Nucleoside 5′-(β,γ-Peroxytriphosphates)†

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ABSTRACT: Procedures for the synthesis, purification, and characterization of β,γ-peroxy analogues of the eight common ribo- and deoxyribonucleoside triphosphates have been developed. Although adenosine 5′-(β,γ-peroxytriphosphate) was stable to conditions in most biochemical systems, incubation of a solution of the analogue at 100 °C led to formation of AMP and ATP, as well as ADP. NAD^+ pyrophosphorylase was the only enzyme among 13 tested for which adenosine 5′-(β,γ-peroxytriphosphate) was a good substrate, but the analogue was an effective inhibitor for a number of kinases. The peroxy compounds tested inactive with *Escherichia coli* RNA polymerase and DNA polymerase I, as well as with wheat germ RNA polymerase II.

Most nucleoside triphosphate utilizing enzymes catalyze reactions involving the phosphate chain. Nucleotide analogues with phosphate chain modifications have been of particular value in the study of such enzymes. Those analogues in which one or another of the phosphorus atoms has been made chiral by sulfur and/or oxygen isotope substitution have been of great value in the elucidation of the stereochemical course of enzymatic reactions and the role of cations in catalysis [for reviews, see Eckstein (1979) and Knowles (1980)]. Replacement of the β,γ-bridge oxygen by a $-CH_2-$ or $-NH-$ group yields analogues that are inert to kinase reactions, while α,β-methylene nucleotide analogues are inert to reactions in which the leaving group is either pyrophosphate or a nucleoside monophosphate (Yount, 1975). We recently communicated the synthesis and preliminary enzymatic testing of the first examples of a new class of nucleoside triphosphate analogues, adenosine 5′-(β,γ-peroxytriphosphate) and guanosine 5′-(β,γ-peroxytriphosphate), in which the β,γ-bridge oxygen is replaced by a peroxide linkage (Rosendahl & Leonard, 1981; Leonard & Rosendahl, 1981). We now describe fully procedures for the synthesis, purification, and characterization of the β,γ-peroxy analogues of the eight common ribo- and deoxyribonucleoside triphosphates plus dUTP. We also describe some of the chemical behavior of these compounds, as well as their behavior in a variety of enzyme systems. It is our hope that these and related analogues will further the understanding of both the binding and reactivity of nucleotides at the active sites of enzymes.

Materials and Methods

Tetrapotassium peroxydiphosphate was obtained from Chem Services, Inc., West Chester, PA. *Escherichia coli* and wheat germ RNA polymerases and *E. coli* DNA polymerase I were gifts of Dr. Judith Jaehning. *E. coli* adenine phosphoribosyltransferase was a gift of Dr. Robert Switzer.

DMF[1] was stirred over KOH for about 16 h and distilled from CaO. Pyridine was distilled first from $KMnO_4$ and then from CaH_2. Morpholine was distilled before use. These solvents and toluene were stored in the dark over Linde 3A molecular sieves. Triethylamine was distilled before use.

Synthesis of Nucleoside 5′-(β,γ-Peroxytriphosphates). (A) Coupling Procedure. Two different methods were used for activation of nucleoside 5′-monophosphates for condensation with PO_2P_i. The morpholidate method (Moffatt & Khorana, 1961) was successful in all cases. The simpler imidazolidate procedure (Hoard & Ott, 1965) was also successful, except for cytidine nucleotides.

If the nucleoside monophosphate had been obtained as the free acid, morpholidate formation was performed exactly as previously described (Moffatt & Khorana, 1961). Morpholinium salts were obtained from the sodium salts (1 mmol) by passage of ~50 mL of aqueous solution over a 100-mL column of Dowex 50-X8, 20–100 mesh, morpholinium form. The eluate was reduced to the recommended volume in vacuo, and 4 equiv of morpholine (0.34 mL, 4 mmol) was added. Morpholidate formation was checked by TLC (cellulose; 2-propanol–0.25 M NH_4HCO_3, 2:1; R_f of starting materials ~0.3, R_f of products ~0.6). After the ether washes in the published procedure, the aqueous layer was dried to a glass, and the morpholidate was coevaporated twice with dry pyridine (~20 mL) and twice with toluene (~20 mL). Because toluene foams under high vacuum, a water aspirator was used to remove the visible liquid toluene before the oil pump was applied. This procedure afforded flaky white solids that dissolved readily in dry DMF (~20 mL). These DMF solutions were then added to PO_2P_i (5 equiv) prepared as described below.

For imidazolidate formation, sodium salts of nucleoside monophosphates (1 mmol) were converted to tri-*n*-butylammonium salts as follows: A 50-mL aliquot of an aqueous solution was passed over a 100-mL column of Dowex 50-X8, 20–100 mesh, pyridinium form. One equivalent of tri-*n*-butylamine (0.24 mL, 1 mmol) was added, and the solution was evaporated to dryness with an oil pump. Free acids of nucleoside monophosphates (1 mmol) were suspended in 15 mL of water, and 1 equiv of tri-*n*-butylamine was added before evaporation. A stirring bar was then added. The residue was coevaporated with dry pyridine and toluene as above. The dried tri-*n*-butylammonium salt was dissolved in ~15 mL of

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[1] Abbreviations: DMF, *N,N*-dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate; KP_i, potassium phosphate buffer; NMN, nicotinamide mononucleotide; PEI–cellulose, poly(ethylenimine)-impregnated cellulose TLC sheets (Brinkmann); PO_2P_i, inorganic peroxydiphosphate; PP_i, inorganic pyrophosphate; P-Rib-PP, phosphoribosyl pyrophosphate; TE, 50 mM Tris-HCl (pH 7.5 at room temperature) + 2 mM NaEDTA; TEAB, triethylammonium bicarbonate, pH 7; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane. AMP·PO_2P designates adenosine 5′-(β,γ-peroxytriphosphate), GMP·PO_2P designates guanosine 5′-(β,γ-peroxytriphosphate), and so on.

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dry DMF, and 2.5 equiv (0.40 g, 2.5 mmol) of 1,1′-carbonyldiimidazole was added. The flask was stoppered and placed in a desiccator. The reaction mixture was stirred for between 2 and 16 h. Imidazolidate formation was verified by TLC as for the morpholidates; the R_f's were similar. When activation was complete, 3 equiv of anhydrous methanol (0.12 mL, 3 mmol) was added, and the mixture was stirred for an additional 30 min before being added to the PO_2P_i (below).

Five equivalents of tetrapotassium peroxydiphosphate (1.73 g, 5 mmol) was dissolved in ~50 mL of water and passed through a 100-mL column of Dowex 50-X8, 20–100 mesh, pyridinium form. One equivalent of tri-*n*-butylamine (1.19 mL, 5 mmol) was added, and the solution was inspissated with an oil pump to ~4 mL of syrup. Addition of pyridine (~20–30 mL) induced crystallization. The solid was dried by coevaporation with pyridine and toluene as described for the morpholidates, and it was then partially dissolved in ~30 mL of dry DMF. The activated monophosphate and a stirring bar were added, and the stoppered flask was placed in a desiccator. The reaction mixture was stirred for ~16–30 h or until TLC on cellulose showed that the activated monophosphate was no longer present.

