2b in 2.5 mL of Me_2SO-d_6 and 200 mg (1.8 mmol) of *t*-BuOK. After the solution was stirred at room temperature for 16 h, the reaction mixture was worked up, yielding 50 mg of a mixture of **1**, **2a**, and **2b** in a ratio of 35:40:25, as was shown by GC-MS. Neither in **1** nor in **2a** or **2b** was deuterium incorporated.

Registry No. 1, 81387-93-1; **1**-*10-d*, 81387-94-2; **2a**, 74543-53-6; **2a**-*10-d*, 81387-95-3; **2b**, 74561-81-2; **2b**-*10-d*, 81444-71-5; **3**, 74543-54-7; **4**, 74543-55-8; **5**, 81444-72-6; **6a**, 74543-56-9; **6b**, 74561-82-3; 4,5,6,7-tetrahydro-2-indanol, 6010-79-3.

Supplementary Material Available: Table IV (thermal parameters), Table V (hydrogen atom parameters) (2 pages). Ordering information is given on any current masthead page.

Synthesis of Chiral [^{18}O]Phosphorothioate Analogues of Adenine Nucleotides

John P. Richard[1] and Perry A. Frey*[2]

Contribution from the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210. *Received October 21, 1981*

Abstract: Syntheses of R_P-ATPγS,$\gamma^{18}O_2$, R_P- and S_P-ADPβS,β^{18}O, and R_P- and S_P-AMPS,^{18}O are described. Coupling of 2′,3′-(methoxymethylidene)-AMP, **1**, with S_P-ADPαS,$\alpha^{18}O_2$ produced P^1-5′-adenosyl P^3-2′,3′-(methoxymethylidene)-5′-adenosyl 1-thio[1-tri$^{18}O_2$]phosphate, **3**. Upon cleavage of the unprotected ribose ring with IO_4^-, reduction of IO_3^- by $HPSO_3^{2-}$, removal of the 2′,3′-methoxymethylidene protecting group at pH 2, and alkaline elimination of the product from the IO_4^--cleaved dialdehyde, R_P-ATPγS,$\gamma^{18}O_2$ was produced and isolated by chromatography in 55-58% yield based on S_P-ADPαS,$\alpha^{18}O_2$. Coupling of AMPS,$^{18}O_2$ with **1** produced (R_P)- and (S_P)-P^1-5′-adenosyl P^2-2′,3′-(methoxymethylidene)-5′-adenosyl 1-thio[1-^{18}O]pyrophosphate, **4a** and **4b**, which were separated by ion-exchange chromatography through DEAE-Sephadex A-25. Periodate cleavage, deprotection, and alkaline elimination of **4a** and **4b** analogous to that described above for **3** produced S_P- and R_P-ADPβS,β^{18}O, respectively. Coupling of AMPS,$^{18}O_2$ with AMP produced (R_P)- and (S_P)- P^1,P^2-bis(5′-adenosyl) 1-thio[1-^{18}O]pyrophosphate, **5a** and **5b**, which were separated by ion-exchange chromatography through DEAE-Sephadex A-25. Nucleotide pyrophosphatase catalyzed hydrolysis of **5a** and **5b** produced R_P- and S_P-AMPS,^{18}O, respectively. These chiral nucleoside and nucleotide [^{18}O]phosphorothioates are useful as substrates for stereochemical studies of phosphotransferases. These compounds and their precursors should also serve as important synthetic precursors of ADP and ATP stereospecifically enriched with heavy isotopes of oxygen at any desired position in the phosphoanhydride system.

The efficacy of chirally substituted phosphorothioates in studies of the stereochemical courses of enzymatic phosphoryl group transfer has been demonstrated in a number of studies of reactions catalyzed by phosphohydrolases, phosphotransferases, and nucleotidyltransferases.[3] An essential prerequisite for such studies is the capability to synthesize phosphorothioates that are both acceptable substrates and chirally substituted at the phosphorus atoms that undergo nucleophilic substitution. The sulfur analogue of ATP used in determining the stereochemical courses of the actions of phosphotransferases has one of the three equivalent oxygen atoms bonded to P_γ replaced by a sulfur atom and another enriched in ^{18}O to produce a chiral phosphorus center at P_γ.

We have recently determined the stereochemical courses of phosphoryl group transfer catalyzed by adenylate kinase,[3d] nucleoside phosphotransferase,[4] nucleoside diphosphate kinase,[3j] and adenosine kinase.[5] In these studies R_P- and S_P-AMPS,^{18}O, R_P- and S_P-ADPβS,β^{18}O, and R_P-ATPγS,γ^{18}O were synthesized and used either as substrates or as reference compounds for the assignment of configurations to products of enzymatic [^{18}O]thiophosphoryl transfer.

The chiral [^{17}O]- and [^{18}O]phosphorothioate analogues of nucleotides are important as starting materials for a number of isotopically enriched nucleotides as well as for stereochemical studies in which they serve as substrates for enzymes. They serve as precursors for nucleotides whose α or β phosphorus atoms are chiral by virture of stereospecific ^{17}O and ^{18}O enrichments. They are also key intermediates in the synthesis of nucleotides having exclusively α-β or β-γ-bridging ^{17}O or ^{18}O. This paper describes in detail the syntheses and configurational analyses of the chiral [^{18}O]phosphorothioate analogues of adenine nucleotides including R_P- and S_P-AMPS,^{18}O (adenosine 5′-[^{18}O]phosphorothioate), R_P- and S_P-ADPβS,β^{18}O (adenosine 5′-(2-thio[2-^{18}O]diphosphate)), and R_P-ATPγS,γ^{18}O (adenosine 5′-(3-thio[3-^{18}O]triphosphate)]. Some of the procedures have been reported briefly in Communications.[3d,6]

Materials and Methods

Enzymes, Coenzymes, and Chemicals. The following enzymes were purchased from Sigma Chemical Co. and used without additional purification: rabbit muscle pyruvate kinase, rabbit muscle adenylate kinase, rabbit muscle lactate dehydrogenase, *E. coli* alkaline phosphatase, *E. coli* acetate kinase, yeast hexokinase, and snake venom (*Crotalus atrox*) nucleotide pyrophosphatase. All nucleotides and nucleosides were purchased from Sigma Chemical Co., as were phosphoenol pyruvate, acetyl phosphate, and DEAE-Sephadex A-25. Thiophosphoryl trichloride and sodium thiophosphate were purchased from Ventron. Deuterium oxide (99.7% enriched) and $H_2{}^{18}O$ (99% enriched) were purchased from BioRad Laboratories. All other chemicals were reagent grade and were obtained from commercial sources.

^{31}P NMR and Mass Spectral Analyses. ^{31}P NMR spectra were obtained on a Bruker HX-90 spectrometer operating at 36.43 MHz and equipped with Fourier transform and proton noise decoupler accessories

(1) Graduate Department of Biochemistry, Brandeis University, Watham, MA 02254.

