(*Z*)-3-(Fluoromethyl)phosphoenolpyruvate: Synthesis and Enzymatic Studies†

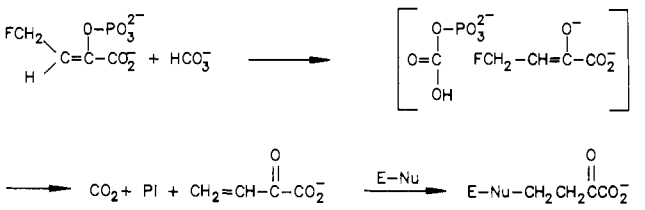
Peter Wirsching and Marion H. O'Leary*
Departments of Chemistry and Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706
Received July 10, 1987; Revised Manuscript Received October 16, 1987

ABSTRACT: (*Z*)-3-(Fluoromethyl)phosphoenolpyruvate has been synthesized in nine chemical steps from glyoxylic acid. The compound is stable at pH 3, but at pH 8 it decomposes within seconds to give 2-oxo-3-butenoate. When 3-(fluoromethyl)phosphoenolpyruvate is added to a solution of phosphoenolpyruvate carboxylase or pyruvate kinase, the enzyme is inactivated over the course of an hour. Identical kinetics of inactivation are observed whether the reaction is initiated by addition of 3-(fluoromethyl)-phosphoenolpyruvate, preformed 2-oxo-3-butenoate, or 4-fluoro-2-oxobutanoate (which rapidly undergoes elimination of fluoride ion to form 2-oxo-3-butenoate). The inactivating species in all cases is believed to be 2-oxo-3-butenoate. The inactivation is completely prevented by the presence of dithiothreitol, which reacts rapidly with 2-oxo-3-butenoate. Studies with competitive inhibitors of both enzymes indicate that inactivation does not occur at the active site.

Incorporation of fluorine into a variety of enzyme substrates and inhibitors has led to useful suicide substrates, some of which have found biomedical uses (Walsh, 1984; Kollonitsch, 1982). The utility of fluorine lies in its similarity in size to hydrogen, coupled to its very different electronic characteristics. Loss of fluorine from such inhibitors can often lead to reactive intermediates that then inactivate the enzyme. For example, fluorine-containing enolates can potentially be used to generate highly reactive Michael acceptors.

Such a reaction is of potential use in the inactivation of enzymes that operate on pyruvic acid or its enolate. Both PEP carboxylase (O'Leary, 1981) and pyruvate kinase (Rose, 1970) operate by mechanisms involving enzyme-bound enolates and so might be amenable to such an approach. The compound monocyclohexylammonium 3-(fluoromethyl)phosphoenolpyruvate (FMe-PEP)[1] was designed to be a mechanism-based inactivator of such enzymes. We expected that in the enzymatic process phosphate would be transferred to the acceptor as usual, after which the resulting enolate would undergo rapid elimination of fluoride, generating the potential Michael acceptor, 2-oxo-3-butenoate, which might then alkylate a nucleophilic amino acid at the active site and inactivate the enzyme (Scheme I). The high reactivity of 2-oxo-3-butenoate has been seen in a number of previous studies (Walsh & Cromartie, 1975; Walsh & Marcotte, 1976; Walsh et al., 1975). In this paper we report the synthesis of FMe-PEP and its interaction with PEP carboxylase and pyruvate kinase.

Scheme I



Experimental Procedures

Materials. Glyoxylic acid monohydrate (Aldrich Chemical Co.), sodium cyanoborohydride (Aldrich), dithiothreitol (Calbiochem), acetone, anhydrous ether, HEPES (U.S. Biochemicals), and methanol were reagent grade materials and were used without further purification. 1,3-Propanedithiol (Aldrich) was distilled under reduced pressure. Oxalyl chloride (Aldrich) was distilled. Pyridine (CCI Co.) was distilled from CaH_2. *tert*-Butyl alcohol (Aldrich) was distilled from CaH_2. 2-Bromo-1-fluoroethane (Fairfield Chemical Co.) was distilled before use. *N*-Bromosuccinimide (Aldrich) was recrystallized from water and dried over P_2O_5 under vacuum. Trifluoroacetic acid (Aldrich) was distilled from P_2O_5 and stored tightly capped over Drierite. Bromine was refluxed over KBr, distilled from KBr, and distilled from BaO. Cyclohexylamine was distilled from CaH_2. Trimethyl phosphite was stirred over sodium metal for 2 days and then distilled from sodium. Benzene was distilled from CaH_2. Dimethylformamide was dried with Na_2SO_4 and distilled from BaO under reduced pressure. Carbon tetrachloride was dried over K_2CO_3. Bromotrimethylsilane was distilled from 1-decene. Dichloromethane was stirred over concentrated H_2SO_4, washed with water, then $NaHCO_3$, and then water, dried with $CaCl_2$, and distilled from CaH_2. Dowex ion-exchange resins (Bio-Rad or Aldrich) were purified by washing with bromine water, 6 M NaOH, and 6 M HCl. Water was purified by a Millipore filtration–deionization system. All solvents used in high-performance liquid chromatography were filtered by using 0.45-μm Gelman Metricel membrane filters and then degassed.

Phosphoenolpyruvate carboxylase was isolated from maize (Díaz et al., 1988) and dialyzed before use. Pyruvate kinase (type II from rabbit muscle), lactate dehydrogenase, malate dehydrogenase, hexokinase (from yeast), glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), PEP, ADP, NAD, and NADH were purchased from Sigma Chemical Co.