(*B*) *Purification.* Because the nucleoside peroxytriphosphates are sensitive to metal ion catalyzed decomposition, glass-distilled water was used throughout the workup. All chromatography was conducted at 4 °C. Other precautions are given below.

The crude purine nucleotide reaction mixtures in DMF were added to ~1 L of water and applied to a 240-mL (5 × 12 cm) column of DEAE-Sephadex A-25 (Pharmacia) equilibrated in 20 mM sodium succinate buffer, pH 5.9. The column was developed with a linear gradient of 2 L of 40 mM $MgCl_2$ and 2 L of 100 mM $MgCl_2$ in succinate buffer. AMP·PO_2P eluted at ~70 mM $MgCl_2$ and GMP·PO_2P at ~77 mM $MgCl_2$. Pooled fractions were diluted with 3 volumes of water and applied to a 150-mL (4 × 12 cm) column of DEAE-Sephadex A-25 equilibrated in 0.1 M TEAB. A 10-mL column of Chelex-100, 200–400 mesh (Bio-Rad), triethylammonium form, in a 20-mL plastic syringe, was placed in the inlet to the column after loading, and 0.1 mL of 10 mM NaEDTA, pH 7, was added to each 18 × 150 mm tube in the fraction collector. The column was developed with a linear gradient of 1.5 L of 0.2 M TEAB and 1.5 L of 0.7 M TEAB. Appropriate fractions were pooled and evaporated to dryness under reduced pressure at 30 °C or below. A few milliliters of 1-butanol was added to control bumping. The resulting residue was coevaporated with ethanol until the odor of triethylamine was no longer detected.

The glassy film remaining in the flask was dissolved in 2–3 mL of methanol. The nucleotide was precipitated as its sodium salt by addition of 15–20 mL of 0.15 M $NaClO_4$ in acetone. The gelatinous precipitate was collected by centrifugation and washed with four 10-mL portions of acetone. A gentle stream of nitrogen was used to remove the acetone. The slightly hygroscopic white (off-white in the case of the guanine nucleotides) solid was transferred to a small vial and crushed with a spatula. The products were stored in a desiccator at −20 °C.

Crude pyrimidine peroxytriphosphate reaction mixtures were diluted in ~1 L of water and applied to a 200-mL (4.5 × 12 cm) column of Dowex 1-X8, 200–400 mesh, Cl^- form. The column was developed with a linear gradient of 2 L of 0.2 M KCl and 2 L of 0.4 M KCl, both in 20 mM sodium succinate, pH 5.9. Pooled fractions were diluted and chromatographed on DEAE-Sephadex A-25 in TEAB, and the products were recovered as their sodium salts, as described for the purine peroxytriphosphates. The Dowex column was washed with 1 L of 1 N HCl and 1 L of water before reuse.

(*C*) *Characterization.* After isolation, the nucleoside β,γ-peroxytriphosphates were characterized by their ultraviolet spectra, phosphate content after ashing, and content of the corresponding mono-, di-, and triphosphates, as follows. Inorganic phosphate was assayed in column fractions after acid hydrolysis and in samples of the isolated products after ashing (Clark & Switzer, 1977). KH_2PO_4 was used as a standard after being dried for several hours at 100 °C.

Nucleotide contamination was checked qualitatively by TLC. Mono- and diphosphate contamination was checked either on silica gel plates in isobutyric acid–concentrated NH_4OH–water, 66:1:33 (system I), or in PEI–cellulose plates in 0.3 M KP_i, pH 7 (system II). The latter system runs faster and is odorless; on a 20-cm sheet it also gave good separation of all four triphosphates. However, neither system separated a peroxytriphosphate from its corresponding natural triphosphate. This separation was obtained on PEI–cellulose TLC plates in 0.2 M KP_i, pH 6, containing 40 mM $MgCl_2$ (system III). It was necessary to run the water front to at least 15 cm above the origin to obtain complete resolution. The buffer was freshly made each time because $MgHPO_4$ began to crystallize within 1–2 h and the resolution deteriorated. R_f's were variable, but a peroxy analogue always ran more slowly than its natural counterpart.

Di- and triphosphate contamination was quantitated enzymatically. Diphosphates were assayed by use of pyruvate kinase and lactate dehydrogenase, with nucleoside diphosphate kinase (which is shown below not to utilize the nucleoside peroxytriphosphates) and ATP (1 mM) as auxiliaries when needed. A typical assay (1 mL) contained 0.1 M Tris-HCl, pH 7.5, 5 mM $MgSO_4$, 10 mM KCl, 2 mM EDTA, 0.5 mM phosphoenolpyruvate, 0.05 mM NADH, plus enzymes. The amount of NAD^+ formed upon addition of peroxytriphosphate was determined from the decrease in absorbance at 340 nm ($\Delta mM\epsilon = 6.22$).

The extent of natural triphosphate contamination was determined by two enzymatic methods, which gave comparable results. The first used P-Rib-PP synthetase and adenine phosphoribosyltransferase:

$$\text{step I: ATP + Rib-5-P} \rightarrow \text{AMP + P-Rib-PP}$$

$$\text{step II: P-Rib-PP + [}^3\text{H]adenine} \rightarrow \text{[}^3\text{H]AMP + PP}_i$$

P-Rib-PP synthetase does not use AMP·PO_2P (below). Since this enzyme is adenine specific, nucleoside diphosphate kinase and ADP (0.01 mM) were used as auxiliaries for the assay of other triphosphates. The assay was conducted essentially as described by Gibson & Switzer (1981). This assay has the advantage of sensitivity (10 pmol of NTP is readily detected), but its disadvantage is that it depends on two enzymes that are not commercially available.

Since hexokinase was unable to utilize AMP·PO_2P (see below), it was possible to use hexokinase and Glu-6-P dehydrogenase, with nucleoside diphosphate kinase and ADP (1 mM) as auxiliaries where necessary, to assay for triphosphate contamination. A typical assay mixture (1 mL) contained TE, 5 mM glucose, 5 mM $MgCl_2$, 0.2 mM $NADP^+$, sufficient enzymes to ensure completion of the reaction in 20 min or less, and enough NMP·PO_2P or dNMP·PO_2P to give an increase in A_{340} of 0.03 or more.

