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Supplementary Material Available: ^{13}C NMR spectra and high-resolution FAB-MS data for 3–5 (3 pages). Ordering information is given on any current masthead page.

Evidence for an Intramolecular, Stepwise Reaction Pathway for PEP Phosphomutase Catalyzed P–C Bond Formation

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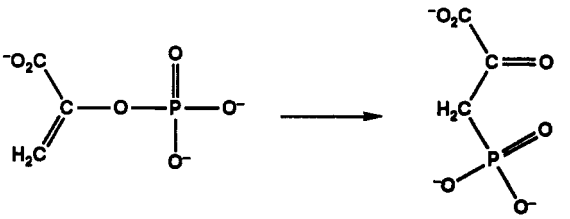
The *Tetrahymena pyriformis* enzyme, phosphoenolpyruvate phosphomutase, catalyzes the rearrangement of phosphoenolpyruvate to the P–C bond containing metabolite, phosphonopyruvate. To distinguish between an intra- and intermolecular reaction pathway for this process an equimolar mixture of [P-^{18}O,C(2)-^{18}O]thiophosphonopyruvate and (all ^{16}O) thiophosphonopyruvate was reacted with the phosphomutase, and the resulting products were analyzed by ^{31}P NMR. The absence of the cross-over product [C(2)-^{18}O]thiophosphonoenolpyruvate in the product mixture was interpreted as evidence for an intramolecular reaction pathway. To distinguish between a concerted and stepwise intramolecular reaction pathway the pure enantiomers of the chiral substrate [^{18}O]-thiophosphonopyruvate were prepared and the stereochemical course of their conversion to chiral [^{18}O]thiophosphoenolpyruvate was determined. The assignments of the phosphorus configurations in the [^{18}O]thiophosphonopyruvate enantiomers reported earlier (McQueney, M. S.; Lee, S.-l.; Bowman, E.; Mariano, P. S.; Dunaway-Mariano, D. *J. Am. Chem. Soc.* **1989**, *111*, 6885–6887) were revised according to the finding that introduction of the ^{18}O label into the thiophosphonopyruvate precursor occurs with retention rather than with (the previously assumed) inversion of configuration. On the basis the observed conversion of (S_p)-[^{18}O]thiophosphonopyruvate to (S_p)-[^{18}O]thiophosphoenolpyruvate and (R_p)-[^{18}O]thiophosphonopyruvate to (R_p)-[^{18}O]thiophosphoenolpyruvate, it was concluded that the PEP phosphomutase reaction proceeds with retention of the phosphorus configuration and therefore by a stepwise mechanism. Lastly, the similar reactivity of the oxo- and thio-substituted phosphonopyruvate substrates (i.e., nearly equal V_{max}) was interpreted to suggest that nucleophilic addition to the phosphorus atom is not rate limiting among the reaction steps.

Introduction

The chemistry of P–C bond formation in biological systems has eluded researchers since the discovery of the first phosphonate natural product over 30 years ago.[1] Laboratory chemical synthesis of P–C linkages typically involve the addition of a nucleophilic phosphorus reactant to a carbon electrophile. If such a strategy were to be used in a biological system, precursors containing phosphorus in the +3 oxidation state would be required. An alternate mode of P–C bond formation might rely on the activation of a carbon acid for nucleophilic addition to the phosphorus atom of a phosphate ester or anhydride. While this latter approach is analogous to the phosphoryl transfer strategy employed in the biosynthesis of organophosphates,[2] the low acidity of carbon acids (as opposed to oxy acids) and the comparatively high energy of the P–C vs P–O bond[3] would pose a particular challenge to the protein catalyst.

Recently, the P–C bond forming enzyme phosphoenolpyruvate (PEP)[4] phosphomutase was isolated in our laboratory from the protozoan, *Tetrahymena pyriformis*.[5] This enzyme catalyzes the rearrangement of PEP to phosphonopyruvate, a reaction which serves as a major entry step into the phosphonate class of natural products.[6,7]



The present study examines the chemical mechanism of this enzymic reaction in the thermodynamically favored,

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(2) For a review, see: Knowles, J. R. *Annu. Rev. Biochem.* **1980**, *49*, 877.

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(4) Abbreviations: NAD^+, nicotinamide adenine dinucleotide; NADH, dihydronicotineamide adenine dinucleotide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; ATPβS, adenosine 5′-(1-thiotriphosphate); ATPβS, adenosine 5′-(2-thiotriphosphate); ADPβS, adenosine 5′-(2-thiodiphosphate); EDTA (ethylenedinitrilo)tetraacetic acid; *p*-TsOH, *p*-toluenesulfonic acid; TEAB, triethylamine bicarbonate; Tris, tris(hydroxymethyl)aminomethane; THF, tetrahydrofuran; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance.

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phosphonopyruvate-forming direction. Herein, we report the results of an oxygen-18 cross-labeling experiment which demonstrates that the rearrangement occurs intramolecularly. In addition, we correct our earlier communication[8] on the stereochemical course of the reaction from inversion to retention of configuration at phosphorus and thus alter our conclusion of a concerted reaction to a stepwise process.

Experimental Methods

General. PEP phosphomutase was prepared according to the method of Bowman et al.[5] Phosphonopyruvate was prepared from phosphonoalanine according to the method of Anderson et al.[9] Ninety-eight percent enriched ^{18}O-labeled water ($H_2{}^{18}O$) was purchased from Cambridge Isotope Laboratories. The enzymes, buffers, and substrates used were purchased from Sigma Chemical Co. Oxalyl chloride, (*S*)-(−)-*N*-(1-phenylethyl)amine, D_2O, and CuI were purchased from Aldrich. Methylphosphonothioic dichloride was purchased from Alpha. All new compounds reported in this paper were judged to be >90% pure by NMR spectroscopic analysis and were isolated as oils unless otherwise specified.

Thiophosphonopyruvate and Racemic [P=^{18}O]Thiophosphonopyruvate. To 80 mg (140 μmol) of the dicesium salt of **13** were added 600 μL (30 mmol) of either H_2O at natural isotope abundance or $H_2{}^{18}O$ (98%) and 24 μL (288 μmol) of 12 N HCl. After 10 s at 25 °C, 400 μL of K^+Hepes (20 mM, pH 8) was added, and the pH of the resulting solution was adjusted to 8 with 1 M KOH. The solutions were concentrated in vacuo. ^{31}P NMR analysis of the residues dissolved in D_2O (pH 8.0) revealed that they contained 84% thiophosphonopyruvate (t, +44.80 ppm, J = 18.2 Hz) and 16% phosphonopyruvate (t, +10.55 ppm, J = 20.4 Hz), or 84% [^{18}O]thiophosphonopyruvate (t, +44.76 ppm, J = 18.2 Hz) and 16% [$^{18}O_2$]phosphonopyruvate (t, +10.50 ppm, J = 20.4 Hz), respectively. Unless stated otherwise the thiophosphonopyruvate preparations were used in the experiments described below without prior removal of the phosphonopyruvate contaminants.

Kinetic Constants for Thiophosphonopyruvate vs Phosphonopyruvate. The initial velocities of PEP phosphomutase catalyzed conversion of phosphonopyruvate to PEP or thiophosphonopyruvate to thiophosphoenolpyruvate were measured at pH 8.0 as a function of the concentration of pure phosphonopyruvate or thiophosphonopyruvate. Thiophosphoenolpyruvate or PEP formation was monitored by using the pyruvate kinase–lactate dehydrogenase coupled assay. All reaction mixtures (25 °C) contained ADP (1 mM), $MgCl_2$ (5 mM), KCl (5 mM), NADH (0.3 mM), pyruvate kinase (75 units), and lactate dehydrogenase (30 units) in K^+Hepes buffer (50 mM, pH 8). The thiophosphonopyruvate K_m = 5 μM and V_m = 16 s^{-1} values and phosphonopyruvate K_m = 20 μM and V_m = 24 s^{-1} values were evaluated from a Lineweaver–Burk plot of the initial velocity data.

[P=^{18}O,C(2)-^{18}O]Thiophosphonopyruvate (2) and [C(2)-^{18}O]Thiophosphonopyruvate (7). Complete ^{18}O exchange at C(2) of either [P=^{18}O]thiophosphonopyruvate (118 μmol) or (all ^{16}O) thiophosphonopyruvate (59 μmol) was accomplished by separately incubating the potassium salts in 250 μL of $H_2{}^{18}O$ (98%) for 24 h at 4 °C.