(2) To whom correspondence should be addressed at the Institute for Enzyme Research, University of Wisconsin, Madison, WI 53706.

(3) (a) Usher, D. A.; Richardson, D. I.; Eckstein, F. *Nature (London)* **1970**, *228*, 663-665. (b) Orr, G. A.; Simon, J.; Jones, S. R.; Chin, G. J.; Knowles, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2230-2233. (c) Sheu, K.-F. R.; Frey, P. A. *J. Biol. Chem.* **1978**, *253*, 3378-3380. (d) Richard, J. P.; Frey, P. A. *J. Am. Chem. Soc.* **1978**, *100*, 7757-7758. (e) Burgers, P. M. J.; Eckstein, F. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4798-4800. (f) Midelfort, C. F.; Sarton-Miller, I. *J. Biol. Chem.* **1978**, *253*, 7127-7129. (g) Bryant, F. R.; Benkovic, S. J. *Biochemistry* **1979**, *18*, 2825-2828. (h) Yee, D.; Armstrong, V. W.; Eckstein, R. *Ibid.* **1979**, *18*, 4116-4120. (i) Gerlt, J. A.; Coderre, J. A.; Wolin, M. S. *J. Biol. Chem.* **1980**, *255*, 331-334. (j) Sheu, K.-F. R.; Richard, J. P.; Frey, P. A. *Biochemistry* **1979**, *18*, 5548-5556. (k) Pliura, D. H.; Schomburg, D.; Richard, J. P.; Frey, P. A.; Knowles, J. R. *Ibid.* **1980**, *19*, 325-329.

(4) Richard, J. P.; Prasher, D. C.; Ives, D. H.; Frey, P. A. *J. Biol. Chem.* **1979**, *254*, 4339-4341.

(5) Richard, J. P.; Carr, M.; Ives, D. H.; Frey, P. A. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1052-1056.

(6) Richard, J. P.; Ho, H.-T.; Frey, P. A. *J. Am. Chem. Soc.* **1978**, *100*, 7756-7757.

0002-7863/82/1504-3476$01.25/0 © 1982 American Chemical Society

and a Nicolet BNC 12 computer. The spectrometer was field-frequency locked at the deuterium resonance of solvent D_2O, and samples were prepared for analysis by dissolving the nucleotide in 1 mL of D_2O, adjusting the pD with NaOD to either 8.4 for most nucleotides or 10.4 for ATPγS, ADPβS, **4a**, and **4b** (Scheme II), and diluting to a final volume of 1.5 mL in 10-mm Wilmad NMR tubes. Proton noise decoupled spectral data were obtained at 37 °C, and spectra were recorded after 500–20 000 scans, depending on nucleotide concentrations, which ranged from 50 to 5 mM. Chemical shifts referenced against an external H_3PO_4 standard.

Thionucleotides were degraded to trimethyl phosphate and trimethyl phosphorothioate as described by Richard et al.[6] We report here the ^{18}O enrichment of trimethyl phosphorothioate as the nucleotide ^{18}O enrichment when it is known that there is no ^{18}O enrichment at a phosphate–thiophosphate bridge position.

Mixtures of trimethyl phosphate and trimethyl phosphorothioate were separated on a 6 ft × 2 mm prepacked Altech nickel column containing 10% SE30 adsorbed on Chromosorb G. The column was attached to a Perkin-Elmer 990 gas chromatograph operating with a helium flow rate of 25 mL/min and an injection port temperature of 120 °C. The following temperature program was used: 90 °C for 6 min, followed by a temperature increase of 12 °C/min to 160 °C, and finally flushing of the column for 30 min at 160 °C. With this program the retention times of trimethyl phosphate and trimethyl phosphorothioate were 6 and 11 min, respectively.

The ^{18}O enrichments were determined from parent peak analysis (M 156 for trimethyl phosphorothioate) after correction of the M + 2 peak for natural-abundance ^{18}O and ^{34}S.

General Methods. Pyridine, dimethylformamide, triethyl phosphate, and 1,4-dioxane were dried over CaH_2 for several days and then distilled. Tri-*n*-butylamine was distilled over KOH pellets. Pyridine and tri-*n*-butylamine were stored over KOH pellets, dimethylformamide and 1,4-dioxane over Linde 4-Å molecular sieves, and triethyl phosphate over 5-Å sieves.

Solutions of triethylammonium bicarbonate were prepared by passing CO_2 through a glass fritted tube into a one M solution of triethylamine. When the pH of this solution had dropped to 7.6, the flask was stoppered and stored at room temperature. Synthetic nucleotides were routinely purified by ion-exchange chromatography through columns of DEAE-Sephadex A-25 eluted with linear gradients of triethylammonium bicarbonate. Fractions containing nucleotides were identified by A_{260}. Peak fractions were pooled, and triethylammonium bicarbonate was removed in vacuo at temperatures below 30 °C by rotary flash evaporation, using a high-capacity pump and a dry ice–acetone-cooled trap. To ensure that all buffer salts were removed, the residue was twice suspended in ethanol and again evaporated. Final residues were dissolved in small volumes of water and stored at −15 °C. Solution of ATPγS and ADPβS, **4a** and **4b** (Scheme II), were adjusted to pH 10.4 before freezing.

Thionucleotides with terminal thiophosphate groups (AMPS, ADPβS, and ATPγS) were distinguished in eluates from thionucleotides with internal sulfur such as ADPαS (adenosine 5′-(1-thiodiphosphate)) or ATPαS (adenosine 5′-(1-thiotriphosphate)) by the ability of terminal phosphorothioates to reduce DTNB (5,5′-dithiobis[2-nitrobenzoate]).[7] Columns were often monitored qualitatively for DTNB-reactive thionucleotides by diluting 0.1 mL of a column fraction into 0.9 mL of a 1.1 mM solution of DTNB and measuring the absorbance at 412 nm. When desired, the amount of thionucleotide present was quantitated by using sufficiently small aliquots of column fractions in the colorimetric analysis and comparing the color changes with a standard curve.

The triethylammonium salts of nucleoside phosphates or phosphorothioates were converted to their respective tri-*n*-octylammonium salts by the following procedure. The triethylammonium salt was freed of water on a rotary evaporator and the residue suspended in methanol. Tri-*n*-octylamine was added (1 molar equiv to 2′,3′-(methoxymethylidene)-AMP and -AMPS and 2 molar equiv to ADPαS) and the suspension stirred until clear. Methanol was removed in vacuo and the nucleotide was dried by evaporating from three successive 1-2-mL additions of dimethylformamide.