Methods. Proton NMR spectra were taken on a Bruker WP-200 or WP-270 FT spectrometer. Carbon NMR spectra were taken on a JEOL FX-200 or Bruker AM-500 spectrometer. Fluorine and phosphorus spectra were taken on a Bruker WP-200 or a Bruker AM-400 instrument. Chemical shifts are given relative to tetramethylsilane, 85% H_3PO_4, or fluorotrichloromethane (for ^{19}F spectra).

† This work was supported by Grant 83-CRCR-1-1297 from the Competitive Research Grants Office, U.S. Department of Agriculture.

[1] Abbreviations: PEP, phosphoenolpyruvate; FMe-PEP, (*Z*)-3-(fluoromethyl)phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

0006-2960/88/0427-1348$01.50/0

IR spectra were recorded on a Beckman Acculab-7 spectrometer. Mass spectra were recorded at 30 eV on a Kratos MS-25 or MS-80. Peak matches were obtained for all compounds. In some cases, the compound underwent self-chemical ionization, and the peak match was made on the M + 1 peak. UV spectra and kinetics were measured on a Cary 118 spectrophotometer equipped with a thermostated cell compartment maintained at 25 °C. High-performance liquid chromatography was carried out with a Waters instrument using an Aminex HPX-87 column eluted with 5 mM H_2SO_4 or 5 mM H_3PO_4.

For syntheses, all glassware and syringes were oven- or flame-dried and cooled under nitrogen. All liquid transfers were accomplished via syringe or cannula. All reactions were conducted under dry nitrogen. Reagents were weighed in a glovebag where necessary.

Syntheses. (*a*) *1,3-Dithiane-2-carboxylic Acid.* A 2000-mL round-bottom flask containing a magnetic stirring bar was charged with glyoxylic acid monohydrate (0.34 mol, 32.0 g) and 1200 mL of benzene. The flask was swirled to dissolve some of the acid, and then *p*-toluenesulfonic acid monohydrate (0.042 mol, 8.0 g) was added. The flask was fitted with a septum, and the contents were stirred, after which 1,3-propanedithiol (0.37 mol, 40.0 g) was added.

The flask was then fitted with a 20-mL Dean–Stark trap and a condenser, after which the mixture was heated to 85–90 °C for about 90 min. After this time, 12 mL of water had been collected. The yellow solution was allowed to cool to room temperature and then was decanted away from the polymeric residue, and 300 mL of saturated $NaHCO_3$ was added. The solution was decanted away from the precipitate into a 2000-mL separatory funnel. The layers were separated, and the organic layer was further extracted with two portions of saturated $NaHCO_3$. The combined aqueous layers were then carefully acidified with 6 M HCl until a white cloudiness appeared, after which this solution was extracted with 200 mL of ethyl acetate. The aqueous layer was then acidified further and extracted. This was repeated until no more cloudiness appeared. A total of about 1400 mL of ethyl acetate was used. The ethyl acetate solution was then dried with $MgSO_4$ and filtered. The solvent was removed on a rotary evaporator, leaving a white solid. Further evaporation overnight using a vacuum pump gave 45.0 g (80%) of a white solid.

^1H NMR ($CDCl_3$): δ 2.10 (2 H, m), 2.65 (2 H, ddd), 3.50 (2 H, ddd), 4.20 (1 H, s), 11.4 (br s). IR ($CDCl_3$): 3100 (br), 1710 (s), 1430 (s), 1420 (s), 1300 (s), 1250 (m), 1185 (m), 940 (m), 825 (w) cm^{-1}. mp 113–115 °C.

(*b*) *tert-Butyl 1,3-Dithiane-2-carboxylate. Procedure A* (*Small Scale*). A 250-mL round-bottom flask containing a magnetic stirring bar was charged with dry dimethylformamide (0.146 mol, 10.7 g) and dry acetonitrile (27 mL). The solution was cooled to −20 °C in a dry ice–acetone bath. A solution of oxalyl chloride (0.054 mol, 6.85 g) in 5 mL of acetonitrile was then added dropwise over 15 min. During this time, the thick, white, Vilsmeier reagent formed. After the mixture was stirred for another 20 min at −20 °C, 1,3-dithiane-2-carboxylic acid (0.049 mol, 8.0 g) was added as a solid, in portions, from a flask that had been attached with Gooch tubing. A clear, yellow solution developed that was stirred 30 min at −20 °C. A solution of *tert*-butyl alcohol (0.113 mol, 8.5 g) in 11 mL of pyridine was then added dropwise via cannula over 30–45 min at −25 °C. The yellow color increased and some solid formed. The mixture was allowed to warm to room temperature in the bath (at 0 °C, redissolution of the solid occurred) and stirred overnight. The brownish solution was then diluted with 160 mL of CH_2Cl_2 and washed, in succession, with 160 mL of 20% $KHCO_3$ and 3 × 100 mL of H_2O. The aqueous layer was then back-extracted with 300 mL of CH_2Cl_2. The combined organic layers were dried with Na_2SO_4, filtered, and evaporated, leaving a dark brown liquid, which was fractionally distilled giving a clear, light yellow liquid that solidified overnight at 4 °C; yield 7.1 g (66%).