PEP Phosphomutase Catalyzed Reactions of [P=^{18}O,C(2)-^{18}O]Thiophosphonopyruvate (2) and [C(2)-^{18}O]Thiophosphonopyruvate (7) to the Corresponding Thiophosphoenolpyruvates. [C(2)-^{18}O]Thiophosphonopyruvate (7) (59 μmol) or [P=^{18}O,C(2)-^{18}O]thiophosphonopyruvate (2) (59 μmol) dissolved in 250 μL of $H_2{}^{18}O$ (98%) was added to 7.5 mL of 20 mM K^+Hepes buffer (pH 8) containing 2.5 mM $MgCl_2$ and 5 units of PEP phosphomutase. The resulting solution was incubated for 10 min at 25 °C and then passed through an Amicon filter (DiaFlo, PM10, 10000 MW cut off) under N_2 pressure. The filtrate was concentrated in vacuo, and the residue obtained was dissolved in a D_2O solution containing 20 mM K^+Hepes (pH 8) and 0.2 M EDTA. The ^{31}P NMR spectrum of the mixture obtained from reaction of [C(2)-^{18}O]thiophosphonopyruvate (7) showed a singlet for thiophosphoenolpyruvate (5) (60%) at +39.443 ppm and one for [C(2)-^{18}O]thiophosphoenolpyruvate (3) (40%) at +39.417 ppm. The ^{31}P NMR spectrum of the product mixture obtained from the PEP phosphomutase catalyzed reaction of [P=^{18}O,C(2)-^{18}O]thiophosphonopyruvate (2) under the same conditions as described above showed a singlet at +39.397 ppm for [P=^{18}O]thiophosphoenolpyruvate (4) (60%) and one at +39.368 ppm for [P=^{18}O,C(2)-^{18}O]thiophosphoenolpyruvate (6) (40%).

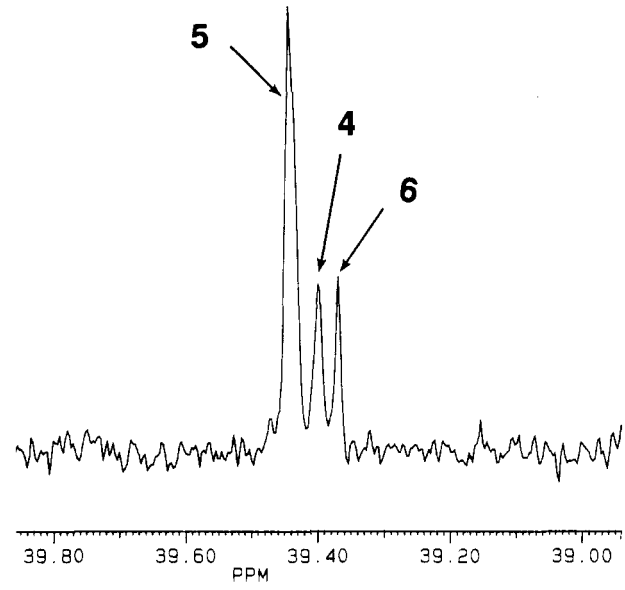

Figure 1. The ^{31}P NMR spectrum of the mixture of isotopically labeled thiophosphoenolpyruvates obtained by PEP phosphomutase catalyzed reaction of a mixture of (all ^{16}O) thiophosphonopyruvate (1) and [P=^{18}O,C(2)-^{18}O]thiophosphonopyruvate (2) (Scheme II). The observed resonances correspond to the thiophosphoenolpyruvate isotopomers **4**, **5**, and **6** as labeled.

PEP Phosphomutase Catalyzed Reaction of an Equimolar Mixture of Thiophosphonopyruvate (1) and [P=^{18}O,C(2)-^{18}O]Thiophosphonopyruvate (2). A mixture of 59 μmol each of (all ^{16}O) thiophosphonopyruvate (1) and [P=^{18}O,C(2)-^{18}O]-thiophosphonopyruvate (2) was reacted at 26 °C for 10 min with 10 units of PEP phosphomutase in 5 mL of 20 mM K^+Hepes (pH 8) containing 2.5 mM $MgCl_2$. The reaction mixture was subjected to the workup procedure described above. The mixture obtained was analyzed by ^{31}P NMR, giving the spectrum displayed in Figure 1.

2-(Trimethylsilyl)ethyl Methylphosphonochloridothioate (9). To a stirred solution of 2-(trimethylsilyl)ethanol (9.78 g, 82.7 mmol) in THF (150 mL) was added, dropwise, *n*-butyllithium (1.2 M, 75.2 mL, 90.2 mmol) at −78 °C. The resulting solution was added to a stirred solution of methylphosphonothioic dichloride **8** (11.2 g, 75.2 mmol) in THF (40 mL) at −78 °C over 1 h. After 7 h at −78 °C, the mixture was warmed to 25 °C and diluted with ether (100 mL) and water (100 mL). The organic layer was separated, washed successively with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was subjected to distillation (80 °C, 0.6 mm) to give 8.43 g (49%) of the silylethyl ester **9**. Spectral data are provided in the supplementary material.

[2-(Trimethylsilyl)ethyl]oxalyl Chloride. To oxalyl chloride (13.6 g, 0.11 mol) was added 2-(trimethylsilyl)ethanol (12.5 g, 0.11 mol), dropwise, with stirring at 0 °C. The mixture was warmed to 25 °C, stirred for 15 h, concentrated in vacuo, and subjected

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to distillation (56 °C, 2.0 mm) to give 13.6 g (62%) of the monoester acid chloride. Spectral data are provided in the supplementary material.

(*S*)-(−)-*N*-Methyl-*N*-(1-phenylethyl)amine (10). To a stirred solution of (*S*)-(−)-*N*-(1-phenylethyl)amine (14.1 g, 0.12 mol) and potassium carbonate (78.8 g, 0.57 mol) in THF (100 mL) was added a solution ethyl chloroformate (18.9 g, 0.18 mol) in THF (30 mL), dropwise, with stirring at 0 °C. After 2 h the reaction mixture was warmed to 25 °C and stirred at this temperature for 6 h. Water (100 mL) was added, and the organic layer was separated and washed with water. The aqueous extracts were washed with ether. The ethereal extracts were dried over anhydrous sodium sulfate and concentrated in vacuo to yield 30.0 g (99%) of the corresponding carbamate. To a stirred mixture of lithium aluminum hydride (4.7 g, 0.12 mol) in THF (100 mL) at 0 °C was added, dropwise, a solution of the carbamate (15.2 g, 79.2 mmol) in THF (40 mL). The solution was warmed to 25 °C and stirred at this temperature for 7 h. Ether (200 mL), water (8 mL), and 0.1 N NaOH (7 mL) were successively added. The mixture was filtered through Celite, and the precipitate was washed with ether (160 mL) and chloroform (80 mL). The filtrate and washings were dried over anhydrous sodium sulfate and concentrated in vacuo, giving a residue which was subjected to distillation (25 °C, 0.01 mm) to yield 9.7 g (91%) of the known[10] phenylethylamine **10**. Spectral data are provided in the supplementary material.

(S_p,S_c)- and (R_p,S_c)-*O*-2-(Trimethylsilyl)ethyl *N*,*P*-Dimethyl-*N*-(1-phenylethyl)phosphonamidothioate (11a and 11b). To a stirred solution of **10** (2.20 g, 16.3 mmol) in THF (100 mL) was added, dropwise, *n*-butyllithium (1.2 M, 11.3 mL, 13.6 mmol) at 78 °C. To this solution was added, dropwise, a solution of **9** (3.13 g, 13.6 mmol) in THF (2 mL). The reaction mixture was stirred for 5 h, after which time ether (20 mL) and water (20 mL) were added. The organic layer was separated, washed successively with water and brine, dried over anhydrous sodium sulfate and concentrated in vacuo. The resulting residue was subjected to flash silica chromatography (4% ether in cyclohexane), affording 3.86 g (86%) of a mixture of the thiophosphonoamidate diastereomers **11a** and **11b**. The diastereomeric mixture was then subjected to HPLC (silica gel, 4% ether in cyclohexane) to give 1.53 g (34%) of the first eluting diastereomer, **11a**, and 1.34 g (30%) of the second eluting diastereomer, **11b**. Spectral data are provided in the supplementary material.

(S_p,S_c)-*S*-2-(4-Nitrophenyl)-2-oxoethyl *N*,*P*-Dimethyl-*N*-(1-phenylethyl)phosphonamidothioate (15). To a stirred solution of **11a** (0.32 g, 0.98 mmol) in acetonitrile (10 mL) was added cesium fluoride (0.74 g, 4.88 mmol). The mixture was stirred at reflux for 5 h. After cooling to 25 °C the solution was filtered through anhydrous sodium sulfate and concentrated in vacuo to yield 0.34 g (88%) of the cesium salt of desilylethylated **11a**. To a solution of this cesium salt (0.13 g, 0.32 mmol) in acetonitrile (4 mL) was added *p*-nitrophenacyl bromide (0.078 g, 0.32 mmol). The solution was stirred at 25 °C for 12 h. Water (1 mL) was added, and the resulting solution was extracted with chloroform. The chloroform extracts were dried over anhydrous sodium sulfate and concentrated in vacuo to yield 0.13 g (93%) of the desired *p*-nitrophenacyl thioester **15** (mp 106–108 °C, benzene). Spectral data, including X-ray data, are provided in the supplementary material.