(*D*) *Stability of AMP·PO_2P.* Rates of ATP, ADP, and AMP formation from AMP·PO_2P during a 20-min incubation at 100 °C (boiling water bath) were determined at 4 and 20

mM AMP·PO_2P. AMP·PO_2P at 0.1 M was diluted into TE at 100 °C to give the desired final concentration. Samples were transferred at intervals into glass 13 × 100 mm tubes in an ice–water bath. ATP and ADP were assayed as described above. AMP was assayed as for ADP, except that adenylate kinase was added. The amount of AMP was calculated from the additional NADH oxidation in the presence of adenylate kinase, assuming that each molecule of AMP allowed oxidation of two molecules of NADH. The fraction of AMP·PO_2P remaining was calculated as 1 − (ATP + ADP + AMP), and the first-order rate constant for loss of AMP·PO_2P was obtained from a plot of log AMP·PO_2P remaining vs. time. Individual rate constants for the formation of the three nucleotide products were calculated by assuming parallel first-order reactions (Frost & Pearson, 1961). The stability of 4 mM AMP·PO_2P was determined in TE + 10 mM $MgCl_2$ and also in 50 mM KP_i, pH 7.5, + 2 mM EDTA. The stability of 10 mM ATP in the same three buffers was also determined.

Enzyme Assays. (*A*) *Hexokinase* (EC 2.7.1.1, from yeast) was tested for activity with AMP·PO_2P in a mixture (50 μL) containing TE, 5 mM $MgCl_2$, 2.5 mM AMP·PO_2P, and 5 mM each of glucose, Fru-6-P, and glycerol. Approximately 2 units of hexokinase was added, and reaction was monitored by TLC (system II). Spectrophotometric assays (1 mL) for activity were conducted in 0.1 M Tris-HCl, pH 8, 5 mM $MgCl_2$, 2 mM EDTA, 0.2 mM $NADP^+$, 5 mM glucose, excess Glu-6-P dehydrogenase, and 0.2 unit of hexokinase. Reaction was initiated by addition of AMP·PO_2P and monitored at 340 nm.

(*B*) *Phosphofructokinase* (EC 2.7.1.11, from rabbit muscle) (~5 units) was tested for activity by TLC in the same mixture as for hexokinase. Spectrophotometric assays (1 mL) were conducted in 0.1 M Tris-HCl, pH 8, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM KCl, 0.2 mM NADH, 1 mM Fru-6-P, 5 units of phosphofructokinase, and excess aldolase, triosephosphate isomerase, and glycerolphosphate dehydrogenase. Reaction was initiated by addition of AMP·PO_2P and monitored at 340 nm.

(*C*) *Glycerokinase* (EC 2.7.1.30, from *Candida mycoderma*) (~0.4 unit) was tested for activity by TLC as described for hexokinase.

(*D*) *Phosphoglycerate kinase* (EC 2.7.2.3, from yeast) was tested for activity spectrophotometrically. The assay (1 mL) contained TE, 5 mM $MgCl_2$, 2.5 mM 3-phosphoglycerate, 0.2 mM NADH, excess glyceraldehyde-3-phosphate dehydrogenase, and 5 units of phosphoglycerate kinase. Reaction was initiated by addition of AMP·PO_2P.

(*E*) *Adenylate kinase* (EC 2.7.4.3, from rabbit muscle) was assayed for activity with AMP·PO_2P in mixtures (50 μL) containing TE, 10 mM $MgCl_2$, 1 mM each of ATP and AMP or 2 mM ADP, ~10^5 cpm [γ-^{32}P]ATP, 15 units of adenylate kinase, and ±5 mM AMP·PO_2P. Aliquots of 5 μL were spotted onto TLC plates (system II) at intervals during an incubation at room temperature. After chromatography, ATP + AMP·PO_2P and ADP regions were cut out and counted. ATP and AMP·PO_2P were also resolved by TLC (system III), and the triphosphate spots were cut out and counted separately. AMP·PO_2P inhibition of ATP synthesis was assayed by the hexokinase–Glu-6-P dehydrogenase coupled assay system. Assay mixtures (1 mL) contained TE, 10 mM $MgCl_2$, 0.5 mg/mL bovine serum albumin, 5 mM glucose, 0.2 mM $NADP^+$, excess coupling enzymes, and the desired levels of ADP and AMP·PO_2P. Reaction was initiated with adenylate kinase. In one experiment, assays at 0.1 mM ADP were also supplemented with 0.2 mM AMP.

(*F*) *Nucleoside diphosphate kinase* (EC 2.7.4.6, from beef liver) was tested for activity with the peroxytriphosphates in mixtures (50 μL each) containing 0.1 M triethanolamine hydrochloride, pH 8, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM mercaptoethanol, 50 μM ATP (including ~10^5 cpm of [γ-^{32}P]ATP), 1 mM NMP·PO_2P, and 0.1 unit of enzyme. Assays of AMP·PO_2P and dAMP·PO_2P contained 1 mM UDP or CDP also. Reactions were monitored as for adenylate kinase. ATP and partner triphosphate (UTP or CTP in AMP·PO_2P or dAMP·PO_2P reactions) spots were cut out and counted. To check for exchange of ^{32}P into the peroxy nucleotides, purines were chromatographed in system III. Pyrimidine peroxytriphosphates were separated from their corresponding triphosphates and from ATP by chromatography on 10 × 20 cm sheets of PEI–cellulose. The short dimension was developed in solvent II, and the plate was dried and cut just below the phosphate front. The long dimension was developed in solvent III, and the nucleotide spots were cut out and counted. AMP·PO_2P inhibition of ^{32}P transfer from [γ-^{32}P]ATP to UDP was measured in mixtures (50 μL each) containing TE, 5 mM $MgCl_2$, 1 mM UDP, ~10^5 cpm labeled ATP, and the desired levels of ATP and AMP·PO_2P. Reaction at room temperature was initiated with enzyme and monitored as before.

(*G*) *Phosphoribosylpyrophosphate synthetase* (EC 2.7.6.1, from *Salmonella typhimurium*) was tested for activity with AMP·PO_2P in place of ATP in 0.1 mL of the mixture used for the standard assay (Switzer & Gibson, 1978). The mixture was incubated with 8 units of enzyme for 20 min at 37 °C and then for 16 h at room temperature, and the reaction was monitored by TLC (system II). AMP·PO_2P inhibition of the enzyme was measured by using the ^{32}P transfer assay (Switzer & Gibson, 1978), except that 2 mM EGTA replaced the EDTA, and ATP and AMP·PO_2P concentrations were varied.

