# Stereochemical Course of Phosphoanhydride Synthesis

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**Abstract:** In the synthesis of phosphoanhydride linkages in nucleoside polyphosphates such as ADP, ATP, and dinucleotide coenzymes according to the procedure introduced by Michelson (ref 1a), a nucleoside monophosphate is first activated by reaction with diphenyl phosphorochloridate in an inert solvent to produce a $P^1$-nucleoside $P^2$-bis(phenyl) pyrophosphate. The latter reacts in pyridine with trialkylammonium salts of phosphoric acid, pyrophosphoric acid, or phosphoric esters to produce the desired phosphoanhydride and diphenyl phosphate. The mechanism by which the coupling of a $P^1$-nucleoside $P^2$-bisphenyl pyrophosphate with phosphates occurs in pyridine has been investigated by analyzing the stereochemical course of the coupling reaction using $R_p$-[$\alpha$-$^{18}$O]AMPS and $S_p$-[$\alpha$-$^{18}$O]AMPS as the nucleoside monophosphates. Configurational analysis of the coupling products showed that the reaction proceeded with epimerization at the chiral phosphorus. It was concluded that the most probable mechanism for the reaction involves nucleophilic covalent catalysis by pyridine.

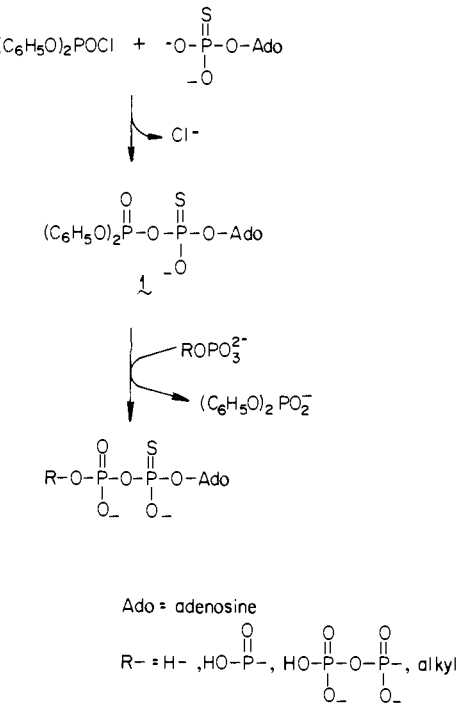
The method introduced by Michelson[1a,b] for synthesizing phosphoanhydride linkages in coenzymes and nucleoside polyphosphates is widely used in the synthesis of sulfur-containing analogues of nucleotides.[2a-i] In this procedure a thiophosphoric ester such as adenosine 5′-phosphorothioate (AMPS)[3] is first activated by reaction with diphenyl phosphorochloridate, producing $P^1$-5′-adenosyl $P^2$-bis(phenyl) 1-thiopyrophosphate (**1** in Scheme I). Phosphate, pyrophosphate, or another phosphoric ester is then combined with **1** either in pyridine or in a mixed solvent containing pyridine. This second step leads to the formation of the desired nucleotide concomitant with the release of diphenylphosphate.

The mechanism by which the reaction of **1** with phosphates and phosphoric esters occurs is unknown. It might involve a direct displacement of diphenylphosphate by $ROPO_3^{2-}$. If so, however, the reaction should proceed in solvents other than pyridine; yet pyridine seems to be required as at least a component of the solvent. The role of pyridine cannot be simply to quench acid produced in the coupling reaction since this step neither produces nor consumes acid under the usual conditions. Inasmuch as the yield of coupling products in timed reactions carried out in mixed solvents depends upon the percentage of pyridine in the solvent,[1a,b] pyridine may participate directly in the reaction mechanism.

One means of obtaining information about the mechanism of the coupling step is to study its stereochemistry. The observation of overall inversion of configuration about P in the activated phosphoryl group of **1** upon coupling to $ROPO_3^{2-}$ would be most easily explained by a direct, single displacement mechanism, in which $ROPO_3^{2-}$ displaces diphenyl phosphate from **1** via a trigonal-bipyramidal transition state. Overall retention or loss of configuration at this center could not be explained by such a mechanism.

To determine this stereochemistry we have used $R_p$-[$\alpha$-$^{18}$O]-AMPS and $S_p$-[$\alpha$-$^{18}$O]AMPS as substrates. Activation by diphenyl phosphorochloridate followed by coupling with AMP or 2′,3′-(methoxymethylidene)-AMP led to overall loss of chirality at the [$^{18}$O]phosphorothioate centers of the coupling products. We have interpreted our results as indicating the involvement of pyridine as a nucleophilic covalent catalyst of the second step in Scheme I.

Scheme I




## Experimental Section

**Materials and Methods.** The enzymes, coenzymes, and chemicals used were described earlier, as were the methods by which solvents were purified and dried for use in synthetic procedures.[4] $R_p$-[$\alpha$-$^{18}$O]AMPS and $S_p$-[$\alpha$-$^{18}$O]AMPS were synthesized by the procedure described in the earlier work.[4] The $^{18}$O enrichments at the $R$ and $S$ positions of the chiral [$^{18}$O]thiophosphoryl groups of these compounds were 87%. 2′,3′-(Methoxymethylidene)-AMP was synthesized as described earlier.[4]

Samples of $R_p$-[$\alpha$-$^{18}$O]AMPS, $S_p$-[$\alpha$-$^{18}$O]AMPS, and 2′,3′-(methoxymethylidene)-AMP prepared as triethylammonium salts were converted to tri-*n*-octylammonium salts so that they would be soluble in anhydrous organic solvents. This was done by dissolving the desired

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(1) (a) Michelson, A. M. *J. Chem. Soc.* **1958**, 1957. (b) Michelson, A. M. *Biochim. Biophys. Acta* **1964**, *91*, 1–13.