(S_p,S_c)- and (R_p,S_c)-2-(Trimethylsilyl)ethyl 3-[[Methyl-(1-phenylethyl)amino][2-(trimethylsilyl)ethoxy]phosphinothioyl]-2-oxopropanoate (12a and 12b). To each stirred solution of **11a** or **11b** (0.51 g, 1.56 mmol) in THF (1 mL) was added, dropwise, *n*-butyllithium (1.2 M, 1.4 mL, 1.7 mmol) in hexanes at −78 °C. After 30 min, cuprous iodide (0.32 g, 1.67 mmol) in THF (5 mL) at −78 °C was added to each of the reaction mixtures. The mixtures were warmed to −30 °C over 1 h and stirred at this temperature for 2 h. Solutions of [2-(trimethylsilyl)ethyl]oxalyl chloride (0.49 g, 2.33 mmol) in ether (2 mL) were added dropwise to each solution. The reaction mixtures were stirred at −30 °C for 7 h and then warmed to 25 °C. Water (7 mL) was added, and the resulting solutions were filtered through Celite. The precipitates were washed with methylene chloride and refiltered. The methylene chloride layers of the filtrate were dried over anhydrous sodium sulfate and concentrated in vacuo. The resulting residues were subjected to chromatography on Florisil (25% ether in cyclohexane) yielding 0.32 g (41%) of **12a** and **12b**, respectively. Spectral data are provided in the supplementary material.

(S_p,S_c)- and (R_p,S_c)-3-[[Methyl(1-phenylethyl)amino]-phosphinothioyl]-2-oxopropanoic Acid (13a and 13b). To each stirred solution of **12a** or **12b** (0.32 g, 0.64 mmol) in dry acetonitrile (2 mL) was added a mixture of cesium fluoride (0.20 g, 1.30 mmol) and 18-crown-6 (0.17 g, 0.64 mmol) in acetonitrile (3 mL) under a nitrogen atmosphere. The mixtures were stirred at 25 °C for 24 h. The precipitates were separated by decantation and washed repetitively with dry acetonitrile to remove 18-crown-6. This afforded 0.21 g (56%) of each of the pure deprotected thiophosphonamidate diastereomers, **13a** and **13b**. Spectral data are provided in the supplementary material.

Preparation of the (*R*)- and (*S*)-[^{18}O]Thiophosphonopyruvates (14a and 14b) by Hydrolysis of 13a and 13b in $H_2{}^{18}O$. Compounds **13a** and **13b** (0.134 g, 0.24 mmol) were separately mixed with $H_2{}^{18}O$ (98%) (1.25 mL, 62.5 mmol) and HCl (12 M, 45 μL, 0.54 mmol) and then immediately quenched with 1.3 mL of D_2O containing K_2HPO_4 (80 mM), $MgCl_2$ (5 mM), dithiothreitol (0.8 mM), and enough KOH to bring the pH of the resulting solutions to 8.0. This procedure generated (*R*)-[^{18}O]-thiophosphonopyruvate (**14a**) (0.17 mmol) and [$^{18}O_2$]-phosphonopyruvate (0.07 mmol) from **13a** and (*S*)-[^{18}O]thiophosphonopyruvate **14b** (0.17 mmol) and [$^{18}O_2$]phosphonopyruvate (0.07 mmol) from **13b**. ^{31}P NMR (D_2O): +44.8 ppm (t, J = 18.2 Hz) for **14a** and **14b** and +10.6 ppm (t, J = 20.4 Hz) for the [$^{18}O_2$]phosphonopyruvate. These samples were used without purification.

Preparation and $H_2{}^{18}O$ Hydrolysis of (S_p,S_c)-2-(Trimethylsilyl)ethyl 3-[[Methyl(1-phenylethyl)amino]phosphinothioyl]-2-oxopropanoate (18a). To a stirred mixture of cesium fluoride (0.11 g, 657 μmol) and 18-crown-6 (0.17 g, 657 μmol) in dry acetonitrile (5 mL) was added a solution of **12a** (0.33 g, 657 μmol) in dry acetonitrile (3 mL). The reaction mixture was stirred at 25 °C for 3 h and then centrifuged (14000g × 2 min). The supernatant was concentrated in vacuo to yield a residue containing the desired monodeblocked derivative **18a** (61%) (^{31}P NMR ($CDCl_3$) +53.7 and +62.4 ppm for the keto and enol forms of **18a**), the enol form of unreacted **12a** (25%) (^{31}P NMR ($CDCl_3$) +81.1 ppm), and the O_c-detrimethylsilylethylated derivative (12%) (^{31}P NMR($CDCl_3$) +76.3 ppm). To the mixture containing the **18a** (400 μmol) was added $H_2{}^{18}O$ (98%) (69 μL) and *p*-TsOH (0.122 g, 640 μmmol). After 20 min at 25 °C the mixture was diluted with 100 mL of Hepes buffer (100 mM), and the pH of the resulting solution was adjusted to 8 with KOH solution. ^{31}P NMR (D_2O) analysis the product mixture revealed that it contained 10% of the desired [^{18}O]thiophosphonopyruvate (+44.8 ppm) along with the O_p-trimethylsilylethyl ester of [^{18}O]thiophosphonopyruvate (35%, +64.0 ppm), thiophosphate (34%, +35.6 ppm), and phosphonopyruvate (12%, +10.6 ppm).

(S_p,S_c)- and (R_p,S_c)-2-Propyl 3-[[Methyl(1-phenylethyl)amino][2-(trimethylsilyl)ethoxy]phosphinothioyl]-2-oxopropanoate (16a and 16b). To each stirred solution of **11a** and **11b** (0.20 g, 0.61 mmol) in THF (1 mL) was added, dropwise, *n*-butyllithium (1.2 M, 0.56 mL, 0.67 mmol) in hexanes at −78 °C. After 30 min, cuprous iodide (0.13 g, 0.67 mmol) in THF (5 mL) was added. The mixtures were warmed to −30 °C over 1 h and stirred at this temperature for 2 h. Solutions of the known[11] (2-propyl)oxalyl chloride (0.12 g, 0.8 mmol) in ether (2 mL) were added dropwise to the mixtures, and each was stirred at −30 °C for 7 h and then warmed to 25 °C. Water (7 mL) was added, and the resulting solutions were filtered through Celite. The precipitates were washed with methylene chloride (20 mL), and the filtrates were refiltered. The methylene chloride layers were separated, dried over anhydrous sodium sulfate, and concentrated in vacuo. The resulting residues were subjected to chromatography on Florisil (30% ether in cyclohexane) to yield (40%) in pure form the individual thiophosphonamidate esters **16a** and **16b**. Spectral data are provided in the supplementary material.

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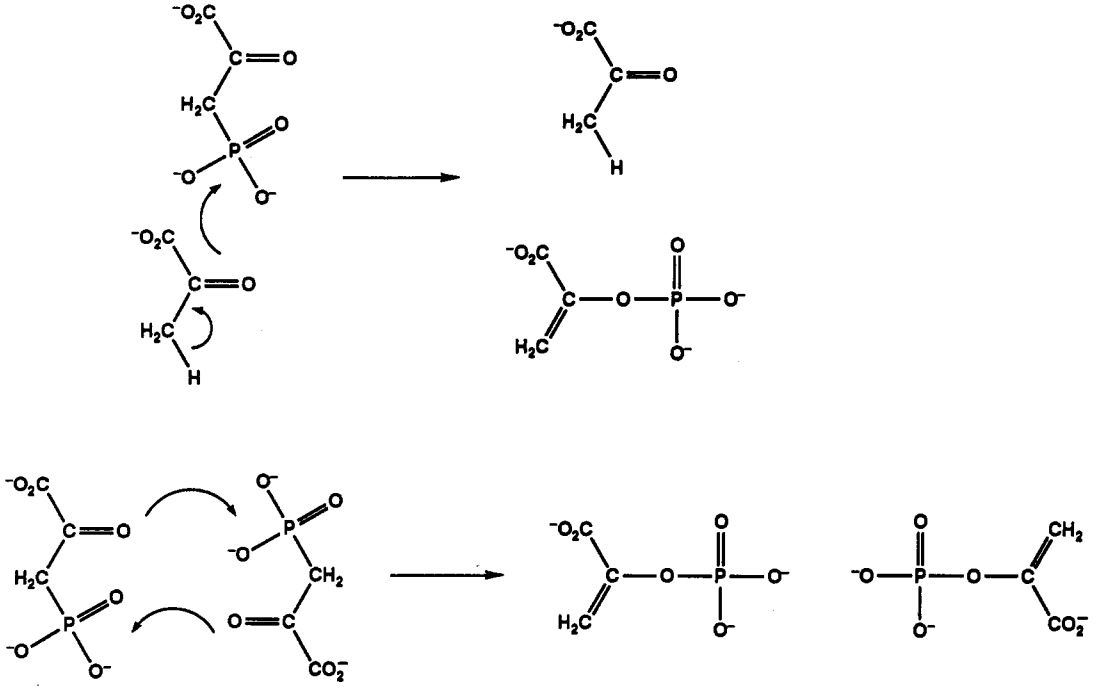
Scheme I. Possible Intermolecular Mechanisms of PEP Mutase Catalysis



(S_p,S_c)- and (R_p,S_c)-2-Propyl 3-[[Methyl(1-phenylethyl)amino]phosphinothioyl]-2-oxopropanoate (17a and 17b). To **16a** and **16b** (90 mg, 0.20 mmol) in dry acetonitrile (2 mL) were added cesium fluoride (32 mg, 0.21 mmol) and 18-crown-6 (54 mg, 0.20 mmol) in dry acetonitrile (3 mL) under a nitrogen atmosphere. The reaction mixtures were stirred for 30 h at 25 °C and concentrated in vacuo to afford the crude esters **17a** and **17b** (85%), respectively. Spectral data are provided in the supplementary material.