The extinction coefficients of **4** and **5** (Schemes II–IV) were determined from the change in absorbance at 260 nm that occurs as nucleotide pyrophosphatase cleaves these compounds to their component mononucleotides. An extinction coefficient of 24 000 was calculated for **4** and **5**, assuming the extinction coefficient 15 000 M^{-1} cm^{-1} for adenine mononucleotides.

AMP was measured by the coupled actions of adenylate kinase, pyruvate kinase, and lactate dehydrogenase as described by Sheu and Frey[8] for AMP and AMPS. When AMP was measured in the presence of AMPS, the amount of adenylate kinase used was adjusted to give a fast response for AMP followed by a much slower second response for the analogue, AMPS.

Enzymatic Synthesis. S_P-ATPαS,$^{18}O_2$ was synthesized by coupled enzymatic phosphorylation of AMPS,$^{18}O_2$ with adenylate kinase and pyruvate kinase.[8] Hexokinase was used to convert S_P-ATPαS,$\alpha^{18}O_2$ to S_P-ADPαS,$\alpha^{18}O_2$. R_P- and S_P-ATPβS were synthesized by stereoselective phosphorylation at the pro-*R* and pro-*S* oxygens of ADPβS catalyzed by acetate kinase and pyruvate kinase, respectively.[6,9]

2′,3′-(Methoxymethylidene)-AMP (1). This compound was synthesized as described by Darlix et al.[10] with a few modifications. The synthesis involved the use of cyanoethyl phosphoric acid to catalyze the conversion of adenosine to 2′,3′-(methoxymethylidene)adenosine in trimethyl orthoformate. Dicyclohexylcarbodiimide was then added to mediate the phosphorylation of the 5′-OH group in 2′,3′-(methoxymethylidene)adenosine by the cyanoethyl phosphate present in the solution, and the resulting 2′,3′-(methoxymethylidene)adenosine 5′-(cyanoethyl phosphate) was treated with base to eliminate acrylonitrile. The product from 5 mmol of adenosine and 40 mmol of cyanoethyl phosphate was purified by chromatography through a 4 × 50 cm column of DEAE-Sephadex A-25. The column was eluted with a 6-L linear gradient of triethylammonium bicarbonate increasing in concentration from 0.15 to 0.35 M, and **1** emerged as the major peak at about 0.28 M triethylammonium bicarbonate. The ^{31}P NMR spectrum revealed the presence of small amounts of other phosphates, and paper electrophoresis in 0.1 M sodium tetraborate at pH 9.2 sometimes revealed the presence of AMP. When AMP was found, it was removed by treatment with 1 equiv of $NaIO_4$ (with respect to **1**) in 50 mM $NaCO_3$·HCl buffer at pH 10.5 for 30 min followed by incubation at 50 °C for an additional 30 min. Final purification over DEAE-Sephadex A-25 with elution conditions identical with those given above yielded a product that was pure as judged by paper electrophoresis and ^{31}P NMR spectroscopy. After the second chromatography step, **1** was immediately converted to its tri-*n*-octylammonium salt, dried by three successive evaporations from 1–2-ml additions of dimethylformamide, and stored at −15 °C as a 100 mM solution in dimethylformamide.

Synthesis of AMPS,$^{18}O_2$. This compound was synthesized by a modification of the procedure of Murray and Atkinson.[11] Adenosine (1.07 g, 4 mmol, dried in vacuo for 12 h over P_2O_5) was dissolved in 10 mL of triethyl phosphate that had been preheated to 100 °C, and the solution was cooled to 0 °C in an ice-water bath. After addition of thiophosphoryl trichloride (1.2 mL, 11.6 mmol), the solution was stirred at 4 °C for 12 h. Unreacted thiophosphoryl trichloride was then removed by distillation at reduced pressure (30 °C (0.1 mm)). $H_2{}^{18}O$ (1 g, 99% enriched) and 1 g of anhydrous sodium acetate were added to the suspension, which was then stirred for 4 h at room temperature. After dilution to 300 mL with H_2O, the product was purified by chromatography through 3.0 × 30 cm column of DEAE-Sephadex A-25 eluted with a linear triethylammonium bicarbonate gradient having a total volume of 4 L that increased in concentration from 0.20 to 0.40 M. AMPS,^{18}O eluted as the major peak free of thiophosphate at 0.2. M buffer. The yield was 2.3 mmol or 63% of theoretical based on adenosine. The ^{18}O enrichment of AMPS,$^{18}O_2$ was determined by analysis of the trimethyl phosphorothioate derivative to be 1.82 atoms excess ^{18}O, or 91% enrichment with respect to each diastereotopic P_α-oxygen position.

Synthesis of R_P-ATPγS,$\gamma^{18}O_2$. The tri-*n*-octylammonium salt of **1** (500 μmol) was dried by three successive evaporations from 1–2-mL additions of dimethylformamide and reacted in 2 mL of dioxane with a 1.5-fold molar excess of diphenyl phosphorochloridate and a 2-fold molar excess of tri-*n*-butylamine for 3 h at room temperature. Dioxane was removed by rotary evaporation and the residue was extracted with 50 mL of a 4:1 mixture of petroleum ether and diethyl ether. The suspension was stirred for 30 min at room temperature; the ether was decanted and excess ether was removed by addition of 2 mL of dioxane followed by rotary evaporation to dryness. The resulting residue was dissolved in 1 mL of pyridine containing 267 μmol of the bis(tri-*n*-octylammonium) salt of S_P-ADPαS,$\alpha^{18}O_2$ and stirred at room temperature for 12 h. After removal of pyridine by rotary evaporation, 5 mL of diethyl ether was added to the reaction mixture, and the resulting suspension was extracted while still in the reaction flask with ca. 30 mL of H_2O. The aqueous layer was adjusted to pH 8–9 with triethylamine and mixed with 3 mL of a 100 mM $NaIO_4$ solution. After 30 min at room temperature, 100 μL of ethylene glycol and 1.2 g of Na_3PSO_3·$12H_2O$ were added, and the pH of the solution was adjusted to 2.0 with 1.0 M HCl and after an addi-

(7) Goody, R. S.; Eckstein, F. *J. Am. Chem.* **1971**, *93*, 6252–6257.
(8) Sheu, K.-F. R.; Frey, P. A. *J. Biol. Chem.* **1977**, *252*, 4445–4448.
(9) Eckstein, F.; Goody, R. S. *Biochemistry* **1976**, *15*, 1685–1691.
(10) Darlix, J. L.; Fromogeot, H. p. M.; Fromogeot, P. *Biochim. Biophys. Acta* **1967**, *145*, 517–519.
(11) Murray, W. A.; Atkinson, M. R. *Biochemistry* **1968**, *7*, 4023–4029.

