Stereochemical Probe for the Mechanism of P-C Bond Cleavage Catalyzed by the *Bacillus cereus* Phosphonoacetaldehyde Hydrolase

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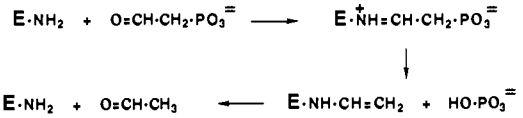
Abstract: The enzyme, the phosphonoacetaldehyde hydrolase (phosphonatase) of *Bacillus cereus*, catalyzes the conversion of phosphonoacetaldehyde to phosphate and acetaldehyde. Previous studies have shown that phosphonatase labilizes the C-P bond in the substrate by forming a protonated Schiff base between an active site lysine and the aldehyde carbonyl group (Olsen, B. H.; Hepburn, T. W.; Moos, M.; Mariano, P. S.; Dunaway-Mariano, D. *Biochemistry* **1988**, *27*, 2229). In this article, we describe the synthesis of stereochemical probes of this C-P bond cleavage reaction. The enantiomers of the chiral [^{17}O,^{18}O](thiophosphono)acetaldehyde have been prepared for this purpose, and an analysis of the stereochemistry of their phosphonatase-catalyzed transformations to [^{16}O,^{17}O,^{18}O]thiophosphate has been carried out. The synthesis of the enantiomers of [^{17}O,^{18}O](thiophosphono)acetaldehyde centered about the preparation and HPLC separation of diastereomeric thiophosphonamide precursors. The absolute phosphorus configurations in the thiophosphonamides were determined by X-ray analysis of a crystalline derivative of the (S_P,S_C) diastereomer. The stereochemistry of the phosphonatase-catalyzed reactions of the chiral (thiophosphono)acetaldehydes was determined to be retention at phosphorus. The results are interpreted in terms of a mechanism involving P-C bond cleavage in a protonated Schiff base intermediate by in-line displacement by an enzyme nucleophile. Subsequent hydrolysis of the resultant acetaldehyde enamine and phosphoenzyme groups then yields acetaldehyde and phosphate.

Introduction

Annually, thousands of tons of synthetic phosphonates are introduced into the environment primarily as detergent additives and agrochemicals.[1] Because of the chemical inertness of the C-P bond, the potential for removal of these environmental phosphonates would be low if it were not for the existence of phosphonate-degrading bacteria.[2] While the current knowledge about microbial phosphonate biodegradation is limited, one pathway for phosphonate catabolism, relying on enzyme-mediated hydrolytic C-P bond cleavage, has been identified.[3,4] To date, three unique C-P bond cleaving enzymes have been characterized. The best understood of these, phosphonoacetaldehyde hydrolase (phosphonatase) from *Bacillus cereus*, catalyzes the hydrolysis of phosphonoacetaldehyde (P-Ald) to acetaldehyde and inorganic phosphate by use of a protonated Schiff base mechanism.[4,5] A second enzyme, phosphonoacetate hydrolase, catalyzes the hydrolysis of phosphonoacetate to acetate and inorganic phosphate.[6] This process proceeds via a metal cation assisted hydrolytic C-P bond cleavage pathway.[6] Finally, the collection of proteins known as C-P lyase[7] catalyzes the hydrolysis of a broad spectrum of alkyl- and arylphosphonates by use of either a single electron reduction or oxidation pathway.[8]

Scheme I



Important information about the detailed mechanisms for these enzyme-catalyzed C-P bond cleavage processes would come from knowledge of the phosphorus stereochemistry (i.e., retention, inversion, or racemization). Previous studies of phosphoryl-transfer enzymes have proven that stereochemical determinations made at phosphorus are particularly powerful in aiding the elucidation of reaction mechanisms. At the beginning of our efforts in this area, no methods were available to determine the stereochemistry of phosphonate hydrolysis reactions in which the C-P bond is cleaved. Thus, our attention focused initially on the development of a general strategy for preparation of chiral probes that could be used to examine not only the stereochemical course of the phosphonatase reaction but also that of model solution reactions of P-Ald and, in addition, could be extended to investigate the phosphonoacetate hydrolase and C-P lyase mechanisms. In this report, we describe the general strategy that we have developed for these purposes. Specifically, methodology for the synthesis of the individual and configurationally assigned enantiomers of [^{17}O,^{18}O](thiophosphono)acetaldehyde ([^{17}O,^{18}O]SP-Ald) and their use in determining the phosphorus stereochemistry of the phosphonatase reaction is presented. In addition, the stereochemistry of this process is compared with that of a model hydrolytic reaction of P-Ald catalyzed by aniline.[9]

The enzyme, phosphonatase isolated from *Bacillus cereus*, catalyzes the dephosphonylation reaction of P-Ald to yield acetaldehyde and phosphate.[4a] Previous studies have demonstrated that the mechanism of this enzymatic process involves activation of C-P bond cleavage by formation of a Schiff base between P-Ald and an active site lysine in phosphonatase.[4a,5,10] These earlier

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(2) Cook, A. M.; Daughton, C. G.; Alexander, M. *J. Bacteriol.* **1978**, *133*, 85. Daughton, C. G.; Cook, A. M.; Alexander, M. *J. Agric. Food Chem.* **1979**, *27*, 1375. LaNauze, J. M.; Rosenberg, H. *Biochim. Biophys. Acta* **1967**, *148*, 811. Fitzgibbon, J.; Braymer, H. D. *Appl. Environ. Microbiol.* **1988**, *54*, 1886. Shinabarger, D. L.; Schmitt, E. K.; Braymer, H. D.; Larson, A. D. *Appl. Environ. Microbiol.* **1984**, *48*, 1049. Wackett, L. P.; Shames, S. L.; Venditti, C. P.; Walsh, C. T. *J. Bacteriol.* **1987**, *169*, 710. Rosenberg, H.; LaNauze, J. M. *Biochim. Biophys. Acta* **1967**, *141*, 79.
(3) Cordeiro, M. L.; Pompliano, D. L.; Frost, J. W. *J. Am. Chem. Soc.* **1986**, *108*, 332. Shinabarger, D. L.; Braymer, H. D. *J. Bacteriol.* **1986**, *168*, 702. Avila, L. Z.; Loo, S. H.; Frost, J. W. *J. Am. Chem. Soc.* **1987**, *109*, 6758. Kishore, G. M.; Jacob, G. S. *J. Biol. Chem.* **1987**, *262*, 12164. Loo, S. H.; Peters, N. K.; Frost, J. W. *Biochem. Biophys. Res. Commun.* **1987**, *148*, 148. Pipke, R.; Amrhein, N.; Jacob, G. S.; Schaefer, J.; Kishore, G. M. *Eur. J. Biochem.* **1987**, *165*, 267.
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(9) Lee, S. L.; Hepburn, T. W.; Mariano, P. S.; Dunaway-Mariano, D. *J. Org. Chem.* **1990**, *55*, 5435.
(10) LaNauze, J. M.; Coggins, J. R.; Dixon, H. B. F. *Biochem. J.* **1977**, *165*, 409.

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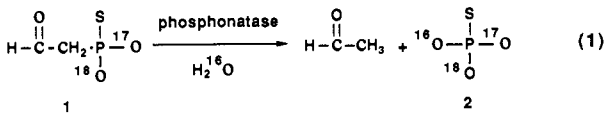
efforts suggest that the overall reaction occurs in three stages. As depicted in Scheme I, the process is initiated by Schiff base formation, which is then followed by sequential C–P bond cleavage and Schiff base hydrolysis.

The goal of the present study was to examine the mechanism for phosphate formation from the protonated Schiff base intermediate. A priori, phosphate could be formed from the phosphonoaldiminium ion intermediate by a direct displacement route with water as the nucleophile. Alternatively, C–P bond cleavage could be assisted by an enzyme nucleophile, in which case phosphate would be produced in a hydrolytic second step. In order to gain insight into the mechanism for the C–P cleavage step in the phosphonatase-catalyzed transformation of P-Ald to acetaldehyde and phosphate, we have investigated the stereochemical course of this reaction at phosphorus. At the outset, we anticipated that reaction would proceed with inversion of stereochemistry if a direct water displacement mechanism for C–P bond cleavage were operating. Alternatively, a mechanism in which enzyme-assisted C–P cleavage is followed by hydrolysis of a transient phosphoenzyme intermediate would involve two displacements and, consequently, is expected to proceed with retention of phosphorus stereochemistry.

Results and Discussion

Design of the Stereochemical Probe. The enantiomers of [^{17}O,^{18}O]SP-Ald (**1**) were selected to study the phosphonatase-catalyzed C–P bond cleavage process owing to the following factors.[11] Firstly, as shown in eq 1, a chiral phosphorus product, [^{18}O,^{17}O,^{16}O]thiophosphate, is formed for which methods of configurational analysis have been previously described.[12,23]

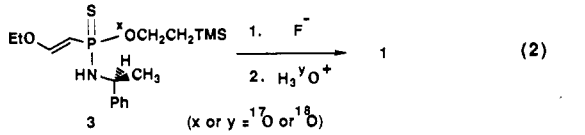


(1)

1 **2**

Secondly, earlier studies have shown that Sp-Ald is a reasonable substrate for phosphonatase (K_m = 50 μM and k_{cat} = 50 min^{-1} at 25 °C, pH 8, vs for P-Ald, K_m = 40 μM and k_{cat} = 1000 min^{-1}).[14] Finally, incorporation of the ^{17}O label in the probe is more economical than the use of $H_2{}^{17}O$ in the phosphonatase reaction.

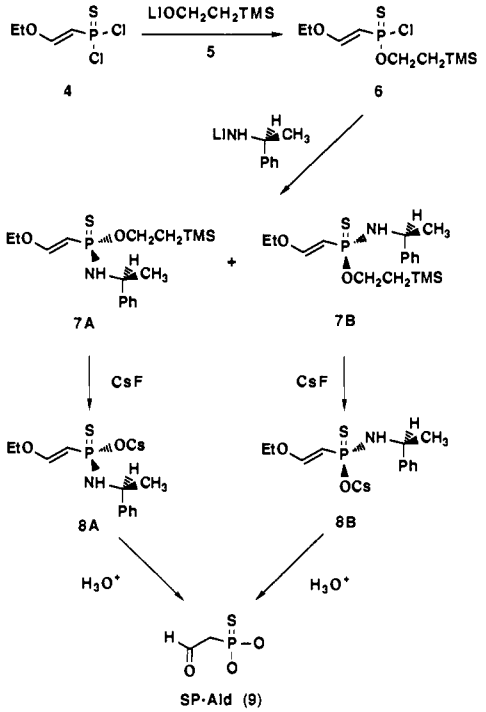
The methodology we have developed for synthesis of the separate enantiomers of [^{17}O,^{18}O]SP-Ald (**1**) involves the preparation, separation, stereochemical identification, and subsequent transformations of the diastereomeric, isotopically labeled thiophosphonamidates **3** (^{x}O or ^{y}O = ^{17}O or ^{18}O). The design advantageously utilizes the fact that the diastereomers **3** contain (1) TMSEt-blocked P oxygens, ^{x}O, which can be liberated by fluoride-induced desilylethylation with retention of phosphorus configuration, (2) blocked acetaldehyde groups capable of being unmasked by acid-catalyzed hydrolysis of the enol ether moieties, and (3) precursors of the final P oxygen, ^{y}O, which can be exposed by hydrolytic P–N bond cleavage using $H_2{}^{y}O$ with predominant inversion[15] of configuration (eq 2).