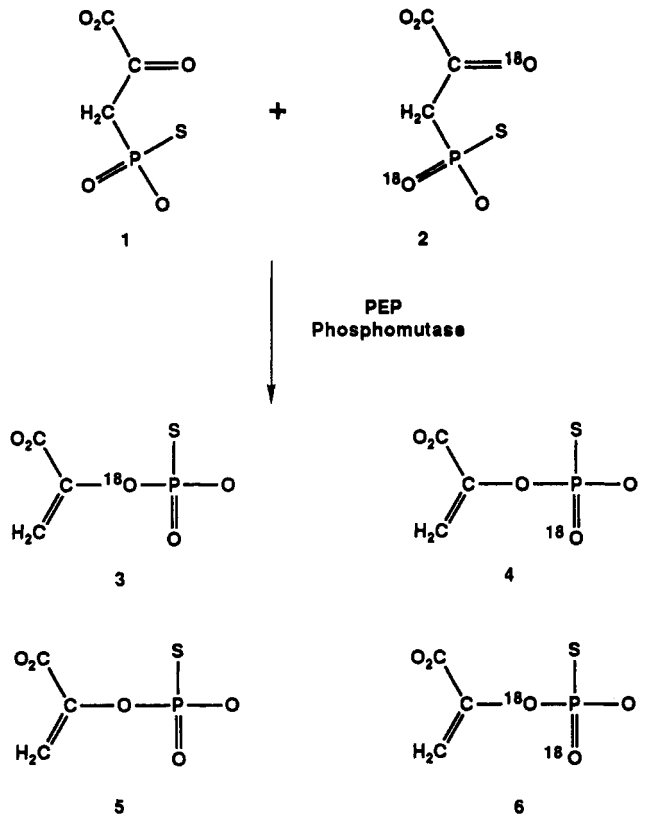
Hydrolysis of 17a and 17b in $H_2{}^{18}O$. The monocesium salts **17a** and **17b** [ca. 0.1 mmol] were separately dissolved in mixtures of $H_2{}^{18}O$ (98%) (250 μL) and THF (25 μL). The resulting solutions were added to 1 mL of Dowex-50 [H^+] resin to which HCl (22 μL, 12 M, 260 μmol) had been added. The mixtures were incubated at 4 °C for 40 min and then diluted with 250 μL of 200 mM HEPES and the pH of the resulting solutions was adjusted to 8. ^{31}P NMR analysis of the product mixtures revealed [^{18}O]-thiophosphonopyruvate in an ca. 10% yield. These mixtures were used in subsequent experiments without purification.

PEP Phosphomutase Catalyzed Conversion of Chiral [^{18}O]Thiophosphonopyruvate to Chiral [^{18}O]Thiophosphoenolpyruvate and Then to [^{18}O]ATPβS. The (*S*)- and (*R*)-[^{18}O]thiophosphonopyruvates (**14a** and **14b**), derived from the $H_2{}^{18}O$ hydrolysis of either **13a** and **13b** (170 μmol) or **17a** and **17b** (17 μmol), were separately reacted at 25 °C with 1.8 units of PEP phosphomutase in a 5-mL solution of 5 mM $MgCl_2$, 0.8 mM dithiothreitol, and 50 mM K^+Hepes (pH 8.0) in 50% D_2O. The formation of [^{18}O]thiophosphoenolpyruvate was complete within 7 h. In the case of the catalyzed reaction of the [^{18}O]-thiophosphonopyruvate generated from the $H_2{}^{18}O$ hydrolysis of **18a**, $MgCl_2$ (to a final concentration of 2.5 mM) and PEP phosphomutase (0.08 units) were added directly to the buffered hydrolysate. The ^{31}P NMR (D_2O, pH 8) analysis of the resulting reaction mixtures revealed the product [^{18}O]thiophosphoenolpyruvate (+39.4 ppm). To these mixtures were added 1 equiv of MgADP and 250–2000 units of pyruvate kinase in 1 mL of K^+HEPES (50 mM, pH 8.0) at 25 °C. The [γ-^{18}O]ATPγS (formed within 12 h) was purified from the reaction mixture by chromatography on a (2 × 40 cm) DEAE Sephadex A-25 column with 1.5 L of a linear gradient of TEAB, 0.15 M to 0.70 M at pH 8.1 serving as the eluant. Fractions containing [γ-^{18}O]ATPγS were pooled and concentrated in vacuo. The residue was dissolved in 10 mL of water and concentrated. This process was repeated four times in order to remove residual TEAB. The yield of the [γ-^{18}O]ATPγS was estimated by ^{31}P NMR to be ca. 70%. The [γ-^{18}O-P]ATPγS (15–110 μmol) was converted to [β-^{18}O]ADPβS by reaction with myokinase (500 units) in a 5-mL solution containing 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 40–250 μmol of AMP, and 50 mM Tris-HCl (pH 8.0). The progress of the reaction was monitored by ^{31}P NMR. After 10 h at 25 °C the reaction was ca. 80% complete. The [β-^{18}O]ADPβS was purified from the reaction mixture by chromatography on a DEAE Sephadex A-25 column (1.5 × 35 cm) equilibrated with 0.10 M TEAB (pH 8.1). The column was eluted with a 1.5 L linear gradient of TEAB (0.10 M to 0.4 M). Fractions containing [β-^{18}O]ADPβS were pooled and concentrated in vacuo. Residual TEAB was removed by dissolving the residue in 10 mL of water followed by concentration in vacuo. The yield of [β-^{18}O]ADPβS was estimated by ^{31}P NMR analysis to be ca. 60%. The [β-^{18}O]ADPβS (7–70 μmol) was converted to (S_p)-[β-^{18}O]ATPβS by incubation in a 5-mL (50% D_2O, 25 °C) solution containing $MgCl_2$ (4 mM), KCl (380 mM), dithiothreitol (0.8 mM), Tris-HCl (40 mM, pH 8), PEP (10–70 μmol), and 250 units of pyruvate kinase. The reaction, monitored by ^{31}P NMR, and was found to be complete in 12 h. The reaction mixture was concentrated in vacuo, and the residue obtained was dissolved in 0.5 mL of D_2O containing EDTA (0.2 M) and Tris-HCl (1 M, pH 8.0) and subjected to ^{31}P NMR analysis. The spectrum of this substance was obtained on a Bruker AM 400 instrument at 160 MHz with a deuterium field lock; spectral width 11363 Hz; acquisition time 1.44 s; pulse width 8 μs; relaxation delay 5 s; number of transients, 3000.

Results and Discussion

The present work was carried out for the purpose of defining the chemical pathway of PEP phosphomutase catalysis. For convenience, the phosphomutase reaction was studied in the thermodynamically favored, PEP-forming direction as opposed to the physiological, phosphonopyruvate forming direction. First, we set out to determine whether the reaction proceeds intramolecularly or intermolecularly. Two intermolecular mechanisms considered are illustrated in Scheme I. One involves phosphoryl transfer from phosphonopyruvate to enzyme-bound pyruvate while the second requires phosphoryl exchange between two phosphonopyruvate molecules bound in a head-to-tail arrangement. Both pathways involve transfer of a phosphoryl group from phosphonopyruvate to the pyruvate unit of a second reactant molecule. We hoped to distinguish this process from the intramolecular phosphoryl transfer reaction by ^{18}O-labeling

Scheme II. Test for an Intramolecular vs Intermolecular Reaction Pathway



1 2 3 4 5 6

both the phosphoryl group and the C(2)-carbonyl in one reactant and reacting it with PEP phosphomutase in the presence of a second unlabeled reactant. ^{18}O-Labeling at the phosphoryl moiety but not at the C(2)-carbonyl (or vice versa) of the product, PEP, would signify an intermolecular reaction while the absence of cross-labeled product would evidence an intramolecular process.

The actual experiment as illustrated in Scheme II was carried out with an equimolar mixture of (all ^{16}O) thiophosphonopyruvate (1) and thiophosphonopyruvate (2) labeled with one ^{18}O at phosphorus and one ^{18}O at C(2). Thiophosphonopyruvate was used in place of phosphonopyruvate to simplify analysis of the ^{18}O-labeled product by ^{31}P NMR.[12] Initial velocity studies of the substrate activity of thiophosphonopyruvate demonstrated that it is turned over at 75% the rate of phosphonopyruvate and that it has a K_m (5 μM) which is 4-fold smaller than that of the phosphonopyruvate (20 μM). Thus, its use as an alternate substrate is well justified.