(2) (a) Eckstein, F.; Goody, R. S. *Biochemistry* **1976**, *15*, 1685–1691. (b) Sheu, K-F. R.; Richard, J. P.; Frey, P. A. *Ibid.* **1979**, *18*, 5548–5556. (c) Richard, J. P.; Ho, H.-T.; Frey, P. A. *J. Am. Chem. Soc.* **1978**, *100*, 7756. (d) Richard, J. P.; Frey, P. A. *Ibid.* **1978**, *100*, 7757. (e) Richard, J. P.; Prasher, D.; Ives, D. H.; Frey, P. A. *J. Biol. Chem.* **1979**, *254*, 4339–4341. (f) Sheu, K-F. R.; Frey, P. A. *Ibid.* **1977**, *252*, 4445–4448. (g) Sheu, K-F. R.; Frey, P. A. *Ibid.* **1978**, *253*, 3378–3380. (h) Goody, R. S.; Eckstein, F. *J. Am. Chem. Soc.* **1971**, *93*, 6252–6257. (i) Brody, R. S.; Frey, P. A. *Biochemistry* **1981**, *20*, 1245–1252.

(3) The abbreviations are: AMPS, adenosine 5′-phosphorothioate; [$\alpha$-$^{18}$O$_2$]AMPS, adenosine 5′-[$^{18}$O$_2$]phosphorothioate; $R_p$- and $S_p$-[$\alpha$-$^{18}$O]AMPS, [$\alpha$-$^{18}$O]-AMPS having the $R$ and $S$ configurations about phosphorus; [$\beta$-$^{18}$O]ADP$\beta$S, adenosine 5′-(2-thio[2-$^{18}$O]diphosphate); $R_p$- and $S_p$-[$\beta$-$^{18}$O]-ADP$\beta$S, [$\beta$-$^{18}$O]-ADP$\beta$S having the $R$ and $S$ configurations about the $\beta$-phosphorus; AMP, adenosine 5′-phosphate; [$\beta$-$^{18}$O]ATP$\beta$S, adenosine 5′-(2-thio[2-$^{18}$O]triphosphate); $R_p$- and $S_p$-[$\beta$-$^{18}$O]ATP$\beta$S, [$\beta$-$^{18}$O]ATP$\beta$S having the $R$ and $S$ configurations about the $\beta$ phosphorus.

(4) Richard, J. P.; Frey, P. A. *J. Am. Chem. Soc.* **1982**, *104*, 3476–3481.

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amount of nucleotide (50–100 μmol) in 10 mL of methanol, adding 1 equiv of tri-*n*-octylamine, and removing the methanol by rotary evaporation in vacuo. To dry the samples further and remove excess triethylamine, the residues were dissolved in 1 mL of dry dimethylformamide and then again evaporated to dryness. This process was repeated two additional times to ensure complete removal of water and triethylamine.

All evaporations were by rotary evaporation in vacuo using a Büchi apparatus and a bath temperature of 30 °C. Nucleotides in pooled chromatographic fractions containing triethylammonium bicarbonate were desalted by rotary evaporation. The residues were repeatedly dissolved in ethanol and again evaporated to ensure that all triethylammonium bicarbonate was removed.

The $^{18}O$ content of [α-$^{18}O_2$]AMPS used in the syntheses of $R_p$-[α-$^{18}O$]AMPS and $S_p$-[α-$^{18}O$]AMPS was determined by chemically transforming the [$^{18}O$]phosphorothioate into [$^{18}O$]trimethylphosphorothioate and measuring its $^{18}O$ content by gas chromatographic mass spectroscopy. $R_p$-[β-$^{18}O$]ATPβS and $S_p$-[β-$^{18}O$]ATPβS were analyzed for bridging and nonbridging $^{18}O$ using the procedure described by Richard et al.[2c] in which the β-phosphoryl group [β-$^{18}O$]ATPβS is chemically transformed into trimethyl[$^{18}O$]phosphorothioate for mass spectroscopic analysis. The same procedure was applied to the analyses of the $R_p$ and $S_p$ epimers of $P^1$,$P^2$-bis(5′-adenosy) 1-thio[1-$^{18}O$]pyrophosphate. The $^{18}O$ enrichments were calculated as the percent abundance of the $m + 2$ species measured as the relative intensities of the parent ions.