3 **1** (2)

Since this strategy is dependent upon the successful preparation and separation of the diastereomers of thiophosphonamidate **3**, our initial efforts focused on these two goals. As outlined in Scheme II, the nonisotopically labeled analogs of these substrates, **7A** and **7B**, can be independently prepared by an efficient sequence starting with the known[16] (ethoxyvinyl)thiophosphonic dichloride (**4**). Accordingly, sequential addition to **4** of lithium (trimethylsilyl)ethoxide (**5**) and lithium (*S*)-α-phenethylamide gives the diastereomeric (R_P,S_C) and (S_P,S_C) thiophosphonamidates **7A** and **7B**. These materials can be readily separated by HPLC (silica gel, Et_2O–cyclohexane).

Scheme II



The efficacy of our methodology also requires a viable sequence for conversion of the thiophosphonamidate diastereomers **3** to the SP-Ald enantiomers **1**. Indeed, model transformations of the ^{16}O-analogs, **7A** and **7B**, can be accomplished by treatment with CsF in refluxing CH_3CN to give the crystalline cesium salts, **8A** and **8B** (Scheme II). Hydrolyses of **8A** and **8B** under a variety of aqueous acid conditions invariably led to the desired SP-Ald **9** in a mixture with products arising from desulfurization and dephosphonylation. Optimal conditions, involving treatment of **8A** or **8B** with 1 M *p*-TsOH in H_2O–THF for 3.5 h at 25 °C, gave SP-Ald **9** in a 50% yield along with P-Ald (16%) and thiophosphate (5%).

Lastly, a technique for determining the phosphorus stereochemistry in the thiophosphonamidate diastereomers, **7A** and **7B** or **8A** and **8B**, was needed. Attempts to correlate ^{1}H, ^{13}C, and ^{31}P NMR spectroscopic parameters with phosphorus stereochemistry in the **7** and **8** diastereomeric pairs were unsuccessful. While several major differences exist in the NMR spectra of **7A** and **8A** (for example, vinyl-H resonances for **7A** at 4.64 and 7.03 ppm vs 4.97 and 7.14 ppm for **7B** and methylene-H resonances for **7A** at 0.97 and 4.00 ppm vs 0.55–0.78 and 3.47–3.93 ppm for **7B**), they are not sufficient to unambiguously characterize absolute configurations at the phosphorus centers. This contrasts with several cyclic thiophosphonamidates studied earlier by Inch and his co-workers,[17] whose configurational assignments were made by NMR analysis.

Attempts to employ X-ray methods to assign stereochemistry to **8A** and **8B** were unsuccessful owing to the poor diffraction characteristics of their crystals. Derivatization of these cesium

(11) For a preliminary communication describing a portion of these results, see ref 9.

(12) (a) Webb, M. R.; Trentham, D. R. *J. Biol. Chem.* **1980**, *255*, 1775. (b) Webb, M. R. *Methods Enzymol.* **1982**, *87*, 301.

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(17) Cooper, D. B.; Hall, C. R.; Inch, T. D. *J. Chem. Soc., Chem. Commun.* **1975**, 721. Harrison, J. M.; Inch, T. D.; Lewis, G. J. *J. Chem. Soc., Perkin Trans. 1* **1975**, 1892.

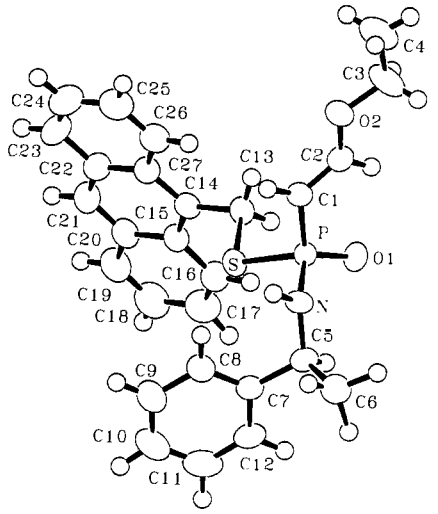
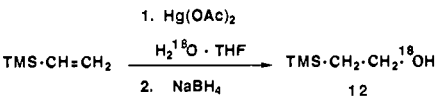
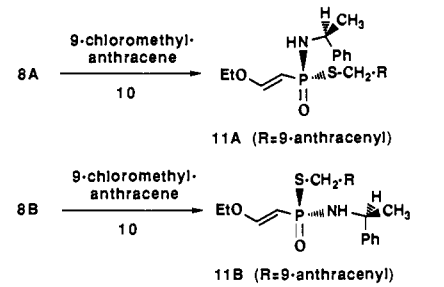


Figure 1. ORTEP drawing of **11B**. The C, N, O, S, and P atoms are shown as 5070 ellipsoids. H-atoms are depicted as spheres with $B = 1.5$ Å.[2]

Scheme III



thiophosphonamidates, however, avoided this problem. Thus, independent treatment of **8A** and **8B** with 9-(chloromethyl)-anthracene (**10**) gave the corresponding (*S*)-alkyl derivatives, **11A** and **11B**. Crystals of **11B**, obtained by slow evaporation of an



acetone–cyclohexane solution, yielded to X-ray diffraction analysis (Figure 1). This analysis demonstrated that **11B** is the (S_P,S_C) diastereomer and, consequently, that **11A** is the (R_P,S_C) diastereomer. By analogy, **8A** and **8B** must be the (R_P,S_C) and (S_P,S_C) thiophosphonamidates, respectively.

Synthesis and Enantiomeric Purity Determinations of the [^{17}O,^{18}O]SP-Ald Enantiomers. An assessment based on the costs of the water isotopomers and the yields of the preparative steps required suggested that the route for synthesis of the [^{17}O,^{18}O]-SP-Ald enantiomers involving preparation and addition of Li^{18}OCH$_2$CH$_2$TMS to **4** and hydrolysis of the intermediate ^{18}O isotopomers of **8** with $H_3{}^{17}O^+$ would be the most economical. Experimentation uncovered an efficient procedure (Scheme III) to generate the alcohol precursor of the Li salt of [^{18}O](trimethylsilyl)ethanol (**12**) via oxymercuration (Hg(OAc)$_2$, $H_2{}^{18}O$–THF) and reduction ($NaBH_4$) of vinyltrimethylsilane.[18] Application of this methodology gave the alcohol in an 83% yield and with >85% ^{18}O label as judged by ^{13}C NMR and GC–MS analysis.

Using the chemistry outlined in Scheme II, addition of Li [^{18}O](trimethylsilyl)ethoxide to dichloride **4** followed by reaction with Li (*S*)-phenethylamide, HPLC separation, CsF-induced disilylethylation, and hydrolysis of the ^{18}O-labeled isotopomers

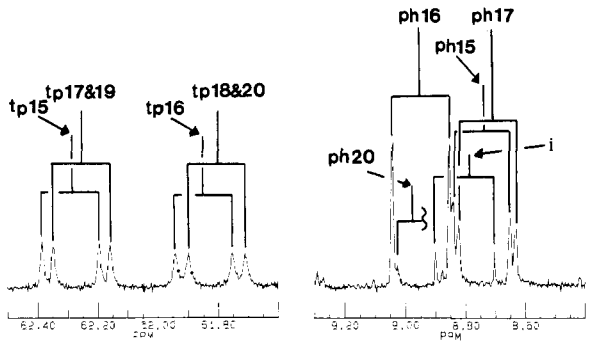


Figure 2. ^{31}P NMR spectrum of the mixed anhydrides derived from **8A** by hydrolysis in 1:1 $H_2{}^{16}O$–$H_2{}^{18}O$ followed by reaction with chloride **14**. The thiophosphonyl ^{31}P resonances are marked by tp and the phosphoryl resonances by ph. The doublet centered at 8.78 ppm (marked as i) was shown by decoupling experiments to be an impurity (possibly *trans* isomer) and not associated with **15–20**.

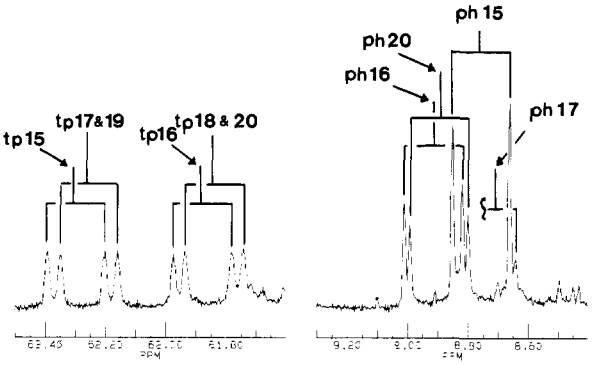
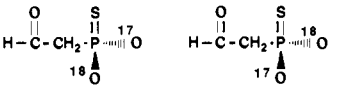


Figure 3. ^{31}P NMR spectrum of the mixed anhydrides derived from **8B** by hydrolysis in 1:1 $H_2{}^{16}O$–$H_2{}^{18}O$ followed by reaction with chloride **14**. The thiophosphonyl ^{31}P resonances are marked by tp and the phosphoryl resonances by ph.

of thiophosphonamidates **8A** and **8B** with 1 M *p*-TsOH in $H_2{}^{17}O$–THF gave the respective (S_P) and (R_P) enantiomers of [^{17}O,^{18}O]SP-Ald (**1**). These substances were contaminated with ca. 12% thiophosphate and 8–28% P-Ald.