Chemical shift standards for product analysis were obtained from separate reactions of [P=^{18}O,C(2)-^{18}O]thiophosphonopyruvate (2) and [C(2)-^{18}O]thiophosphonopyruvate (7) with PEP phosphomutase in H_2O. Within the time frame required to complete the phosphomutase-catalyzed reaction of 7 and 2, 60% of the ^{18}O label at the C(2)-carbonyl had been exchanged with ^{16}O from solvent H_2O. Hence, a 4:6 mixture of [P=^{18}O,C(2)-^{18}O]-thiophosphoenolpyruvate (6) and [P=^{18}O]thiophosphoenolpyruvate (4) was generated from 2, and a 4:6 mixture of [C(2)-^{18}O]thiophosphoenolpyruvate (3) and (all ^{16}O) thiophosphoenolpyruvate (5) were formed from 7. Importantly, the ^{31}P NMR resonances from these products were well-resolved, revealing a 0.026 ppm upfield isotopic shift for ^{18}O located in the bridge position and a 0.046 ppm upfield isotopic shift for ^{18}O located in the nonbridge position on the phosphorus.

The test reaction was carried out with an equal molar ratio of (all ^{16}O) thiophosphonopyruvate (1) and [P=^{18}O,C(2)-^{18}O]thiophosphonopyruvate (2). Factoring in the expected 60% loss of ^{18}O label at the C(2) position of 2, we estimated that the ratio of the isotopomers 5, 3, 4, and 6 produced via the intramolecular pathway to be 1:0:0.6:0.4 and the ratio produced via one of the intermolecular pathways to be 1:0.25:1:0.25. ^{31}P NMR analysis of the product mixture (Figure 1) gives a 1:0:0.6:0.4 ratio of these substances, thus, demonstrating that the PEP phosphomutase catalyzed rearrangement of phosphonopyruvate to PEP occurs via an intramolecular mechanism. Consistent with this conclusion is the fact that we found no evidence that the rate law governing the reaction contains a squared term in phosphonopyruvate[5] as would be required by the reaction between two phosphonopyruvate molecules. Likewise, endogenous pyruvate did not stimulate the reaction with phosphonopyruvate nor did the PEP generated from phosphonopyruvate and PEP phosphomutase preequilibrated with [^{14}C]pyruvate (data not shown) contain the ^{14}C label. Thus we were unable to detect a substrate/cofactor role for pyruvate.

Stereochemistry of the PEP Phosphomutase Reaction. The second goal of our investigations of PEP phosphomutase catalysis was to determine whether the intramolecular rearrangement of phosphonopyruvate proceeds by a concerted or stepwise mechanism. Such a determination could be made by elucidation of the stereochemical course of the catalyzed reaction. Specifically, orbital topology considerations[15] suggest that a concerted route (pathway A in Scheme III) for suprafacial C to O 1,3-phosphoryl migration should proceed through a Mobius transition state with inversion of configuration at phosphorus. This prediction is based on the analogy gained from studies[16] of 1,3-sigmatropic rearrangements in carbon systems. In contrast, metaphosphate formed in the dissociative pathway (pathway B, Scheme III) should rebond to the pyruvate unit at C(3) using the same face of the phosphorus from which the pyruvate unit dissociated. Thus, retention of stereochemistry at phosphorus is predicted. The two-step, oxaphosphetane mechanism (pathway C, Scheme III) is also expected to proceed with retention of configuration based upon the information gained from studies of the Wittig reaction and other reactions which occur through analogous carba- and azaphosphetane intermediates.[17] We anticipate that pathway D involving initial transfer of the phosphoryl grouping to the internal carboxyl group should also proceed with overall retention of configuration at phosphorus since it is comprised of two

(12) Our preliminary ^{31}P NMR studies of ^{18}O-labeled phosphoenolpyruvate, however, revealed that the presence of ^{18}O at the C(2)-O-P bridge position causes an isotopic shift[13] of the same magnitude as when the ^{18}O is positioned at the P–O nonbridging position (viz 0.025 ppm). Because of this we were unable to use the ^{18}O analogues of the natural substrate, phosphonopyruvate, in this experiment. Studies of electron distributions in thiophosphates[14] have demonstrated that the nonbridging oxygen atoms have, at the expense of the sulfur atom, more double-bond character than do the oxygen atoms of their phosphate counterparts. Hence, we anticipated that we would be able to resolve the ^{31}P NMR respoances for the thiophosphoenolpyruvates isotopomers 3–6 of Scheme II.

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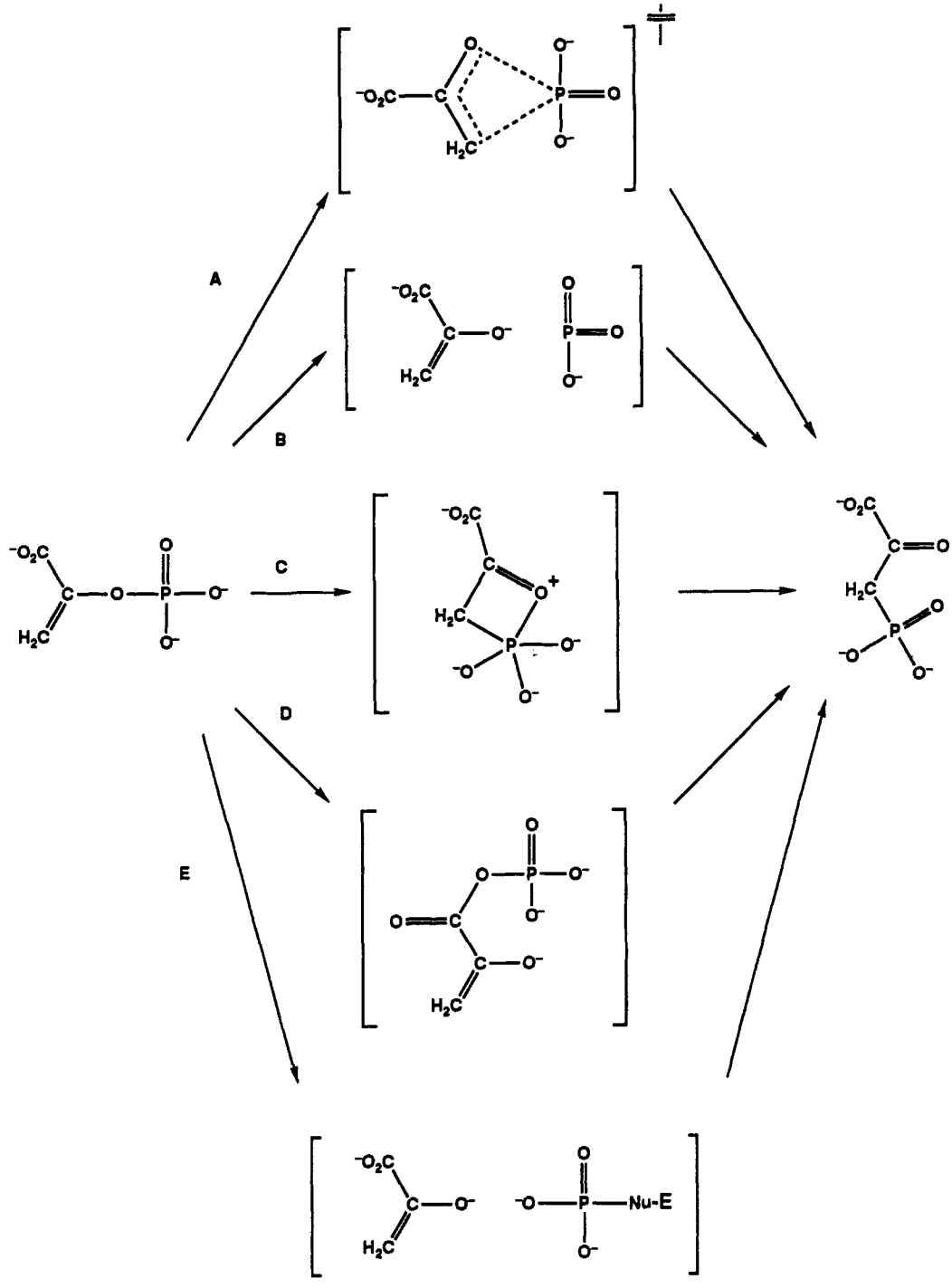
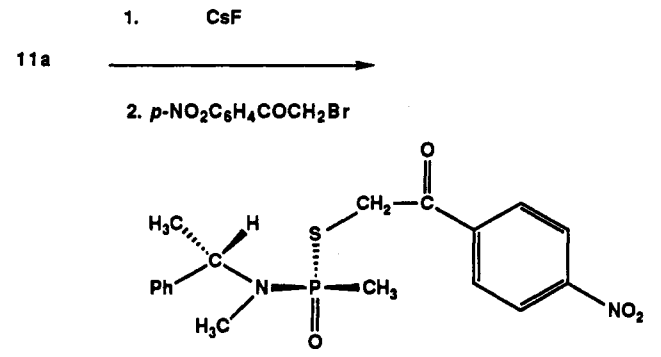
Scheme III. Possible Intramolecular Mechanisms for PEP Phosphomutase Catalysis



individual steps, each of which should preserve the original phosphorus configuration. Finally, the double displacement pathway (pathway E, Scheme III) should also display net retention of the phosphorus configuration owing to the fact that two sequential, in-line displacements are involved and each should invert the configuration at phosphorus.