**Synthesis of $P^1$-5′-Adenosyl $P^2$-2′,3′-(Methoxymethylidene)-5′-adenosyl 1-Thio[1-$^{18}O$]pyrophosphate.** The tri-*n*-octylammonium salt of $R_p$-[α-$^{18}O$]AMPS (55 μmol) was dissolved in 0.3 mL of triethyl phosphate. Diphenyl phosphorochloridate (72 μmol) was added to this solution. Tri-*n*-butylamine (25 μL) was added to neutralize HCl produced in the reaction, which was permitted to proceed for 4 h at room temperature. The solvent was removed by rotary evaporation. The residue was extracted by adding 20 mL of a 3:1 mixture of petroleum ether (bp 30–60 °C) and diethyl ether, stirring the suspension, and permitting it to stand at 4 °C for 30 min. The ether layer was decanted, and excess ether was removed by adding 1 mL of dioxane and then removing it by rotary evaporation to dryness. The residue was dissolved with 0.3 mL of a pyridine solution containing 120 μmol of the tri-*n*-octylammonium salt of 2′,3′-(methoxymethylidene)-AMP. After 12 h at room temperature, pyridine was removed by rotary evaporation, and 3 mL of diethyl ether was added to the residue. After extracting this suspension in the reaction flask with 15 mL of water, the pH of the aqueous layer was adjusted to 8.5 by addition of triethylamine.

This procedure is essentially that described earlier except that $R_p$-[α-$^{18}O$]AMPS was substituted for [α-$^{18}O_2$]AMPS and the scale was reduced by a factor of 10. Since the product yield was reproducibly good and the stereochemical analysis did not require the purification of $P^1$-5′-adenosyl $P^2$-2′,3′-(methoxymethylidene)-5′-adenosyl 1-thio[1-$^{18}O$]pyrophosphate, it was not purified at this stage. It was instead transformed directly into [β-$^{18}O$]ADPβS as described in the following.

**Synthesis of [β-$^{18}O$]ADPβS.** The aqueous solution of $P^1$-5′-adenosyl $P^2$-2′,3′-(methoxymethylidene)-5′-adenosyl 1-thio[1-$^{18}O$]pyrophosphate prepared in the preceding section was treated with 75 μmol of $NaIO_4$ for 10 min at room temperature. Ethylene glycol (25 μL) and 0.4 g of $Na_3PSO_3$ were added to the solution to reduce iodate and periodate to $I^-$. The pH was adjusted to 2.0 by adding 0.1 M HCl, and the solution was set aside at room temperature for 20 min to allow for the hydrolytic removal of the methoxymethylidene groups. The pH was then adjusted to 10.5 using NaOH, and the solution was placed at 50 °C for 30 min to effect β-elimination of [β-$^{18}O$]ADPβS from the periodate-cleaved nucleoside.[2c,d,5a,b] The final volume was adjusted to 40 mL.

[β-$^{18}O$]ADPβS was purified from the above solution by anion-exchange chromatography through a 1.5 × 45 cm column of DEAE Sephadex A-25. After absorption of nucleotides, the column was eluted with a linear gradient of triethylammonium bicarbonate increasing from 0.2 M (1.0 L) to 0.6 M (1.0 L). [β-$^{18}O$]ADPβS, obtained in 30% yield based on $R_p$-[α-$^{18}O$]AMPS, was identified by $A_{260}$ measurements and reactivity with 5,5′-dithiobis(2-nitrobenzoate), as well as by its elution position and reactivity as a substrate for acetate kinase and pyruvate kinase. Pooled fractions containing [β-$^{18}O$]ADPβS were desalted as described above.

**Synthesis of $P^1$,$P^2$-Bis(5′-adenosyl) 1-Thio[1-$^{18}O$]pyrophosphate and Separation of $S_p$ and $R_p$ Epimers.** The tri-*n*-octylammonium salt of $S_p$-[α-$^{18}O$]AMPS (55 μmol) was dissolved in 0.2 mL of triethyl phosphate and combined with 96 μmol of diphenyl phosphorochloridate. Tri-*n*-butylamine (25 μL) was added to neutralize HCl produced in the reaction. After 4 h at ambient temperature, 8 mL of petroleum ether (30–60 °C) and 2 mL of diethyl ether were added; the reaction mixture was stirred and placed at 4 °C for 30 min. The ether layer was decanted and 0.2 mL of dioxane added to the residue. The dioxane and residual ether were removed by rotary evaporation. This residue contained the activated [α-$^{18}O$]AMPS, $P^1$-5′-adenosyl $P^2$-bis(phenyl) 1-thio[1-$^{18}O$]-pyrophosphate as a mixture of epimers differing in configuration at $P^1$.

The dry tri-*n*-octylammonium salt of AMP (133 μmol) was dissolved in 0.2 mL of pyridine and combined with the activated $S_p$-[α-$^{18}O$]AMPS prepared above. The reaction proceeded for 12 h at ambient temperature, after which pyridine was removed by rotary evaporation. The residue was shaken with 1 mL of diethyl ether and 5 mL of water and the aqueous layer adjusted to pH 8 with triethylamine.

