A Practical Enzymatic Synthesis of (S_P)-Adenosine 5′-*O*-(1-Thiotriphosphate) ((S_P)-ATP-α-S)[1]

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(S_P)-Adenosine 5′-*O*-(1-thiotriphosphate) ((S_P)-ATP-α-S) is an analogue of ATP useful in studying the mecha-

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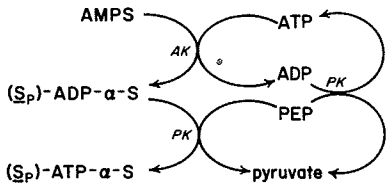
Scheme I. Enzymatic Synthesis of (S_P)-ATP-α-S



nisms of nucleotidyl transfer reactions and for introducing the phosphothioate group into RNA.[3] ATP-α-S was first synthesized by a stereorandom chemical method that produced the S_P and R_P diastereomers in nearly equal amounts.[4] Subsequently, a small-scale (<1 mmol) stereospecific synthesis of (S_P)-ATP-α-S was reported that used a coupled enzyme system comprising adenylate kinase (AK) and pyruvate kinase (PK) (Scheme I).[5,6] We have developed this procedure into a convenient synthesis of (S_P)-ATP-α-S, applicable to preparations on a 20-mmol scale, by using PAN-immobilized enzymes (PAN is a copolymer of acrylamide and *N*-acryloxysuccinimide) and simplifying the synthesis of the starting material and the isolation of the product. The starting material, adenosine 5′-*O*-(monothiophosphate) (AMPS), is prepared by a straightforward thiophosphorylation procedure that provides a solution of AMPS of sufficient purity to be used directly, without purification, in the subsequent enzymatic reaction. The phosphoenol pyruvate[11] required in this synthesis is easily prepared, and the enzymes required are inexpensive, easily immobilized, and readily recycled.

Experimental Section

General Procedures. UV measurements were made at 25 °C with a Perkin-Elmer Model 552 spectrophotometer. ^{31}P NMR spectra were determined at 121.5 MHz on a Bruker Model WM 300 spectrometer. NMR samples were prepared in 1.0 M Tris–HCl, pH 8.5, containing 50 mM EDTA and 20% D_2O. Chemical shifts are reported relative to external 1 M H_3PO_4 in D_2O. HPLC analyses were carried out on a Waters Associates system equipped with a differential ultraviolet detector operating at 254 nm, using a Waters Radial-PAK C-18 column (5 mm × 10 cm, 10-μm particle size). The mobile phase was 5 mM Waters PIC Reagent A containing 10–20% acetonitrile.

Materials. Enzymes, biochemicals, and Dowex-1×8 (200–400 mesh) were obtained from Sigma. Thiophosphoryl chloride was obtained from Aldrich and distilled before use. Triethyl phosphate was obtained from Aldrich and was distilled from BaO before use. PAN was prepared as described previously.[7]

Adenosine 5′-*O*-(Monothiophosphate) (AMPS). Adenosine (13.4 g, 50 mmol) and 120 mL of triethyl phosphate were added to a 250-mL flask equipped with a magnetic stirring bar, condensor, and inlet for argon. The suspension (under argon) was heated rapidly to reflux and maintained at that temperature until a homogenous solution was obtained (ca. 4 min).[8] The mixture was cooled to 5 °C, and 2,6-dimethylpyridine[9] (16.1 g, 150 mmol) and thiophosphoryl chloride (15.3 g, 90 mmol) were then added sequentially. After 45 min the resulting suspension was poured into petroleum ether (bp 35–57 °C, 500 mL), and the solvents were decanted. The residual white solid was washed with petroleum ether (2 × 300 mL) and hydrolyzed by stirring with water (100 mL) at 4 °C for 1 h. The solution was adjusted to pH 7.0 with 5 N KOH and extracted with diethyl ether (2 × 100 mL) and petroleum ether (2 × 100 mL). The resulting aqueous solution (200 mL) contained 40 mmol (80% yield) of AMPS and was used in the following reaction without further purification;[10] ^{31}P NMR δ −58.7 (s).

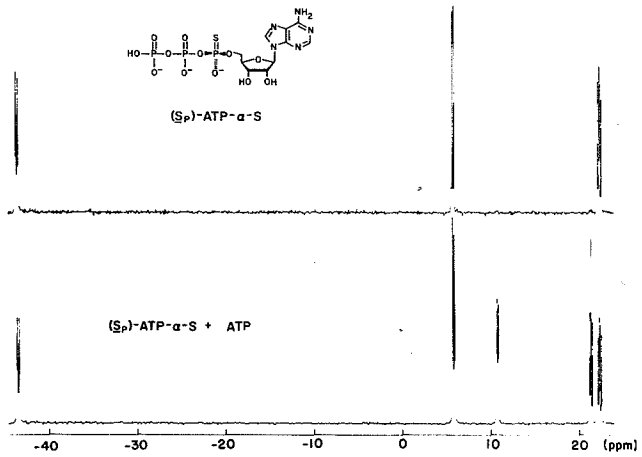


Figure 1. (Upper) 121.5 MHz ^{31}P NMR spectrum of (S_P)-ATP-α-S, 50 mM in 1.0 M Tris–HCl (pH 8.5) (4:1 H_2O:D_2O). (Lower) ^{31}P NMR spectrum of (S_P)-ATP-α-S and ATP under similar conditions.