Table I. ^{31}P Nuclear Magnetic Resonance Data on Nucleotides

nucleotide	source	chemical shift,[a] ppm P_α	P_β	$J_{\alpha-\beta}$, Hz
5a	synthesis (Scheme III)	43.61 (d)	−11.88 (d)	28.08
R_P-ADPαS	IO_4^--base degradation of 5a	40,61 (d) (lit. 40.68[b])	−6.73 (d)	31.74
5b	synthesis (Scheme III)	43.26 (d)	−11.86 (d)	26.86
S_P-ADPαS	IO_4^--base degradation of 5b	41.16 (d) (lit. 41.08[b])	−6.70 (d)	31.13
4a	synthesis (Scheme II)	43.54 (d)	−12.18 (d)	28.08
4b	synthesis (Scheme II)	43.17 (d)	−12.20 (d)	27.47

[a] Positive values signify shifts *downfield* from H_3PO_4 and d signifies a doublet. [b] The literature source is ref 8.

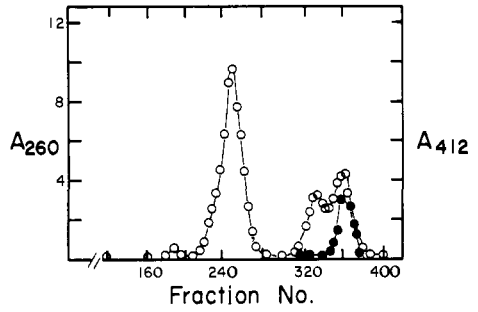


Figure 1. Chromatographic separation of **4a** and **4b**. The reaction mixture was applied to a 3.0 × 170 cm column of DEAE-Sephadex A-25 and eluted with a linear gradient of triethylammonium bicarbonate, 12 L in total volume and increasing from 0.2 to 0.4 M. Fractions were collected and their A_{260} measured and plotted on the left ordinate (○). Aliquots of fractions were analyzed for DTNB-positive materials (AMPS), and the corresponding A_{412} were plotted on the right ordinate (●), showing that AMPS coeluted with **4a**. Pooled fractions 316–340 yielded 65 μmol of **4b** and fractions 352–384 yielded 70 μmol of **4a** plus 30 μmol of AMPS. AMPS was removed as described in the text: the overall yield of **4a** + **4b** was 35%. The band eluting in fractions 220–260 was unreacted excess 2′,3′-(methoxymethylidene)-AMP.

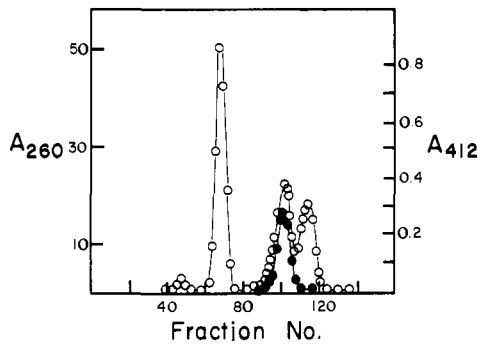


Figure 2. Chromatographic separation of **5a** and **5b**. The reaction mixture was applied to a 2.0 × 100 cm column of DEAE-Sephadex A-25 and eluted as described in Figure 1. Fractions were analyzed as in Figure 1. Unreacted AMPS coeluted with **5b** and was removed as described in the text. The overall yield of **5a** + **5b** was 39%. The band eluting in fractions 60–80 was unreacted excess AMP.

tional 30 min at room temperature to 10.5 with 6.0 M NaOH. This solution was incubated for 30 min at 50 °C and then diluted to a final volume of 150 mL. The product was purified by chromatography through a 2.5 × 30 cm column of DEAE-Sephadex A-25 with a linear elution gradient of triethylammonium bicarbonate having a total volume of 3 L that increased in concentration from 0.3 to 0.7 M. R_P-ATPγS,γ$^{18}O_2$ eluted as a sharp, well-resolved peak at 0.58 M buffer. The yield was 58% of theoretical based on S_P-ADPαS,$^{18}O_2$. The ^{31}P NMR spectrum exhibited a P_α doublet at −11.32 ppm, a P_β quartet at −23.54 ppm, and a P_γ doublet at 43.08 ppm, with coupling constants $J_{\alpha\beta}$ = 20.75 Hz and $J_{\beta\gamma}$ = 30.52 Hz, in agreement with reported values.[12]

Synthesis of R_P- and S_P-ADPβS,β^{18}O. The dry tri-*n*-octylammonium salt of AMPS,$^{18}O_2$ (410 μmol) was activated with diphenyl phosphorochloridate as described above for **1**, substituting triethyl phosphate for dioxane as the activation solvent. Following extraction with 4:1 petroleum ether–diethyl ether, the activated AMPS,$^{18}O_2$ was dissolved with 2 mL of pyridine containing 820 μmol of the tri-*n*-octylammonium salt of **1**. After 12 h at room temperature, pyridine was removed by rotary evaporation in vacuo and 10 mL of diethyl ether was added to the residue. This suspension was extracted in the reaction flask with 40 mL of H_2O, and the pH of the extract was adjusted to 8–9 with triethylamine. Products were purified by ion-exchange chromatography, with results illustrated in Figure 1. It was found that the slower migrating isomer of the coupled product, the R_P epimer **4a** (Scheme II), coeluted with unreacted AMPS,^{18}O. This contaminant was removed in the subsequent periodate–base degradation of the R_P epimer to S_P-ADPβS,β^{18}O. After removal of buffer salts, **4a** and **4b** (Scheme II) were dissolved in minimum volumes of water and their pHs adjusted to 10 before freezing and storing at −15 °C. The ^{31}P NMR spectral data for **4a** and **4b** are given in Table I. The R_P epimer **4a** was converted to S_P-ADPβS,β^{18}O by the following procedure. **4a** (64 μmol) (with 23 μmol of AMPS) was dissolved in 10 mL of water and the pH adjusted to 8–9. Sodium periodate (1.3 mL of a 100 mM solution) was added, and after 10 min at room temperature the excess periodate was quenched with 100 μL of ethylene glycol. This solution was immediately applied to a 1.5 × 12 cm column of DEAE-Sephadex A-25 that was washed with 100 mL of 0.1 M triethylammonium bicarbonate followed by 100 mL of 0.6 M triethylammonium bicarbonate. The 0.6 M eluate was evaporated to remove triethylammonium bicarbonate as described under General Methods. The residue was dissolved in 10 mL of H_2O and the pH adjusted to 2.0 with 1.0 M HCl. After 40 min at room temperature, the pH was adjusted to 10.5 with 1 M NaOH. The solution was buffered at pH 10.5 by further addition of 10 mL of 100 mM Na_2CO_3·HCl and incubated at 50 °C for 30 min. S_P-ADPβS,β^{18}O was purified by chromatography through a 1.5 × 45 cm column of DEAE-Sephadex A-25 with a linear triethylammonium bicarbonate elution gradient having a total volume of 2 L and increasing in concentration from 0.25 to 0.45 M. S_P-ADPβS,β^{18}O was the last major peak to be eluted from the column, appearing at 0.33 M buffer. After removal of buffer, S_P-ADPβS,β^{18}O was dissolved in a minimum volume of water and the pH was adjusted to 10 before storing at −15 °C. The yield was 78% of theoretical based on **4a**. R_P-ADPβS,β^{18}O was synthesized from the corresponding S_P epimer **4b** by the same procedure. The ^{31}P proton spin decoupled NMR spectrum of ADPβS,β^{18}O (the spectra of the S_P and R_P epimers are identical) exhibited a P_α doublet at −11.73 ppm and a P_β doublet at +32.55 ppm with $J_{\alpha\beta}$ = 31.74 Hz, in agreement with values reported by Jaffe and Cohn.[12] The ^{18}O enrichment of ADPβS,β^{12}O was determined by analysis of the trimethyl thiophosphate derivative to be 0.86 atom excess ^{18}O.