$R_p$- and $S_p$-$P^1$,$P^2$-bis(5′-adenosyl) 1-thio[1-$^{18}O$]pyrophosphate were purified from the aqueous layer and separated from each other by anion-exchange chromatography. The aqueous solution was passed into a 3 × 160 cm column of DEAE Sephadex A-25 that had been equilibrated with 0.1 M triethylammonium bicarbonate. Elution of the column was with a linear gradient of triethylammonium bicarbonate increasing in concentration from 0.2 to 0.4 M, formed from 6 L of 0.2 M triethylammonium bicarbonate in the mixing chamber and 6 L of 0.4 M of the same salt in the other chamber. The chromatography was at 4 °C, and fractions approximately 12 mL in volume were collected. Measurements of $A_{260}$ were made on selected fractions, with the results illustrated in Figure 1. The large band eluted between fractions 220 and 280 was unreacted AMP present in excess in the reaction mixture. The next two major bands were the desired epimers.

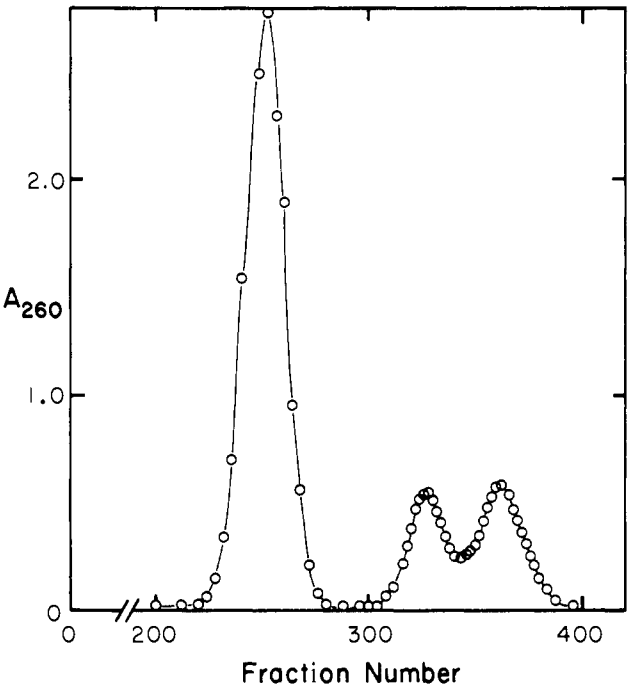
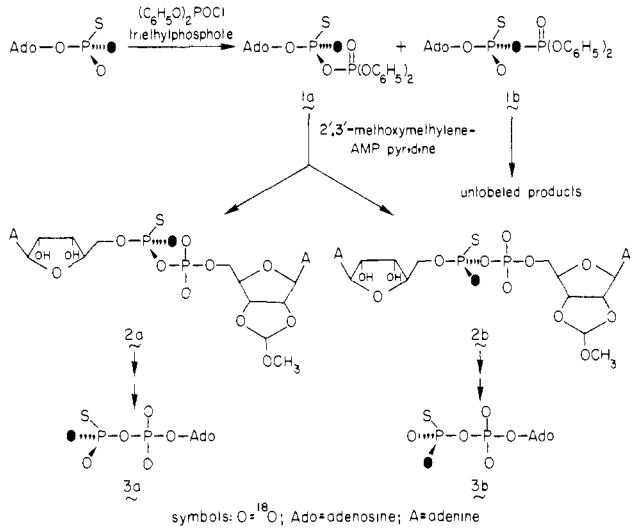



**Figure 1.** Chromatographic separation of the $R_p$ and $S_p$ epimers of $P^1$, $P^2$-bis(5′-adenosyl) 1-thio[1-$^{18}O$]pyrophosphate. The chromatographic conditions are described in the Experimental Section. The two peaks eluted between fractions 300 and 400 were the two epimers. Oxygen-18 analyses of conservative fraction pools from these two peaks are given in Table II. Symbols: O–O, $A_{260}$.

The last band eluted was contaminated by a small amount of unreacted $S_p$-[α-$^{18}O$]AMPS, which was removed as follows. Fractions 350 through 382 were pooled and the buffer salts removed by rotary evaporation as described above. The nucleotides (300 $A_{260}$ units) were dissolved in a solution containing 0.5 mM ATP, 50 mM KCl, 5 mM $MgCl_2$, 50 mM Tris HCl buffer at pH 8.0, 4 mM phosphoenolpyruvate, and 0.1 mM dithioerythritol in a total volume of 3 mL. The AMPS in this solution was phosphorylated to ATPαS by addition of excess adenylate kinase and pyruvate kinase, as described by Sheu and Frey.[2f] The progress of the phosphorylation was monitored by measuring the pyruvate produced from phosphoenol pyruvate using the lactate dehydrogenase assay method described by Sheu and Frey.[2f] When the pyruvate concentration reached a constant value corresponding to the AMPS contamination, 1 unit of alkaline phosphatase was added to catalyze the hydrolysis of phosphoenol pyruvate. This hydrolysis was monitored by measurements of pyruvate production. As soon as pyruvate production ceased, the solution was applied to a 1.5 × 30 cm column of DEAE

(5) (a) Whitfield, P. R.; Markham, R. *Nature (London)* **1953**, *171*, 1151–1152. (b) Brown, D. M.; Fried, M.; Todd, A. R. *Chem. Ind. (London)* **1953**, 352–353.

Scheme II




Sephadex A-25, which was then eluted with a linear gradient of triethylammonium bicarbonate increasing from 0.2 to 0.4 M (0.5 L of each solution; 1.0 L total volume). $R_p$-$P^1$,$P^2$-Bis(5′-adenosyl) 1-thio[1-$^{18}$O]-pyrophosphate emerged as a single band in a yield of 260 $A_{260}$ units.