Since the enantiomeric compositions of these chiral probes will have a direct impact on the evaluation of the stereochemical course of the phosphonatase-catalyzed C–P bond cleavage reaction, a determination of the ee of these substances was necessary. The general method developed by Cullis and his co-workers[19] utilizing ^{31}P NMR analysis of mixed anhydride diastereomers generated by reaction of [^{16}O,^{18}O]thiophosphate monoesters with enantiomerically pure *cis*-2-chloro-1,3,2-oxazaphospholidin-2-one **14** (derived from (–)-ephedrine) appeared applicable for this purpose. Accordingly, the diastereomeric thiophosphonamidates, **8A** and **8B**, were separately transformed into equimolar mixtures of Sp-Ald and either (if 100% ee) or both (if <100% ee) of the [^{16}O,^{18}O]-SP-Ald enantiomers (**13A** and **13B**) by treatment with 1 M *p*-TsOH in 1:1 (exact) $H_2{}^{16}O$ and $H_2{}^{18}O$ (Scheme IV). The resulting mixtures were then reacted with chloride **14** to generate mixtures of isotopomeric and diastereomeric anhydrides **15–20**. From **8A**, a mixture containing the diastereomeric, SP-Ald-derived anhydrides, **15** and **16**, along with a single set of [^{18}O]-labeled anhydrides, **17** and **18**, will be obtained if the [^{16}O,^{18}O]SP-Ald produced by hydrolysis is enantiomerically pure. If, on the other hand, [^{16}O,^{18}O]SP-Ald is racemized, then mixtures of **15** and **16**

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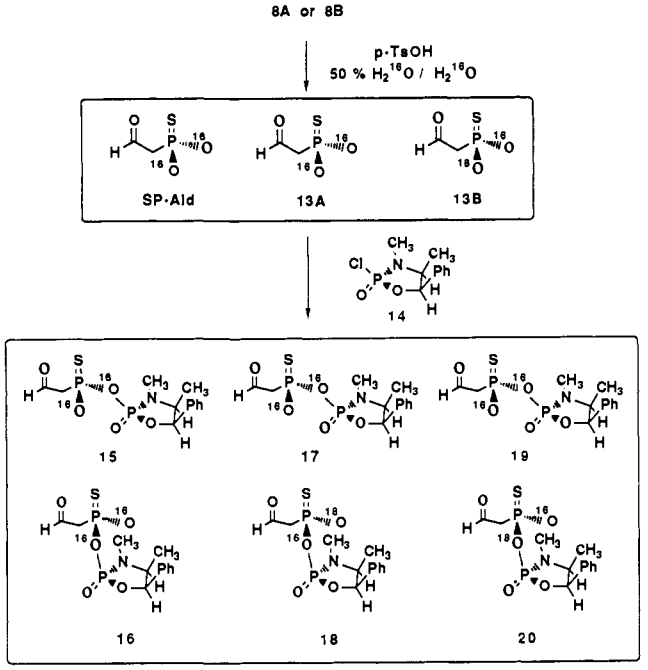
(19) Cullis, P. M.; Iagrossi, A. *J. Am. Chem. Soc.* **1986**, *108*, 7869.

Scheme IV



along with two sets of ^{18}O-labeled anhydride diastereomers, **17,18** and **19,20**, will be formed. Compositions of the anhydride mixtures will be different when **8B** is the starting material; here **15** and **16** will be obtained along with either **19,20** (if [^{16}O,^{18}O]SP-Ald is enantiomerically pure) or both **19,20** and **17,18** (if ee <100%).

^{31}P NMR analysis of the anhydride mixtures allows a quantitative evaluation of both the isotopomer and diastereomer compositions (Figures 2 and 3). The thiophosphonyl (tp) and phosphoryl (ph) ^{31}P resonances in the all-[^{16}O] diastereomers **15** and **16** occur as doublets in the 60 and 8 ppm regions, respectively. In contrast, since the ^{18}O labels in anhydrides **17** and **20** occupy bridge positions, both their thiophosphonyl and phosphoryl resonances will be shifted (ca. 0.02 ppm)[20] upfield of those for **15** and **16**. When, on the other hand, the ^{18}O labels occupy nonbridge positions as in **18** and **19**, then only the thiophosphonyl ^{31}P resonances will be shifted upfield by ca. 0.04 ppm.[20] While the trend of greater upfield shifts by nonbridge ^{18}O oxygens is often seen in the ^{31}P NMR spectra of phosphates and their thio analogs,[20,21] the effects on the thiophosphonyl resonances in **17–20** are nearly equivalent, and consequently, a determination of the **17** vs **19** and/or **18** vs **20** anhydride diastereomer ratio by integrations in the 60 ppm region is prevented.

However, an analysis of the phosphoryl (ph) regions of these spectra at ca. 8 ppm (Figures 2 and 3) provides conclusive information about enantiomeric purities. Specifically, the ratios of the phosphoryl doublets for **15** and **17** can be accurately measured as can those for **16** and **20**. Since an exactly 1:1 $H_2{}^{16}O$–$H_2{}^{18}O$ ratio was used in the **8A** and **8B** hydrolysis reactions, the areas under the ph-**15** and ph-**17** resonances would be equivalent if **8A** had undergone completely enantiospecific (inversion) hydrolysis. To the extent that a second enantiomer is formed from **8A**, the ph-**15** resonance would be enlarged relative to ph-**17**. Indeed, the latter situation is observed, and from the ratio of peak areas (Figure 2), the enantiomeric purity of [^{16}O,^{18}O]SP-Ald isomers formed from **8A** is determined to be 87:13. A similar analysis of the spectrum of the anhydride mixture derived from **8B** gives an enantiomer ratio of 86:14. Thus, the acid-catalyzed hydrolysis reactions of the ^{18}O-labeled thiophosphonamidates [^{18}O]-**8A** and [^{18}O]-**8B** in $H_2{}^{17}O$ occur with high but incomplete enantiospecificity (inversion predominating), and as a result, the purities of the chiral labeled probes **1** are ca. 72% ee.

(20) Cohn, M.; Hu, A. *J. Am. Chem. Soc.* **1980**, *102*, 913.
(21) Frey, P. A.; Sammons, R. D. *Science* **1985**, *228*, 541.

Table I. Isotopomer (**21–24**) Distributions for (S_P)-ATPβS Derived from Phosphonatase-Catalyzed Reactions of (S_P)-**1** and (R_P)-**1** Followed by Enzymatic Transformation and ^{31}P NMR Analysis

isotopomer	theoretical for 100% inversion	theoretical for 100% retention	obsd
	From (S_P)-**1** → [^{16}O,^{17}O,^{18}O]Thiophosphate		
21	0.82	0.82	0.81
22	1.00	0.79	0.79
23	0.79	1.00	1.00
24	0.46	0.46	0.45
	From (R_P)-**1** → [^{16}O,^{17}O,^{18}O]Thiophosphate		
21	0.98	0.98	0.98
22	0.81	1.00	1.00
23	1.00	0.81	0.81
24	0.46	0.46	0.44

Stereochemical Course of Phosphonatase-Catalyzed C–P Bond Cleavage. With the enantiomers of [^{17}O,^{18}O]SP-Ald, (R_P)-**1** and (S_P)-**1**, in hand and their enantiomeric purities determined, an evaluation of the phosphorus stereochemistry for phosphonatase-catalyzed C–P bond cleavage was undertaken. Execution of this required selection of a method to analyze the stereochemistry of the isotopically labeled thiophosphate, **2**, produced in the individual reactions of (R_P)-**1** and (S_P)-**1**. Two procedures have been described previously for this purpose: a chemical method by Lowe and his co-workers[22] and an enzymatic method reported independently by Webb and Trentham[12] and Tsai.[13] The chemical method, which utilizes formation and ^{31}P NMR analysis of diastereomeric and isotopomeric thiophospholanes, is generally considered to be more accurate owing to a low degree of oxygen isotope exchange (washout).[22a] However, its implementation requires separate steps for generation and further conversion of the labeled thiophosphate. This is problematic in our application since high concentrations of thiophosphate produced in the phosphonatase-catalyzed reaction inhibit the enzyme. The enzymatic method for thiophosphate stereochemical analysis, on the other hand, employs an initial enzyme-catalyzed reaction sequence to transform thiophosphate to adenosine 5′-(3-thiotriphosphate) (ATPγS), which can be coupled to the phosphonatase process and, thus, used to avoid build-up of inhibitory concentrations of thiophosphate.

The overall enzymatic sequence for analysis of isotopically labeled thiophosphate involves its conversion to adenosine 5′-(2-thiotriphosphate) (ATPβS) via consecutive formation of (1) ATPγS by glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase (inversion of P stereochemistry), (2) adenosine 5′-(2-thiodiphosphate) (ADPβS) by myokinase (inversion), and (3) (S_P)-ATPβS by pyruvate kinase catalyzed phosphorylation of the *pro*-S oxygen. The major drawback of this methodology, as pointed out by Webb,[12b] results from the generally observed large degree of washout associated with the hydrolytic instability of glycerate 1-thiophosphate 3-phosphate formed in the initial step. Fortunately, washout can be both accounted for in the ultimate ^{31}P NMR analysis and minimized by using high concentrations of the coupling enzyme, phosphoglycerate kinase.

In practice, the ATPγS generated from [^{16}O,^{17}O,^{18}O]thiophosphate will be composed of a mixture of isotopomers **21–24** in addition to those containing ^{17}O due to isotopic washout and the fact that the ^{17}O label is <100%. The theoretical distributions of **21–24** expected from each of the thiophosphate enantiomers (see Table I) can be calculated by taking into account the enantiomeric purities of the precursor SP-Ald, the amount of contaminating achiral thiophosphate present in the precursor SP-Ald,[23] the isotopic compositions of the $H_2{}^{17}O$ water utilized to

(22) (a) Arnold, J. R. P.; Bethell, R. C.; Lowe, G. *Bioorg. Chem.* **1987**, *15*, 250. (b) Arnold, J. R. P.; Lowe, G. *J. Chem. Soc., Chem. Commun.* **1986**, 865.
(23) ^{31}P NMR analysis of these reaction mixtures prior to the addition of phosphonatase allowed determination of the amount of contaminating achiral thiophosphate present in the SP-Ald samples.

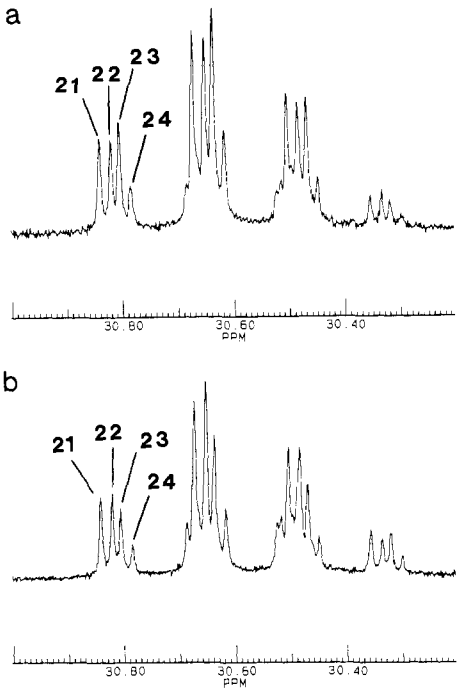
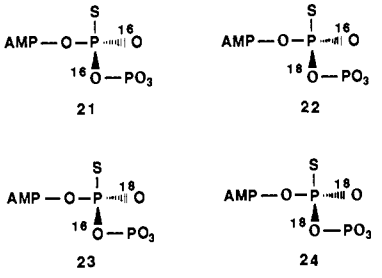


Figure 4. P_β region of ^{31}P NMR spectra of (S_P)-ATPβS derived from thiophosphate arising from phosphonatase-catalyzed dephosphonylation of (a) (S_P)-[^{17}O,^{18}O]SP-Ald and (b) (R_P)-[^{17}O,^{18}O]SP-Ald. The small triplet seen in spectra a and b arises from the P_β resonance of the contaminating (R_P)-ATPβS isomer.

