A New Method for Synthesizing Phosphoanhydrides: Synthesis of Adenosine 5′-(2-Thiodiphosphate) and Adenosine 5′-(2-Thiotriphosphate)†

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ABSTRACT: A new method for synthesizing adenosine 5′-(2-thiotriphosphate), ATPβS, in excellent yield is described. Reaction of crude adenosine 5′-phosphorodichloridate with 2′,3′-(methoxymethylidene)adenosine 5′-(2-thiodiphosphate) in hexamethylphosphoramide produced P^1-(5′-adenosyl) P^3-[5′-2,3′-(methoxymethylidene)adenosyl] 2-thiotriphosphate in 65% yield. The latter compound was converted to ATPβS in at least 87% yield by periodate cleavage of the adenosyl group, reduction of IO_3^- and excess IO_4^-, removal of the methoxymethylidene group at pH 2, and elimination of the periodate-cleaved adenosyl group at pH 10.5. The new phosphoanhydride coupling reaction involves the formation of P^1-(5′-adenosyl) P^3-[5′-2′,3′-(methoxymethylidene)adenosyl] 2-thiocyclotriphosphate, as shown by the fact that product workup in $H_2{}^{18}O$ leads to the formation of ATPβS with ^{18}O at both P_α and P_γ. A convenient synthesis of adenosine 5′-(2-thiodiphosphate), ADPβS, is also described. Coupling of adenosine 5′-phosphorothioate with 2′,3′-(methoxymethylidene)-AMP produces P^1-(5′-adenosyl) P^2-[5′-2′,3′-(methoxymethylidene)adenosyl] 1-thiopyrophosphate. Treatment of the crude coupling product with periodate, a reducing agent, weak acid (pH 2), and dilute base (pH 10.5) followed by chromatographic purification produced ADPβS in an overall yield of 55–60%.

The compounds ADPβS[1] and ATPβS have been widely used in stereochemical studies of phosphotransferases and ATP-dependent synthetases (Eckstein & Goody, 1976; Jaffe & Cohn, 1978a,b, 1979; Rossomondo et al., 1979; Lerman & Cohn, 1980; Burgers & Eckstein, 1980). Syntheses of these compounds were reported by Goody & Eckstein (1971) and Eckstein & Goody (1976), who adapted a general procedure introduced by Michelson for synthesizing phosphoanhydrides (Michelson, 1964) to the preparation of sulfur-containing nucleoside phosphoanhydrides. In the synthesis of ADPβS, AMP was chemically activated by reaction with diphenyl phosphorochloridate, producing P^1-diphenyl P^2-(5′-adenosyl) pyrophosphate. This intermediate reacted with *S*-(2-carbamoylethyl) phosphorothioate in pyridine to produce P^1-(5′-adenosyl) P^2-*S*-(2-carbamoylethyl) 2-thiopyrophosphate, which was converted by treatment with NaOH to ADPβS in an overall yield of 35%. In the synthesis of ATPβS, 2-cyanoethyl phosphate was chemically activated by reaction with diphenyl phosphorochloridate. The resulting P^1-diphenyl P^2-(2-cyanoethyl) pyrophosphate was converted by reaction with ADPβS in pyridine to P^1-(5′-adenosyl) P^3-(2-cyanoethyl) 2-thiotriphosphate. Removal of the cyanoethyl group by base-catalyzed elimination led to ATPβS in 12% overall yield. In contrast to most applications of the Michelson procedure, the reported yields, especially of ATPβS, were low. We attempted without success to improve the yield of ATPβS and ultimately traced the cause of the low yield to the instability of ADPβS in pyridine.

Pyridine is an excellent solvent for supporting the coupling of diphenyl phosphorochloridate activated phosphoric esters with phosphates, because it participates directly in the reaction mechanism as a nucleophilic catalyst (Richard & Frey, 1983). The reaction is poor in solvents that lack this capacity. Moreover, in our experience ADPβS is unstable in many of the solvents commonly used in the nucleotide synthesis field. To be fully successful in this case, the Michelson method would have to be modified by adopting a more active nucleophilic catalyst to promote the coupling reaction at a rate that exceeds the decomposition rate for ADPβS under the reaction conditions.

In this paper we describe a simplified new procedure for synthesizing ATPβS in very good yield. The method should be applicable to the synthesis of any nucleoside triphosphate. We also describe a convenient, high-yield synthesis of ADPβS, an intermediate in the synthesis of ATPβS.

Experimental Procedures

Materials. Barium oxide, calcium hydride, diphenyl phosphorodichloridate, hexamethylphosphoramide, 4-Å molecular sieves, phosphorus oxychloride, sodium periodate, thiophosphoryl trichloride, tri-*n*-butylamine, triethyl phosphate, triethyl orthoformate, and tri-*n*-octylamine were purchased from Aldrich Chemical Co. DEAE-Sephadex A-25 and adenosine were purchased from Sigma Chemical Co. and Boehringer Mannheim, respectively. Dowex AG-50 ion-exchange resin and deuterium oxide (88.7% ^2H) were purchased from Bio-Rad Laboratories. $H_2{}^{18}O$ was purchased from Monsanto Research Corp., Mound Laboratory. AMPS and 2′,3′-(methoxymethylidene)-AMP were synthesized by published procedures (Richard & Frey, 1982).