## Results

The overall phosphoanhydride synthesis in Scheme I involves the substitution of a phosphate or phosphoric ester group for one of the two phosphate oxygens in AMPS. These oxygens are diastereotopic and, therefore, sterically distinct. The actual substitution occurs in the second step, preceded in the first step by the activation of one of the oxygens with diphenyl phosphorochloridate. The activation step does not cleave any bonds to phosphorus, so that the stereochemistry of the overall coupling sequence in Scheme I should reflect the stereochemistry of the second step.

The stereochemical course of the phosphoanhydride synthesis was determined by two different methods. The first analysis began with the activation of $R_p$-[$\alpha$-$^{18}$O]AMPS by reaction with diphenyl phosphorochloridate in triethyl phosphate, converting it to a mixture of **1a** and **1b** (Scheme II). After removing triethyl phosphate, this mixture was combined with 2′,3′-(methoxymethylidene)-AMP in pyridine. Reaction of **1a** could have led to the $^{18}$O-enriched coupling products **2a** or **2b**, depending on whether the reaction proceeded with retention or inversion of configuration at phosphorus. Epimerization at the chiral phosphorus would have led to a mixture of **2a** and **2b**. Reaction of **1b** led to coupling products lacking $^{18}$O which, while they diluted the $^{18}$O in isolated products, did not interfere in the stereochemical analysis.

To determine whether the $^{18}$O-enriched coupling product was **2a** or **2b** or a mixture of the two, it was converted to [$\beta$-$^{18}$O]-ADP$\beta$S using a procedure that did not involve cleavage of any bonds to phosphorus. This consisted of periodate oxidation of the unprotected adenosyl moiety, reduction of excess periodate and iodate, removal of the 2′,3′-methoxymethylidene protecting group by treatment with dilute acid and alkaline $\beta$-elimination of [$\beta$-$^{18}$O]ADP$\beta$S from the cleaved ribosyl ring.[2c] The configuration at $P_\beta$ in [$\beta$-$^{18}$O]ADP$\beta$S was analyzed by stereoselective enzymatic phosphorylation to $R_p$-[$\beta$-$^{18}$O]ATP$\beta$S, using acetyl phosphate and acetate kinase and to $S_p$-[$\beta$-$^{18}$O]ATP$\beta$S using phosphoenolpyruvate and pyruvate kinase.[2c] Analysis of these products for bridging and nonbridging $^{18}$O established the configuration at $P_\beta$ in [$\beta$-$^{18}$O]ADP$\beta$S. Thus phosphorylation of $R_p$-[$\beta$-$^{18}$O]ADP$\beta$S, **3b** in Scheme II, would produce $R_p$-[$\beta$-$^{18}$O]ATP$\beta$S with exclusively bridging $^{18}$O and $S_p$-[$\beta$-$^{18}$O]ATP$\beta$S with exclusively nonbridging $^{18}$O.

The analysis for bridging and nonbridging $^{18}$O in [$\beta$-$^{18}$O]ATP$\beta$S involved its chemical transformation according to Scheme III to trimethyl phosphorothioate derived from $P_\beta$ and trimethyl phosphate derived from $P_\alpha$ and $P_\gamma$ by a procedure that partitioned

Scheme III

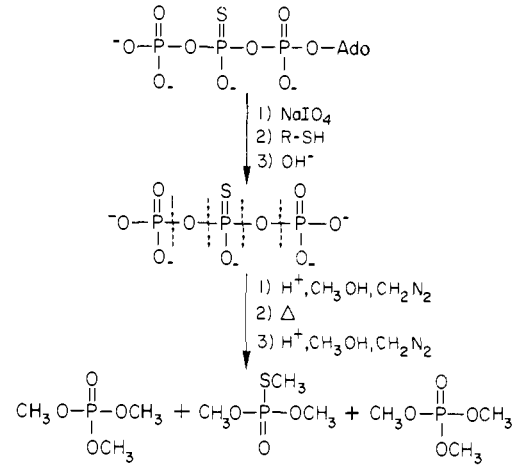



Table I. Configurational Analysis of [$\beta$-$^{18}$O]ADP$\beta$S Synthesized from $R_p$-[$\alpha$-$^{18}$O]AMPS

| compound analyzed[a] | $^{18}$O enrichment[b] |
|---|---|
| $S_p$-[$\beta$-$^{18}$O]ATP$\beta$S | 31.1 |
| $R_p$-[$\beta$-$^{18}$O]ATP$\beta$S | 32.5 |
| calculated for epimerization | 34.6 |

[a] The synthesis of [$\beta$-$^{18}$O]ADP$\beta$S from $R_p$-[$\alpha$-$^{18}$O]AMPS is described in the Experimental Section. The resulting [$\beta$-$^{18}$O]-ADP$\beta$S was stereoselectively phosphorylated to either $S_p$- (first entry) or $R_p$-[$\beta$-$^{18}$O]ATP$\beta$S, which was analyzed for bridging and nonbridging $^{18}$O. [b] $S_p$- and $R_p$-[$\beta$-$^{18}$O]ATP$\beta$S were chemically transformed to trimethyl phosphorothioate as described in the Experimental Section; and trimethyl phosphorothioate was analyzed by gas chromatographic mass spectrometry. The tabulated values are the % of the $e/m = m + 2$ parent ions corresponding to $^{18}$O-enriched species.