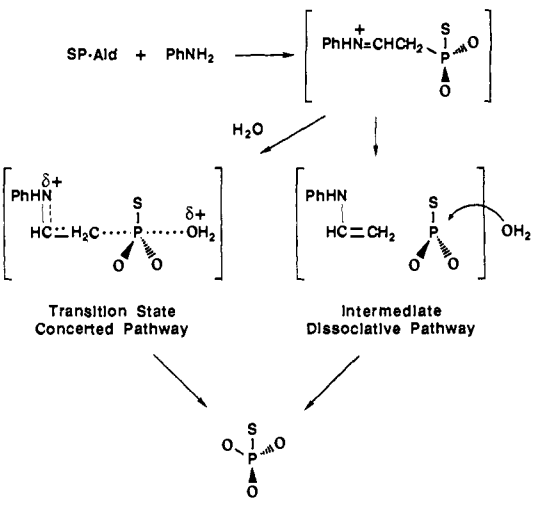
produce SP-Ald, and the extent of washout[24] in the enzymatic sequence.



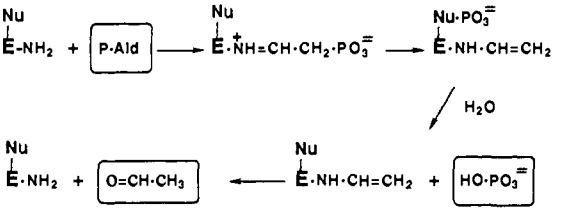
To probe the stereochemical course of the phosphonatase reaction, the [^{17}O,^{18}O]SP-Ald enantiomers, (R_P)-**1** and (S_P)-**1** (40–50 μmol), were individually treated first with glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase (in the presence of glyceraldehyde 3-phosphate, adenosine 5′-diphosphate (ADP), and nicotinanide adenine dinucleotide (NAD)[23]) followed by phosphonatase (20 units) in $H_2{}^{16}O$ to yield ATPγS. The γ-thiophosphoryl moiety of ATPγS was then transferred to adenosine 5′-monophosphate (AMP) by use of myokinase to give, after DEAE-Sephadex A-25 chromatography, pure ADPβS. Finally, pyruvate kinase catalyzed phosphorylation of ADPβS by phosphoenolpyruvate (PEP) resulted in formation of (S_P)-ATPβS, composed of a mixture of isotopomers **21**–**24**, which was analyzed by ^{31}P NMR spectroscopy. The β-phosphorus resonances for the constituent isotopomers occur as triplets in the 30 ppm regions of the spectra (Figure 4) and have chemical shifts appearing in descending order (**21**, 30.654, > **22**, 30.634, > **23**, 30.618, > **24**, 30.597 ppm) as a result of the number and position (bridge or nonbridge) of the ^{18}O labels. Integrations of the resonances corresponding to **21**–**24** give the observed distributions of these isotopomers recorded in Table I. Comparisons of the observed and predicted ratios of **21**–**24** demonstrate that [^{17}O,^{18}O]SP-Ald enantiomers (R_P)-**1** and (S_P)-**1** undergo enantiospecific (>90%) phosphonatase-catalyzed C–P bond cleavage

(24) The extent of washout can be determined by accounting for the larger than predicted amount of the all-^{16}O isomer **21**.

Scheme V



Scheme VI



to produce (R_P)- and (S_P)-[^{16}O,^{17}O,^{18}O]thiophosphate, respectively, and thus that *this process occurs with retention of configuration at phosphorus.*

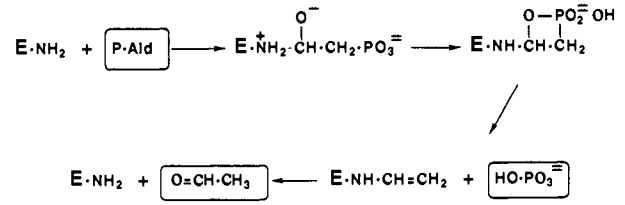
Mechanistic Analysis. In a previous study,[9] we probed the aniline-catalyzed hydrolysis reaction of the enantiomers of [^{17}O,^{18}O]SP-Ald as a potential chemical model for the phosphonatase process. In that effort, we found that in the presence of a 20-fold excess of aniline the [^{17}O,^{18}O]SP-Ald enantiomers were quantitatively converted to [^{16}O,^{17}O,^{18}O]thiophosphate within 3 h at 25 °C. Importantly, the dethiophosphonylation reaction was observed to occur with >90% inversion of configuration at phosphorus. These earlier results suggest that the mechanism for C–P bond cleavage in the protonated aniline–(thiophosphono)-acetaldehyde Schiff base intermediate involves either a concerted displacement with an in-line arrangement of the nucleophile (water) and the leaving group (PhNHCH=CH_2) or a dissociative process via a tightly paired metathiophosphate intermediate (Scheme V).

As our current results demonstrate, the phosphonatase-catalyzed reaction of [^{17}O,^{18}O]SP-Ald occurs with >90% retention of the phosphorus configuration. These contrasting behaviors allow us to rule out an in-line displacement by water in a Schiff base intermediate in the enzymatic C–P bond cleavage process since inversion of stereochemistry would have been observed. While adjacent attack by water would have resulted in the observed retention of configuration, no precedent exists for the operation of this type of mechanism in either our chemical model[9] or in known enzymic phosphoryl-transfer reactions.[25]

Our results necessitate an alternative mechanism for the phosphonatase process. One reasonable possibility involves the participation of enzyme covalent catalysis. Accordingly, formation of the requisite[4a,5] Schiff base intermediate by reaction of P-Ald with the active site lysine could be followed by attack of a second nucleophilic enzyme residue on phosphorus with in-line displacement to produce a phosphoenzyme intermediate (Scheme

(25) Eckstein, F. *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 423. Knowles, J. R. *Annu. Rev. Biochem.* **1980**, *49*, 877. Frey, P. A. *Adv. Enzymol. Rel. Areas Mol. Biol.* **1989**, *62*, 119. McQueney, M. S.; Lee, S.-I.; Swartz, W. H.; Ammon, H. L.; Mariano, P. S.; Dunaway-Mariano, D. *J. Org. Chem.* **1991**, *56*, 7121. Freeman, S.; Seidel, H. M.; Schwalbe, C. H.; Knowles, J. R. *J. Am. Chem. Soc.* **1990**, *112*, 8149.

Scheme VII



VI). Subsequent in-line substitution at the phosphoenzyme center by water would then produce phosphate with overall retention of the phosphorus configuration. In this way, the phosphonatase mechanism would parallel that of the more than 17 enzymes to date which have been documented[25a] to proceed through phosphoenzyme intermediates with retention of phosphorus stereochemistry. The PEP to phosphonopyruvate converting enzyme PEP mutase has also been recently shown to catalyze phosphoryl transfer with retention of configuration.[25b]

If the double displacement mechanism depicted in Scheme VI is operable for phosphonatase catalysis, the participation of two active site residues (the lysine amine and a nucleophile) are required. While double covalent catalysis of this type is unusual, several examples of enzymes, believed to catalyze reactions by use of two different active site residues to covalently link substrates, have been described. These include kynureninase,[26] squalene synthetase,[27] and the mannitol-specific enzyme II of the PEP-dependent phosphotransferase system.[28]

Another mechanistic alternative for the phosphonatase reaction is outlined in Scheme VII. In this pathway, addition of the active site lysine amine group to the carbonyl carbon of P-Ald would lead to the oxaphosphetane intermediate in a fashion analogous to Wittig and Horner–Emmons type reactions. Subsequent pseudorotation and simultaneous fragmentation of the C–O and C–P bonds in this intermediate would generate phosphate and the enzyme–acetaldehyde enamine, previously identified by $NaBH_4$ trapping as part of the evidence supporting a Schiff base mechanism for phosphonatase.[4,5,29] As predicted by the apical entry–departure rule,[30] the C–P bond cleavage reaction via the oxaphosphetane intermediate must proceed only after pseudorotation at phosphorus, and consequently, it must occur with retention of phosphorus configuration. Close chemical analogs of this process are found in Wittig type reactions, which have been demonstrated by many workers[31] to occur with retention of configuration at phosphorus. Moreover, direct measurements have been made on the pseudorotation rates in oxaphosphetanes,[32] and these have shown that this process is rapid (ca. 6×10^4 min^{-1}) relative to the phosphonatase turnover rate (ca. 1×10^3 min^{-1}). The double displacement mechanism shown in Scheme VI predicts that phosphate would be formed with oxygen incorporation from solvent H_2O, while the Wittig type mechanism displayed in Scheme VII predicts that oxygen added during phosphate formation derives from the C-2 carbonyl oxygen of P-Ald. Recent results[33] of experiments determining ^{18}O incorporation into phosphate in the reaction of C-2 ^{18}O-labeled P-Ald in H_2O show that the Wittig type pathway is not operable in the phosphonatase-catalyzed reaction.

Conclusion

While the aniline-catalyzed hydrolysis of [^{17}O,^{18}O]SP-Ald occurs with inversion of configuration at phosphorus, the phosphonatase-catalyzed process proceeds with retention of stereochemistry. These results are interpreted in terms of a double displacement mechanism proceeding via protonated Schiff base and phosphoenzyme intermediates (Scheme VI) for this enzymatic C–P cleavage reaction.

Experimental Section

General. Melting points are recorded uncorrected. ^1H NMR spectra were recorded at 200 and 400 MHz, and chemical shifts are reported in parts per million relative to tetramethylsilane (δ 0.00) or chloroform (δ 7.24) as internal standards for samples in $CDCl_3$ or relative to 3-(trimethylsilyl)-1-propanesulfonic acid (disodium salt) (δ 0.00 for Me_3Si resonance) as an external standard for samples in D_2O. ^{13}C NMR spectra were obtained at operating frequencies of 50 or 100 MHz, and chemical shifts are reported in ppm relative to $CDCl_3$ (δ 77.0) as an internal standard for organic-soluble compounds or relative to 1,4-dioxane (δ 66.5) as an external standard for samples in D_2O. ^{31}P NMR spectra were recorded at operating frequencies of 81 and 162 MHz, and chemical shifts are reported in ppm relative to 85% (δ 0.0) phosphoric acid as an external standard.