Purification of Solvents. Triethyl phosphate was mixed with barium oxide and allowed to stand for 24 h before being distilled in vacuo. Redistilled triethyl phosphate was stored over molecular sieves (4 Å) in the dark. Thiophosphoryl

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[1] Abbreviations: ADPβS, adenosine 5′-(2-thiodiphosphate); ATPβS, adenosine 5′-(2-thiotriphosphate); 2′-dATPαS, 2′-deoxyadenosine 5′-(1-thiotriphosphate); 2′,3′-(methoxymethylidene)-ADPβS, 2′,3′-(methoxymethylidene)adenosine 5′-(2-thiodiphosphate); NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; AMPS, adenosine 5′-phosphorothioate; [α,γ-^{18}O]ATPβS, ATPβS with ^{18}O bonded to P_α and P_γ.

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chloride and phosphorus oxychloride were redistilled and desiccated under N_2. Pyridine was mixed with calcium hydride overnight, redistilled, and stored over potassium hydroxide in the dark. *N,N*-Dimethylformamide was dried by mixing it with powdered barium oxide and allowing the mixture to stand overnight. It was then distilled from alumina. Redistilled *N,N*-dimethylformamide was stored over molecular sieves (4 Å) in the dark. Tri-*n*-butylamine was stirred with calcium hydride for 12 h and then distilled in vacuo. Redistilled tri-*n*-butylamine was desiccated under N_2. Triethylamine was redistilled before use. Hexamethylphosphoramide was distilled in vacuo prior to being used. Ethyl ether, if not from a freshly opened can, was passed through an alumina (Basic X, type WB-5) column just prior to being used. Dioxane was stored overnight with molecular sieves (4 Å) and distilled. Redistilled dioxane stored over BaO in the dark was percolated through an alumina column prior to use. Small aliquots of alumina-treated dioxane were tested for the presence of peroxides. They were mixed with equal volumes of water, and a few crystals of KI were added. A yellow color from oxidation of I^- to I_2 signaled the presence of peroxides. The alumina treatment was repeated until a colorless clear solution was obtained in the KI test. Other solvents were obtained from commercial suppliers and used without further purification.

Chromatography. Nucleotides synthesized in this research were routinely purified by ion-exchange column chromatography through DEAE-Sephadex A-25 with triethylammonium bicarbonate in the elution gradients. DEAE-Sephadex A-25 ion exchanger was prepared by permitting the exchanger to swell in 1.0 M triethylammonium bicarbonate for several hours. After the exchanger was packed in a column having the desired dimensions, the column of ion exchanger was washed first with 4 volumes of 1.0 M $KHCO_3$ and then with 4 volumes of 0.1 M triethylammonium bicarbonate buffer at pH 7.6. Regeneration of the column was by a similar procedure.

Triethylammonium bicarbonate was prepared by bubbling CO_2 gas generated from dry ice through a glass filter and into a 1.0 M aqueous solution of triethylamine with stirring until the pH of the solution reached 7.6. The stock solution was stored at room temperature in a tightly capped bottle.

Nucleotides in solution at pH 7.6 with ionic strength at 0.1 M or less were absorbed to columns of DEAE-Sephadex A-25 that had been equilibrated with 0.1 M triethylammonium bicarbonate at pH 7.6 (see above). Nucleotides and thionucleotides were eluted from the columns with linear gradients of triethylammonium bicarbonate at pH 7.8. The gradients were prepared by adding equal volumes of two different concentrations of triethylammonium bicarbonate buffer to two identical flasks connected by a bridge filled with the buffer of lower concentration. The latter buffer was stirred continuously while the gradient was drawn from this flask to feed the column. Fractions were collected, and those containing nucleotides were identified by A_{260} measurements. Peak fractions were pooled and the buffer salts removed by flash evaporation in a rotary evaporator connected to a vacuum pump. The bath temperature was kept below 30 °C at all times. The initial residue was twice taken up in 95% ethanol and again evaporated to ensure that all the buffer salts were removed. The final residue was dissolved in a minimum amount of water and stored at −15 °C after the pH had been adjusted to 10 by addition of triethylamine.

In preparation for further use in synthesis, the triethylammonium salts of nucleoside phosphorothioates were converted to tri-*n*-octylammonium salts to render them soluble in organic solvents. The triethylammonium salt of a nucleotide was passed through a column of Dowex 50 (pyridinium form) that had at least at 20-fold excess of exchange capacity. The flow through contained the pyridinium salt of the nucleotide, which was evaporated to dryness by rotary evaporation in vacuo. The residue was dissolved in a minimum volume of methanol; and tri-*n*-octylamine was added to the solution (1 equiv for all nucleoside monophosphates or monophosphorothioates; 2 equiv for nucleoside diphosphates or thiodiphosphates). After the mixture was stirred until it became clear, methanol was removed in vacuo. The residue was desiccated with P_2O_5 under vacuum for 20 h at 25 °C to remove traces of methanol and water.