$^{18}$O in the $P_\beta$-$P_\gamma$ bridge between trimethyl phosphorothioate and trimethyl phosphate.[2c] Nonbridging $^{18}$O bonded to $P_\beta$ remained associated with $P_\beta$ in trimethyl phosphorothioate. Mass spectrometric analysis of these compounds revealed their $^{18}$O contents as the ratios of parent ions, $m/e$ 156 (and 158 for $^{18}$O-enriched species) for trimethyl phosphorothioate and $m/e$ 140 (and 142 for $^{18}$O-enriched species) for trimethyl phosphate.

The data obtained in this analysis starting with $R_p$-[$\alpha$-$^{18}$O]-AMPS containing 87% enrichment with $^{18}$O in the $R$ oxygen are presented in Table I. Approximately half of the $^{18}$O is lost in the conversion of $R_p$-[$\alpha$-$^{18}$O]AMPS to the coupling products. The loss of $^{18}$O should not be exactly half, since the oxygens should not react with diphenyl phosphorochloridate at exactly the same rates. Data on the relative amounts of $R_p$ and $S_p$ ADP$\beta$S resulting from the coupling of AMPS with phosphate indicate about 45/55 as the ratio of products, consistent with slightly different activation rates. Assuming for the present purpose that the activation rates are about equal, it can be expected that the enrichment of $m/e$ 158 in trimethyl phosphorothioate would be about 44.3% for one isomer of [$\beta$-$^{18}$O]ATP$\beta$S in which $^{18}$O is nonbridging and about 22.1% in the other isomer if $^{18}$O is bridging. This would be the result if bridging $^{18}$O were equally partitioned between $P_\gamma$ and $P_\beta$ in the degradation of [$\beta$-$^{18}$O]ATP$\beta$S. Earlier data confirm that this partitioning is approximately even.[2c] The data in Table I show that the actual $^{18}$O enrichments in trimethyl phosphorothioate were intermediate between the extremes and the *same* for the two isomers. Moreover, the values are those that would be expected if the chiral [$^{18}$O]phosphorothioate center were *epimerized* during the coupling step.

Table I strongly indicates that the coupling step is accompanied by epimerization. The fact that the activations of the two diastereotopic oxygens in $R_p$-[$\alpha$-$^{18}$O]AMPS probably do not occur at exactly equal rates does not weaken this interpretation of the data. Thus, if one of the two oxygens were activated at a rate 20% faster than the other, the $^{18}$O enrichment at the nonbridging

Table II. $^{18}O$ Enrichments in Epimers of $P^1,P^2$-Bis(5'-adenosyl) 1-Thio[1-$^{18}O$]pyrophosphate Synthesized from $S_p$-[$\alpha$-$^{18}O$]AMPS

| compound | $^{18}O$ enrichment[a] |
|---|---|
| $S_p$ | 50.0 |
| $R_p$ | 49.3 |
| calculated for epimerization | 43.9 |

[a] The epimers of $P^1,P^2$-bis(5'-adenosyl) 1-thio[1-$^{18}O$]-pyrophosphate were separated and degraded to trimethyl phosphorothioate as described in the Experimental Section. Their derivatives were then subjected to gas chromatographic mass spectral analysis. The tabulated values are the % abundance of the $e/m = m + 2$ parent ions corresponding to $^{18}O$-enriched species in the derivatives.

$P_\beta$ position in one epimer of [$\beta$-$^{18}O$]ATP$\beta$S could be as small as 37% or as large as 53%, depending upon the relative rates. The enrichment in trimethyl phosphorothioate derived from the other epimer with bridging $^{18}O$ would be 18.5 or 26.5%. Therefore, the enrichments could not be the same in samples of trimethyl phosphorothioate derived from $R_p$- and $S_p$-[$\beta$-$^{18}O$]ATP$\beta$S unless the chirality of the [$^{18}O$]phosphorothioate group is lost through epimerization. The stereochemistry is, therefore, neither inversion nor retention but epimerization.

To verify this conclusion we redetermined the stereochemistry by an alternative procedure. The $R_p$ and $S_p$ epimers of **2a** and **2b** in Scheme II are known to be separable by ion-exchange chromatography through DEAE Sephadex A-25.[2c,4] If they were to be separated and analyzed for their $^{18}O$ contents, a finding of equivalent enrichment in both epimers would demonstrate that epimerization had occurred. Since the corresponding epimers resulting from the coupling of AMP with **1a** are more easily separated than **2a** and **2b**, AMP was used in place of 2',3'-(methoxymethylidene)-AMP in the coupling step.

The details of the procedure for coupling $S_p$-[$\alpha$-$^{18}O$]AMPS with AMP and separating the $R_p$ and $S_p$ epimers of $P^1,P^2$-bis(5'-adenosyl) 1-thio[1-$^{18}O$]pyrophosphate are described in the Experimental Section. The $^{18}O$ enrichments are given in Table II. The data confirm that coupling of **1a** (and **1b**) with AMP in pyridine is accompanied by epimerization at phosphorus.