The stereochemistry of the PEP phosphomutase reaction was determined by preparing the enantiomers of chiral [^{18}O]thiophosphonopyruvate for use as probes and analyzing the configurations of the corresponding [^{18}O]thiophosphoenolpyruvate enantiomers produced as products. The synthetic route to the individual enantiomers of chiral [^{18}O]thiophosphonopyruvate (**14a** and **14b**) that was developed is outlined in Scheme IV. It involves the elaboration of methylphosphonothioic dichloride **8** to the S_p,S_c and R_p,S_c diastereomers of *O*-2-(trimethylsilyl)ethyl *N*,*P*-dimethyl-*N*-(1-phenylethyl)phosphonamidothioate (**11a** and **11b**). Reaction of dichloride **8** with the lithium alkoxide of 2-(trimethylsilyl)ethanol provided a monoester (**9**) which was then condensed with the lithium amide of (*S*)-*N*-methyl-*N*-(1-phenylethyl)amine (**10**) to give a mixture of **11a** and **11b**. Separation of these diastereomers was accomplished by HPLC. In order to determine the configuration at phosphorus in each of the diastereoisomers, the crystalline *p*-nitrophenacyl derivative **15** was prepared from **11a** by removal of the (trimethylsilyl)ethyl protecting group followed by S-alkylation. X-ray analysis of **15** provided the structure shown in Figure 2 and, thus, the absolute stereochemistry at phosphorus (S_p) in **11a**.

Conversion of the S_p,S_c and R_p,S_c diastereomers **11a** and **11b** to the respective R_p and S_p enantiomers, **14a** and **14b**, was accomplished (Scheme IV) by coupling the individual



15

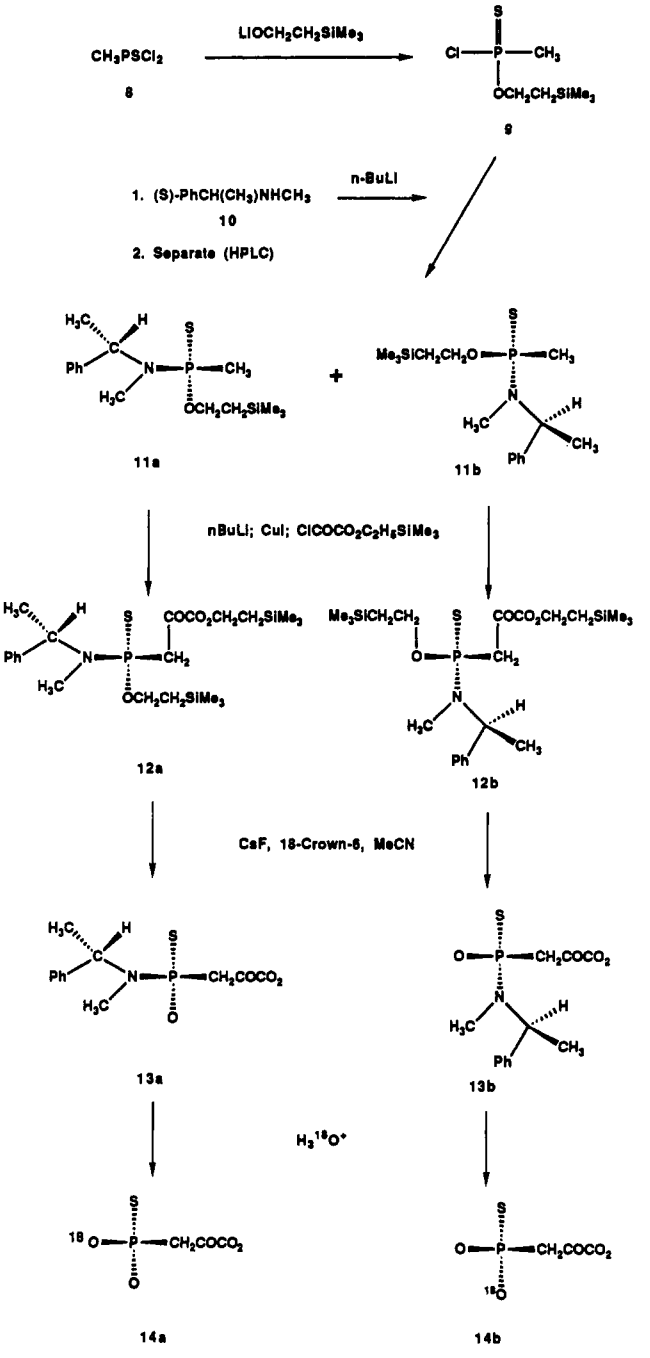
lithium cuprates derived from **11a** and **11b** with the (trimethylsilyl)ethyl half ester of oxalyl chloride to introduce the pyruvyl moiety in **12a** and **12b**.[18] This step was followed by deprotection of both of the silylethyl ester functionalities to form **13a** and **13b**, respectively, and finally, hydrolytic displacement of the amine substituent by reaction in acidic $H_2{}^{18}O$.

The hydrolysis step, leading from **13a** and **13b** to **14a** and **14b**, respectively, proceeds with retention of the phosphorus configuration as a result of neighboring group participation (shown in Scheme V) by the carboxyl substituent. Our original communication[8a] of this work did not take into account the role of the carboxyl group in the hydrolysis reaction, and thus, the stereochemical assignments made to the [^{18}O]thiophosphonopyruvate enantiomers generated from **13a** and **13b** were incorrectly[19] based on the assumption of inversion rather than retention of configuration phosphorus.

Precedence for carboxyl group participation in the hydrolysis of **13a** and **13b** is found in the demonstrated role of the carboxyl substituent in accelerating the rate of hydrolysis of the *O*-benzyl ester of PEP[20] illustrated in Scheme V. The catalytic effect of the carboxyl function on the hydrolysis rates of **13a** and **13b** is evident from the comparatively slower rates of hydrolysis of the *P*-(2-ethoxyethenyl)-*N*-(1-phenylethyl)[^{18}O]phosphonamidothioate enantiomers. While the hydrolyses of **13a** and **13b** in 0.5 M HCl (2 equiv of protons) are complete within 10 s at 25 °C, the hydrolyses of the phosphonamidothioate enantiomers lacking the carboxyl substituent require 3.5 h in 1 M *p*-TsOH (5 equiv of protons) at 25 °C.[21] The participation of the carboxyl function in the hydrolyses of **13a** and **13b** is also reflected by the product composition. Whereas the hydrolyses of the *P*-(2-ethoxyethenyl)-*N*-(1-phenylethyl)[^{18}O]phosphonamidothioate enantiomers occurs with predominantely inversion of configuration at phosphorus, accompanied by a significant level of racemization (~30%),[21] the hydrolyses of **13a** and **13b** produce enantiomerically pure products (see Figure 3).

To verify that the hydrolysis of **13a** and **13b** occurs by the pathway shown in Scheme V we prepared the carboxy ester adducts **17a** and **17b** (see Scheme VI). Since the carboxyl group assisted hydrolysis in **17a** and **17b** should be blocked, we expected that the rates of hydrolysis of **17a** and **17b** would be significantly slower than those of **13a** and **13b**. This was in fact observed. Under the same reaction conditions used for **13a** and **13b**, hydrolyses of **17a**

Scheme IV. Synthesis of the *S* and *R* Enantiomers of Chiral [^{18}O]Thiophosphonopyruvate



and **17b** required ~30 min. Furthermore, we expected that hydrolysis at the phosphorus centers of **17a** and **17b** would proceed with inversion of configuration[22] and therefore produce [^{18}O]thiophosphonopyruvate enantiomers having the mirror image configuration of that produced from **13a** and **13b**, respectively. Because the hydrolysis of **17a** and **17b** in acidified $H_2{}^{18}O$ removed both the amine and isopropyl substituents, the hydrolysis products from **17a** and **17b** could be directly analyzed, as describe below, in parallel with the [^{18}O]thiophosphonopyruvate enantiomers generated from **13a** and **13b**.