Synthesis of R_P- and S_P-AMPS,^{18}O. AMPS,$^{18}O_2$ (650 μmol) was activated with diphenyl phosphorochloridate and coupled to the tri-*n*-octylammonium salt of AMP by the same procedure described above, except for the substitution of AMP for **1**. Products were purified by ion-exchange chromatography, with the results depicted in Figure 2. The faster migrating isomer of the coupled product, the S_P epimer **5b** (Scheme III), cochromatographed with unreacted AMPS,$^{18}O_2$. AMPS,$^{18}O_2$ was, therefore, converted to ATPαS,$^{18}O_2$ by the coupled actions of adenylate kinase and pyruvate kinase.[8] **5b** was then separated from ATPαS,$^{18}O_2$ by chromatography through a 3.0 × 30 cm column of DEAE-Sephadex A-25, which was eluted with a linear gradient having a total volume of 3 L and consisting of triethylammonium bicarbonate increasing in concentration from 0.2 to 0.4 M. The recovery of **5b** was quantitative; however, it was isolated along with unreacted phosphoenolpyruvate. Contaminating phosphoenolpyruvate was removed by the action of alkaline phosphatase in the following reaction mixture prepared at pH 8.0 in a volume of 25 mL to contain 10 mM $MgCl_2$, 50 mM Tris·HCl, 125 μmol of **5b** with 185 μmol of phosphoenolpyruvate, and 40 units of alkaline phosphatase. The formation of pyruvate was monitored by determining NADH oxidation after dilution of 25 μL of the reaction mixture into a solution containing NADH and lactate dehydrogenase. After 90 min the reaction was complete and **5b** was repurified by ion-exchange chromatography as described above.

(12) Jaffe, E. K.; Cohn, M. *Biochemistry* **1978**, *17*, 561–569.

5a and **5b** (Scheme III) were converted to R_P-AMPS,^{18}O and S_P-AMPαS,^{18}O, respectively, by the action of nucleotide pyrophosphatase. The reaction mixtures consisted of 87.5 μmol of **5a** or **5b**, 10 mM $MgCl_2$, 50 mM Tris·HCl buffer at pH 8.0, and 1 mg of Sigma nucleotide pyrophosphatase in a total volume of 7 mL. During 60 min at 37 °C, the AMP resulting from hydrolysis increased in concentration and reached a plateau corresponding to the appearance of 85 μmol (97%). The reaction mixture was passed into a 3 × 30 cm column of DEAE-Sephadex A-25, which was eluted with 2-L linear gradient formed from 1 L each of 0.1 and 0.4 M triethylammonium bicarbonate. Fractions of 24-mL volume were collected; AMP appeared in fractions 17–24 (81 μmol, 93%) while AMPαS,^{18}O appeared in fractions 29–39 (87 μmol, 100%).

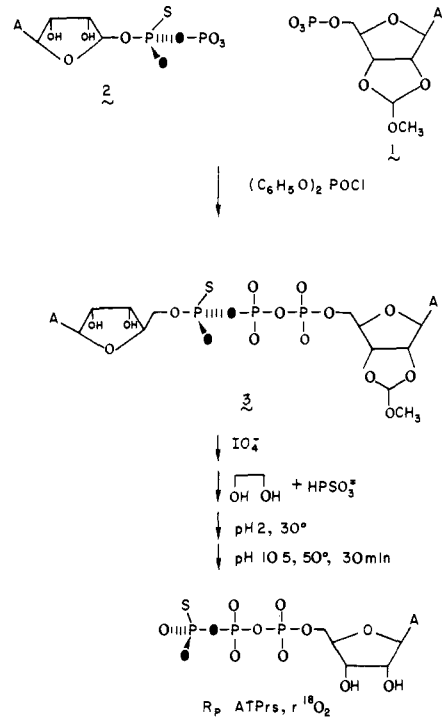
Periodate–Base Degradation of 5a and 5b. **5a** and **5b** (250 μmol) was dissolved in 100 mL of 20 mM Na_2CO_3 at pH 10.5, 2.2 mL of a 100 mM solution of $NaIO_4$ was added, and the solution was incubated first at room temperature for 30 min and then at 50 °C for 30 min. Products were purified by chromatography through a 2.5 × 50 cm column of DEAE-Sephadex A-25 with a 4-L linear elution gradient of triethylammonium bicarbonate increasing in concentration from 0.25 to 0.45 M. ADPβS and ADPαS were cleanly separated, eluting at 0.38 and 0.39 M buffer concentration, respectively. ADPαS and ADPβS were each isolated in 24% yield.

Results and Discussion

AMPS,$^{18}O_2$. The synthesis of AMPS described by Murray and Atkinson[11] was adapted to the synthesis of AMPS,$^{18}O_2$ by modifying the hydrolytic workup to make it compatible with the use of $H_2{}^{18}O$ in limited amounts and by the use of ion-exchange chromatography to purify the product. The isotopic enrichment of ^{18}O in AMPS,$^{18}O_2$ was about 90% of that in the $H_2{}^{18}O$ used in the hydrolytic workup. The 10% dilution of ^{18}O might be attributed to the presence of sodium acetate in the hydrolysis of 5′-adenosyl thiophosphoryl dichloride, the initial thiophosphorylation product; that is, acetate might participate as a nucleophilic catalyst and in this way serve as a source of ^{16}O. Alternatively, dilution might have resulted from incomplete hydrolysis in $H_2{}^{18}O$ with the consequence that hydrolysis went to completion in the subsequent workup and chromatography in H_2O. The latter appears to be the most probable cause of dilution, since Brody and Frey substituted pyridine for acetate as the base in the $H_2{}^{18}O$ hydrolysis in the synthesis of dAMPS,$^{18}O_2$ and found about the same diluton of isotope.[13] A longer hydrolysis with $H_2{}^{18}O$ would probably result in higher enrichment. It is important to remove as much $PSCl_3$ as possible before beginning the hydrolysis because the resulting HCl can exceed the amount of acetate in the hydrolytic reaction mixture, causing it to become acidic.