Procedure B (*Large Scale*). The 1,3-dithiane-2-carboxylic acid (0.242 mol, 40.0 g) was converted to the sodium salt by adding 1 equiv of NaOH in 25 mL of water to a suspension of the acid in 60 mL of water, adjusting the pH to 7 with 6 M HCl and 6 M NaOH and lyophilizing. The resulting solid was powdered and further dried for 6–8 h at 50–60 °C using a vacuum pump. The off-white, amorphous solid was put in a 100-mL round-bottom flask.

A 500-mL, three-neck, round-bottom flask containing a magnetic stirring bar was charged with 240 mL of dry benzene and oxalyl chloride (0.275 mol, 34.9 g). The flask was fitted with a septum and a condenser and connected to the 100-mL flask containing the sodium 1,3-dithiane-2-carboxylate via Gooch tubing. For the first stage of the reaction, no water was used in the condenser. The flask was immersed in an ice bath, and the sodium 1,3-dithiane-2-carboxylate was added over about 2 h (vigorous bubbling and foaming occurred if the addition was too rapid). After the addition, the yellow mixture was allowed to warm to room temperature with stirring. The two outer necks were stoppered, and the mixture was heated at 80–85 °C for 1 h. During this time, the mixture turned dark orange. After cooling to room temperature, the suspension was filtered through a sintered-glass funnel into a 500-mL round-bottom flask. A translucent, orange solution was obtained. The solvent was removed over several hours by using a stream of nitrogen at 35–40 °C. The resulting dark orange acid chloride was dissolved in 180 mL of dry ether.

A 500-mL round-bottom flask having a sidearm with a stopcock was fitted with a mechanical stirrer. The flask was immersed in an ice bath and charged with pyridine (0.266 mol, 21.0 g) and *tert*-butyl alcohol (0.29 mol, 21.5 g). The ethereal acid chloride was then added dropwise with rapid stirring over about 90 min. A reaction occurred almost immediately, and after a few minutes, a yellow precipitate of pyridinium chloride developed. After the addition was complete, the bath was removed and the mixture stirred 15 h, during which time it turned pink. A 100-mL portion of 0.1 M HCl was then added that dissolved the solid. The layers were separated, and the ether layer was washed with 0.1 M HCl (6 × 50 mL), 5% $NaHCO_3$ (2 × 100 mL), and brine (2 × 50 mL). The ether was dried with $MgSO_4$, filtered, and then evaporated on a rotary evaporator, leaving an orange liquid. The liquid was carefully distilled (with the aid of a heat gun) through base-washed glassware giving a clear, slightly yellow liquid. Upon standing at room temperature, the liquid solidified, yielding 28.0 g (52%) of a yellow solid.

^1H NMR ($CDCl_3$): δ 1.50 (9 H, s), 2.10 (2 H, m, $^2J_{ae}$ = 14.1 Hz), 2.60 (2 H, ddd, $^3J_{ea}$ = 5.5, $^3J_{ee}$ = 8.6, $^2J_{ea}$ = 13.7 Hz), 3.40 (2 H, ddd, $^3J_{ae}$ = 3.3, $^3J_{aa}$ = 10.4, $^2J_{ae}$ = 13.7 Hz), 4.10 (1 H, br s). IR ($CDCl_3$): 2990 (s), 1720 (s), 1480 (w), 1460 (w), 1430 (w), 1370 (m), 1300 (s), 1140 (s), 960 (w), 850 (m) cm^{-1}. ^{13}C NMR (acetone-d_6): δ 25.86, 26.48, 27.97, 41.98, 82.11, 169.38. MS: calculated for $C_9H_{16}O_2S_2$, M_r 220.0588; found, 220.0569. bp 72–75 °C (0.3 mmHg). mp 42–44 °C.

(*c*) *tert-Butyl 2-(2′-Fluoroethyl)-1,3-dithiane-2-carboxylate.* A 500-mL, three-neck round-bottom flask was charged with

oil-free NaH (0.126 mol, 3.0 g) and fitted with a mechanical stirrer and septa, and 175 mL of dry benzene was added. The stirring suspension was cooled in an ice bath. A solution of *tert*-butyl 1,3-dithiane-2-carboxylate (0.127 mol, 28.0 g) and 2-bromo-1-fluoroethane (0.146 mol, 18.6 g) in 53 mL of dry dimethylformamide was then added over 2.5 h (the rate of addition is crucial). During this time, the gray suspension turned yellow. The mixture was stirred at room temperature for 18 h, and then 100 mL of water was added. The layers were separated, and the organic layer was washed with 7 × 50 mL of water. The solvent was removed in a hood by using a stream of nitrogen and a 35–40 °C oil bath. The resulting dark yellow liquid was eluted through a 2.5 × 10 cm column of Florisil with ether as eluant. A light yellow, semitranslucent liquid (29.3 g, 86%) was obtained. This material is stable for weeks at room temperature.

^{1}H NMR ($CDCl_3$): δ 1.52 (9 H, s), 2.0 (2 H, m), 2.44 (2 H, dt, $^3J_{FH}$ = 20.7, $^3J_{HH}$ = 6.39 Hz), 2.69 (2 H, ddd, $^2J_{ea}$ = 14.3, $^3J_{ee}$ = 8.15, $^3J_{ea}$ = 3.31 Hz), 3.33 (2 H, ddd, $^2J_{ea}$ = 14.3, $^3J_{aa}$ = 11.9, $^3J_{ae}$ = 2.64), 4.70 (2 H, dt, $^2J_{FH}$ = 46.9, $^3J_{HH}$ = 6.39 Hz). IR ($CDCl_3$): 2990 (s), 2920 (m), 2900 (m), 1720 (s), 1480 (w), 1430 (w), 1400 (w), 1370 (m), 1260 (s), 1150 (s), 850 (w), 680 (w) cm^{-1}. ^{13}C NMR (acetone-d_6): δ 25.99, 28.04, 28.14, 39.78 (d, $^2J_{FC}$ = 22.09 Hz), 51.26, 80.70 (d, $^1J_{FC}$ = 165.3 Hz), 82.94, 169.9. MS: calculated for $C_{11}H_{19}FO_2S_2$, M_r 266.0806; found, 266.0811. ^{19}F NMR (acetone-d_6) δ −216.7.