^{31}P NMR Analysis. Proton spin-decoupled ^{31}P NMR spectra of nucleotides were obtained on 2–2.5-mL samples consisting of 1–5 mM nucleotide dissolved in 40% D_2O at pH 9–10 and containing 50 mM EDTA. The spectrometer was field frequency locked to the deuterium resonance of the solvent. Spectra were obtained on a Bruker 200-MHz, a Bruker 360-MHz, and a Nicolet 200-MHz spectrometers. All chemical shifts were related to that of 1 N (0.33 M) H_3PO_4 dissolved in D_2O as an external reference.

Synthesis of ADPβS. An aqueous solution containing 1 mmol of the triethylammonium salt of 2′,3′-(methoxymethylidene)-AMP was dried by flash evaporation in a rotary evaporator. After the residue was dissolved in 20 mL of methanol, 1 mmol of tri-*n*-octylamine (0.45 mL) was added to the solution. The mixture was stirred until it became clear. After methanol was removed by rotary evaporation, the residue was further dried by twice dissolving it in anhydrous *N,N*-dimethylformamide and removing the solvent by rotary evaporation. The dried 2′,3′-(methoxymethylidene)-AMP was desiccated in vacuo over P_2O_5 for 24 h in preparation for coupling to P^1-diphenyl P^2-(5′-adenosyl) 2-thiopyrophosphate prepared as follows. The triethylammonium salt of AMPS (2 mmol) was dried by rotary evaporation to remove water. Methanol (40 mL) and tri-*n*-octylamine (2 mmol, 0.9 mL) were added to the nucleotide. The mixture was stirred until a clear solution was obtained and then dried by rotary evaporation. The residue was further dried by twice dissolving it in anhydrous *N,N*-dimethylformamide and removing the solvent by rotary evaporation. The AMPS was then dried in vacuo over P_2O_5 for 24 h.

The mono tri-*n*-octylammonium salt of AMPS was dissolved in 8 mL of triethyl phosphate, and to the solution were added diphenyl phosphorochloridate (3 mmol, 0.64 mL) and tri-*n*-butylamine (4 mmol, 1 mL). The flask was stoppered and the mixture stirred at ambient temperature for 3 h. A mixture consisting of 150 mL of petroleum ether (bp 60–80 °C) and 50 mL of diethyl ether (new can) was added to the solution with stirring at the end of the 3-h period. This mixture was kept at 0 °C for 30 min. The ether layer was decanted, and 4 mL of dioxane was added to the residue. The solution was evaporated to dryness in a rotary evaporator. The dried mono tri-*n*-octylammonium salt of 2′,3′-(methoxymethylidene)-AMP was dissolved in 2 mL of pyridine and transferred to the flask containing activated AMPS and the reaction mixture stirred at ambient temperature for 16 h. After removal of pyridine by rotary evaporation, diethyl ether (20 mL) was added to the residue and water used to extract nucleotides until the A_{260} values of aqueous extracts were less than 0.5. The pH of the combined aqueous extract was immediately adjusted to 8 by addition of NaOH to avoid losing the methoxymethylidene group. A total of 3 mmol of $NaIO_4$ was added and the solution kept at ambient temperature for 30 min, at which time 30

mmol of 2-mercaptoethanol was added to quench the periodate degradation and reduce IO_4^- and IO_3^- to I^-. The pH of the solution was adjusted to 2 by adding HCl; and after 100 min at 25 °C, the pH was adjusted to 10.5 by addition of NaOH. This solution was heated at 50 °C for 30 min. The solution was diluted to 2 L and divided into two 1-L aliquots, each of which was passed through a 4 × 50 cm column of DEAE-Sephadex A-25 in the HCO_3^- form. Nucleotides were eluted from each column with a triethylammonium bicarbonate gradient having a total volume of 7 L (3.5 L of each component) and increasing from 0.2 to 0.45 M. Fractions 23 mL in volume were collected at 16-min intervals; and selected fractions were analyzed for A_{260} and reactivity with 5,5′-dithiobis(2-nitrobenzoate). ADPβS appeared as a prominent band in fractions 240–280. Pooled fractions were desalted as described above, yielding ADPβS in an overall yield of 58%.

The ^{31}P NMR spectrum (proton spin decoupled) of ADPβS exhibited a P_α doublet at −11.79 ppm and a P_β doublet at 32.82 ppm with $J_{\alpha,\beta}$ = 31.74 Hz, in agreement with the values reported for [β-^{18}O]ADPβS (Jaffe & Cohn, 1978a). Thin-layer chromatography also showed that the ADPβS prepared in this manner comigrates with authentic [β-^{18}O]ADPβS prepared by the procedure of Richard & Frey (1982). ADPβS was also quantitatively converted to ATPβS by the two enzymatic phosphorylating systems phosphoenolypyruvate/pyruvate kinase and acetyl phosphate/acetate kinase.

Synthesis of 2′,3′-(Methoxymethylidene)-ADPβS. P^1-(5′-Adenosyl) P^2-[5′-2′,3′-(methoxymethylidene)adenosyl] 1-thiopyrophosphate was synthesized as described by Richard & Frey (1982), except that the R_P and S_P epimers were not separated. The mixture was converted to 2′,3′-(methoxymethylidene)-ADPβS by the following procedure.