***R*$_P$-ATPγS,$\gamma^{18}O_2$.** The synthetic route to R_P-ATPγS,$\gamma^{18}O_2$ is depicted in Scheme I. The key reactants are 2′,3′-(methoxymethylidene)-AMP (**1**) and S_P-ADPαS,$\alpha^{18}O_2$, which are coupled together by the Michelson procedure[14] to **3**, the key intermediate. **3** possesses the requisite chemical properties for systematic high-yield transformation into R_P-ATPγS,$\gamma^{18}O_2$. The chiral phosphorus is straightforwardly transformed into P_γ of ATPγS by cleaving the ribose ring of the unblocked adenosyl group with IO_4^- and then subjecting the resulting 2′,3′-dialdehyde to mildly alkaline conditions resulting in the β elimination of the chiral [^{18}O]phosphorothioate from C-5′. Periodate–base degradation of ribonucleotides was first described by Brown et al. and Whitfield and Markham.[15] The methoxymethylidene group protects the other blocked adenosine from being attacked by IO_4^- while being easily removed at the appropriate time under conditions sufficiently mildly acidic that the sulfur is not lost to acid-catalyzed hydrolysis. The precursor of the chiral [^{18}O]phosphorothioate group in R_P-ATPγS,$\gamma^{18}O_2$ is P_α of S_P-ADPαS$^{18}O_2$, **i** in Scheme I. This compound is prepared in essentially quantitative yield from AMPS,$^{18}O_2$ by stereospecific phosporylation to S_P-ATPαS,$^{18}O_2$ with phosphoenolpyruvate in the presence of adenylate kinase and pyruvate kinase and a trace of ATP[8] followed by dephosphorylation to **2** with glucose and hexokinase.

Scheme I



Several complications arose during the development of procedures to cleave **3**. In the cleavage with periodate, unreacted IO_4^-, as well as IO_3^- produced in the cleavage process, rapidly desulfurized the thionucleotides under the acidic conditions required to remove the methoxymethylidene groups and, at a slower rate, even under neutral or slightly alkaline conditions. This was controlled by quenching excess periodate with glycol and reducing the resulting iodate with a large excess of thiophosphate as soon as the cleavage of the nucleoside by periodate was complete. In addition, because of the hydrolytic lability of the γ-phosphorothioate group of ATPγS in acid, removal of the methoxymethylidene group in acid according to Smith et al.[16] had to be carried out prior to alkaline elimination of ATPγS from the periodate-cleaved **3**. All the steps in Scheme I, diphenyl phosphorochloridate activation of **1** and coupling with **2**, cleavage of the unprotected ribose with IO_4^-, reductive removal IO_3^- and excess IO_4^-, removal of the methoxymethylidene protecting group in acid, and finally alkaline elimination of R_P-ATPγS,$\gamma^{18}O_2$ from the cleaved ribose ring, were carried out in sequence without isolating intermediates. The product was obtained reproducibly and in good yield (55–58%) after chromatographic purification. In one experiment, **3** was purified and partially characterized by ^{31}P NMR spectroscopy.[3d]

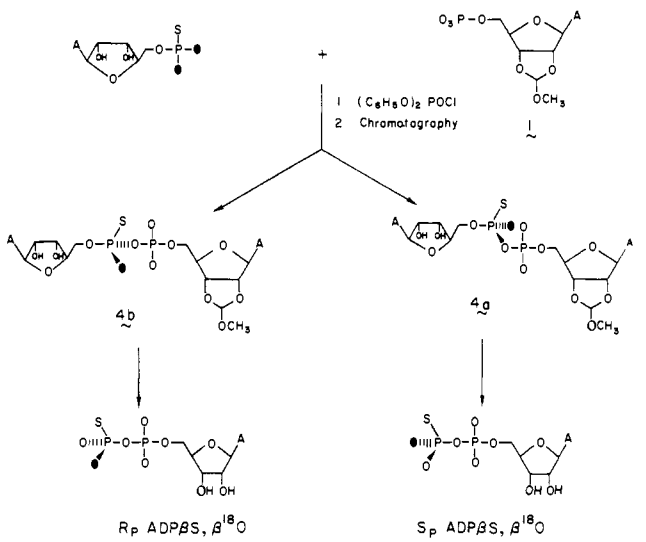
The ^{31}P NMR spectrum of the product R_P-ATPγS,$\gamma^{18}O$ was in agreement with the spectrum published by Jaffe and Cohn[12] and it served as a substrate for adenosine kinase[15] and adenylate kinase.[3j] The products of these reactions, AMPS,^{18}O and ADPβS,$\beta^{18}O$, were fully enriched in ^{18}O and were greater than 97% chirally pure as judged by ^{31}P NMR and mass spectral analysis.

***R*$_P$- and *S*$_P$-ADPβS,$\beta^{18}O$.** The synthetic route to R_P- and S_P-ADPβS,$\beta^{18}O$ is outlined in Scheme II. Activation of AMPS,$^{18}O_2$ with diphenyl phosphorochloridate and coupling with **1** by the Michelson procedure[14] produces a mixture of epimers **4a** and **4b** that can be separated by ion-exchange chromatography through a column of DEAE-Sephadex A-25 (Figure 1).

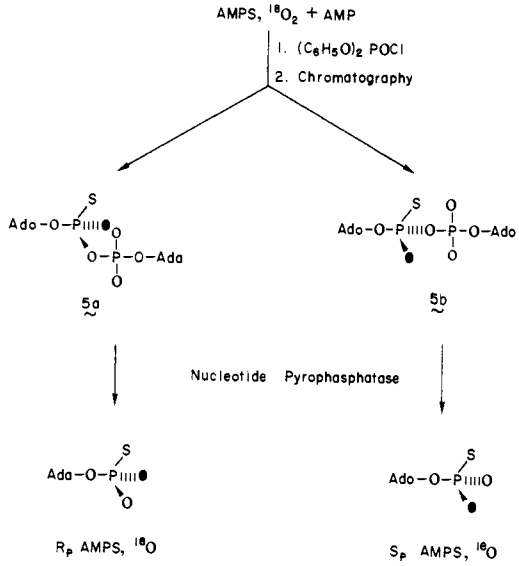
Consecutive treatment with periodate followed by acid and then by base converted **4a** and **4b** to S_P- and R_P-ADPβS,$\beta^{18}O$, re-

(13) Brody, R. S.; Frey, P. A. *Biochemistry* **1981**, *20*, 1245–1252.
(14) Michelson, A. M. *Biochim. Biophys. Acta* **1968**, *91*, 1–13.
(15) (a) Brown, D. M.; Fried, M.; Todd, A. R. *Chem. Ind. (London)* **1953**, 352–353. (b) Whitfield, P. R.; Markham, R. *Nature (London)* **1953**, *171*, 1151–1152.
(16) Smith, M. M.; Reeve, A. E.; Huang, R. C. C. *Biochemistry* **1978**, *17*, 493–500.