(*d*) *tert-Butyl 2-Oxo-4-fluorobutanoate.* [For a comparable procedure, see Corey and Erickson (1971).] A 1-L Erlenmeyer flask containing a magnetic stirring bar was charged with *N*-bromosuccinimide (0.21 mol, 37.2 g) and 410 mL of 97% aqueous acetone (398 mL of acetone, 12 mL of water). The solution was chilled in ice and stirred vigorously. A solution of *tert*-butyl 2-(2′-fluoroethyl)-1,3-dithiane-2-carboxylate in 35 mL of acetone was then added dropwise over 50 min. The solution turned first yellow, then orange, and then deep red before fading back to yellow-orange. After the addition, the solution was stirred 5 min. It was then diluted with 250 mL of 1:1 CH_2Cl_2/hexane. The orange color was then discharged by adding a minimum amount of saturated Na_2SO_3. The aqueous layer was then removed and the organic layer washed with saturated $NaHCO_3$ (4 × 75 mL) and brine (2 × 75 mL). The solvent was dried with $MgSO_4$, filtered, and carefully removed on a rotary evaporator until about 20 mL of liquid remained. The remainder of the solvent was evaporated by using a stream of nitrogen and a 40 °C water bath. A yellow, lachrymatory liquid containing some white solid remained. This residue was then diluted with 100 mL of ether and 10 mL of 5% HCl was added to dissolve the solid. The ether layer was then washed with 5% HCl (2 × 10 mL), 5% $NaHCO_3$ (2 × 10 mL), and brine (1 × 10 mL). The solvent was dried with $MgSO_4$, filtered, partially removed with a rotary evaporator, and then the remainder evaporated with a stream of nitrogen in a hood. A yellow, oily, heterogeneous liquid remained.

The crude, oily liquids from several preparations were combined and eluted through a 2.5 × 6 cm column of Florisil with 75 mL of pentane. This gave a biphasic eluate, which was separated, with both layers containing product. The pentane was removed from the upper layer, giving a yellow, oily liquid. The bottom layer was dissolved in 100 mL of ether and washed and dried as above. The ether was removed, giving a yellow liquid. The column was then further eluted with ether, from which crystallized white needles.

The above yellow liquids were combined, dissolved in 75 mL of ether, and dried with $MgSO_4$. After filtration, the ether was removed and the product was distilled by using a one-piece simple distillation unit fitted with a fraction collector. After a forerun at 28–30 °C, 0.5 mmHg, a clear, colorless liquid (8.34 g, 47%) was collected in a flask cooled with dry ice. Simpler workups did not provide material of comparable purity.

^{1}H NMR ($CDCl_3$): δ 1.57 (9 H, s), 3.20 (2 H, dt, $^3J_{FH}$ = 23.8, $^3J_{HH}$ = 5.95 Hz), 4.77 (2 H, dt, $^2J_{FH}$ = 46.3, $^3J_{HH}$ = 5.95 Hz). IR ($CDCl_3$): 2995 (s), 1750 (sh), 1730 (s), 1380 (s), 1120 (s), 1100 (s) cm^{-1}. ^{13}C NMR ($CDCl_3$): δ 27.8, 39.8 (d, $^2J_{FC}$ = 22.0 Hz), 78.0 (d, $^1J_{FC}$ = 166 Hz), 84.4, 159.9, 191.8 (d, $^3J_{FC}$ = 4.5 Hz). MS: calculated for $C_8H_{13}FO_3$, M_r 176.0845; found, M_r + 1 177.0926. bp 44–45 °C (0.5 mmHg).

(*e*) *2-Oxo-4-fluorobutanoic Acid.* [The procedure used was analogous to that of Mosher and Nimitz (1981).] A 100-mL round-bottom flask containing a magnetic stirring bar was charged with 50 mL of dry trifluoroacetic acid. The flask was cooled to 0 °C, and the *tert*-butyl ester was added dropwise. The solution was stirred for a further 50 min and then 20 min at room temperature. The trifluoroacetic acid was then removed from the lightly colored solution by using a stream of nitrogen at 40 °C and then an aspirator. This material was then Kugelrohr-distilled from the reaction flask, giving pure, white, hygroscopic crystals (4.67 g, 93%). This material is stable for months over Drierite at −20 °C. It should be handled in a glovebag.