P^1-(5′-Adenosyl) P^2-[5′-2′,3′-(methoxymethylidene)-adenosyl] 1-thiopyrophosphate (300 μmol) was dissolved in 30 mL of water and the pH adjusted to 8.0. $NaIO_4$ (450 μmol) was added to the solution. After being stirred at ambient temperature for 30 min, 5 mmol of 2-mercaptoethanol was added, and the pH was adjusted to 10.5 with NaOH. The reaction mixture was maintained at 50 °C for 30 min and then diluted with water to a final volume of 200 mL in preparation for DEAE-Sephadex A-25 column chromatography through a 2.5 × 40 cm column of DEAE-Sephadex A-25. A 3.5-L linear gradient of triethylammonium bicarbonate increasing from 0.1 to 0.5 M was used to elute the compounds. Fractions of 23 mL were collected at a rate of 1.5 mL/min. The product appeared as an isolated band of 5,5′-dithiobis(2-nitrobenzoate)-sensitive and A_{260}-absorbing material in fractions 119–142, which were pooled and desalted. The pooled fractions contained 223.3 μmol of 2′,3′-(methoxymethylidene)-ADPβS, an overall yield of 74.4% on the basis of an extinction coefficient of 24 000 $M^{-1}\cdot cm^{-1}$ for P^1-(5′-adenosyl) P^2-[5′-2′,3′-(methoxymethylidene)adenosyl] 1-thiodiphosphate and 15 000 $M^{-1}\cdot cm^{-1}$ for 2′,3′-(methoxymethylidene)-ADPβS.

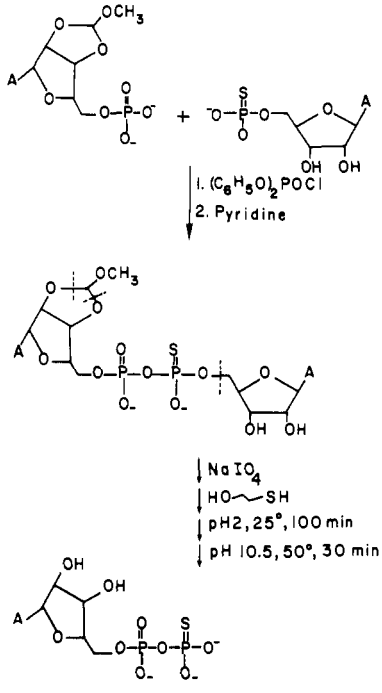
The 2′,3′-(methoxymethylidene)-ADPβS obtained gave a positive reaction with 5,5′-dithiobis(2-nitrobenzoate), gave an identical UV absorption spectrum as that of 2′,3′-(methoxymethylidene)AMP, and was not degraded by $NaIO_4$. The ^{31}P NMR spectrum of 2′,3′-(methoxymethylidene)-ADPβS gave two doublets with chemical shifts and a coupling constant essentially identical with those of ADPβS: P_α, −11.07 ppm; P_β, 33.05 ppm; $J_{\alpha,\beta}$ = 31.70 Hz.

Synthesis of P^1-(5′-Adenosyl) P^3-[5′-2′,3′-(Methoxymethylidene)adenosyl] 2-Thiotriphosphate. This compound was prepared as an intermediate in the overall synthesis of ATPβS. It was produced either by phosphorylation of adenosine with $POCl_3$ and reaction of the crude adenosine 5′-phosphorodichloridate with 2′,3′-(methoxymethylidene)-ADPβS followed by aqueous workup or by reaction of $POCl_3$ with 2′,3′-(methoxymethylidene)adenosine and coupling of the resulting 5′-phosphorodichloridate with ADPβS and aqueous workup. The same basic procedure for both routes produced comparable yields of the compound.