Molecular distillations were performed with a Kugelrohr apparatus. Preparative chromatographic separations were accomplished by using the following adsorbents: flash column chromatography, Merck EM type 60 (230–400 mesh) silica gel; column chromatography, Fisher Florisil (100–200 mesh); column chromatography, Alcoa type F-20 alumina (80–200 mesh); anion exchange column chromatography, diethylaminoethyl (DEAE) Sephadex A-25 or DEAE-Sephadex A-50; gel filtration column, Sephadex G-10 and G-150 (40–120 μm bead size). Preparative high-performance liquid chromatography (HPLC) separations were carried out on the following columns: reversed phase, Perkin Elmer C-18; silica gel, Du Pont ZORBAX SIL (21.1 mm i.d. × 25 cm). Fast protein liquid chromatography (FPLC) was with a Pharmacia Mono Q HR 5/5 anion exchange column.

All organic layers obtained in the workup of preparative reactions were dried with anhydrous Na_2SO_4. All moisture-sensitive reactions were conducted in oven-dried (125 °C) or flame-dried glassware under an atmosphere of dry nitrogen, and transfers were performed by syringe or cannula.

The 48.6% $H_2{}^{17}O$ (containing 48.6 atom % ^{17}O, 30.7 atom % ^{18}O, and 20.7 atom % ^{16}O), 45% $H_2{}^{17}O$ (containing 45.0 atom % ^{17}O, 34.8 atom % ^{18}O, and 20.2 atom % ^{16}O), and 98.0 atom % $H_2{}^{18}O$ were obtained from Cambridge Isotope Laboratories. From Mound Stable Isotopes was obtained 97.3% $H_2{}^{18}O$. All other reagents, obtained from Aldrich and Sigma, were of commercial grade and used without further purification unless noted. The following enzymes were obtained from Sigma: glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 4545 U/mL), 3-phosphoglycerate phosphokinase (yeast, 5880 U/mL), lactate dehydrogenase (pig muscle, 940 U/mg), myokinase (rabbit muscle, 1000 U/mg), and pyruvate kinase (rabbit muscle, 500 U/mg). All enzymes obtained as ammonium sulfate suspensions were dialyzed against 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 8.0, containing 1 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA) for 2 h before use. Isolation and purification of phosphonatase were performed by the methods described previously.[5]

Synthesis of (Ethoxyvinyl)thiophosphonic Dichloride (4).[16] A solution of ethyl vinyl ether (7.1 g, 0.74 mol) in benzene (7.5 mL) was added dropwise over 1 h to a benzene (150 mL) solution containing PCl_5 (41.5 g). After the addition was complete, the reaction mixture was stirred for 12 h at 25 °C and then at 60 °C for 0.5 h. After cooling, the mixture was saturated with hydrogen sulfide gas until a transparent solution was obtained. The mixture was then concentrated in vacuo to give 18.0 g of the crude material, which was fractionally distilled (83 °C, 1 mmHg) to give 11.4 g (56%) of **4** as a colorless liquid: IR (neat) 2970, 1590, 675 cm^{-1}; ^1H NMR ($CDCl_3$) 1.40 (t, J = 7.1 Hz, CH_3, 3 H), 4.02 (q, J = 7.1 Hz, CH_2, 2 H), 5.71 (dd, J = 12.8, 16.7 Hz, CHP, 1 H), 7.52 (dd, J = 12.8, 15.7 Hz, OCH, 1 H); ^{13}C NMR ($CDCl_3$) 14.3 (CH_3), 68.2 (CH_2), 105.2 (d, J = 134.8 Hz, CHP), 165.4 (d, J = 29.5 Hz, CHCP); ^{31}P NMR ($CDCl_3$) 73.5 (dd, J = 15.7, 16.7 Hz); EIMS m/e (relative intensity) 204 (M^+, 100), 179 (24), 141 (18), 133 (30), 115 (18), 75 (82); HRMS m/e 203.9331 (M^+, calcd for $C_4H_7OPSCl_2$ 203.9332).

Synthesis of *O*-[2-(Trimethylsilyl)ethyl] (Ethoxyvinyl)thiophosphonochloridate (6). To a stirred solution of 2-(trimethylsilyl)ethanol (0.498

(26) Palcic, M. M.; Antoun, M.; Tanizawa, K.; Soda, K.; Floss, H. G. *J. Biol. Chem.* **1985**, *260*, 5248.

(27) Beytia, E. D.; Quershi, A. A.; Porter, J. W. *J. Biol. Chem.* **1973**, *248*, 1856.

(28) Muller, E. G.; Khandekar, S. S.; Knowles, J. S.; Jacobson, G. R. *Biochemistry* **1990**, *29*, 6892.

(29) Phosphonatase inactivation experiments using [2-^{3}H]P-Ald and $NaBH_4$ followed by trypsin digestion and amino acid sequencing allowed us to identify N^{ϵ}-ethyllysine (ref 5). Thus, the Schiff base remaining after dephosphonylation was trapped by reduction rather than being the Schiff base formed between P-Ald and the active site lysine. While not recognized at that time, the P-Ald/$NaBH_4$-induced inactivation of phosphonatase is consistent with both the Schiff base and oxaphosphetane mechanisms.

(30) Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1*, 70.

(31) Blade-Font, A.; Vanderwerf, C. A.; McEwen, W. E. *J. Am. Chem. Soc.* **1960**, *82*, 2396. Gerlt, J. A.; Coderre, J. A. *J. Am. Chem. Soc.* **1980**, *102*, 4531. Baraniak, J.; Lesiak, K.; Sochacki, M.; Stec, W. J. *J. Am. Chem. Soc.* **1980**, *102*, 4533. Stec, W. J. *Acc. Chem. Res.* **1983**, *16*, 411.

(32) Vedejs, E.; Marth, C. F. *J. Am. Chem. Soc.* **1989**, *111*, 1519.

(33) Lee, S. L. Ph.D. Dissertation, University of Maryland, College Park, MD, 1991.

g, 4.22 mmol) in THF (5 mL) was added, dropwise, *n*-butyllithium (3.60 mmol) in hexane at 0 °C. The resulting solution was then added to a solution of (ethoxyvinyl)thiophosphonic dichloride (**4**) (0.666 g, 3.27 mmol) at −78 °C. The mixture was stirred at 25 °C for 2 h. Ether was then added, and the solution was washed successively with water and brine, dried, and concentrated in vacuo. The residue was subjected to molecular distillation (70 °C, 0.005 mmHg) to give 0.642 g (74%) of **6**: IR (neat) 2945, 2890, 1600, 1210 cm^{-1}; ^1H NMR ($CDCl_3$) 0.04 (s, $SiCH_3$, 9 H), 1.09 (t, J = 8.4 Hz, CH_2Si, 2 H), 1.33 (t, J = 7.1 Hz, CH_3, 3 H), 3.93 (q, J = 7.1 Hz, CH_2OC, 2 H), 4.01–4.47 (m, $POCH_2$, 2 H), 5.35 (dd, J = 11.2, 13.1 Hz, PCH, 1 H), 7.43 (dd, J = 13.1, 14.8 Hz, PCCH, 1 H); ^{13}C NMR ($CDCl_3$) −1.5 ($SiCH_3$), 14.3 (CH_3), 19.0 (d, J = 8.2 Hz, $POCCH_2$), 65.5 (d, J = 7.5 Hz, $POCH_2$), 67.3 (CH_2OC), 99.8 (d, J = 157.7 Hz, CHP), 164.8 (d, J = 30.0 Hz, CHCP); ^{31}P NMR ($CDCl_3$) 86.6; EIMS *m/e* (relative intensity) 258 (M^+ − 28, 12), 243 (19), 229 (19), 189 (33), 73 (100); HRMS *m/e* 258.0065 (M^+ − 28, calcd for $C_7H_{16}O_2PClSiS$ 258.0066).

Synthesis of [^{18}O][2-(Trimethylsilyl)ethyl] (Ethoxyvinyl)thiophosphonochloridate ([^{18}O]-6). This material was prepared in the same manner as described for **6** except that [^{18}O](trimethylsilyl)ethanol **12** was used. The residue obtained was subjected to molecular distillation (70 °C, 0.005 mmHz) to give 1.496 g of [^{18}O]-**6** from 2.574 g of **4** (46%): CIMS *m/e* (relative intensity) 289 ([M + 1], 16), 263 (40), 261 ([M − 28]$^+$ + 1, 100); HRMS (EI) *m/e* 260.0114 ([M − 28]$^+$, calcd for $C_7H_{16}{}^{16}O_1{}^{18}O_1SiPClS$ 260.0109), the observed ratio of peaks 261 vs 259 is equal to ^{18}O:^{16}O = 90:10.

Synthesis of the (R_P,S_C)- and (S_P,S_C)-*O*-[2-(Trimethylsilyl)ethyl] *N*-(1-Phenylethyl)(ethoxyvinyl)thiophosphonamidates (7A and 7B). To a stirred solution of (*S*)-1-phenylethylamine (0.273 g, 2.25 mmol) in THF (5 mL) was added, dropwise, *n*-butyllithium (2.24 mmol) in hexane at 25 °C. To this solution was added *O*-[2-(trimethylsilyl)ethyl] (ethoxyvinyl)thiophosphonochloridate (**6**) (0.537 g, 1.87 mmol). The mixture was stirred at 25 °C for 3 h. Ether was then added, and the solution was washed successively with water and brine, dried, and concentrated in vacuo. The residue was subjected to flash silica gel chromatography (10% ether–hexane) to afford 0.532 g (76%) of a mixture of diastereomers **7A** and **7B**. The diastereomeric mixture was then subjected to HPLC chromatography (silica gel, 7.5% ether–cyclohexane) to give 0.230 g of the isomer eluting first, **7A** (retention time = 30.5 min), and 0.292 g of the isomer eluting second, **7B** (retention time = 35.0 min).