(S_P)-Adenosine 5′-*O*-(1-Thiotriphosphate) ((S_P)-ATP-α-S). A solution (800 mL) containing potassium phosphoenol pyruvate[11] (PEP, 11.0 g, 100 mmol), AMPS (38 mmol, 190 mL of 0.2 M solution), dithiothreitol (1.43 g, 9.3 mmol), $MgCl_2$·$6H_2O$ (0.08 g, 0.4 mmol), and ATP (0.1 mmol, 2.0 mL of 50 mM solution) was placed in a 2-L round-bottomed flask equipped with a magnetic stirring bar, pH electrode, and argon atmosphere and deoxygenated with argon and adjusted to pH 8.0 with 5 N KOH. Adenylate kinase (EC 2.7.4.3, 3000 U[12]) and pyruvate kinase (EC 2.1.1.40, 5100[12] U) coimmobilized in 250 mL of PAN gel[7] were added, and the resulting suspension was heated to 37 °C. The reaction mixture was maintained at pH 8.0 by the occasional addition of 5 N KOH. After 5 days the enzyme-containing polyacrylamide gel was separated by decantation. The recovered enzymatic activities (calculated as the percentage of starting activities) were AK, 63% and PK, 84%. The turnover numbers (mole of product/mole of enzyme) were AK, 1.0×10^5 and PK, 2.9×10^5.

The phosphate-containing species were adsorbed from the supernatant onto Dowex-1 (250 g, 200–400 mesh, HCO_3^- form) at 4 °C, and the resin was washed with deionized water (1.5 L). Inorganic thiophosphate, PEP, ATP, and other impurities were desorbed by washing with 0.01 M HCl–0.02 M NaCl solution (4 L). (S_P)-ATP-α-S was then desorbed by washing the resin with 0.02 M HCl–0.8 M NaCl solution (3.5 L). The eluent was adjusted to pH 8.0 with 5 N KOH and $BaCl_2$ (34.0 g, 166 mmol) was added. The precipitate was collected by centrifugation and washed se-

(1) Supported by the National Institutes of Health, GM 30367.
(2) Damon Runyon–Walter Winchell Cancer Fund Postdoctoral Fellow, 1983–1985.
(3) Eckstein, F. *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 423–439 and references therein.
(4) Eckstein, F.; Goody, R. S. *Biochemistry* **1976**, *15*, 1685–1691.
(5) Frey, P. A.; Sheu, K. F. R. *J. Biol. Chem.* **1977**, *252*, 4445–4448.
(6) Jaffe, E. K.; Cohn, M. *Biochemistry* **1978**, *17*, 652–657.
(7) Pollak, A.; Blumenfeld, H.; Wax, M.; Baugh, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324–6336. (PAN is a copolymer of acrylamide and *N*-acryloxysuccinimide.)
(8) We have found it essential to start with homogeneous solutions of adenosine in order to obtain reproducibly high yields of AMPS. At the concentration used here adenosine becomes soluble in triethyl phosphate at ca. 160–170 °C. Control experiments showed adenosine does not decompose in the short time (<5 min) during which it is heated.
(9) 2,6-Dimethylpyridine was found to promote more rapid and complete thiophosphorylation than pyridine under these reaction conditions.
(10) This solution contained AMPS (40 mmol), inorganic thiophosphate (10 mmol), triethyl phosphate (20 mmol), and other unidentified impurities that did not contain phosphorus. The composition of this solution remained constant over a 24-h period when stored at 4 °C.
(11) Hirschbein, B. L.; Mazenod, F. P.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 3766–6769.
(12) Enzymes were assayed according to Bergmeyer et al. Bergmeyer, H. U. "Methods of Enzymatic Analysis"; Verlag Chemie, New York, 1974. Assays were carried at 25 °C. One unit (U) of enzymatic activity is defined as that amount of enzyme that catalyzes the formation of 1 μmol of product/min at 25 °C. Note that the activities of AK and PK were measured with use of their natural substrates. The activities of the enzymes with the nucleotide phosphothioates are considerably lower. At 1.0 mM MgATP, the apparent K_m for AK-catalyzed phosphorylation of AMPS is 0.31 mM and that for AMP is 0.11 mM at pH 8.0 and 27 °C. The apparent V_{max} for AMP is 39 times that for AMPS.[5]

quentially with water (200 mL), 50% aqueous EtOH (200 mL), and acetone (200 mL). After drying, the isolated solid (17.3 g) contained 90% $Ba_2(S_P)$-ATP-α-S[13] by weight (20 mmol, 53% yield from AMPS), no detectable (R_P)-ATP-α-S, and <3% ATP (determined by HPLC and ^{31}P NMR);[13] ^{31}P NMR δ −43.5 (d, P_α), +5.8 (d, P_γ), +22.3 (dd, P_β); $J_{P_\alpha\text{-}P_\beta}$ = 27.2 Hz, $J_{P_\beta\text{-}P_\gamma}$ = 20.0 Hz (Figure 1).

Registry No. (S_P)-ATP-α-S, 58976-48-0; (S_P)-ATP-α-S·2Ba, 88157-74-8; AMPS, 19341-57-2; adenosine, 58-61-7; PEP, 138-08-9; ATP, 56-65-5; PK, 9001-59-6; AK, 9013-02-9.

(13) The $Ba_2(S_P)$-ATP-α-S was solubilized for analysis by stirring with 2 equiv of $(NH_4)_2SO_4$ (pH 8.0, 4 °C, 1 h). The $BaSO_4$ was removed by centrifugation.