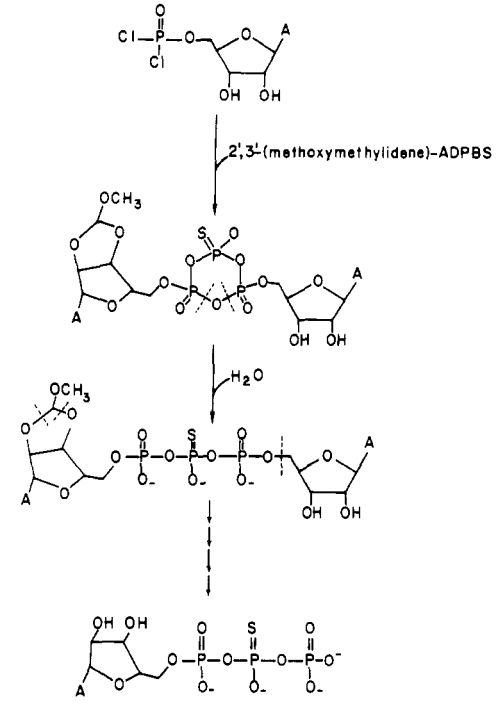
The triethylammonium salt of 2′,3′-(methoxymethylidene)-ADPβS (or of ADPβS) (100 μmol) in aqueous solution was concentrated by rotary evaporation and the resulting residue dissolved in 2 mL of methanol. Two equivalents (200 μmol) of tri-*n*-butylamine was added and methanol removed by rotary evaporation in vacuo. The residue was dissolved in 2 mL of water, frozen as a thin film on the wall of the flask, and lyophilized to dryness (12 h). Separately, adenosine [or 2′,3′-(methoxymethylidene)adenosine] (250 μmol), which had been desiccated over P_2O_5 in vacuo at 110 °C overnight, was dissolved in 0.7 mL of triethylphosphate by cautiously swirling the suspension in a distillation flask over an open flame. The clear solution was immediately cooled in an ice–water bath and $POCl_3$ (325 μmol) added. The reaction mixture was stirred magnetically at room temperature for 30 min. Triethyl phosphate, unreacted phosphorus oxychloride, and HCl generated in the reaction were removed by vacuum distillation at 35–40 °C into a receiving flask cooled in an ice–water bath. The lyophilized tri-*n*-butylammonium salt of 2′,3′-(methoxymethylidene)-ADPβS (or ADPβS) was dissolved in 1.0 mL of hexamethylphosphoramide and transferred to the flask containing the crude adenosine 5′-phosphorodichloridate together with a 0.5-mL hexamethylphosphoramide wash. The reaction flask was sealed and the solution stirred at room temperature for 2 h. Diethyl ether (20 mL) was added and the resulting suspension centrifuged. After the supernatant fluid was decanted the precipitate was dissolved with 10 mL of 1 M triethylammonium bicarbonate and stirred at ambient temperature for 1 h. The solution was diluted to 150 mL and applied to a 2.5 × 40 cm column of DEAE-Sephadex A-25 in the HCO_3^- form. The column was eluted with a linear gradient of triethylammonium bicarbonate increasing in concentration from 0.15 to 0.5 M and formed from 1.2 L of each component. The flow rate was 1 mL min^{-1}, and 12-mL fractions were collected. The desired products appeared in fractions 149–179 as a prominent band detected by A_{260} measurements. Fractions 50–80 contained a less prominent band of AMP [or 2′,3′-(methoxymethylidene)-AMP]. Fractions 149–179 were pooled and desalted as described above. The yield of P^1-(5′-adenosyl) P^3-[5′-2′,3′-(methoxymethylidene)adenosyl] 2-thiotriphosphate ranged between 60 and 65%, on the basis of the thionucleotide used. The major product was judged to be the above-name compound on the basis of its ^{31}P NMR spectrum and its nearly quantitative conversion to ATPβS upon sequential treatment with $NaIO_4$, 2-mercaptoethanol, acid (pH 2), and base (pH 10.5), as described below. The proton spin-decoupled ^{31}P NMR spectrum consisted of two triplets—one centered at −11.71 ppm assigned to P^1 and P^3 and a second at 30.65 ppm assigned to P^2—in a 2:1 integration ratio with a coupling constant of 24.91 Hz.

Conversion of P^1-(5′-Adenosyl) P^3-[5′-2′,3′-(Methoxymethylidene)adenosyl] 2-Thiotriphosphate to ATPβS. The triethylammonium salt of P^1-(5′-adenosyl) P^3-[5′-2′,3′-(methoxymethylidene)adenosyl] 2-thiotriphosphate (240 A_{260} units) in 2 mL of aqueous solution was adjusted to pH 8.4. Sodium metaperiodate (15 μmol) was added with stirring. After reaction at ambient temperature for 20 min, 150 μmol of 2-mercaptoethanol was added, and the pH was adjusted to

Scheme I



Scheme II



2.0 by addition of 1 N HCl. The solution was maintained at ambient temperature for 120 min and then readjusted to pH 10.5 by addition of 1 N NaOH. The solution was then heated to and maintained at 50 °C in a water bath for 20 min. It was diluted with water to 20 mL and subjected to ion-exchange chromatography through a 1.5 × 15 cm column of DEAE-Sephadex A-25 in the HCO_3^- form. The column was eluted with a linear gradient of triethylammonium bicarbonate increasing in concentration from 0.2 to 0.6 M and formed from 350 mL of each component. Fractions 4.5 mL in volume were collected at 10-min intervals. A single prominent band of A_{260}-positive material appeared in fractions 28–36, which were pooled and desalted. The product was identified as the expected R_P and S_P epimer mixture of ATPβS by its ^{31}P NMR spectrum and by its use as a substrate for yeast hexokinase, which accepted the S_P epimer as a substrate. The yield of ATPβS was 86%.

Results and Discussion

Synthesis of ADPβS. The method described above and outlined in Scheme I for synthesizing ADPβS is a simplification of one previously described for the syntheses of (R_P)-[β-^{18}O]ADPβS and (S_P)-[β-^{18}O]ADPβS (Richard & Frey, 1982). Elimination of a time-consuming column chromatography step shortens and accelerates the procedure and improves the overall yield. Instead of separating the intermediate P^1-(5′-adenosyl) P^2-[5′-2′,3′-(methoxymethylidene)-adenosyl] 1-thiopyrophosphate into its P^1 epimers, the crude coupling product is converted directly into ADPβS, which is purified in an overall yield of 55–60%. The procedure is reproducible and convenient and, perhaps because of the mild deblocking conditions, produces a good yield. It is also easily adapted to the synthesis of 2′,3′-(methoxymethylidene)-ADPβS by eliminating the acid treatment to remove the methoxymethylidene group. Either ADPβS or 2′,3′-(methoxymethylidene)-ADPβS can be used in the synthesis of ATPβS described below.