7A: IR (neat) 3282, 2954, 1620, 1610 cm^{-1}; ^1H NMR ($CDCl_3$) 0.00 (s, $SiCH_3$, 9 H), 0.93–1.09 (m, CH_2Si, 2 H), 1.15 (t, J = 7.0 Hz, $OCCH_3$, 3 H), 1.43 (d, J = 7.0 Hz, $PhCCH_3$, 3 H), 3.31 (dd, J = 9.7, 9.7 Hz, NH, 1 H), 3.53 (q, J = 7.0 Hz, CH_2OC, 2 H), 3.85–4.18 (m, $POCH_2$, 2 H), 4.30–4.42 (m, PhCH, 1 H), 4.64 (dd, J = 12.4, 13.2 Hz, CHP, 1 H), 7.03 (dd, J = 13.0, 13.0 Hz, CHCP, 1 H), 7.17–7.34 (m, aromatic CH, 5 H); ^{13}C NMR ($CDCl_3$) −1.6 ($SiCH_3$), 14.4 (CH_3CO), 19.1 (d, J = 6.9 Hz, $POCCH_2$), 25.4 (d, J = 6.0 Hz, $PNCCH_3$), 51.6 (PhCH), 62.3 (d, J = 5.5 Hz, $POCH_2$), 65.3 (CH_2OC), 95.8 (d, J = 155.1 Hz, PCH), 125.9, 126.8, 128.2 (aromatic CH), 145.1 (aromatic C), 160.9 (d, J = 22.4 Hz, PCCH); ^{31}P NMR ($CDCl_3$) 70.7; EIMS *m/e* (relative mass) 371 (M^+, 0.3), 270 (11), 191 (18), 179 (5), 120 (100), 105 (29), 73 (21); HRMS *m/e* 371.1494 (M^+, calcd for $C_{17}H_{20}O_2NSiPS$ 371.1504).

7B: IR (neat) 3280, 2950, 1625, 1608, 1195 cm^{-1}; ^1H NMR ($CDCl_3$) −0.13 (s, $SiCH_3$, 9 H), 0.48–0.64 (m, CHH_ASi, 1 H), 0.69–0.86 (m, CHH_BSi, 1 H), 1.28 (t, J = 7.0 Hz, CH_3, 3 H), 1.40 (d, J = 7.1 Hz, $PhCCH_3$, 3 H), 3.36 (dd, J = 10.0, 12.6 Hz, NH, 1 H), 3.40–3.54 (m, $POCHH_A$, 1 H), 3.86 (q, J = 7.0 Hz, CH_2OC, 2 H), 3.85–4.00 (m, $POCHH_B$, 1 H), 4.24–4.37 (m, PhCH, 1 H), 4.97 (dd, J = 12.6, 13.1 Hz, CHP, 1 H), 7.14 (dd, J = 12.6, 13.0 Hz, CHCP, 1 H), 7.15–7.32 (m, aromatic CH, 5 H); ^{13}C NMR ($CDCl_3$) δ −1.7 ($SiCH_3$), 14.2 (CH_3CO), 18.8 (d, J = 7.9 Hz, $POCCH_2$), 25.4 (d, J = 6.7 Hz, $PNCCH_3$), 51.9 (PNCH), 62.2 (d, J = 6.7 Hz, $POCH_2$), 65.9 (CH_2OC), 95.7 (d, J = 155.3 Hz, CHP), 125.8, 126.9, 128.3 (aromatic CH), 145.1 (aromatic C), 161.3 (d, J = 22.0 Hz, PCCH); ^{31}P NMR ($CDCl_3$) 70.4; EIMS *m/e* (relative intensity) 371 (M^+, 0.1), 270 (6), 191 (12), 120 (100), 105 (32), 73 (27); HRMS *m/e* 371.1503 (M^+, calcd for $C_{17}H_{30}O_2NSiPS$ 371.1504).

Synthesis of the (R_P,S_C)- and (S_P,S_C)-[^{18}O][2-(Trimethylsilyl)ethyl] *N*-(1-Phenylethyl)(ethoxyvinyl)thiophosphonamidates ([^{18}O]-7A and [^{18}O]-7B). These substances were prepared from [^{18}O]-**6** using the procedure described for **7A** and **7B**. HPLC chromatography (silica gel, 7% ether–cyclohexane) gave 0.290 g of the isomer eluting first, [^{18}O]-**7A**, and 0.303 g of the isomer eluting second, [^{18}O]-**7B**. [^{18}O]-**7A** CIMS *m/e* (relative intensity) 374 ([M + 1]$^+$, 27), 346 (37), 272 (85), 193 (46), 120 (100), the observed ratio of peaks 374 vs 372 is equal to ^{18}O:^{16}O = 86:14. [^{18}O]-**7B**: CIMS *m/e* (relative intensity) 374 ([M + 1]$^+$, 13), 346 (14), 328 (24), 272 (18), 249 (56), 192 (22), 120 (100), the observed ratio of peaks 374 vs 372 is equal to ^{18}O:^{16}O = 83:17.

Synthesis of the Cesium (R_P,S_C)- and (S_P,S_C)-*N*-(1-Phenylethyl)-(ethoxyvinyl)thiophosphonamidates (8A and 8B). Cesium fluoride (5 equiv) was added to solutions of the individual diastereomers **7A** and **7B** of the *O*-(2-trimethylsilylethyl) *N*-(1-phenylethyl)(ethoxyvinyl)thiophosphonamidate (1 equiv) in acetonitrile (5 mL). Each mixture was then stirred at reflux for 5 h, cooled to 25 °C, and filtered through glass wool. The filtrate from each was concentrated in vacuo to give a solid residue, which was slowly crystallized first from toluene with external hexane and again from toluene to afford the pure, individual cesium thiophosphonamidates **8A** and **8B** both as white needles.

8A: 0.267 g from 0.229 g (0.62 mmol) of **7A** (100%); mp 63–64 °C; IR ($CHCl_3$) 3350, 2940, 1595, 1090 cm^{-1}; ^1H NMR ($CDCl_3$) 1.21 (t, J = 7.0 Hz, CH_3CO, 3 H), 1.37 (d, J = 6.8 Hz, CCH_3, 3 H), 3.22 (bs, NH, 1 H), 3.65 (dq, J = 2.7, 7.0 Hz, CH_2O, 2 H), 4.31–4.37 (m, NCH, 1 H), 4.89 (dd, J = 12.0, 13.2 Hz, CHP, 1 H), 6.89 (dd, J = 11.9, 13.2 Hz, CHCP, 1 H), 7.11–7.36 (m, aromatic H, 5 H); ^{13}C NMR ($CDCl_3$) 14.5 (CH_3CO), 25.8 ($PhCCH_3$), 51.7 (NCH), 64.8 (CH_2OC), 105.1 (d, J = 129.4 Hz, CHP), 126.5, 126.6, 128.4 (aromatic CH), 147.4 (d, J = 4.9 Hz, aromatic C), 156.8 (d, J = 19.0 Hz, CHCP); ^{31}P NMR ($CDCl_3$), 50.2; FABMS *m/e* (relative intensity) 536 ([M + Cs]$^+$, 11), 404 ([M + 1]$^+$, 10), 133 (Cs^+, 100), 105 (14); HRMS(FAB) *m/e* 403.9847 ([M + 1]$^+$, calcd for $C_{12}H_{18}O_2NPSCs$ 403.9850).

8B: 0.308 g from 0.285 g (0.766 mmol) of **7B** (99.8%); mp 80–81 °C; IR ($CHCl_3$) 3250, 2930, 1590, 1090 cm^{-1}; ^1H NMR ($CDCl_3$) 1.26 (t, J = 7.0 Hz, CH_3CO, 3 H), 1.34 (d, J = 6.8 Hz, $PhCCH_3$, 3 H), 3.42 (broad s, NH, 1 H), 3.71 (q, J = 7.0 Hz, CH_2OC, 2 H), 4.09–4.19 (m, NCH, 1 H), 4.91 (dd, J = 13.3, 13.3 Hz, CHP, 1 H), 6.88 (dd, J = 13.3, 11.5 Hz, CHCP, 1 H), 7.16–7.32 (m, aromatic H, 5 H); ^{13}C NMR ($CDCl_3$) 14.1 (CH_3CO), 25.1 (d, J = 7.0 Hz, $PhCCH_3$), 51.9 (NCH), 64.3 (CH_2OC), 104.0 (d, J = 130.3 Hz, CHP), 126.2, 128.0, 128.2 (aromatic CH), 147.5 (aromatic C), 156.1 (d, J = 17.4 Hz, CHCP); ^{31}P NMR ($CDCl_3$) 48.5; FABMS *m/e* (relative intensity) 536 ([M + Cs]$^+$, 6), 404 ([M + 1]$^+$, 2), 133 (Cs^+, 100); HRMS(FAB) *m/e* 403.9847 ([M + 1]$^+$, calcd for $C_{12}H_{18}O_2NPSCs$ 403.9850).

Synthesis of the Cesium (R_P,S_C)- and (S_P,S_C)-[^{18}O]-*N*-(1-Phenylethyl)(ethoxyvinyl)thiophosphonamidates ([^{18}O]-8A and [^{18}O]-8B). These substances were prepared independently from [^{18}O]-**7A** and [^{18}O]-**7B** by the method described for **8A** and **8B**. The filtrate from each was concentrated in vacuo to give a solid residue which was slowly crystallized first from toluene with external hexane and then again from toluene to afford the pure cesium thiophosphonamidates [^{18}O]-**8A** and [^{18}O]-**8B** as white needles. [^{18}O]-**8A**: 0.398 g from 0.367 g (0.984 mmol) of [^{18}O]-**7A** (99.9%); ^{31}P NMR ($CDCl_3$) 48.81:48.85 (^{18}O:^{16}O = 89:11); FABMS *m/e* (relative intensity) 538 ([M + Cs]$^+$, 13), 406 ([M + H]$^+$, 2), 133 (Cs^+, 100), the observed ratio of peaks 538:536 is equal to ^{18}O:^{16}O = 84:16. [^{18}O]-**8B**: 0.442 g from 0.399 g (1.07 mmol) of [^{18}O]-**7B** (100%); FABMS *m/e* (relative intensity) 536 ([M + Cs]$^+$, 6), 404 ([M + 1]$^+$, 2), 133 (Cs^+, 100), the observed ratio of peaks 538:536 is equal to ^{18}O:^{16}O = 84:16.

