the oxygens on the β-phosphorus. In Fig. 2 are shown the corresponding pH-titration curves for AMP · PN(Me)P. It closely resembles that for AMP · PNP. Thus, whatever is causing the β-phosphorus to shift so dramatically is operating in both compounds. Also, since AMP · PN(Me)P can be considered as a "locked" imido tautomer of AMP · PNP (*24*), this similar pH-titration behavior lends further

TABLE 1

V_{max} and K_m Values for ATP, ADP, and Their Corresponding Imido and *N*-Methylimido Analogs in the Creatine Kinase Reaction

	V_{max} (mM · min^{-1})	K_m (mM)	V_{max}/K_m (min^{-1})
	Forward direction (ATP consumption)		
ATP	0.790 ± 0.056	0.094 ± 0.009	8.40 ± 1.00
AMPNPP	N.D.[a]	N.D.[a]	
AMPN(Me)PP	N.D.[a]	N.D.[a]	
	Reverse direction (ATP production)		
ADP	0.297 ± 0.024	0.012 ± 0.002	24.8 ± 4.58
AMPNP	0.248 ± 0.017	0.386 ± 0.029	1.902 ± 0.081
AMPN(Me)P	0.129 ± 0.019	1.71 ± 0.35	0.075 ± 0.091

Note. See text for assay conditions.

[a] Not detectable in the coupled enzyme assayed used. With the PEI-cellulose TLC assay (*21*), however, phosphocreatine was unequivocally detected as a product when the analogs replaced ATP as substrates.

support to our earlier conclusion that AMP · PNP exists in solution largely, if not exclusively, as the imido tautomer shown in structure **1.**

pH-titration curves for both AMPN(Me)P and AMPN(Me)PP, again measured using ^{31}P NMR, are shown in Figs. 3 and 4, respectively. They provide convincing evidence for the proposed structures.

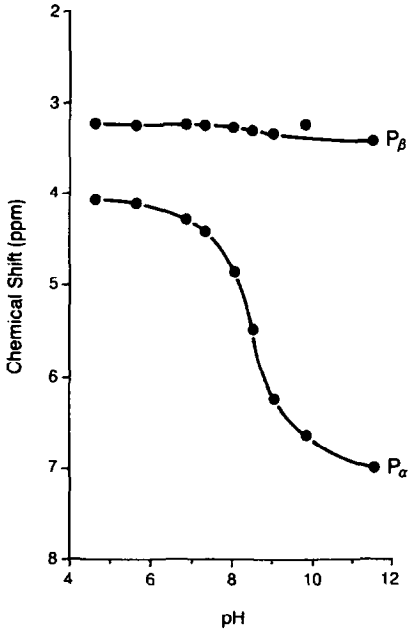


FIG. 3. ^{31}P NMR titration curves for the α- and β-phosphoryl groups of AMPN(Me)P.

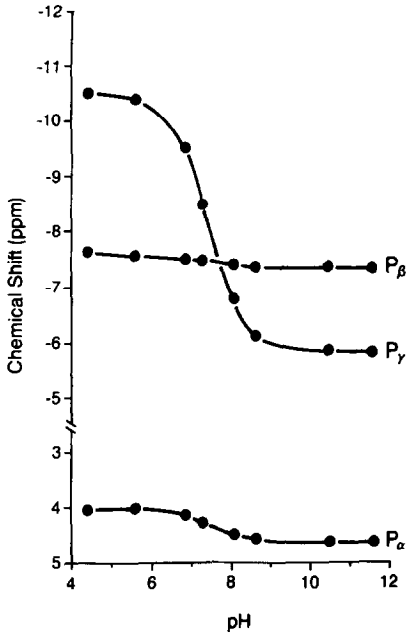


FIG. 4. ^{31}P NMR titration curves for the α- β-, and γ-phosphoryl groups of AMPN(Me)PP.

Enzyme Kinetic Studies in the Creatine Kinase Reaction

In Table 1 are shown V_{max}, K_m, and V_{max}/K_m values for some of these purine nucleotide analogs. AMP · PN(Me)P appears to be rejected by the enzyme since we could detect activity neither as a substrate nor as an inhibitor. Both AMPN(Me)P and AMPN(Me)PP were substrates, however. AMPN(Me)P showed ca. 40% of the V_{max} value of ADP under the same conditions, whereas its K_m value was substantially higher. Accurate values for these kinetic parameters could be detected using the coupled assay with hexokinase for which the AMPN(Me)PP produced is also a substrate. In the reverse direction, however, correspondingly accurate kinetic parameters could not be measured since the AMPN(Me)P generated was not a sufficiently good substrate for pyruvate kinase. Nevertheless, AMPN(Me)PP was shown to generate phosphocreatine from creatine using the PEI-cellulose TLC assay system of Rowley and Kenyon (*21*).

In Table 1 also are included kinetic data for AMPNP as a substrate in the creatine kinase reaction. Comparisons of these data were those for the corresponding *N*-methylated analog indicates, not too surprisingly, that creatine kinase is susceptible to steric hindrance from the *N*-methyl group. On the other hand, the reasonably high relative V_{max} value for AMPN(Me)P shows its potential for interesting interactions with other ADP-utilizing enzymes in future studies.

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