Synthesis of ATPβS. Two methods for synthesizing ATPβS are described in the literature, both of which involve the phosphorylation of ADPβS. Enzymatic phosphorylation using a high-energy phosphorylating substrate and the appropriate enzyme produces one or the other P_β epimer of ATPβS with a high degree of stereoselectivity. Suitable systems include phosphoenol pyruvate/pyruvate kinase and acetyl phosphate/acetate kinase (Eckstein & Goody, 1976; Richard et al., 1978).

A nonenzymatic phosphorylation method was described by F. Eckstein and R. S. Goody in which they coupled ADPβS with 2-cyanoethyl phosphate and treated the resulting P^1-(5′-adenosyl) P^3-(2-cyanoethyl) 2-thiotriphosphate with base to eliminate the 2-cyanoethyl group and produce ATPβS as a mixture of P_β epimers. This procedure produced a low yield of coupling product. Our efforts to improve the yields were unsuccessful. Slightly modified efforts substituting 2′,3′-(methoxymethylidene)-AMP for 2-cyanoethyl phosphate were similarly unsuccessful. We ultimately traced the low coupling yield to the instability of ADPβS in pyridine, the coupling solvent. The decomposition products included AMP as the major ultraviolet-absorbing material, as indicated by thin-layer chromatography of aliquots from pyridine solutions of ADPβS. These experiments revealed the presence of AMP within a few minutes and complete decomposition within 30 min. Since the coupling reaction required hours to go to completion, it could not compete effectively with the decomposition of ADPβS to AMP.

Similar decomposition of ADPβS was observed in solvents such as dimethyl sulfoxide and dimethylformamide. Such solvents would not support coupling by the Michelson procedure, which appears to involve the direct participation of pyridine as a nucleophilic covalent catalyst (Richard & Frey, 1983). The instability of ADPβS in these solvents underscored the importance of having a coupling reaction that proceeds rapidly enough to compete with decomposition.

It occurred to us that direct coupling of 2′,3′-(methoxymethylidene)-ADPβS to adenosine 5′-phosphorodichloridate, according to Scheme II, might afford an experimentally simple, high-yield route to ATPβS. Adenosine 5′-phosphorodichloridate would be generated by reaction of adenosine with $POCl_3$ in triethylphosphate, a standard procedure for synthesizing AMP. Reaction of crude adenosine 5′-phoshoro-

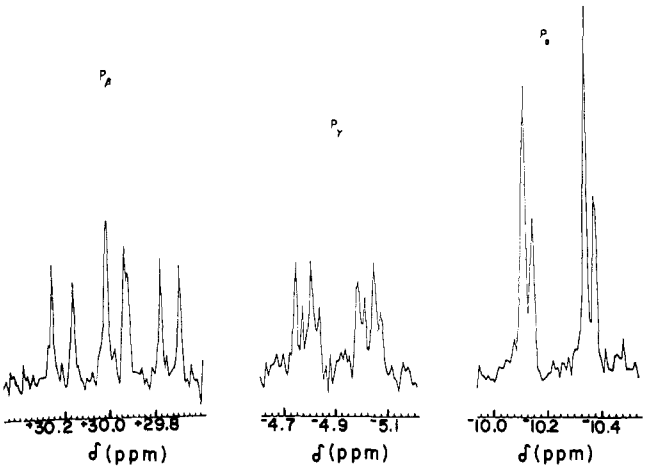


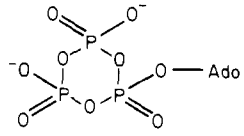
FIGURE 1: ^{31}P NMR spectrum of [α,γ-^{18}O]ATPβS. Shown are the P_α, P_β, and P_γ regions of the proton spin decoupled ^{31}P NMR spectrum of [α,γ-^{18}O]ATPβS. The sample was prepared by the procedure outlined in Scheme II with $H_2{}^{18}O$ for the aqueous workup. The spectrum was obtained on a Bruker 360-MHz spectrometer under the conditions described under Experimental Procedures. The spectrum was obtained at 145.8 MHz in the Fourier-transform mode. Spectral parameters were as follows: 16K memory, spectral width 8000 Hz, 544 scans, 24-μs pulse, 1.0-s acquisition time, and flip angle 45°. Chemical shifts and coupling constants are given in the text.

dichloridate with 2′,3′-(methoxymethylidene)-ADPβS should produce P^1-(5′-adenosyl) P^3-[5′-2′,3′-(methoxymethylidene)-adenosyl] 2-thiocyclotriphosphate (see Scheme II). Upon aqueous workup, the latter would undergo hydrolysis by cleavage of the bonds indicated in Scheme II, producing P^1-(5′-adenosyl) P^3-[5′-2′,3′-(methoxymethylidene)adenosyl] 2-thiotriphosphate. This compound could be converted to ATPβS in very high yield by periodate cleavage and β-elimination of the unblocked adenosyl moiety and removal of the 2′,3′-(methoxymethylidene) group.

This synthetic route produces ATPβS in overall yields of 55–60% on the basis of the thionucleotide as a starting material. The method works equally well starting with ADPβS and 2′,3′-(methoxymethylidene)adenosine. Since ADPβS can be synthesized in 55–60% yield from adenosine, the overall yield of ATPβS from adenosine via Schemes I and II is 30–36%.