^{1}H NMR ($CDCl_3$): δ 3.37 (2 H, dt, $^3J_{FH}$ = 24.4, $^3J_{HH}$ = 5.64 Hz), 4.82 (2 H, dt, $^2J_{FH}$ = 46.2, $^3J_{HH}$ = 5.64 Hz), 5.52 (br s). IR ($CDCl_3$): 3400 (w), 2960 (w), 1765 (s), 1735 (s), 1350 (m), 1250 (m), 1100 (m), 1050 (m), 870 (s) cm^{-1}. ^{13}C NMR (D_2O): (keto isomer) δ 39.07, 78.81 (d, $^1J_{FC}$ = 160.6 Hz), 174.31, 196.0; (hydrate) 39.07 (d, $^2J_{FC}$ = 19.06 Hz), 80.35 (d, $^1J_{FC}$ = 158.9 Hz), 92.68, 174.29. MS: calculated for $C_4H_5FO_3$, M_r 120.0221; found, M_r + 1 121.0303. ^{19}F NMR (376.50 MHz, D_2O): (keto) δ −221.7 (tt, $^2J_{FH}$ = 45.8, $^3J_{FH}$ = 25.4 Hz); (hydrate) −221.2 (tt, $^2J_{FH}$ = 46.8, $^3J_{FH}$ = 28.3 Hz). bp 80–85 °C (0.3 mmHg).

(*f*) *2-Oxo-3-bromo-4-fluorobutanoic Acid.* A 25-mL three-neck, round-bottom flask containing a magnetic stirring bar was charged with 2-oxo-4-fluorobutanoic acid (0.0166 mol, 2.0 g). The flask was fitted with a small condenser, septa, and a nitrogen inlet tube. A 3.5-mL portion of dry CCl_4 was added and the flask immersed in an oil bath at 55 °C. After about 10 min, a solution of dry Br_2 (0.020 mol, 3.2 g) in 1.5 mL of CCl_4 was added dropwise over about 15 min. After a short induction period, bubbling occurred. The septum was removed from the condenser and a slow stream of nitrogen blown through the system. The evolution of HBr was evident. The orange-red solution was stirred for another 45 min at 55–60 °C. The solution was then allowed to cool to room temperature and the solvent removed with a stream of nitrogen at 40 °C. 1,2-Dichloroethane (2 mL) was then added and the process repeated. This procedure was repeated 5 more times using a rotary evaporator to remove all solvent, excess Br_2, and HBr. A semitranslucent, orange liquid remained that was Kugelrohr-distilled, giving a clear, light green liquid which solidified overnight in a freezer to a pale green solid (3.15 g, 95%). This material was 90–95% pure and could be used as such. Attempts to purify it were not successful. This material is extremely hygroscopic and should be handled in a glovebag. It liquifies after a short while at room temperature. It can be stored for months over Drierite at −20 °C with no apparent decomposition.

^{1}H NMR (CD_3CN) (ABX system with fluorine coupling): δ 4.81 (1 H, $^2J_{FH}$ = 46.2, $^2J_{HH}$ = 10.37, $^3J_{HH}$ = 5.75 Hz), 4.87 (1 H, $^2J_{FH}$ = 46.2, $^2J_{HH}$ = 10.37, $^3J_{HH}$ = 5.96 Hz), 5.35 (1 H, $^3J_{FH}$ = 14.5, $^3J_{HH}$ = 5.75, $^3J_{HH}$ = 5.96 Hz). IR (neat): 3200 (s), 1740 (s), 1460 (w), 1390 (m), 1330 (m), 1250 (m), 1120 (m), 1020 (s) cm^{-1}. ^{13}C NMR (acetone-d_6): δ 44.63 (d, $^2J_{FC}$ = 21.90 Hz), 82.03 (d, $^1J_{FC}$ = 174.7 Hz), 160.7, 186.1. MS: calculated for $C_4H_4BrFO_3$, M_r 199.9306; found, M_r + 1 200.9386. ^{19}F NMR (acetone-d_6): δ −217.7. bp 100–105 °C (0.3 mmHg).

(*g*) *Dimethyl 2-(Fluoromethyl)-1-carboxyvinyl Phosphate.* A 5-mL round-bottom flask containing a magnetic stirring bar was charged with trimethyl phosphite (1.7 mmol, 210 mg), and the flask was cooled in an ice bath. A solution of the bromo acid (1.08 mmol, 216 mg) in 3.0 mL of dry ether was added dropwise over 10–15 min. The solution was stirred 3 h and then allowed to warm. During this time, a white precipitate (an impurity or byproduct) developed on the walls of the flask. The solution was then filtered through glass wool and transferred into a dry 10-mL conical flask. The ether was removed by using a stream of nitrogen and a 40 °C water bath. A vacuum pump was then used to remove residual solvent and phosphite. A clear, colorless, viscous liquid remained (234 mg) that was an 80:20 mixture of *Z*/*E* isomers.

^{1}H NMR (acetone-d_6): (*Z* isomer) δ 3.88 (6 H, d, $^3J_{PH}$ = 11.46 Hz), 5.23 (2 H, ddd, $^2J_{FH}$ = 46.28, $^3J_{HH}$ = 6.17, $^5J_{PH}$ = 2.65 Hz), 6.62 (1 H, dtd, $^3J_{FH}$ = 13.88, $^3J_{HH}$ = 6.17, $^4J_{PH}$ = 1.99 Hz); (*E* isomer) 3.75 (6 H, d, $^3J_{PH}$ = 11.02 Hz), 5.41 (2 H, ddd, $^2J_{FH}$ = 47.37, $^3J_{HH}$ = 5.07, $^5J_{PH}$ = 3.31 Hz), 6.35 (1 H, dtd, $^3J_{FH}$ = 19.84, $^3J_{HH}$ = 5.07, ^4JP_H = 2.65 Hz). IR ($CDCl_3$): 2940 (s), 2820 (s), 1730 (s), 1670 (m), 1450 (m), 1250 (s), 1050 (s), 850 (m) cm^{-1}. MS: calculated for $C_6H_{10}FO_6P$, M_r 228.0196; found, 228.0199.