(*H*) *NAD^+ pyrophosphorylase* (EC 2.7.7.7.1, from pig liver) was assayed with AMP·PO_2P as follows. Conversion of AMP·PO_2P (0.05–0.5 mM) to ATP by exchange was assayed in the presence of enzyme, 1 mM NMN, and 1 mM PP_i. The assay also contained TE, 10 mM $MgCl_2$, 5 mM glucose, 0.2 mM $NADP^+$, plus hexokinase and Glu-6-P dehydrogenase, and the reaction was monitored at 340 nm. ATP formation was visualized directly by TLC (system III) in a mixture containing TE, 5 mM $MgCl_2$, 10 mM PP_i, 2.5 mM NMN, 5 mM glucose, 5 mM AMP·PO_2P, and enzyme. The rate of exchange of ^{32}P between [γ-^{32}P]ATP and PP_i was measured under the conditions for the spectrophotometric assay of AMP·PO_2P–PP_i exchange, except that labeled ATP (0.5 mM) replaced AMP·PO_2P and the coupling enzymes and their substrates were omitted. At timed intervals, 5-μL aliquots were added to tubes containing 0.5 mL of cold 5% $HClO_4$. The samples were worked up as for the ^{32}P transfer assay of P-Rib-PP synthetase (Switzer & Gibson, 1978). NAD^+ synthesis was assayed in mixtures containing TE, 5 mM $MgCl_2$, 1 mM NMN, 50 mM ethanol, alcohol dehydrogenase, and ATP or AMP·PO_2P (0.01–1 mM). Reaction was initiated by addition of enzyme and monitored at 340 nm. AMP·PO_2P and ATP synthesis by NAD^+ pyrophosphorylase was monitored by TLC (system III) in mixtures containing TE, 5 mM $MgCl_2$, 2.5 mM NAD^+, and 10 mM PO_2P_i or PP_i.

(*I*) *Firefly luciferase* was assayed for activity with AMP·PO_2P in the Du Pont Luminescence Biometer. The assay cocktail contained TE, 1 mM DTT, 10 mM $MgSO_4$, 0.05 mM luciferin, 0.3 mg/mL bovine serum albumin, and purified luciferase (Boehringer). The height of the light flash produced when ATP was injected into the assay mixture was

proportional to the concentration of ATP (McElroy & Seliger, 1961); the same was true for AMP·PO_2P injections. Preincubations of nucleotide solutions with hexokinase and P-Rib-PP synthetase were conducted in the standard assay mixture for P-Rib-PP synthetase (Switzer & Gibson, 1978), supplemented with 5 mM glucose.

(*J*) *Aminoacylation* of yeast $tRNA^{Phe}$ by its cognate synthetase (EC 6.1.1.20) was assayed in mixtures (50-μL total volume) containing 0.1 M Tris-HCl, pH 7.5, 0.1 M KCl, 60 mM $MgCl_2$, 0.12 mg/mL bovine serum albumin, 60 mM mercaptoethanol, 20 μM [^{3}H]-L-Phe, 0.3 μM tRNA, 5 mM glucose, and 1 mM ATP or AMP·PO_2P. Mixtures supplemented with crystalline hexokinase (~0.1 mg) were preincubated at 37 °C for 10 min before reaction was initiated by addition of 5 μL of enzyme at 0.5 unit/mL. At timed intervals (0–5 min), 10-μL aliquots were removed and added to 2 mL of cold 5% TCA. Precipitates were collected on Millipore HAWP filters. The filters were washed 5 times with ~2 mL of 5% TCA and rinsed with ethanol. They were then dried and counted.

(*K*) *E. coli RNA polymerase* (EC 2.7.7.6) was assayed in mixtures (25-μL total volume) containing 50 mM Tris-HCl, pH 7.9, 0.2 M NaCl, 10 mM $MgCl_2$, 4 mM DTT, 0.5 mM each of three unlabeled NTP's or two unlabeled NTP's and one NMP·PO_2P, 0.1 mM [α-^{32}P]NTP, and 55 μg/mL T4 DNA. Enzyme (10 μL/assay) was diluted in 50 mM Tris-HCl, pH 7.9, containing 10% glycerol, 60 mM NaCl, 0.1 mM EDTA, and 2 mg/mL bovine serum albumin. Some reactions were supplemented with 20 mM K_2HPO_4, 5 mM Rib-5-P, and ±80 units/mL P-Rib-PP synthetase. Reaction at 37 °C was initiated by addition of enzyme. After 10 min, each reaction mixture was applied to a 1.5-cm^2 piece of Whatman DE-81 paper. The squares were washed 6 times in 0.5 M Na_2HPO_4, twice in water, and once in ethanol. They were then dried and counted.

(*L*) *Wheat germ RNA polymerase II* (EC 2.7.7.6) was assayed in mixtures (20-μL total volume) containing 50 mM Tris-HCl, pH 7.9, 0.1 M KCl, 10 mM $MgCl_2$, 4 mM DTT, 0.5 mM each of CTP, UTP, and ATP or AMP·PO_2P, 0.1 mM [α-^{32}P]GTP, 20 mM K_2HPO_4, and 0.15 mg/mL heat-denatured calf thymus DNA. Some reactions were supplemented with 5 mM Rib-5-P and/or 60 units/mL P-Rib-PP synthetase. The polymerase (5 μL/assay) was diluted in 20 mM Tris-HCl, pH 7.9, 1 mM EDTA, plus 20% glycerol. Reactions were incubated for 10–20 min at 30 °C and worked up as for the *E. coli* enzyme.

(*M*) *E. coli DNA polymerase I* (EC 2.7.7.7) was assayed in a total volume of 25 μL. The cocktail (20 μL/assay) contained 125 mM Tris-HCl, pH 7.5, 12.5 mM $MgSO_4$, 2.5 mM EDTA, 1.25 mM DTT, 25 mM KH_2PO_4, 6.25 mM Rib-5-P, 0.25 μM each dGTP, dTTP, and dATP or dAMP·PO_2P, 0.86 μM [α-^{32}P]dCTP, and 0.1 mg/mL activated calf thymus DNA; some assays were also supplemented with 22 units/mL P-Rib-PP synthetase. Assays were preincubated for 10 min at 37 °C before addition of polymerase (5 μL/assay), which was diluted in 10 mM KP_i, pH 6.9, containing 0.1 mM EDTA, 1 mM DTT, 5% glycerol, and 1 mg/mL bovine serum albumin. After 30 min at 37 °C, reaction mixtures were worked up as for the RNA polymerases.

Results

Synthesis, Purification, and Properties of β,γ-Peroxytriphosphates. We have prepared β,γ-peroxy analogues of ATP, GTP, UTP, CTP, dATP, dGTP, dUTP, dCTP, and dTTP from inorganic peroxydiphosphate and the mononucleotide. Synthesis of these compounds in 50–70% yield was straightforward, except that use of 1,1′-carbonyldiimidazole as a condensing agent with cytidine nucleotides gave products whose absorption spectrum was shifted 20 nm to the red. No such problem attended the use of the morpholidate. The purine peroxytriphosphates were largely separated from the corresponding triphosphates by a $MgCl_2$ gradient on DEAE-Sephadex A-25; Mg^{2+} has been shown to bind to AMP·PO_2P 30 times more weakly than to ATP (Rosendahl & Leonard, 1981). A second chromatography on DEAE-Sephadex A-25 in TEAB was used both to remove nonvolatile salts and to complete the separation of AMP·PO_2P from PO_2P_i. The pyrimidine peroxytriphosphates cochromatographed on DEAE-Sephadex A-25 with PO_2P_i under all conditions tested, but Dowex-1 was found to retard the nucleotides sufficiently to afford separation. At pH 5.9, at which pH the charge difference between natural and peroxytriphosphates would be expected to be maximal (Creaser & Edwards, 1972), a KCl gradient separated the pyrimidine peroxytriphosphates from both PO_2P_i and the corresponding triphosphates. A second chromatography was then used to remove nonvolatile salt.