If inversion or retention of configuration had occurred in the coupling step, either the $R_p$ or the $S_p$ epimer of $P^1,P^2$-bis(5'-adenosyl) 1-thio[1-$^{18}O$]pyrophosphate would have been enriched in $^{18}O$ at a level comparable to that in $S_p$-[$\alpha$-$^{18}O$]AMPS from which it had been synthesized, and the other epimer would not have contained $^{18}O$. The observed presence of $^{18}O$ in both epimers could arise only from a mechanism that permits the chiral phosphorus to undergo epimerization during the coupling step.

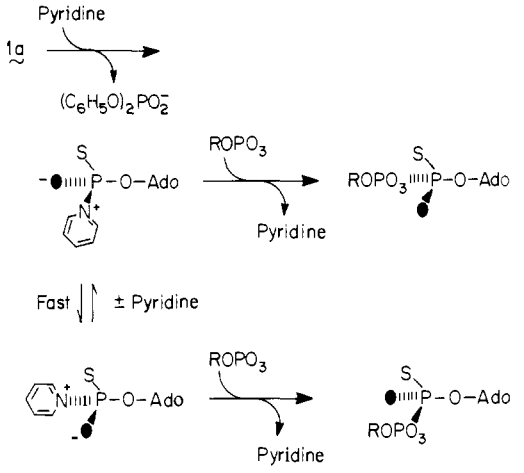
The calculated $^{18}O$ enrichments corresponding to epimerization in Tables I and II were obtained on the basis of the following assumptions: (a) the reactions of the $R$ and $S$ oxygens in $R_p$- or $S_p$-[$\alpha$-$^{18}O$]AMPS with diphenyl phosphorochloridate occur at the same rates; (b) a coupling intermediate undergoes epimerization to equilibrium; (c) the epimerization equilibrium constant is 1. It is unlikely that all of these assumptions are exactly true, although they are reasonable approximations for our purposes. The fact that the calculated and measured values are not identical is not considered to be significant, relative to the question of whether epimerization has reached equilibrium.

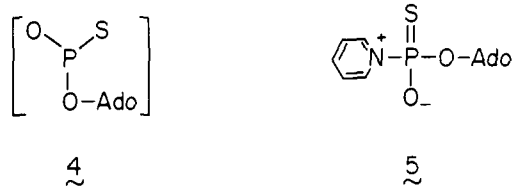
The $^{18}O$ enrichments differ in the two experiments because in Table II all of the $^{18}O$ in the epimers of $P^1,P^2$-bis(5'-adenosyl) 1-thio[1-$^{18}O$]pyrophosphate is detected, whereas in Table I only the nonbridging $^{18}O$ in [$\beta$-$^{18}O$]ATP$\beta$S plus the fraction of bridging $^{18}O$ that is partitioned to trimethyl phosphorothioate is detected in the derivative.

## Discussion

Epimerization of the chiral phosphorothioate center during the coupling of $R_p$- and $S_p$-[$\alpha$-$^{18}O$]AMPS to phosphoric esters is best rationalized on the basis of the existence of a reaction intermediate in the second step of Scheme I. A direct displacement by an $S_N2$ mechanism would proceed with inversion of configuration and so can be excluded. The most probable intermediates would be **4** and **5**. The metaphosphorothioate **4** would result from the spontaneous expulsion of diphenyl phosphate from the activation intermediate **1a** and **1b**. This is an electrophilic species that would quickly react with a nucleophile such as a phosphoric ester. Since the coordination about phosphorus in **4** is planar, it could be captured by reaction at either face to form both epimers of the product.

Scheme IV



4 5

The mechanism above offers no explanation for the special efficacy of pyridine as a solvent for this reaction. The involvement of **5** as an intermediate would explain the effect of pyridine. Acting as a nucleophilic catalyst, pyridine would displace diphenyl phosphate from **1a** or **1b**, forming **5** which would in turn react with a phosphate to expel pyridine and generate a phosphoanhydride. Such a mechanism is chemically reasonable. Pyridine at solvent concentrations is fairly nucleophilic and electrically neutral. Phosphoric esters, on the other hand, are anions under the reaction conditions. It can be expected that the rates with which they would react with the anionic phosphorothioate center in **1a** or **1b** would be minimized by electrostatic repulsion, so they might not compete effectively with pyridine. The intermediate **5**, being electrically neutral (zwitterionic) and having a positive charge on pyridine, the prospective leaving group, should be more reactive with phosphoric esters than **1a** and **1b**. Pyridine in this way fulfills the classical requirements of a good nucleophilic catalyst.

In an ordinary double displacement involving nucleophilic catalysis, the stereochemical consequences are expected to be overall retention of configuration. In the present case, however, the intermediate **5** can be expected to exhibit high electrophilic reactivity with pyridine, the solvent, as well as with phosphates. If its reaction rate with pyridine exceeds that with phosphates, the chiral reaction center will be epimerized prior to the formation of the phosphoanhydride product. Scheme IV illustrates epimerization in the reaction of **1a** by this mechanism.