(*h*) *Bis(trimethylsilyl) 2-(Fluoromethyl)-1-[((trimethylsilyl)oxy)carbonyl]vinyl Phosphate.* [The procedure used was analogous to that of Bartlett and Chouinard (1983).] The product from the above reaction (234 mg, 1.02 mmol) was dissolved in 5.6 mL of dry CH_2Cl_2, and bromotrimethylsilane (5.3 mmol, 810 mg) was added. After 90 min, the solvent was removed with a stream of nitrogen at 40 °C. The excess bromotrimethylsilane and residual HBr were then removed with a vacuum pump, giving an 80:20 mixture of *Z*/*E* isomers (388 mg, 92%).

^{1}H NMR (acetone-d_6): (*Z* isomer) δ 0.05 (27 H, s), 5.21 (2 H, ddd), 6.55 (1 H, dtd); (*E* isomer) 0.05 (27 H, s), 5.38 (2 H, ddd), 6.33 (1 H, dtd).

(*i*) *Monocyclohexylammonium 3-(Fluoromethyl)-phosphoenolpyruvate.* The tris(trimethylsilyl) ester (388 mg, 0.93 mmol) was dissolved in 2.9 mL of dry ether and transferred to a dry test tube. A solution of cyclohexylamine (0.74 mmol, 73.7 mg) in 0.13 mL of methanol was then added dropwise to the solution. A small amount of fine, white precipitate developed and was immediately centrifuged. The tube was tightly capped and stored for 18 h at 4 °C. Overnight, a coarse, white solid developed. The ether solution was removed with a Pasteur pipet and the solid rinsed with ether in the tube. The ether was removed and the solid dried first with a stream of nitrogen and then with a vacuum pump for several hours. The white, flaky solid (184 mg, 80%) could then be separated from the pellet. This material, which was only the *Z* isomer, contained 8% of an impurity (visible as an NMR resonance just upfield of the vinylic proton) that persisted even after slow recrystallization from methanol–ether (1:1).

^{1}H NMR (D_2O): δ 1.0–2.1 (10 H, m), 3.14 (1 H, br s), 5.23 (2 H, ddd, $^2J_{FH}$ = 46.05, $^3J_{HH}$ = 6.17, $^5J_{PH}$ = 2.42 Hz), 6.57 (1 H, dtd, $^3J_{FH}$ = 14.98, $^3J_{HH}$ = 6.17, $^4J_{PH}$ = 2.42 Hz). IR (KBr): 3050 (bs), 2940 (w), 2860 (w), 2610 (w), 2560 (w), 1690 (m), 1655 (m), 1625 (m), 1545 (m), 1460 (m), 1285 (m), 1215 (s), 1090 (s), 915 (s) cm^{-1}. ^{13}C NMR (D_2O, 10 °C): δ 78.06 (d, $^1J_{FC}$ = 155.0 Hz), 123.11 (dd, $^3J_{PC}$ = 5.37, $^2J_{FC}$ = 21.45 Hz), 140.06 (dd, $^2J_{PC}$ = 7.18, $^3J_{FC}$ = 3.60 Hz), 165.98; (cyclohexylammonium) 23.78, 24.26, 30.29, 50.29. ^{19}F NMR (D_2O): δ −220.5 (ddt, $^2J_{FH}$ = 46.2, $^3J_{FH}$ = 13.8, $^5J_{PF}$ = 5.93 Hz). ^{31}P NMR (D_2O): δ −3.56 (d, $^5J_{PF}$ = 5.81 Hz). mp 126–128 °C dec.

(*j*) *2-Oxo-3-butenoate.* This compound was prepared quantitatively from FMe-PEP or 2-oxo-4-fluorobutanoic acid by addition of a known amount of the precursor to a phosphate or HEPES buffer at pH (pD) 8, 20 °C.

(*k*) *2-Hydroxy-3-butenoic Acid* (*Vinylglycolic Acid*). This compound was prepared from acrolein and NaCN by using the procedure of Glattfeld and Hoen (1935).

(*l*) *4-Fluoro-2-hydroxybutanoic Acid.* This compound was prepared by reduction of 4-fluoro-2-oxobutanoic acid with $NaCNBH_3$.

(*m*) *Reaction of 2-Oxo-3-butenoate with Dithiothreitol.* A solution of FMe-PEP (0.023 mmol, 7.0 mg) and dithiothreitol (0.024 mmol, 3.7 mg) in 1.5 mL of water was adjusted to pH 8 with NaOH. After 5 min, the pH was lowered to 2 by adding Dowex 50 (H^+). The mixture was filtered through glass wool and the pH raised to 8 with NaOH. The solution was lyophilized, leaving a yellow solid, which was taken up in D_2O and examined by ^{1}H NMR.

(*n*) *Decomposition of FMe-PEP.* The decomposition of FMe-PEP was monitored by adding aliquots of FMe-PEP solution at low pH to a solution containing lactate dehydrogenase and NADH and observing the decrease in absorbance at 340 nm in solutions containing 50 mM HEPES buffer, pH 7.0 or 8.0, 0.2 mM NADH, and lactate dehydrogenase.

(*o*) *Decomposition of 2-Oxo-4-fluorobutanoic Acid.* The decomposition of 2-oxo-4-fluorobutanoic acid was followed by observing the increase in absorbance at 260 or 240 nm in the presence of 300 mM HEPES buffer, pH 7.0 or 8.0. The decomposition of 2-oxo-4-fluorobutanoic acid in aqueous solution (104 mM in D_2O, pD 2.1) was monitored in an NMR tube by ^{1}H NMR.