The Reaction Mechanism. According to Scheme II, an intermediate in the synthesis is P^1-(5′-adenosyl) P^3-[5′-2′,3′-(methoxymethylidene)adenosyl] 2-thiocyclotriphosphate. In the synthetic procedure, this intermediate is not isolated, but its formation is demonstrated by the ^{31}P NMR spectrum of [α,γ-^{18}O]ATPβS produced when $H_2{}^{18}O$ was used for the aqueous workup. Relevant features of this spectrum appear in Figure 1, showing the presence of ^{18}O at both P_α and P_γ. Note that both positions are equivalently enriched with ^{18}O. The signal for P_α is a doublet centered at −10.23 ppm. The signal slightly upfield, centered at −10.26 ppm, is that of P_α in [α-^{18}O]ATPβS. The signal for P_γ is a pair of doublets, one doublet for P_γ in (R_P)-ATPβS centered at −4.93 ppm and the other for P_γ in (S_P)-ATPβS centered at −4.88 ppm. Note the presence of a second pair of doublets shifted 0.02 ppm upfield. These signals are assigned to P_γ in (R_P)-[γ-^{18}O]ATPβS and (S_P)-[γ-^{18}O]ATPβS. The signal for P_β is a pair of triplets, one for (R_P)-ATPβS and the other for (S_P)-ATPβS centered at 30.09 and 30.02 ppm, respectively. There is no evidence of ^{18}O at the P_β position. The coupling constants in this spectrum are $J_{\alpha,\beta}$ = 26.87 Hz and $J_{\beta,\gamma}$ = 28.89 Hz. This spectrum is in accord with that originally reported for ATPβS by Jaffe & Cohn (1978a). And the detection of ^{18}O at the α- and γ-positions exploits the effect of ^{18}O in shifting the ^{31}P signals upfield (Cohn & Hu, 1978; Lowe & Sproat, 1978).

The ratios of intensities for the ^{18}O-shifted to unshifted signals in Figure 1 are 1:1.8 and 1:1.6 for P_α and P_β, respectively, showing comparable incorporation into the two positions. This is as expected on the basis of what is known about the orientations of hydrolytic opening in cyclotriphosphates. In P^1-(5′-adenosyl) cyclotriphosphate, spontaneous hydrolysis



proceeds by reaction with H_2O at P^2 and P^3, which are almost chemically equivalent, and cleavage of the indicated bonds to form ATP. An example is the reaction of 2′-dATPαS with iodate or periodate in $H_2{}^{18}O$. The sulfur is first oxidatively activated by the oxidizing agent and then internally displaced by the P_γ-phosphoryl group, forming P^1-(5′-adenosyl) cyclotriphosphate as an intermediate. This reacts with water to form exclusively [γ-^{18}O]-2′-dATP (Sammons, 1982).

The orientation of ring opening is in this case governed by the stability of the prospective leaving group rather than by the electrophilic reactivity of the phosphorus. P^2 and P^3 in the intermediate are less electrophilic than P^1 but, nevertheless, react preferentially. The best leaving group in the molecule is the P^1-phosphoryl group at neutral pH. Since this can leave only when attacked by water is at P^2 or P^3, it appears that the direction of cleavage is governed by the stability of the leaving group. This suggests that bond cleavage to the leaving group is well advanced in the transition state. Other examples of this are found in the work of Webb (1980) and Lowe et al. (1983).

The situation is altered in the case of dialkyl cyclotriphosphates such as that shown in Scheme II (Sammons, 1982). In these cases, the stability of the leaving group will be very similar regardless of which phosphorus reacts with water, so no selectivity on this basis can be expected. However, the two alkylated phosphoryl groups can be expected to exhibit higher electrophilic reactivity in neutral aqueous solution than the unalkylated negatively charged group. Therefore, water reacts as a nucleophile preferentially and almost equally at these two positions, with consequent ring opening and cleavage of the bonds indicated in Scheme II. This reaction pattern is similar to that observed by Sammons (1982) in the oxidative desulfurization of P^1-(5′-adenosyl) P^3-*S*-(2-carbamoylethyl) 1,3-dithiotriphosphate.

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Registry No. AMPS·(Et)$_3$N, 81415-28-3; AMPS·[CH$_3$(CH$_2$)$_7$]$_3$N, 81415-30-7; ADPβS, 35094-45-2; ADPβS·(Et)$_3$N, 89231-85-6; ADPβS·(Bu)$_3$N, 89231-87-8; (*S*)-ATPβS, 59261-36-8; (*R*)-ATPβS, 59261-35-7; 2′,3′-(methoxymethylidene)-AMP triethylammonium salt, 89255-64-1; P^1-diphenyl-P^2-(5′-adenosyl) 2-thiopyrophosphate, 89231-78-7; 2′,3′-(methoxymethylidene)-AMP trioctylammonium salt, 81671-39-8; P^1-(5′-adenosyl)-P^2-[5′-2′,3′-(methoxymethylidene)-adenosyl] 1-thiopyrophosphate, 89231-79-8; 2′,3′-(methoxymethylidene)-ADPβS, 89231-80-1; adenosine, 58-61-7; adenosine 5′-phosphorodichloridate, 34051-17-7; 2′,3′-(methoxymethylidene)-adenosine, 16658-10-9; 2′,3′-(methoxymethylidene)adenosine 5′-phosphorodichloridate, 89231-81-2; 2′,3′-(methoxymethylidene)-ADPβS triethylammonium salt, 89231-84-5; 2′,3′-(methoxymethylidene)-ADPβS tributylammonium salt, 89231-86-7; P^1-(5′-adenosyl)-P^3-[5′-2′,3′-(methoxymethylidene)adenosyl] 2-thiotriphosphate triethylammonium salt, 89231-83-4.