The nucleoside 5′-(β,γ-peroxytriphosphates) had ultraviolet absorption spectra indistinguishable from those of their natural counterparts. Phosphate contents (assuming the same extinction coefficients as for the parent nucleotides) were approximately 3 mol of P_i/mol of nucleotide. All samples of peroxynucleotides examined contained detectable NDP and NTP impurities. The level of NDP contamination in freshly prepared NMP·PO_2P samples typically ranged between 0.5 and ~3%; cytosine and guanine compounds seemed more susceptible to decomposition during workup than adenine or uracil compounds. The isolated sodium salts do not appear to be indefinitely stable; a GMP·PO_2P sample that originally contained 2% GDP was found to contain 4.3% GDP when reassayed 7 months later. The sample had been stored in a desiccator at −20 °C, but it had been warmed to room temperature and opened several times in the interim. Triphosphate contamination of the β,γ-peroxy purine nucleotide samples averaged 1.7% (range 0.6–4.1%); two cycles of $MgCl_2$ gradient chromatography afforded AMP·PO_2P containing 0.5% ATP. Triphosphate contamination of samples of β,γ-peroxy pyrimidine triphosphates ranged between 0.3 and 0.8%. While these contaminants were presumed to have negligible effect upon enzyme inhibition studies, their presence means that tests for enzyme activity with the peroxy analogues must be interpreted carefully.

The stability of ATP (10 mM) was compared to that of AMP·PO_2P (4 mM) at 100 °C in three different buffers. These were (buffer A) TE, (buffer B) TE + 10 mM $MgCl_2$, and (buffer C) 50 mM KP_i + 2 mM EDTA, pH 7.5. As expected, ATP was hydrolyzed to ADP; in 20 min at 100 °C, the fraction of ADP was increased by 0.16 in buffer A, by 0.12 in buffer B, and by 0.10 in buffer C. If one assumes that hydrolysis was pseudo first order, the corresponding observed rate constants were (buffer A) 1.4×10^{-4} s^{-1}, (buffer B) 1.1×10^{-4} s^{-1}, and (buffer C) 8.2×10^{-5} s^{-1}. The latter values suggest slight stabilization of ATP in the presence of either Mg^{2+} or inorganic phosphate. AMP formation was negligible.

At 100 °C in each of the three buffers, AMP·PO_2P was significantly less stable than ATP (Table I). Plots of log AMP·PO_2P remaining vs. time were linear, and as expected for a first-order reaction, the rate constants in buffer A at 4 mM and at 20 mM AMP·PO_2P were essentially the same. AMP·PO_2P was equally stable in buffers A and C, without divalent cations, but inclusion of $MgCl_2$ (buffer B) increased the rate of AMP·PO_2P loss 8-fold. In order of predominance,

Table I: Stability of AMP·PO_2P at 100 °C

buffer[a]	initial [AMP·PO_2P] (mM)	rate constants (s^{-1}) for AMP·PO_2P loss	AMP formation	ADP formation	ATP formation
A	4	6.3×10^{-4}	4.3×10^{-4}	2.3×10^{-4}	1.1×10^{-5}
	20	6.8×10^{-4}	4.3×10^{-4}	2.5×10^{-4}	1.3×10^{-5}
B	4	5.0×10^{-3}	4.2×10^{-3}	1.6×10^{-3}	4.3×10^{-4}
C	4	6.0×10^{-4}	4.8×10^{-4}	2.2×10^{-4}	4.8×10^{-6}

[a] Buffer compositions are given in the text.

the products of AMP·PO_2P decomposition were AMP, ADP, and ATP. In any given reaction, the products were present in constant proportion over time, indicating that their formation followed the same rate law and that no product was derived from another but only from the original. In buffers A and C, the AMP:ADP:ATP ratio was approximately 1:0.5:~0.02. Addition of Mg^{2+} (buffer B) gave preferential enhancement of the rate of ATP formation, so that the observed AMP:ADP:ATP ratio was 1:0.4:0.1.

The rates of AMP·PO_2P decomposition were measured at 100 °C, which made them fast enough to be experimentally convenient. At 4 °C, no change in the level of ATP contamination of an AMP·PO_2P solution in TE was observed over a period of 2 weeks. Some ADP formation was apparent by TLC, however. The finding that AMP·PO_2P was stable to overnight incubation in the presence of several different enzymes, Mg^{2+} and cosubstrates indicates that the rates of ADP and ATP formation are quite slow at room temperature. However, an implication of the relatively rapid rearrangement of ATP at 100 °C is that if an enzyme is used to remove the last traces of a contaminating triphosphate from a NMP·PO_2P sample and if the enzyme is incompatible with the system in which the sample is to be used, the enzyme must be inactivated by some means other than boiling. Acid precipitation is not a suitable alternative because the peroxy linkage is acid labile (Creaser & Edwards, 1972).

We have also observed that AMP·PO_2P reacts rapidly with Mn^{2+} (the observed half-life at 37 °C in TE with 10 mM AMP·PO_2P and 20 mM $MnCl_2$ was about 5 min) to give MnO_2 and ADP. We would also expect the β,γ-peroxytriphosphates to be incompatible with other easily oxidized transition metal ions. This sensitivity makes it advisable to include chelators such as EDTA or EGTA in buffers and to supplement them with an excess of the desired cations. Dithiothreitol also appears to react slowly with AMP·PO_2P; upon overnight incubation at room temperature in TE + 20 mM DTT, somewhat more than half of 10 mM AMP·PO_2P was converted to ADP, while decomposition in the absence of DTT was negligible. AMP·PO_2P was also incompatible with hydrazine, which is used as a trapping agent in some assays.

Enzyme Studies. There was no detectable utilization of AMP·PO_2P by any kinase tested. Neither hexokinase, phosphofructokinase, nor glycerokinase gave detectable ADP from AMP·PO_2P (as judged by TLC) upon overnight (18 h) incubation with cosubstrate, under conditions in which comparable amounts of ATP reacted completely in under 10 min. One can therefore estimate that reaction was at least 1000 times slower for the analogue than for ATP. In spectrophotometric assays with hexokinase or phosphofructokinase, reaction went to 1–2% of completion and then appeared to stop; addition of further aliquots of AMP·PO_2P produced further incremental changes in A_{340}. With phosphoglycerate kinase, whose equilibrium favors 3-phosphoglycerate, the progress and apparent equilibrium point of a reaction containing 1 mM AMP·PO_2P were indistinguishable from those of a reaction containing 15 μM ATP, whereas addition of 1 mM ATP produced much more NADH oxidation. These results are most readily understood if the AMP·PO_2P is presumed to be inactive but contaminated with ~1–2% ATP. With each of these kinases, AMP·PO_2P inhibition was competitive with respect to ATP. K_i values were comparable to the K_m values determined for ATP (Table II).