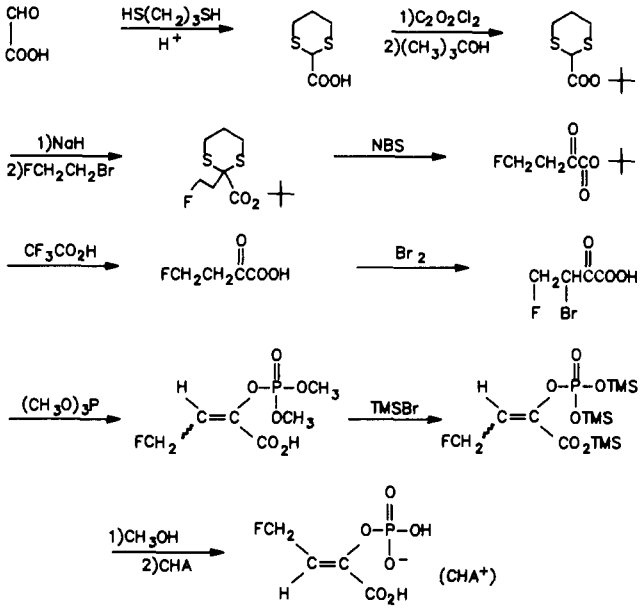
(*p*) *Reaction of 2-Oxo-3-butenoate with N-Acetyl-L-cysteine.* The disappearance of 2-oxo-3-butenoate was followed by monitoring the decrease in absorbance at 240 or 250 nm in the presence of 300 mM HEPES buffer, pH 8.0, 0.3 mM 2-oxo-3-butenoate, and excess *N*-acetyl-L-cysteine (1–1.5 mM).

Kinetics. (*a*) *Lactate Dehydrogenase.* All reactions were followed by observing the decrease in NADH absorbance at 340 nm in the presence of 300 mM HEPES buffer, pH 8.0, 0.2 mM NADH, and lactate dehydrogenase.

(*b*) *PEP Carboxylase.* Activity was measured by observing the decrease in absorbance at 340 nm in the presence of 300 mM HEPES, pH 8.0, 0.2 mM NADH, 5 mM $NaHCO_3$, 1 mM PEP, 5 mM $MgCl_2$, and 12.7 units/mL malate dehydrogenase. In some cases, Mn^{2+} was used in place of Mg^{2+}.

(*c*) *Pyruvate Kinase.* Enzyme activity was measured by observing the decrease in absorbance at 340 nm in the presence of 300 mM HEPES, pH 7.8, 0.2 mM NADH, 1 mM ADP, 0.3 mM PEP, 100 mM KCl, 4 mM $MgCl_2$, and 13.3 units/mL lactate dehydrogenase or else by measuring ATP formation in the presence of 300 mM HEPES, pH 7.8, 1 mM NAD, 1 mM ADP, 0.5 mM PEP, 5 mM glucose, 3 mM $MnCl_2$, 12 units/mL hexokinase, and 10 units/mL glucose-6-phosphate dehydrogenase.

Scheme II



RESULTS

Syntheses. Monocyclohexylammonium (*Z*)-3-(fluoromethyl)phosphoenolpyruvate was synthesized in nine chemical steps starting from glyoxylic acid (Scheme II). This route also provided 2-oxo-4-fluorobutanoic acid.

Commercially available glyoxylic acid hydrate was azeotropically thioacetalized with 1,3-propanedithiol. Esterification of the acid produced either via the acid chloride or by using a Vilsmeier reagent gave the *tert*-butyl ester. The sodium anion of this dithiane was then cleanly alkylated with 1-bromo-2-fluoroethane, giving good yields (>80%) of the protected ester. The hydrolytic unmasking of the dithianyl group with *N*-bromosuccinimide resulted in the isolation of the sensitive α-keto ester with <50% recovery. Attempted bromination of the ester resulted in 50% cleavage. Instead, the ester-protecting group was removed by using trifluoroacetic acid (Mosher & Nimitz, 1981). The free acid was then subjected to bromination. Perkow reaction with trimethyl phosphite cleanly produced the phosphoenolpyruvate framework in 95% yield as an 80:20 mixture of *Z*/*E* isomers, according to ^{1}H NMR, along with 5% of a separable byproduct whose identity is unknown. Conversion of this compound to the tris(trimethylsilyl) ester proceeded smoothly in spite of the generation of HBr in the solution. Hydrolysis of the latter compound was accomplished with 3 equiv of sodium ethoxide (Sekine et al., 1982). This reaction also resulted in formation of 30% of an unsaturated decomposition product. Alternatively, treatment of the tris ester with 1 equiv of cyclohexylamine in methanol afforded FMe-PEP with only minimal decomposition. The minor *E* isomer apparently decomposed during this last treatment, and only the *Z* isomer was obtained.

In general, synthesis of PEP derivatives may be approached either via carboxylic esters of pyruvic acid or via the free acids. The esters tend to be more stable, less hygroscopic, and more easily purified than the free acids. The above synthesis was carried through to the penultimate step with the methyl ester, the ethyl ester, and the *tert*-butyl ester. The basic hydrolysis conditions used to hydrolyze the former two esters caused decomposition of the compound, and ultimately the route described in Scheme II was used.