Scheme II



Scheme III



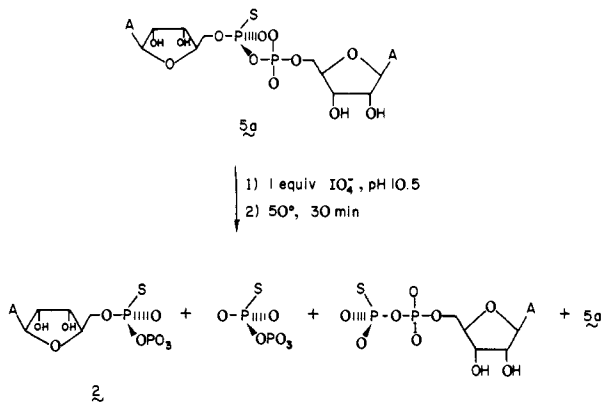
spectively. Again iodate had to be removed; however, in this case quenching with excess sodium thiophosphate proved to be impractical because an unknown product cochromatographing with ADPβS,β^{18}O resulted. Iodate was instead removed by ion-exchange chromatography prior to the acid-catalyzed deprotection step.

Since the conversion of **4a** and **4b** to ADPβS,β^{18}O does not alter the configuration of P_β, the epimeric composition of these compounds must be the same; i.e., synthetic R_P- and S_P-ADPβS,β^{18}O are greater than 95% chirally pure.

The configuration of **4a** and **4b** at the chiral phosphorus could not be known from the synthetic procedure and had to be determined. For this purpose samples of **4a** and **4b** unenriched with ^{18}O were converted to **5a** and **5b** (Scheme III) by subjecting them to pH 2.0 for 30 min to remove the methoxymethylidene protecting groups. The configurations of the resulting samples of **5a** and **5b** were assigned by correlation of their ^{31}P NMR chemical shifts with those of authentic samples synthesized as described below.

***R*P- and *S*P-AMPS,^{18}O.** We synthesized R_P- and S_P-AMPS,^{18}O by the route depicted in Scheme III. Activation of AMPS,$^{18}O_2$ with diphenyl phosphorochloridate and coupling with AMP produced **5a** and **5b** as an epimeric mixture. After separation by chromatography (Figure 2), the P_α configurations of samples not enriched with ^{18}O were assigned by degrading them to samples of ADPαS, the configurations of which could be assigned by their ^{31}P NMR chemical shifts.[8] The degradations were as outlined in Scheme IV. A sample of **5a** (or **5b**) was reacted with 1 equiv

Scheme IV



of IO_4^- and then heated at pH 10,5 for 30 min. Since neither ribosyl ring in **5a** was protected, cleavage by IO_4^- was nearly random, and all the possible periodate–base degradation products were obtained. ADPαS and ADPβS were each obtained in yields of approximately 24%, in close correspondence with the theoretical yields resulting from essentially random periodate cleavage. The P_α configurations of the two ADPαS samples from **5a** and **5b** were assigned by their ^{31}P NMR chemical shifts, given in Table I, which agreed with the values reported earlier.[8] On this basis **5a** was identified as the R_P epimer and **5b** as the S_P epimer, and the order in which they were eluted from the ion-exchange column was **5b** followed by **5a**. The ^{31}P NMR chemical shifts corresponding to the chiral phosphorus centers in **5a** and **5b** and in **4a** and **4b** given in Table I were found to differ, as reported for other epimeric thionucleotides.[8]

Nucleotide pyrophosphatase (dinucleotide nucleotidohydrolase, E.C. 3.6.1.9) catalyzed the cleavage of both **5a** and **5b** to AMP and AMPS,^{18}O. To verify that the configurations at phosphorus were unchanged by this procedure, the two samples were stereospecifically phosphorylated at the pro-*R* oxygens by the coupled actions of adenylate kinase and pyruvate kinase,[8] producing two samples of S_P-ATPαS,α^{18}O. Phosphorylation of R_P-AMPS,^{18}O must produce S_P-ATPαS,α^{18}O enriched exclusively in the $P_{\alpha,\beta}$ bridge oxygen, while S_P-AMPS,^{18}O leads to ATPαS,α^{18}O with enrichment only in the nonbridging P_α position. To determine whether ^{18}O was bridging or nonbriding, the two samples of S_P-ATPαS,α^{18}O were degraded to the corresponding triphosphorothiate, permethylated with diazomethane, hydrolyzed to dimethyl phosphate and methyl phosphorothioate, and again methylated with diazomethane to a mixture of trimethyl phosphate and trimethyl phosphorothioate. The hydrolysis step partitioned bridging ^{18}O into both trimethyl phosphate and trimethyl phosphorothioate while nonbridging ^{18}O remained associated exclusively with trimethyl phosphorothioate. Gas chromatograpic–mass spectrometric analysis revealed that the ^{18}O enrichments in the samples of trimethyl phosphorothioate derived from **5a** and **5b** corresponded to 0,3. and 0.88 mol of ^{18}O per mol, respectively, compared with 0.86 mol of ^{18}O per mol of **5a** and **5b**. This meant that **5a** and **5b** were cleaved by the action of nucleotide pyrophosphatase to R_P and S_P-AMPS,^{18}O, respectively, i.e., with retention of configuration.

Discussion

The chiral [^{18}O]phosphorothioate analogues of ATP, ADP, and AMP whose synthesis are described here have been used to determine the stereochemical courses of adenylate kinase,[3d] nucleoside diphosphate kinase,[3j] nucleoside phosphotransferase,[4] adenosine kinase,[5] glycerokinase,[3k] and polynucleotide kinase.[17] The synthetic methods are generally applicable to the synthesis of chiral nucleoside and nucleotide [^{18}O]phosphorothioates and dinucleoside pyrophosphorothioates and triphosporothioates for a broad range of research applications. The method described

(17) Bryant, F. F.; Benkovic, S. J.; Sammons, D.; Frey, P. A. *J. Biol. Chem.* **1981**, *256*, 5965–5966.

here for synthesizing R_P-ATPγS,$\gamma^{18}O_2$ has been adopted by Webb and Trentham in their studies of the stereochemical courses of ATPases;[18] and these workers have also adapted the method to the synthesis of chiral ATPγS,γ^{17}O,^{18}O. The yields are good when appropriate precautions are taken to exclude water from the phosphoanhydride coupling reaction mixtures, and the method described for the synthesis of R_P- and S_P-ADPβS,β^{18}O has been adapted to the efficient synthesis of ADPβS from AMPS and **1**.[19]