As illustrated in Scheme VII, samples of chiral [^{18}O]-thiophosphonopyruvate were converted to chiral [^{18}O]-

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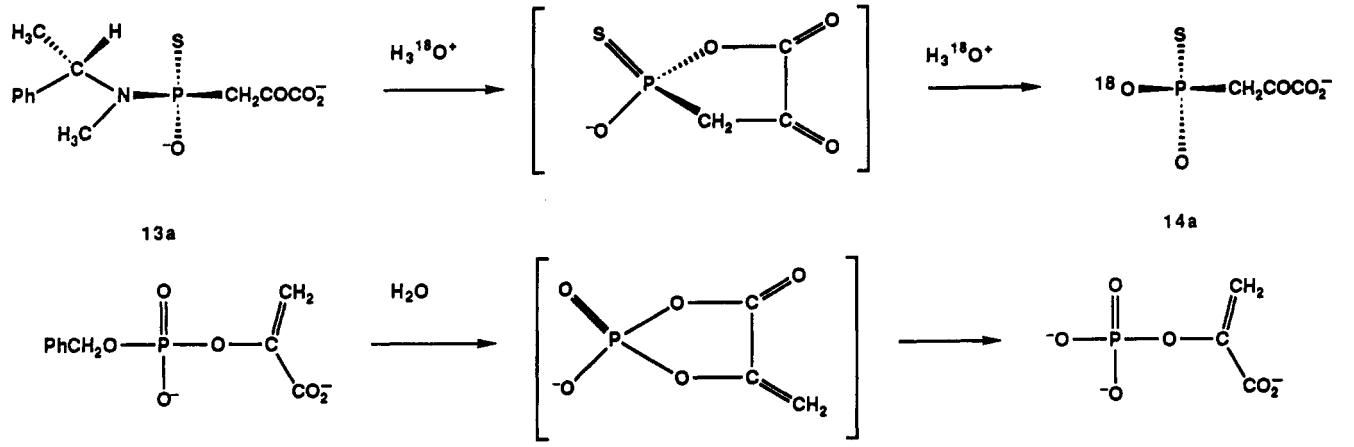
Scheme V. Carboxylate Participation in Phosphonamidothioate 13a and *O*-Benzyl Phosphate Ester Hydrolysis



Scheme VI. Preparation of the Carboxy Ester Adducts of 13a and 13b

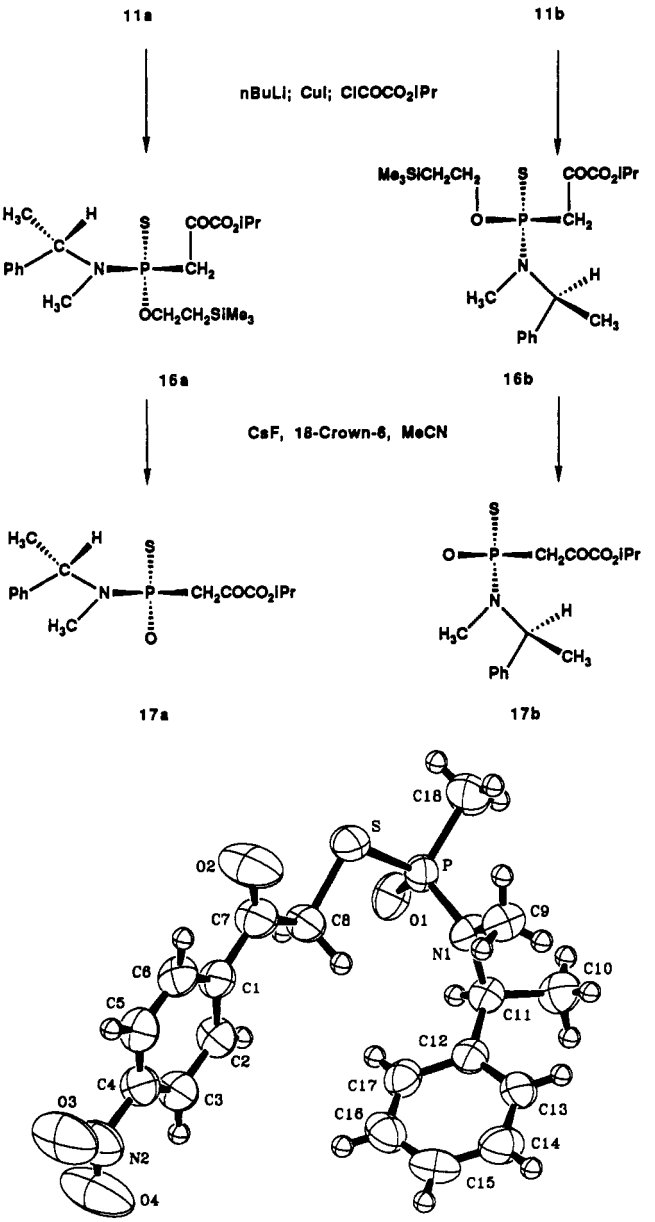


Figure 2. X-ray structure of the crystalline derivative 15 derived from the phosphonamidiothioate 11a.

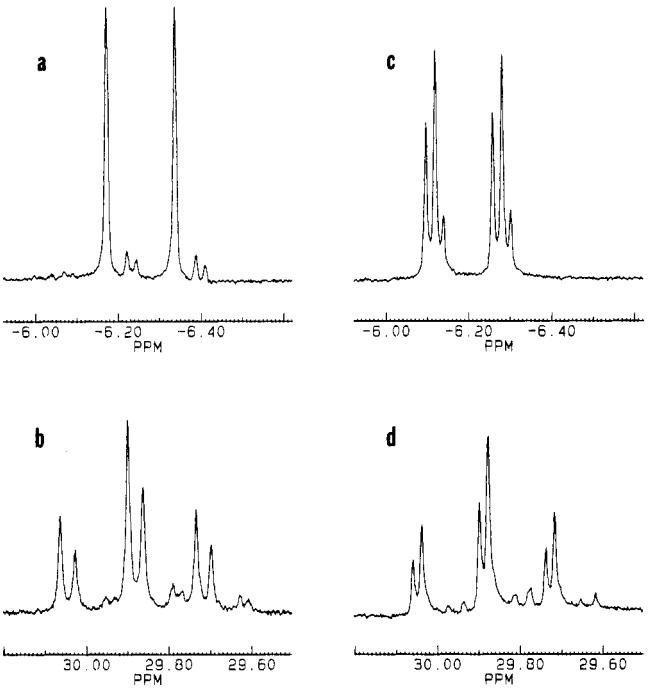


Figure 3. The γ-P region (a) and β-P region (b) of the ^{31}P NMR spectra of a 1:1 mixture of (all ^{16}O) (S_p)-ATPβS and the (S_p)-[^{18}O]ATPβS isotopomer derived from the (R_p)-[^{18}O]thiophosphoenolpyruvate enantiomer formed via PEP phosphomutase reaction of the (R_p)-thiophosphonopyruvate enantiomer **14a**; the γ-P region (c) and β-P region (d) of the ^{31}P NMR spectra of a 1:1 mixture of (all ^{16}O) (S_p)-ATPβS and the (S_p)-[^{18}O]ATPβS isotopomer derived from the (S_p)-[^{18}O]thiophosphoenolpyruvate enantiomer formed via PEP phosphomutase reaction of the (S_p)-[^{18}O]thiophosphonopyruvate enantiomer **14b**. See text for further details.

thiophosphoenolpyruvate with PEP phosphomutase and then to [β-^{18}O]ATPβS and/or [β, γ-^{18}O]ATPβS (with retention of configuration at phosphorus) using the methodology reported by Frey and co-workers.[23]

The ^{31}P NMR spectra recorded for 1:1 mixtures of (all ^{16}O) (S_p)-ATPβS and the (S_p)-[^{18}O]ATPβS samples generated from **14a** and **14b** derived from **13a** and **13b** are shown in Figure 3. The (S_p)-[^{18}O]ATPβS generated from **14a** is a single isotopomer which, based upon the observed isotopic shift 0.037 ppm, is ^{18}O-labeled at the (nonbridge) β-P=O position. The (S_p)-[^{18}O]ATPβS generated from **14b** is also a single isotopomer, which based upon the observed isotopic shift of 0.021 ppm, is ^{18}O-labeled at the (bridge) β,γ-P–O position. Hence, as represented in