Preparation of (Thiophosphono)acetaldehyde (SP-Ald) 9 from Dichloride 4. To a solution of (ethoxyvinyl)thiophosphonic dichloride (**4**) (300 mg, 1.47 mmol) in aqueous acetonitrile (1:1, 50 mL) was added a solution of sodium hydroxide (0.1 g, 4 mmol) in distilled, deionized water (10 mL). The resulting solution was then titrated with 1 N sodium hydroxide until a pH of 7.5 was maintained for 15 min. Acetonitrile was removed in vacuo, and the remaining aqueous solution was added to Dowex 50 (H^+ form) resin (ca. 3 mL) at 4 °C. The solution was then swirled for ca. 1 min, filtered through glass wool, and allowed to stand at 4 °C for 18 h until hydrolysis was observed to be complete by ^{31}P NMR analysis. The pH of the solution was then adjusted to 8.0 with NaOH (0.1 N), and the water was removed in vacuo to give 160 mg (59%) of a white solid observed to be a mixture of (thiophosphono)-acetaldehyde (SP-Ald, **9**) and phosphonoacetaldehyde (P-Ald) (3:1): ^1H NMR (D_2O) 3.25 (dd, J = 4.0, 17.7 Hz, 2 H), 9.69 (t, J = 4.0 Hz); ^{13}C NMR (D_2O) 59.3 (d, J = 75.0 Hz, CH_2P), 206.9 (CHO); ^{31}P NMR (D_2O, pH 8.0) 43.8 (t, J = 17.7 Hz). A solution (4 mL) of the crude (thiophosphono)acetaldehyde **9** (12 mM) in a K^+HEPES (100 mM, pH 8.0), and $MgCl_2$ (10 mM) buffer was treated with phosphonatase (1.0 U). The reaction was monitored by ^{31}P NMR spectroscopy and stopped when all contaminating P-Ald had been converted to phosphate. The solution was then filtered through an Amicon dialysis membrane, concentrated to 0.5 mL, and chromatographed on a Sephadex G-10 column (200 × 1 cm). The column was eluted with K^+HEPES buffer (10 mM, pH 8.0) at 4 °C, and the eluent was collected in 1-mL fractions. The fractions were assayed by adding an aliquot (50 μL) from each to a solution (2 mL) of semicarbazide hydrochloride (1%) in sodium acetate (2%) and monitoring the change in absorbance at 253 nm. The fractions testing positive for aldehyde were pooled and then concentrated in vacuo to give a solid, which was characterized by ^{31}P NMR spectroscopy to contain **9** (43 ppm) as the only phosphorus-containing species.

Preparation of (Thiophosphono)acetaldehyde (SP-Ald) 9 from the Phosphonamidates 8A and 8B. The cesium *N*-(1-phenylethyl)(ethoxyvinyl)thiophosphonamidates **8A** and **8B** (39 mg, 0.095 mmol) were independently dissolved in anhydrous THF (200 μL) containing 384 μL of 1.5 M *p*-toluenesulfonic acid (0.576 mmol) in H_2O. The mixtures were stirred at 25 °C for 3.5 h, followed by addition of 2 M HEPES (0.4 mL) and D_2O (1.0 mL). The pH of the resulting mixture was adjusted to 8 with potassium hydroxide. ^{31}P NMR analysis showed that this mixture consisted of (thiophosphono)acetaldehyde (SP-Ald, **9**) (41.6 μmol) and phosphonoacetaldehyde (P-Ald) (12.2 μmol).

Preparation of (S_P)- and (R_P)-[^{17}O,^{18}O](Thiophosphono)acetaldehydes ((S_P)-1 and (R_P)-1). The (S_P,S_C)- and (R_P,S_C)-[^{18}O] diastereomers of cesium *N*-(1-phenylethyl)(ethoxyvinyl)thiophosphonamidates [^{18}O]-**8B** and [^{18}O]-**8A** (29 mg, 0.071 mmol) were converted to (S_P)-**1** and (R_P)-**1** by using the procedure described for the preparation of SP-Ald from **8A** and **8B** and 48.6% $H_2{}^{17}O$.

Synthesis of (R_P,S_C)- and (S_P,S_C)-*S*-(9-Anthracenylmethyl) *N*-(1-Phenylethyl)(ethoxyvinyl)thiophosphonamidates (11A and 11B). Solutions of thiophosphonamidates **8A** and **8B** (0.061 g, 0.15 mmol) and 9-(chloromethyl)anthracene (0.035 g, 0.15 mmol) in anhydrous acetonitrile (4 mL) were independently stirred at 25 °C for 2 h. The mixtures were then poured into 20 mL of water and extracted with chloroform. The chloroform layers were dried and concentrated, giving residues which were subjected to flash chromatography on silica gel (50% hexane–ethyl acetate) to yield pure (R_P,S_C)- and (S_P,S_C)-*S*-(9-anthracenylmethyl) *N*-(1-phenylethyl)(ethoxyvinyl)thiophosphonamidates **11A** (66%, mp 153 °C) and **11B** (85%, mp 180–181 °C). Crystals of **11B** suitable for X-ray analysis were obtained by slow evaporation of an acetone–cyclohexane solution at 25 °C.

11A: IR ($CDCl_3$) 3360, 3020, 2940, 1595, 1580 cm^{-1}; ^1H NMR ($CDCl_3$) 1.25 (t, J = 7.1 Hz, CH_3, 3 H), 1.51 (d, J = 3.4 Hz, CH_3, 3 H), 3.22 (dd, J = 9.7, 9.7 Hz, NH, 1 H), 3.70 (q, J = 7.1 Hz, CH_2O, 2 H), 4.52–4.74 (m, PhCH, 1 H), 4.79 (dd, J = 13.5, 16.1 Hz, PCH, 1 H), 4.97 (dd, J = 8.3, 16.2 Hz, $PSCHH_A$, 1 H), 5.03 (dd, J = 8.1, 16.1 Hz, $PSCHH_B$, 1 H), 7.22–7.34 (m, phenyl H, 5 H), 7.37–8.39 (m, anthracene H, 9 H); ^{13}C NMR ($CDCl_3$) 14.3 (CH_3), 25.6 (d, J = 5.3 Hz, CH_3CNP), 27.3 (CH_2S), 51.0 (CHPh), 66.3 (CH_2O), 94.5 (d, J = 146.9 Hz, CHP), 124.3, 125.0, 126.0, 126.3, 127.1, 127.7, 128.5, 129.0 (aromatic CH), 128.7, 129.8, 131.5, 145.0 (aromatic C), 163.2 (d, J = 19.6 Hz, CHCP); ^{31}P NMR ($CDCl_3$) 35.8; EIMS m/e (relative intensity) 461 (M^+, 0.4), 206 (33), 191 (56), 165 (22), 120 (23), 106 (100), 77 (91); HRMS m/e 461.1578 (M^+, calcd for $C_{27}H_{28}NO_2PS$ 461.1578).

11B: IR ($CDCl_3$) 3350, 3015, 2940, 1600, 1580, 1185 cm^{-1}; ^1H NMR ($CDCl_3$) 1.24 (t, J = 7.1 Hz, CH_3, 3 H), 1.50 (d, J = 6.8 Hz, CH_3, 3 H), 3.09 (dd, J = 9.1, 9.1 Hz, NH, 1 H), 3.68 (q, J = 7.1 Hz, CH_2O, 2 H), 4.53–4.61 (m, PhCH, 1 H), 4.81 (dd, J = 13.7, 15.8 Hz, PCH, 1 H), 4.99 (d, J = 8.4 Hz, CH_2SP, 2 H), 7.18 (dd, J = 12.8, 13.7 Hz, CHCP, 1 H), 7.22–7.30 (m, phenyl H, 5 H), 7.41–8.38 (m, anthracene H, 9 H); ^{13}C NMR ($CDCl_3$) 14.3 (CH_3), 25.4 (d, J = 3.5 Hz, CH_3CNP), 27.5 (CH_2S), 51.2 (CHPh), 66.4 (CH_2O), 95.2 (d, J = 146.5 Hz, CHP), 124.4, 125.1, 126.1, 126.2, 127.2, 127.7, 128.6, 129.1 (aromatic CH), 128.8, 129.9, 131.6 (aromatic C), 144.9 (d, J = 5.2 Hz, aromatic C), 163.1 (d, J = 19.5 Hz, CHCP); ^{31}P NMR ($CDCl_3$) 36.4; EIMS m/e (relative intensity) 461 (M^+, 2), 191 (100), 165 (20), 120 (20), 106 (69), 77 (24); HRMS m/e 461.0774 (M^+, calcd for $C_{27}H_{28}NO_2PS$ 461.1578).

X-ray Crystal Structure Determination of (S_P,S_C)-*S*-(9-Anthracenylmethyl) *N*-(1-Phenylethyl)(ethoxyvinyl)thiophosphonamidate (11B). Cell parameter and X-ray intensity measurements were performed by Molecular Structure Corp. on a Rigaku AFC5R diffractometer with Cu radiation (graphite monochromator, 12-kW rotating anode generator, Cu Kα λ = 1.5418 Å). Platelike crystal, 0.07 × 0.1 × 0.2 mm; cell parameters and orientation matrix from 25 automatically centered reflections with 41.1 < θ < 50.7°. Monoclinic space group $P2_1$; a = 5.137 (6), b = 20.370 (7), c = 11.624 (4) Å, β = 94.77 (5)°, V = 1212 Å^3, ρ_{calcd} = 1.264 g cm^{-3} for $C_{27}H_{28}NO_2PS$ (mol wt = 461) and Z = 2. F_{000} = 488 e; 2θ–θ scans, θ range = 1.21 + 0.3 tan θ, θ_{max} = 115.2°, θ scan speed = 32 deg min^{-1}; weak reflections ($I < 10\sigma(I)$) rescanned twice, stationary background counts taken on each side of a reflection, 2:1 peak:background counting time, μ = 19.7 cm^{-1}. Azimuthal scan empirical absorption correction, transmission factor range 0.86–1.00; three standard intensities measured at 150 reflection intervals remained constant, 1958 total data, 1740 unique, R_{int} = 0.016, 1640 data with $I > 3\sigma(I)$. All crystallographic calculations were performed with the TEXSAN program system[34] at the University of Maryland on Digital Equipment Corp. MicroVax II computers. Structure solved with MITHRIL[35] link incorporated in TEXSAN; full-matrix least-squares refinement, $\sum(\sigma^2(F_o)(F_o - F_c)^2$ minimized. Anisotropic temperature factors for C, N, O, P, S, and H atoms were included at calculated positions and isotopic TFs were refined, although data with $I < 3\sigma(I)$ were excluded from refinement. Final R, R_w, goodness-of-fit, and min and max $\Delta\rho$ were 0.035, 0.045, 1.62, –0.23, and 0.31 e Å^{-3}; atomic scattering factors (supplied by TEXSAN system) were from the International Tables for X-ray Crystallography.[36] Tables of atomic coordinates and bond lengths and angles are given as supplementary material. The PLOTMD program[37] was used to display the ORTEP[38] drawing of the structure (Figure 1) on a VaxStation II monitor, label the drawing of the structure, and prepare a print file for a Hewlet-Packard Laser-Jet II printer.