Table II: Competitive Inhibition by AMP·PO_2P with Respect to ATP

enzyme	K_m(ATP) (μM)	K_i(AMP·PO_2P) (μM)	K_i/K_m
hexokinase[a]	100	90	0.9
phosphofructokinase[a]	80	200	2.5
phosphoglycerate kinase[a]	500	400	0.8
glycerokinase[a]	60	140	2.3
nucleoside diphosphate kinase	550	3100	5.6
phosphoribosylpyrophosphate synthetase	38	33	0.87

[a] These values were determined by M. S. Rosendahl (1982).

Since the adenylate kinase reaction is freely reversible (Noda, 1973), we examined the influence of AMP·PO_2P on the equilibrium distribution of ^{32}P from [γ-^{32}P]ATP between ATP + AMP·PO_2P and ADP in adenylate kinase reaction mixtures. If AMP·PO_2P were to react, its presence would displace the equilibrium in favor of triphosphate. Addition of 5 mM AMP·PO_2P to reactions containing 2 mM adenine nucleotide had essentially no effect on the position of the diphosphate–triphosphate equilibrium. After 14-h incubation, TLC showed that AMP·PO_2P was still present in the reaction mixtures. However, the amount of radioactivity migrating with AMP·PO_2P (~6% of the total) was no greater than in parallel reactions without AMP·PO_2P. These results suggest that adenylate kinase could neither use AMP·PO_2P as a phosphoryl donor nor exchange ^{32}P into the analogue. Because of the kinetic complexity of the enzyme (Noda, 1973), a complete steady-state analysis of inhibition of AMP·PO_2P was not attempted. However, the ATP synthesis reaction was quite sensitive to AMP·PO_2P; at 0.1 mM ADP, 0.01 mM AMP·PO_2P was required to give 50% inhibition, while, at 1 mM ADP, an $I_{0.5}$ of 0.2 mM was obtained. An AMP·PO_2P inhibition curve was also constructed at 0.1 mM ADP and 0.2 mM AMP (which gave 45% inhibition). When the activity without AMP·PO_2P was taken as 100% for each curve, the inhibition profiles were indistinguishable. This suggests that AMP had no effect on AMP·PO_2P binding in a false ternary complex.

Nucleoside diphosphate kinase was tested for activity with AMP·PO_2P or dAMP·PO_2P by observing equilibration of ^{32}P between 0.05 mM [γ-^{32}P]ATP and 1 mM UDP or CDP. Since the reaction is freely reversible, participation of either analogue should displace the equilibrium in favor of adenine nucleotide. Small displacements were observed, but they were too small to reflect reaction of the peroxy compounds, and we attribute them to di- and triphosphate contamination. In similar experiments with the other seven NMP·PO_2P's and

dNMP·PO_2P's, the distribution of ^{32}P between ATP and other triphosphates was again consistent with negligible reaction of the analogues. Furthermore, the peroxy nucleotide isolated by TLC from such reactions contained no more than 2.5% of the ^{32}P, suggesting that the enzyme did not catalyze ^{32}P exchange with these compounds. AMP·PO_2P was a relatively weak competitive inhibitor with respect to ATP (Table II).

As judged by TLC, no AMP was formed from AMP·PO_2P upon overnight incubation in the presence of 80 units/mL phosphoribosylpyrophosphate synthetase, which was shown afterward to have retained most of its activity. This corresponds to a specific activity of less than 3×10^{-4} unit/mg of protein, which is much less than the specific activity of 110 units/mg for ATP under standard conditions. AMP·PO_2P was an effective competitive inhibitor with respect to ATP (Table II).

NAD^+ pyrophosphorylase activity with AMP·PO_2P was demonstrated in three ways. First, in a reaction dependent upon enzyme, NMN, and PP_i, AMP·PO_2P was converted to ATP, presumably by the following reactions:

$$\text{step I: } \text{AMP·PO}_2\text{P} + \text{NMN} \rightarrow \text{NAD}^+ + \text{PO}_2\text{P}_i$$

$$\text{step II: } \text{NAD}^+ + \text{PP}_i \rightarrow \text{ATP} + \text{NMN}$$

$$\text{net: } \text{AMP·PO}_2\text{P} + \text{PP}_i \rightarrow \text{ATP} + \text{PO}_2\text{P}_i$$

ATP synthesis was followed spectrophotometrically with hexokinase and Glu-6-P dehydrogenase; when AMP·PO_2P was the limiting component, approximately the expected stoichiometry was observed. ATP formation was also visualized directly by TLC at sufficiently high nucleotide concentrations. At 1 mM NMN, 1 mM PP_i, and 0.5 mM AMP·PO_2P, the initial rate of ATP formation (determined spectrophotometrically) was 0.02 unit/mg of protein, which was 3 times slower than ^{32}P exchange between [γ-^{32}P]ATP and PP_i under similar conditions. Second, NAD^+ synthesis from AMP·PO_2P and NMN was assayed with ethanol and alcohol dehydrogenase to reduce NAD^+ as it was formed. Though quantitative reduction of high concentrations of NAD^+ (0.1 mM) could not be obtained, the extent of reaction was such that the known ATP contamination could not account for it. Furthermore, the reactions were not detectably biphasic; the rates appeared constant for several minutes and then declined gradually, just as for ATP. We therefore feel confident that we were assaying reaction of AMP·PO_2P, not ATP. With this assay, the K_m for AMP·PO_2P was 210 μM, and V_{max} was 0.056 unit/mg of protein; the corresponding values for ATP were 55 μM and 0.062 unit/mg. This confirms the previous finding (Rosendahl & Leonard, 1981) that AMP·PO_2P is a good substrate for NAD^+ pyrophosphorylase. The differences between the reported values and those presented here are probably due to differences in enzyme lot or in assay conditions (Rosendahl, 1982). In a third type of experiment, it was also possible to demonstrate AMP·PO_2P synthesis from NAD^+ and PO_2P_i; the reaction was estimated by TLC to be more than 100 times slower than ATP synthesis from NAD^+ and PP_i. This reaction, though slow, could be exploited for enzymatic synthesis of radiolabeled AMP·PO_2P, starting from a suitably labeled NAD^+.