Epimerization of the chiral phosphorothioate rules out the $S_N2$ single displacement mechanism for this coupling reaction carried out in pyridine. Scheme IV, involving covalent catalysis by pyridine, appears to be the most probable mechanism for coupling and epimerization because it also accounts for the fact that the reaction proceeds best in this solvent. This is also in accord with the conclusion of Mikolajczyk that pyridine and imidazole serve as nucleophilic catalysts in the hydrolysis of optically active O-ethyl ethylphosphonochlorothionate, which undergoes hydrolysis with

racemization in the presence of these bases.[6]

Another widely used procedure for synthesizing phosphoanhydrides is that introduced by Khorana and associates in which a nucleoside phosphoramidate reacts with a phosphate in pyridine to form the phosphoanhydride and an amine.[7] This reaction is reported to proceed best in tertiary amines, and pyridine is generally used as the solvent. It is conceivable that pyridine may serve as a nucleophilic catalyst in supporting the coupling of phosphoramidates to phosphates by a mechanism similar to that outlined in Scheme IV.

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**Registry No.** $R_p$-[$\alpha$-$^{18}$O]AMPS trioctylammonium salt, 87226-42-4; [$\beta$-$^{18}$O]ADPBS, 87302-51-0; $S_p$-[$\alpha$-$^{18}$O]AMPS trioctylylammonium salt, 87226-43-5; AMP trioctylammonium salt, 69098-20-0; $S_p$-[$\beta$-$^{18}$O]-ATPBS, 87226-46-8; $R_p$-[$\beta$-$^{18}$O]ATPBS, 87226-47-9; $P^1$-5′-adenosyl-$P^2$-2′,3′-(methoxymethylene)-5′-adenosyl 1-thio[1-$^{18}$O]pyrophosphate, 87302-50-9; diphenylphosphorochloridate, 2524-64-3; 2′,3′-(methoxymethylene)-AMP trioctylammonium salt, 81671-39-8; ($R_p$)-$P^1$-5′-adenosyl-$P^2$-bis(phNyl) 1-thio[1-$^{18}$O]pyrophosphate, 87226-44-6; ($S_p$)-$P^1$-5′-adenosyl-$P^2$-bis(phenyl) 1-thio[1-$^{18}$O]pyrophosphate, 87226-45-7; ($R_p$)-$P^1$,$P^2$-bis(5′-adenosyl) 1-thio[1-$^{18}$O]pyrophosphate, 69010-06-6; ($S_p$)-$P^1$,$P^2$-bis(5′-adenosyl) 1-thio[1-$^{18}$O]pyrophosphate, 68973-42-2.

# Crystal Structures, Molecular Conformations, Infrared Spectra, and $^{13}$C NMR Spectra of Methylproline Peptides in the Solid State

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**Abstract:** Sterically hindered amino acids can form peptides of defined conformation. We have investigated the conformational influences of the methyl groups in *N*-acetyl, *N*′-methylamide derivatives of the seven isomeric monomethylprolines. X-ray crystallography demonstrated two molecular conformations ($\alpha_R$ or $P_{II}$) for these peptides. For each of the conformations, there are two types of intermolecular hydrogen bonding: either the proline CO or the acetyl CO accepts a hydrogen bond from the one NH. The previously determined crystal structure of the AcProNHMe fits one of the classes observed for the methylproline peptides. Infrared spectra of the crystalline peptides can be correlated with their molecular conformations and types of hydrogen bonding. The amide II frequencies differentiate the $\alpha_R$ and $P_{II}$ conformers. The amide I frequencies reveal the hydrogen-bond acceptor. The amide A frequencies seem to indicate the hydrogen-bond strengths. High-resolution $^{13}$C NMR of the crystalline peptides also can be correlated with their conformations. The chemical shift differences between $C^\beta$ and $C^\gamma$ reveal the peptide $\psi$ angles.

## Introduction

Peptide chains have a variety of conformations which are energetically competitive. In proteins and polypeptides, specific conformers are stabilized by long-range cooperative effects. For oligopeptides which lack these long-range effects, covalent restrictions have been introduced to reduce their flexibility. Methyl derivatives of proline provide one class of amino acids which will give conformationally restricted peptides. We previously have reported spectroscopic and thermodynamic studies in solution for *N*-acetyl, *N*′-methylamide derivatives of the seven isomeric monomethylprolines (AcMeProNHMe).[2]

The structure and conformational nomenclature of the AcMeProNHMe are illustrated in Figure 1. There are five accessible conformational regions for these peptides.[3] The three regions for the trans peptide bond isomers will be referred to as $\alpha_R$, $C_7$, and $P_{II}$ which have peptide $\psi$ dihedral angles of ca. −60, 80, and 150°, respectively. The two regions for the cis peptide bond isomers have $\psi$ angles of ca. −60 and 150°, respectively. In solution most of the peptides have about 75% trans isomer. In nonpolar solutions the intramolecularly hydrogen-bonded $C_7$ conformer dominates, while the $P_{II}$ conformer is most prevalent in aqueous solution. Steric effects alter both the distribution of $\psi$ values and cis–trans isomerism.[2]

In the solid state, intermolecular hydrogen bonding and crystal packing produce long-range cooperative effects which stabilize particular conformations among those sterically allowed. Methyl group placement influences both molecular conformation and crystal packing. Atomic coordinates have been determined by X-ray diffraction for the seven isomeric AcMeProNHMe. The

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