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Synthesis and Biologic Activity of a C-Ring Analogue of Vitamin D_3: Biologic and Protein Binding Properties of 11α-Hydroxyvitamin D_3†

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ABSTRACT: The influence of C-ring substituents on the biologic activity and protein binding properties of vitamin D_3 has not been systematically investigated. To this end, we dehydrogenated cholesta-5,7-dien-3β-ol (**1**) to the 5,7,9(11)-triene (**3**). After protection of the 5,7-diene with a 4-phenyl-1,2,4-triazoline-3,5-dione Diels–Alder adduct, oxidation of the unprotected 9(11)-olefin gave epoxide **5**. Reverse Diels–Alder and reductive ring opening of epoxide **5** gave cholesta-5,7-diene-3β,11α-diol (**6**). Photolysis of **6** to the previtamin followed by thermal rearrangement resulted in 11α-hydroxyvitamin D_3 (**8**). We found that vitamin **8** increased calcium transport at a dose of 500 pmol/rat but failed to increase bone calcium mobilization at a dose as high as 50000 pmol/rat. Under the same conditions, corresponding doses of vitamin D_3 and 25-hydroxyvitamin D_3 increased bone calcium mobilization and intestinal calcium transport. The new vitamin analogue, **8**, was slightly less efficient (B-50 = 6.8×10^{-8} M) than 25-hydroxyvitamin D_3, 24(*R*),25-dihydroxyvitamin D_3, and 25(*S*),26-dihydroxyvitamin D_3 (7.1×10^{-9} M, 7.7×10^{-9} M, and 7.9×10^{-9} M, respectively) in displacing radiolabeled 25-hydroxyvitamin D_3 from rat plasma vitamin D binding protein. On the other hand, vitamin analogue **8** showed significantly greater binding efficiency than 1α-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3, and vitamin D_3 (B-50 = 2.5×10^{-6} M, 9.84×10^{-8} M, and 5.46×10^{-7} M, respectively), under these same conditions. Vitamin analogue **8** displayed approximately the same efficiency as vitamin D_3 in displacing radiolabeled 1,25-dihydroxyvitamin D_3 from a chick intestinal cytosol receptor but was less effective than 25-hydroxyvitamin D_3, 24(*R*),25-dihydroxyvitamin D_3, 25(*S*),26-dihydroxyvitamin D_3, 1α-hydroxyvitamin D_3, and 1,25-dihydroxyvitamin D_3. We conclude that introduction of an 11α-hydroxyl group into the C-ring of vitamin D_3 results in a vitamin analogue with moderate vitamin D_3 agonist activity in the intestine but no activity with respect to bone calcium mobilization at the levels tested. 11α-Hydroxyvitamin D_3 does not have improved binding affinity to the intestinal cytosol receptor when compared to vitamin D_3. The new vitamin analogue shows significantly greater binding affinity to plasma vitamin D binding protein than vitamin D_3 (6.79×10^{-8} M vs. 5.46×10^{-7} M) or 1α-hydroxyvitamin D_3 (6.79×10^{-8} M vs. 2.5×10^{-6} M), suggesting that the presence of an extra hydroxyl group sufficiently removed from the 3β-hydroxyl is important in the binding of vitamin D analogues to vitamin D binding protein.

The vitamin D_3 endocrine system plays a central role in calcium and phosphorus homeostasis in many species (DeLuca & Schnoes, 1976, 1983). In order to act physiologically, vitamin D_3 first undergoes C-25-hydroxylation in the liver and subsequently C-1-hydroxylation in the kidney (DeLuca & Schnoes, 1976, 1983). 25-Hydroxyvitamin D_3 is considerably more active than vitamin D_3 in vivo, and 1,25-dihydroxyvitamin D_3 in turn is more active than 25-hydroxyvitamin D_3. Thus, the introduction of a hydroxyl group at C-25 and an α-hydroxyl group at C-1 plays an important role in determining the biological properties of the vitamin D_3 molecule (DeLuca & Schnoes, 1976, 1983). It is also known that shortening or lengthening of the side chain (Holick et al., 1975; Lam et al., 1975; Norman et al., 1979; Koizumi et al., 1979, Esvelt & DeLuca, 1981), alteration of the side chain by introduction of various functionalities (Onisko et al., 1979), expansion of the A-ring (Gerdes et al., 1981), alteration of the triene structure (Wecksler & Norman, 1980), and removal of or prevention of the introduction of hydroxyl groups at C-1, C-25, or C-3 decrease the biological activity of vitamin D_3 (Okamura et al., 1974; Lam et al., 1974; DeLuca & Schnoes, 1976, 1983). In contrast, certain C-24-, C-26-, and C-27-

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