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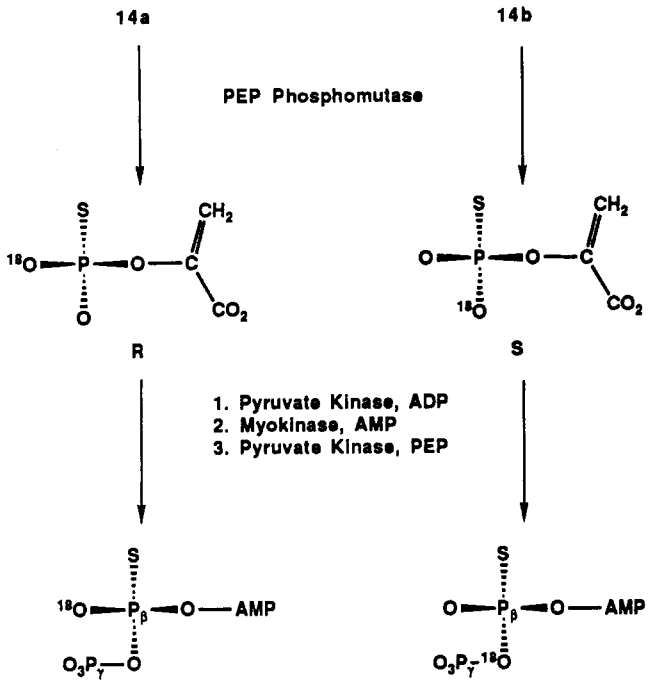
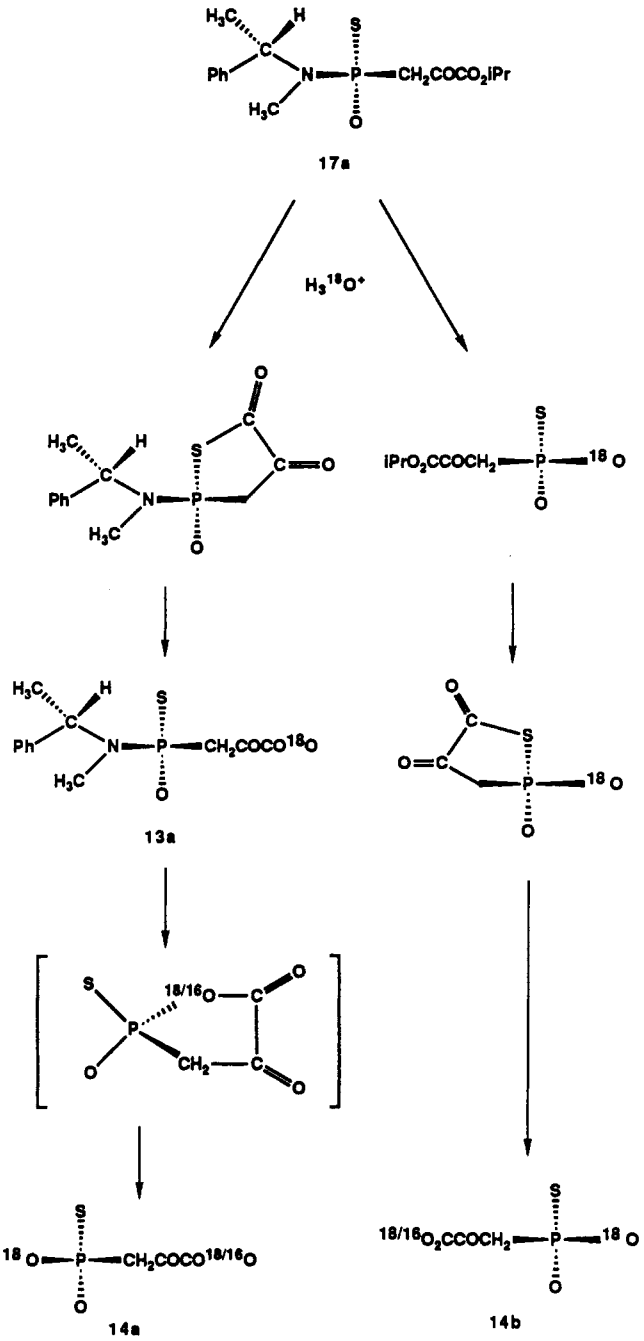
Scheme VII. Stereochemical Analysis of the PEP Phosphomutase Catalyzed Conversion of [^{18}O]Thiophosphonopyruvate to [^{18}O]Thiophosphoenolpyruvate



Scheme VII, the [^{18}O]thiophosphoenolpyruvate derived from **14a** has the *R* configuration and that derived from **14b** has the *S* configuration. Assuming that hydrolyses of **13a** and **13b** to **14a** and **14b** occurred with retention of configuration and, therefore, that **14a** has the *R* configuration and **14b** the *S* configuration, the PEP phosphomutase catalyzed reaction is thus shown to take place with retention of configuration. This conclusion is consistent with that reached by Knowles and co-workers.[19]

The ^{31}P NMR spectra recorded (but not shown) for 1:1 mixtures of (all ^{16}O) (S_p)-ATPβS and the (S_p)-[^{18}O]ATPβS samples generated from the [^{18}O]thiophosphonopyruvate derived from the hydrolyses of the carboxy ester adduct **17a** and **17b** (Scheme VI) revealed a mixture of (S_p)-[^{18}O]ATPβS isotopomers. Specifically, 55% of the (S_p)-[^{18}O]ATPβS derived from **17a** was ^{18}O-labeled at the (nonbridge) β-P=O position while 45% was ^{18}O-labeled at the (bridge) β,γ-P-O position. Likewise, 57% of the (S_p)-[^{18}O]ATPβS formed from **17b** was ^{18}O-labeled at the (bridge) β,γ-P-O position and 43% at the (nonbridge) β-P=O position. In light of the demonstrated stereospecific conversion of a single enantiomer of [^{18}O]thiophosphonopyruvate to a single (S_p)-[^{18}O]ATPβS isotopomer (Figure 3) these results suggest that the hydrolyses of **17a** and **17b** occur with roughly 45% inversion of configuration at phosphorus and 55% retention, in contrast to the 100% retention of configuration noted for the hydrolyses of **13a** and **13b**. Hence, the effect of the esterification of the carboxyl group of **13a** and **13b** on the stereochemical outcome of the hydrolysis reaction at phosphorus is evident. We did not observe a 100% reversal of the stereochemical course of the reaction, i.e., **17a** → **14b** and **17b** → **14a** only, due to loss of the ester function via the competing hydrolytic pathway shown in Scheme VIII. Specifically, attack of the sulfur atom in **17a** or **17b** at C(1) resulted in thiolactone formation and loss of the isopropyl substituent. Ring opening in this thiolactone was found to occur by attack of the $H_2{}^{18}O$ at C(1) (we did not observe incorporation of a second ^{18}O label at the phosphorus center in the product), thus generating **14a** from **17a** and **14b** from **17b**. This thiolactone-forming pathway was found to be the preferred pathway of hydrolysis of less sterically hindered ester adducts of **13a** or **13b**. For example, selective removal of the (trimethylsilyl)ethyl protecting group from the thiophosphonamidate moiety of **12a** followed by hydrolysis in acidified $H_2{}^{18}O$ resulted in a 86:14 mixture of **14a** and **14b** (as determined by using the procedure for stereochemical analysis described above).

Scheme VIII. Opposing Pathways for the Hydrolysis of the Thiophosphonamidate 17a in Acidified $H_2{}^{18}O$



Conclusions

The observed molecularity and stereochemical course of the PEP phosphomutase catalyzed rearrangement of phosphonopyruvate to phosphoenolpyruvate has important mechanistic implications. First, the demonstration of an intramolecular reaction pathway eliminates the two intermolecular pathways in which reaction occurs between either pyruvate and phosphonopyruvate or two molecules

of phosphonopyruvate. Second, the observed retention of configuration at phosphorus rules out the operation of a concerted, pericyclic mechanism. This leaves four intramolecular, stepwise mechanisms (B–E in Scheme III) as being possible for the PEP phosphomutase reaction. Of these four mechanisms, that proceeding via the phosphoenzyme intermediate (pathway E in Scheme III) is the most well precedented.[2] Finally, the similar substrate activity of phosphonopyruvate and thiophosphonopyruvate suggests that, independent of mechanism, nucleophilic attack at the phosphorus is not involved in the rate-limiting step for this rearrangement reaction.[24]

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Supplementary Material Available: Synthetic procedures, spectroscopic data, and NMR spectra of all new compounds reported and X-ray crystallographic data for **15** (37 pages). Ordering information is given on any current masthead page.

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Swertipunicoside. The First Bisxanthone *C*-Glycoside

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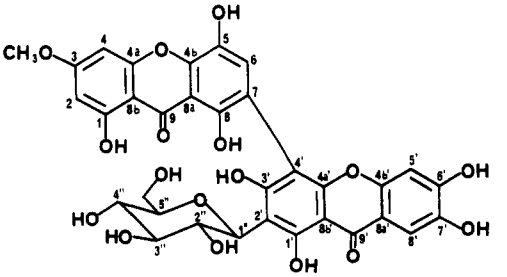
The first bisxanthone *C*-glycoside, swertipunicoside, was isolated from the whole plant of *Swertia punicea* Hemsl. and its structure elucidated through spectroscopic, particularly selective INEPT NMR, analysis as 1,5,8-trihydroxy-3-methoxy-7-(1′,3′,6′,7′-tetrahydroxy-9′-oxo-4′-xanthyl)xanthone 2′-*C*-β-D-glucopyranoside.

Introduction

Seventy-nine of the 170 species of the genus *Swertia* (Gentianaceae) are distributed in China, particularly in the southwestern area.[1] About 20 species of *Swertia* have been used in Chinese traditional medicine for the treatment of hepatic, choleric, and inflammatory diseases.[2,3] *Swertia mileensis* is claimed to be especially efficacious for viral hepatitis.[4] In India, *S. chirata* is used as antimalarial, liver tonic, laxative, febrifuge, stomachic, and bitter tonic.[5,6] The herb of *S. purpurascens* is used in Pakistan as a substitute of *S. chirata*,[7] and in Japan, *S. japonica* is an important bitter stomachic.[8] In previous phytochemical studies, xanthone derivatives,[9–12] flavonoids,[6,13,14] iridoid glycosides,[15–17] and triterpenoids[18,19] have been reported as the main constituents of this genus. Recently, we reported the structure of a new dimeric xanthone, swertiabisxanthone-I, from *S. macrosperma*.[20]

Swertia punicea tastes extremely bitter, possesses the ability to reduce fever and detoxify, and is used in the southwestern part of China for the treatment of hepatogenous jaundice and cholecystitis. No chemical studies have previously been reported for *S. punicea*. Investigation of the whole plant of *S. punicea* has led to the isolation, from the *n*-BuOH fraction of the EtOH extract, of the first member of a new series of natural products, a bisxanthone *C*-glucoside, swertipunicoside (1). The structure was elucidated by a series of NMR experiments, especially the selective INEPT technique.[21–23]



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