A recent advance in the chemistry of phosphorothioates should make the chiral nucleoside [^{18}O]phosphorothioates described here increasingly important as synthetic precursors in the synthesis of ATP specifically enriched with isotopic oxygen at any position of the triphosphate moiety. It has been found that ATPβS,β-cyanoethyl-ADPαS and cyclohexyl propyl pyrophosphorothioate are desulfurized by reaction with cyanogen bromide in water at neutral to alkaline pH.[19,20] The reaction leads to the substitution of ^{18}O for sulfur in $H_2{}^{18}O$ and, in the case of β-cyanoethyl-ADP, with inversion of configuration.[20] By combining highly stereoselective and stereospecific phosphorylation[6,8] of R_P- and S_P-AMPS,^{18}O and R_P- and S_P-ADPβS,β^{18}O with desulfurization by cyanogen bromide, it should be possible for the first time to introduce isotopic oxygen specifically either into the P_α–P_β or the O_β–P_γ bridging positions of ATP or into either diastereotopic position at P_α or P_β of ATP. Similarly, R_P- and S_P-ADPα^{18}O as well as ^{18}O-bridging ADP(α–β)^{18}O an be synthesized. These specifically enriched species of ATP and ADP, heretofore inaccessible, will greatly facilitate isotopic studies probing the mechanisms of enzyme action.

The phosphorus configurations assigned in this paper are based upon the ^{31}P NMR chemical shifts of P_α in samples of R_P- and S_P-ADPαS produced in the chemical degradations of **4a**, **4b**, **5a**, and **5b**. Since the shifts for the epimers of ADPαS are known from earlier work, the configurational assignments are secure. Table I exemplifies the importance of exercising caution in the assignment of configurations by the use of ^{31}P NMR data. Note that the P_α chemical shift for R_P-ADPαS is downfield from that for the S_P epimer; this relationship extends to the epimers of ATPαS[8] and to the uridine series as well.[3c,j] The relationship does *not* extend to compounds **4a** and **4b** or **5a** and **5b**, since in these compounds the P_α chemical shifts for the S_P epimers are downfield from those for the R_P epimers. Therefore, any configurational assignments for **4a** and **4b** or **5a** and **5b** based on their relative P_α chemical shifts and the corresponding relative shifts for other nucleotides would have been erroneous. The measurement of ^{31}P NMR chemical shifts is a powerful technique for distinguishing the epimers of thionucleotides, but assignments of absolute configuration can be made only by chemical correlation with compounds of known configuration.

Acknowledgment. We are grateful to Dr. Charles Cottrell for his assistance in obtaining nuclear magnetic resonance spectra and to Richard Weisenberger for his assistance in obtaining mass spectral data, This research was supported by Grant No. GM24390 from the National Institute of General Medical Sciences.

Registry No. 1, 68973-49-9; **1** trioctylammonium salt, 81671-39-8; **2**, 68973-47-7; **3**, 68973-51-3; **4a**, 69010-05-7; **4b**, 68973-40-0; **5a**, 81671-40-1; **5b**, 81738-72-9; AMPS,$^{18}O_2$, 68973-48-8; AMPS,$^{18}O_2$ trioctylammonium salt, 81687-72-1; *R*p-ATPγS,$\gamma^{18}O_2$, 68973-46-6; *S*p-ADPαS,$\alpha^{18}O_2$ trioctylammonium salt, 81687-74-3; *R*p-ADPβS,β^{18}O, 69182-10-1; *S*p-ADPβS,β^{18}O, 68973-41-1; *R*p-AMPS,^{18}O, 71067-08-8; *S*p-AMPS,^{18}O, 71067-07-7; AMP trioctylammonium salt, 69098-20-0; *S*p-ADPαS,$\alpha^{18}O_2$, 81687-75-4; *R*p-ADPαS,$\alpha^{18}O_2$, 81687-76-5; adenosine, 58-61-7; 2′,3′-(methoxymethylidene)adenosine, 16658-10-9; 2,3-(methoxymethylidene)adenosine-5′-cyanoethylphosphate, 81671-41-2.

(18) Webb, M. R.; Trentham, D. R. *J. Biol. Chem.* **1980**, *255*, 8629–8632.
(19) Ho, H.-T.; Frey, P. A., unpublished results.
(20) Sammons, R. D.; Frey, P. A. *J. Biol. Chem.* **1982**, *257*, 1138–1141.

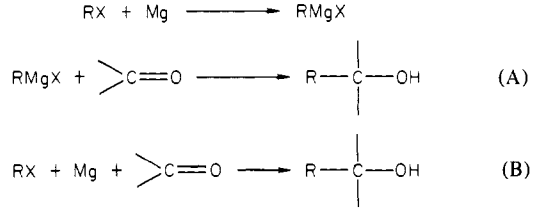
The Barbier Synthesis: A One-Step Grignard Reaction?

Gérard Molle and Pierre Bauer*

Contribution from the Institut de Topologie et de Dynamique des Systèmes, Université Paris VII, associé au C.N.R.S., 75005 Paris, France. *Received June 3, 1981. Revised Manuscript Received January 7, 1982*

Abstract: Counter to generally accepted theory, it is demonstrated that the Barbier synthesis does not necessarily involve the in situ formation of an organometallic compound. In certain cases, there is a radical pathway in which the anion radical ($R^{\cdot}X^{-}$) resulting from the attack by a halogenated derivative on lithium is directly trapped by the ketone or by the ketyl radical on the metal surface before the organometallic compound forms. This pathway can be unique, as when 1-bromoadamantane condenses with adamantanone or hexamethylacetone. However, by extension of the Barbier synthesis to other "cage-structure" compounds homologous to adamantane, it is seen that the radical pathway can compete with the organometallic pathway and that this competition is principally determined by the stability of the cage radicals generated at the metal–solution interface. An optimum yield can be attained in this type of synthesis by choosing the Grignard reaction or the Barbier reaction, depending on the nature of the halogenated cage derivatives in use.

The Grignard reaction,[1] which involves a first step for the synthesis of an organometallic compound and a second step for the reaction of the latter with a substrate (A), chronologically follows the Barbier synthesis,[2] which yields the end product in a single step (B). So long as use of the Grignard reaction was restricted to magnesium, it was preferred over the Barbier reaction and was studied to a greater extent from the viewpoint of synthesis



and from that of the reaction mechanisms. However, with the recent use of other metals, particularly lithium,[3-7] there has been

(1) Grignard, V. *C. R. Hebd. Seances Acad. Sci.* **1900**, *130*, 1322.
(2) Barbier, P. L. *C. R. Hebd. Seances Acad. Sci.* **1898**, *128*, 110.

0002-7863/82/1504-3481$01.25/0 © 1982 American Chemical Society