After the ATP contamination of AMP·PO_2P was recognized, AMP·PO_2P was retested for its ability to serve as a substrate for firefly luciferase. Although there was some variation in replicate experiments, AMP·PO_2P gave on the average ~5% of the flash height observed for the same amounts of ATP. Control experiments with ATP showed that pretreatment with hexokinase and excess glucose, or with P-Rib-PP synthetase and Rib-5-P, reduced the flash height by ~99%, as expected. Similar preincubation of AMP·PO_2P with either enzyme to degrade contaminating ATP, which represented 2% of the total adenine nucleotide, reduced the flash height by approximately 50%. Parallel incubations supplemented with [γ-^{32}P]ATP showed that over 90% of the ATP in the AMP·PO_2P had been degraded. This suggests that only about half the light observed with untreated AMP·PO_2P could be attributed to ATP contamination. It appears, therefore, that AMP·PO_2P may indeed be a substrate for firefly luciferase, but if so, it is a much poorer one than the first experiments suggested (Rosendahl & Leonard, 1981). Given the low apparent activity of AMP·PO_2P and the difficulty in obtaining it completely free of ATP, further kinetic analysis was not attempted.

AMP·PO_2P was not a substrate for the aminoacylation of yeast $tRNA^{Phe}$ by its cognate synthetase, since the low level of activity seen with the analogue was completely eliminated when the assay was conducted in the presence of hexokinase and glucose to degrade contaminating ATP. AMP·PO_2P was somewhat inhibitory to the enzyme; the inhibition was not kinetically simple. Complex inhibition of the *E. coli* phenylalanyl-tRNA synthetase by ATP analogues has also been observed (Santi et al., 1971).

E. coli RNA polymerase appeared unable to use AMP·PO_2P, GMP·PO_2P, or UMP·PO_2P as a nucleotidyl donor. The low level of activity seen with these compounds was probably due to NTP contamination, since the time course and extent of reaction seen with each analogue were indistinguishable from those in a reaction mixture containing the natural triphosphate at a concentration equivalent to the known level of NTP contamination of the analogue. Furthermore, when AMP·PO_2P was assayed in the presence of P-Rib-PP synthetase and Rib-5-P to degrade contaminating ATP, incorporation of ^{32}P from [α-^{32}P]CTP into polymer was reduced to background. A sample of UMP·PO_2P containing 2.5% UTP gave a 40-fold higher apparent K_m and the same V_{max} when compared to UTP, which suggests that there was no kinetically significant interaction between the enzyme and the analogue.

Wheat germ RNA polymerase II also appeared to be unable to utilize AMP·PO_2P. The analogue appeared to give 15% of the activity seen at the same concentration of ATP; however, this level of incorporation was comparable to that in reactions to which ATP had not been added, and so it may largely have reflected ATP contamination of the other NTP's. Preincubation of mixtures containing AMP·PO_2P with both P-Rib-PP synthetase and Rib-5-P (but not with either alone) reduced ^{32}P incorporation 5-fold, to a level indistinguishable from that of similarly treated mixtures containing ATP. This level was ~3–4-fold higher than a no enzyme control; it could reflect ^{32}P incorporation into sequences lacking A and/or polymerization supported by the low steady-state level of ATP in the mixture, since P-Rib-PP synthetase was not inactivated after the preincubation.

When *E. coli* DNA polymerase I was tested for activity with dAMP·PO_2P, the results were very similar to those obtained with wheat germ RNA polymerase II. dAMP·PO_2P appeared to give 43% of the activity seen in a parallel reaction containing dATP. This level of activity was only 20% higher than in reactions to which dATP had not been added deliberately. The inclusion of P-Rib-PP synthetase and Rib-5-P in the assay reduced ^{32}P incorporation to the same level with either compound, suggesting that the apparent activity of dAMP·PO_2P was related to dATP contamination rather than to utilization of the analogue. The amount of polymerization in the presence

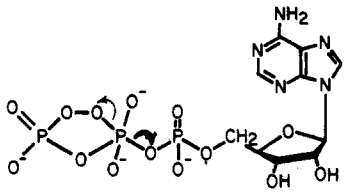

FIGURE 1: Proposed intermediate in rearrangement and fragmentation of AMP·PO_2P.

of P-Rib-PP synthetase was 60% of that in the control without dATP but some 80-fold higher than in a control mixture without enzyme. Some of the difference between the controls could reflect incorporation into sequences lacking dA. It is also likely, since the K_m of DNA polymerase I is "low" (Kornberg & Kornberg, 1974), that the enzyme was able to scavenge dATP efficiently even though its steady-state concentration was very low.

Discussion

The nucleoside β,γ-peroxytriphosphates differ from the natural nucleoside triphosphates by having two, rather than one, oxygen atoms between P_β and P_γ. Because the additional oxygen atom is bonded to its neighbor oxygen in peroxide linkage, these analogues are sensitive to acids and to such odd-electron cations as Mn^{2+} and to reductants such as hydrazine; the nucleotide product is the corresponding diphosphate. Despite these sensitivities, however, the peroxynucleotides are stable to the conditions in most biochemical systems. We have also found that at 100 °C, AMP·PO_2P undergoes not only decomposition to ADP but also rapid fragmentation to AMP and slower rearrangement to ATP. To account for formation of the latter products, we propose a five-membered cyclic intermediate formed by attack of a P_γ oxygen on P_β (Figure 1). The preferred route of productive decomposition yields AMP (heavy arrow), while the pathway indicated by the light arrow yields adenosine 5′-(γ-peroxytriphosphate). AMP would be expected to form faster than adenosine 5′-(γ-peroxytriphosphate) if AMP were the better leaving group. This is likely because the peroxy hydrogen of peroxymonophosphoric acid is less acidic than the corresponding hydrogen of orthophosphoric acid (Creaser & Edwards, 1972). Decomposition of the initially formed adenosine 5′-(γ-peroxytriphosphate) would then yield ATP. The occurrence of similar rearrangement and fragmentation processes in DMF solution during NMP·PO_2P synthesis could account both for the observed triphosphate contamination and for the observation that the major nucleotide byproduct was nearly always the monophosphate.

Although the nucleoside 5′-(β,γ-peroxytriphosphates) have an unexpectedly rich chemistry of which it is well to be aware, we were primarily interested in these compounds as alternate substrates or as inhibitors in enzyme systems. Since only one of the enzymes tested (NAD^+ pyrophosphorylase) was active with these analogues, it is clear that the peroxy modification can have a drastic effect on reactivity. It is possible that the presence of the additional O atom results in steric crowding at the active site in a way that inhibits reaction. The different electronic properties of the peroxide rather than the phosphoanhydride grouping may contribute; for the reason discussed above, adenosine 5′-(β-peroxydiphosphate) is probably a poorer leaving group than the ADP anion, which might explain why the kinases tested were unable to use AMP·PO_2P. Work with the isomers of ATPβS [e.g., Jaffe & Cohn (1978)] and β,γ-Co(III)$(NH_3)_4$-ATP [e.g., Li et al. (1978)] suggests that many enzymes utilize selectively only one stereoisomer of a six-membered chelate ring between a cation and the β- and γ-phosphates of a nucleoside triphosphate. If a β,γ-chelate ring were to form with a peroxytriphosphate, it would be seven membered, which might be unacceptable. However, since AMP·PO_2P was in many cases quite a potent inhibitor (see Results and Table II), its inertness was not a consequence of general failure to bind to the enzymes. We therefore hope that these compounds will find use as inhibitors or possibly as analogues of allosteric effectors in other systems, even if they are only rarely substrates.