Synthesis of [^{18}O]-2-(Trimethylsilyl)ethanol (12). To a solution of mercuric acetate (3.99 g, 12.5 mmol) in $H_2{}^{18}O$ (10 g) and anhydrous THF (10 mL) was added trimethylvinylsilane (1.254 g, 12.5 mmol). The resulting solution was stirred for 30 min at 25 °C. To the mixture was added 3 M NaOH (10 mL) followed by 0.5 M $NaBH_4$/3 M NaOH (10 mL). Stirring was continued for 15 min. The aqueous phase was separated, saturated with K_2CO_3, and then extracted with THF (3 × 10 mL). The combined organic layers were dried over K_2CO_3, filtered, and fractionally distilled to give 1.23 g of [^{18}O]-2-(trimethylsilyl)ethanol (**12**) (82%, bp 71–73 °C, 35 mmHg): ^1H NMR ($CDCl_3$) 0.39 (s, $SiCH_3$, 9 H), 0.90–0.98 (m, CH_2Si, 2 H), 3.72 (t, J = 8.5 Hz, OCH_2, 2 H); ^{13}C NMR ($CDCl_3$) –1.5 ($SiCH_3$), 22.0 (CH_2Si), 59.80, 59.82 ($^{18}OCH_2$:$^{16}OCH_2$ = 87.4:12.6); GCMS (CI) m/e (relative intensity) 101 (M – $H_2{}^{18}O$ + 1, 100), 93 (M – 28 + 1, 56), 73 (33), the observed ratio of peaks 93 vs 91 is equal to $(CH_3)_3Si^{18}OH$:$(CH_3)_3Si^{16}OH$ = 88:12.

Preparation of 3,4(*S*)-Dimethyl-5(*S*)-phenyl-1,3,2-oxazaphospholidin-2-one Derivatives (R_P)- and (S_P)-[^{18}O](Thiophosphono)acetaldehydes 13A and 13B. To solutions of thiophosphonamidates **8A** and **8B** (15 mg, 0.037 mmol) in anhydrous THF (0.075 mL) was added 1.5 M *p*-toluenesulfonic acid in 50% $H_2{}^{18}O$ (0.15 mL, 0.225 mmol). The mixtures were stirred for 3.5 h at 25 °C, adjusted to pH 8 with 10 N KOH, and concentrated in vacuo to give white solids. To these were added *cis*-2-chloro-3,4(*S*)-dimethyl-5(*S*)-phenyl-1,3,2-oxazaphospholidin-2-one (**14**) (9 mg, 0.037 mmol) and 18-crown-6 (21 mg, 0.080 mmol) in anhydrous CH_3CN (2 mL). The mixtures were stirred at 25 °C for 12 h and then concentrated in vacuo. The residues were directly subjected to stereochemical analysis by ^{31}P NMR spectroscopy (Figures 2 and 3) to determine the compositions of the mixtures of isotopomeric and diastereomeric anhydrides **15–20** and, thus, the enantiomeric purities of the [^{18}O](thiophosphono)acetaldehyde products.

From **8A**. **15**: ^{31}P NMR ($CDCl_3$) 62.3 (d, J_{PP} = 30.9 Hz, ^{18}O shift = 6.0 Hz), 8.74 (d, J_{PP} = 30.5 Hz, ^{18}O shift = 3.0 Hz). **16**: ^{31}P NMR ($CDCl_3$) 61.8 (d, J_{PP} = 30.6 Hz, ^{18}O shift = 7.0 Hz), 8.94 (d, J_{PP} = 30.6 Hz). From **8B**. **15**: ^{31}P NMR ($CDCl_3$) 62.3 (d, J_{PP} = 29.9 Hz, ^{18}O shift = 7.0 Hz), 8.74 (d, J_{PP} = 29.9 Hz). **16**: ^{31}P NMR ($CDCl_3$) 61.9 (d, J_{PP} = 30.5 Hz, ^{18}O shift = 6.4 Hz), 8.90 (d, J_{PP} = 30.2 Hz, ^{18}O shift = 2.9 Hz).

Phosphonatase Stereochemistry. Enzymatic Conversion of the [^{17}O,^{18}O](Thiophosphono)acetaldehydes (R_P)-1 and (S_P)-1 to [γ-^{17}O,^{18}O]ATPγS. A solution of phosphonatase (20 U) was concentrated in an Amicon concentrator to 2 mL and then dialyzed in a collodian bag against 500 mL of buffer (100 mM HEPES, 10 mM $MgCl_2$, 0.1 mM DTT, pH 8.0) for 12 h. Freshly prepared enantiomers of [^{17}O,^{18}O]-(thiophosphono)acetaldehyde [(R_P)-**1** and (S_P)-**1**] (ca. 60–70 μmol) were each incubated separately in solutions (3.0 mL, pH 8.0) containing 75 mM HEPES, 15 mM DTT, 1.5 mM EDTA, 30 mM $MgCl_2$, 45 mM ADP, 45 mM glyceraldehyde 3-phosphate, 0.75 mM NAD, 75 mM pyruvate, lactate dehydrogenase (400 U), glyceraldehyde-3-phosphate dehydrogenase (2000 U), and 3-phosphoglycerate kinase (2000 U). The nonchiral thiophosphate contaminant in each of the starting substrates was converted to ATPγS in 15 min. The ^{31}P NMR spectra of the mixture showed that ca. 8 μmol formed in each case. To each of the resulting mixtures was then added phosphonatase, bringing the total volume to 4.5 mL, pH 8.0. ADP (67.5 μmol), glyceraldehyde 3-phosphate (67.5 μmol), and $MgCl_2$ (45 μmol) were added each hour. The reactions were monitored by ^{31}P NMR spectroscopy and stopped after 6 h. The reaction mixtures were then separately loaded on a DEAE-Sephadex A-25 column eluted with a triethylammonium bicarbonate

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gradient (TEAB, 0.15-0.7 M, 1 L each) to give samples of the pure ATPγS (ca. 20-50 μmol), which were then transformed into [β-^{17}O,^{18}O]ATPβS as described below.

Enzymatic Conversion of ATPγS to ADPβS. The purified ATPγS (40-45 μmol) samples (see above) were converted individually to ADPβS samples by reaction with myokinase (750-1000 U) in solutions containing 50 mM tris(hydroxymethyl)aminomethane (Tris), 10 mM $MgCl_2$, 1 mM DTT, and 75 mM AMP (1.5-3.0 mL, pH 8.0). The progress of each reaction was monitored by ^{31}P NMR spectroscopy. After 15 h at 25 °C, the reactions were determined to be ca. 75% complete. The product, ADPβS (15-35 μmol), was purified by DEAE-Sephadex A-25 column chromatography (0.1-0.4 M TEAB, pH 8.0, 500 mL each). The purified ADPβS samples were then converted to ATPβS as described below.

Stereoselective Conversion of [β-^{17}O,^{18}O]ADPβS to (S_P)-[β-^{17}O,^{18}O]-ATPβS. The [β-^{17}O,^{18}O]ADPβS (15-35 μmol) samples from the above reactions were converted to (S_P)-[β-^{17}O,^{18}O]ATPβS samples by reaction in solutions containing 10 mM ADPβS, 40 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 380 mM KCl, 0.8 mM DTT, 15 mM PEP, and pyruvate kinase (250 U/mL). The progress of each reaction was monitored by ^{31}P NMR spectroscopy and found to be complete in 15 h. The reactions were terminated by passing the mixtures through Chelex 100 (2 mL, pH 8.0), washing with deionized water (8 mL). The solutions were concentrated in vacuo, and the residues obtained were dissolved in D_2O (0.8 mL) containing 50 mM EDTA, 10 mM DTT, and 200 mM Tris (pH 8.0). Carbon tetrachloride (0.25 mL) was added to each solution to precipitate residual enzyme. The aqueous layers were subjected to ^{31}P NMR analysis. The spectra of these substances were obtained on a Bruker AM 400 instrument at 160 MHz with a deuterium field lock; spectral width 7936 Hz, acquisition time 4.19 s, pulse width 3 μs, relaxation delay 0.5 s, number of transients, 5000-11 300 (see Figures 4 and 5 for plots of the $^{31}P_\beta$ regions of these spectra).

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Supplementary Material Available: Tables of X-ray crystallographic data for **11B**, including fractional coordinates, bond lengths and angles, and anisotropic parameters (6 pages); table of observed and calculated structure factors (12 pages). Ordering information is given on any current masthead page.

Dimethylallyltryptophan Synthase. An Enzyme-Catalyzed Electrophilic Aromatic Substitution

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Abstract: Dimethylallyltryptophan (DMAT) synthase catalyzes the alkylation of L-tryptophan at C(4) by dimethylallyl diphosphate (DMAPP) in the first pathway-specific step in the biosynthesis of ergot alkaloids. The mechanism of the reaction was studied with analogs of both substrates. Five 7-substituted derivatives of *N*-acetyltryptophan (**2**, Z = OCH_3, CH_3, F, CF_3, and NO_2) were synthesized. The L enantiomers of the free amino acids were obtained by selective hydrolysis of the racemate using aminoacylase from *Aspergillus*. In addition, the *E* and *Z* fluoromethyl and difluoromethyl analogs of DMAPP (**1**, Y = CH_3, CH_2F, CHF_2) were prepared. Rates of the enzyme-catalyzed reactions were measured for the dimethylallyl derivatives with L-tryptophan and for the L-tryptophan derivatives with DMAPP. In addition, the relative reactivities of the methanesulfonate derivatives of the DMAPP analogs were determined for solvolysis in aqueous acetone. A Hammett plot for the tryptophan analogs gave a good linear correlation with $\rho = -2.0$. In addition, a Hammett plot of the logarithms of the relative rates of solvolysis and enzyme-catalyzed alkylation gave a positive linear correlation. These results indicate that the prenyl-transfer reaction catalyzed by DMAT synthase is an electrophilic aromatic substitution and is mechanistically similar to the electrophilic alkylation catalyzed by farnesyl diphosphate synthase.

Prenyltransferases comprise a family of enzymes that catalyze the alkylation of electron-rich acceptors by the hydrocarbon moieties of allylic isoprene diphosphates.[1] These reactions are the major building steps in isoprene metabolism and lead to a number of essential metabolites, including sterols,[2] dolichols,[3] ubiquinones,[4] and prenylated proteins.[5] A variety of functional groups, such as carbon-carbon double bonds,[1,2] aromatic rings,[4,6,7] alcohols,[8] amines,[9] and mercaptans,[5] can serve as prenyl acceptors. Although several different mechanisms have been proposed for various prenyl-transfer reactions, all of the transformations can be explained as electrophilic alkylations of the acceptors by the allylic diphosphates.[1,10]

Farnesyl diphosphate synthase is the only prenyltransferase for which questions concerning the mechanism of bond formation have been extensively explored. Poulter and Rilling[11] discovered that the enzyme catalyzes hydrolysis of its allylic substrate by a direct displacement at carbon, and linear free energy studies with alternate substrates indicate that there is substantial development of positive charge in the allylic moiety during prenyl transfer.[12] Experiments with bisubstrate analogs of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) suggest that the enzyme catalyzes a stepwise reaction via carbocationic intermediates.[13]

Dimethylallyltryptophan synthase catalyzes alkylation of C(4) in L-tryptophan (**2**-H) by DMAPP (**1**-CH_3,OPP).[7,14] This is the

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