Acknowledgments

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Registry No. AMP·PO_2P, 80589-10-2; GMP·PO_2P, 80589-11-3; UMP·PO_2P, 87713-23-3; CMP·PO_2P, 87713-24-4; dAMP·PO_2P, 87713-25-5; dGMP·PO_2P, 87713-26-6; dUMP·PO_2P, 87713-27-7; dCMP·PO_2P, 87713-28-8; dTMP·PO_2P, 87713-29-9; PO_2P_i, 13825-81-5; AMP·*X*Na, 13474-03-8; GMP·*X*Na, 13474-02-7; UMP·*X*Na, 7545-48-4; CMP·*X*Na, 13718-47-3; dAMP·*X*Na, 50611-39-7; dGMP·*X*Na, 87713-30-2; dUMP·*X*Na, 68982-99-0; dCMP·*X*Na, 75652-48-1; dTMP·*X*Na, 75652-49-2; AMP·*X*morpholine, 78112-73-9; GMP·*X*morpholine, 87713-31-3; UMP·*X*morpholine, 87713-32-4; CMP·*X*morpholine, 87713-33-5; dAMP·*X*morpholine, 87713-34-6; dGMP·*X*morpholine, 87713-35-7; dUMP·*X*morpholine, 87713-36-8; dCMP·*X*morpholine, 87713-37-9; dTMP·*X*morpholine, 87713-38-0; AMP·*X*Bu_3N, 40246-02-4; GMP·*X*Bu_3N, 87713-39-1; UMP·*X*Bu_3N, 87713-40-4; CMP·*X*Bu_3N, 87713-41-5; dAMP·*X*Bu_3N, 87713-42-6; dGMP·*X*Bu_3N, 87713-43-7; dUMP·*X*Bu_3N, 87713-44-8; dCMP·*X*Bu_3N, 87713-45-9; dTMP·*X*Bu_3N, 32909-03-8; 1,1′-carbonyldiimidazole, 530-62-1; ATP, 56-65-5; ADP, 58-64-0; 5′-AMP, 61-19-8; NAD pyrophosphorylase, 9032-70-6; nucleoside diphosphate kinase, 9026-51-1; phosphoribosylpyrophosphate synthetase, 9015-83-2.

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Selective Inhibition of Thrombin by (2*R*,4*R*)-4-Methyl-1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl)]-2-piperidinecarboxylic Acid†

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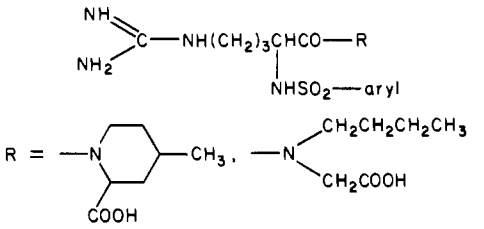
ABSTRACT: The potency of thrombin inhibition by 4-methyl-1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)-sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid (MQPA) depended on the stereoconformation of the 2-piperidinecarboxylic acid moiety. K_i values for bovine α-thrombin were 0.019 μM with (2*R*,4*R*)-MQPA, 0.24 μM with (2*R*,4*S*)-MQPA, 1.9 μM with (2*S*,4*R*)-MQPA, and 280 μM with (2*S*,4*S*)-MQPA. (2*R*,4*R*)-MQPA of the four stereoisomers of MQPA was also the most potent inhibitor for other trypsin-like serine proteases with K_i values of 5.0 μM for trypsin, 210 μM for factor Xa, 800 μM for plasmin, and 1500 μM for plasma kallikrein. Examination of the potency of thrombin inhibition by arginine derivatives related to MQPA in structure suggested the presence of a specific binding site for the carboxamide portion (C-terminal side). The relative inhibitory potency of the four stereoisomers of MQPA for trypsin was nearly identical with that for thrombin, suggesting that the specific binding site for the carboxamide portion is present in both enzymes. Modification of thrombin by phosphopyridoxylation or the presence of heparin did not significantly alter the binding of MQPA.

A series of N^2-substituted L-arginine ester and amide derivatives has been examined for inhibitory effects on thrombin. Potent inhibition of thrombin has been found with arginine derivatives having two hydrophobic moieties, an aromatic moiety such as the substituted naphthalenesulfonyl group as an N^2-substituent of arginine and alkylamines or cyclic amines such as butylamine or 4-methylpiperidine as an amine component of the carboxamide portion (Okamoto et al., 1980; Kikumoto et al., 1980a). On the basis of these observations, it has been suggested that thrombin possesses three binding sites corresponding to (1) the guanidino group, (2) the aromatic N^2-substituent, and (3) the hydrophobic carboxamide portion. A typical compound of this type of thrombin inhibitors, 1-(N^2-dansyl-L-arginyl)-4-ethylpiperidine, exhibited a highly specific inhibition of thrombin among trypsin-like serine proteases (Hijikata et al., 1979; Nesheim et al., 1979). These inhibitors, however, were too toxic to be used as antithrombotic agents in man.

In an effort to obtain less toxic thrombin inhibitors, it has been found that introduction of a carboxyl group into the carboxamide portion of N^2-substituted L-arginine amide derivatives resulted in less toxic derivatives without a loss of inhibitory effect. A structure–activity study of the carboxyl-containing N^2-substituted L-arginine derivatives showed that the carboxyl group should be introduced at the carbon adjacent to the amide nitrogen, as shown in the following general formula, to provide maximum inhibition of thrombin (Kikumoto et al., 1980b).



The stereochemical study of one such inhibitor, 4-methyl-1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid (MQPA),[1] indicated that the stereoconformation of the 4-methyl-2-piperidinecarboxylic acid portion is important for its binding to thrombin, (2*R*,4*R*)-MQPA, the synthetic thrombin inhibitor No. 805, being the most potent thrombin inhibitor (Okamoto et al., 1981).[2] In this paper, the inhibitory effect of (2*R*,4*R*)-MQPA

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[1] Abbreviations: MQPA, 4-methyl-1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid; QPA, 1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid; MQP, 4-methyl-1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]piperidine; QP, 1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]piperidine; 4MPA, 4-methyl-2-piperidinecarboxylic acid; H-D-Phe-Pip-Arg-pNA, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide; Bz-Ile-Glu-Gly-Arg-pNA, *N*-benzoyl-L-isoleucyl-L-glutamyl (γ-OH and γ-OCH_3)-glycyl-L-arginine-*p*-nitroanilide; H-D-Pro-Phe-Arg-pNA, H-D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide; H-D-Val-Leu-Lys-pNA, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide; Tos-Gly-Pro-Arg-pNA, *N*-tosyl-glycyl-L-prolyl-L-arginine-*p*-nitroanilide; Tris, tris(hydroxymethyl)-aminomethane.

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