Stability of FMe-PEP. This compound was completely stable as a solid for over a year at −20 °C or for up to 3 days at room temperature as a solution in D_2O at pD 2.5. However, it decomposed rapidly at 25 °C at pH 6 or above. The decomposition product is formed cleanly and is a substrate for lactate dehydrogenase. The half-life for decomposition is 7.8 s at pH 7.0, 25 °C, and 5.4 s at pH 8.0. The NMR spectrum of the product produced following decomposition in D_2O at pD 8 showed only vinylic resonances corresponding to 2-oxo-3-butenoate.

Decomposition of FMe-PEP might give 4-fluoro-2-oxobutanoate or might proceed in a concerted fashion directly to 2-oxo-3-butenoate. Both the latter compounds are good substrates for lactate dehydrogenase (vide infra). A ^{19}F NMR spectrum of a solution following decomposition of FMe-PEP in the presence of lactate dehydrogenase and NADH showed only inorganic fluoride and no fluorine-containing organic products. Thus, it appears that decomposition proceeds directly to give 2-oxo-3-butenoate.

Stability of 4-Fluoro-2-oxobutanoate. The ^{1}H NMR spectrum of this compound at pH 2.3 in D_2O (104 mM, pD 2.1) showed the presence of keto and hydrate forms in the ratio 1:3. At pH 2.1, decomposition to 2-oxo-3-butenoate occurred with a half-time of several hours. No deuterium exchange into 4-fluoro-2-oxobutanoate or the product was observed. At higher pH, decomposition was faster but could be followed by observing the increase in absorbance at 240–260 nm due to formation of 2-oxo-3-butenoic acid. The half-life for decomposition was 162 s at pH 7.0, 25 °C, and 52 s at pH 8.0.

Synthesis and Stability of 2-Oxo-3-butenoate. This compound could be generated quantitatively from either FMe-PEP or 4-fluoro-2-oxobutanoate in aqueous solution at pH 8. The compound has a strong UV absorbance at 240 nm. It elutes as a single peak on cation-exchange high-performance liquid chromatography with an elution time similar to that of other α-keto acids. 2-Oxo-3-butenoate is a good substrate for lactate dehydrogenase (vide infra) and is quantitatively reduced when generated from either FMe-PEP or 4-fluoro-2-oxobutanoate. In aqueous solution, 2-oxo-3-butenoate slowly decomposes ($t_{1/2}$ > 1 h at pH 8) to give a complex mixture of products. 2-Oxo-3-butenoate has previously been implicated by Meister et al. (1976) as an elimination product from the action of L-amino acid oxidase on methionine sulfoximine. The half-life for decomposition of approximately 60 min at pH 8.0 reported in that work is consistent with that found here.

2-Oxo-3-butenoate reacts rapidly upon addition of 1.4–1.8 equiv of *N*-acetyl-L-cysteine, methyl L-cysteinate, or mercaptoacetic acid, with the vinylic resonances in the NMR spectrum disappearing within 5 min. The reaction of 2-oxo-3-butenoate with 1 equiv of dithiothreitol in H_2O gave a 2:1:1 mixture of mono-, di-, and unalkylated (and oxidized) derivatives of DTT that was isolated and completely characterized by ^{1}H NMR.

The reaction of *N*-acetyl-L-cysteine with 2-oxo-3-butenoate (0.2–0.3 mM) was followed spectrophotometrically at 240 or 250 nm under pseudo-first-order conditions. The half-life for the reaction was 5.4 s in the presence of 1.0 mM *N*-acetyl-L-cysteine.

Reactions of Lactate Dehydrogenase. Both 4-fluoro-2-oxobutanoate and 2-oxo-3-butenoate are substrates for lactate dehydrogenase at pH 8.0, 25 °C. The reactions were followed by observing the decrease in absorbance at 340 nm due to the disappearance of NADH, and the amount of NADH used was always equal to the amount of substrate added. In studying 4-fluoro-2-oxobutanoate, it was necessary to use low concentrations of the compound and high enzyme concentrations to ensure that reduction occurred, rather than decomposition. Kinetic parameters for 4-fluoro-2-oxobutanoate, 2-oxo-3-

Table I: Kinetic Constants for Substrates of Lactate Dehydrogenase at pH 8.0 in 300 mM HEPES Buffer, 25 °C

compound	K_m (mM)	V_{max}
pyruvate	0.11	178
α-oxobutanoate	1.3	90
4-fluoro-2-oxobutanoate	0.21	12
2-oxo-3-butenoate	1.3	560

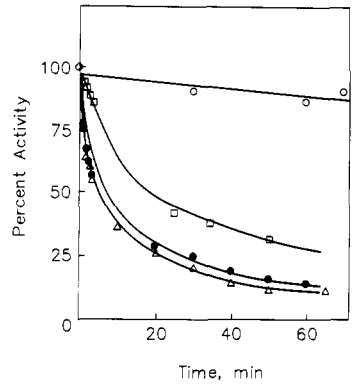


FIGURE 1: Inactivation of PEP carboxylase by FMe-PEP, 4-fluoro-2-oxobutanoate, and 2-oxo-3-butenoate at pH 8.0, 25 °C, in the presence of 5 mM $NaHCO_3$, 1 mM $MnCl_2$, and 0.05 mM EDTA: (○) control; (●) 2 mM 2-oxo-3-butenoate; (□) 2 mM FMe-PEP; (△) 2 mM 4-fluoro-2-oxobutanoate.

butenoate, pyruvate, and α-oxobutanoate under similar conditions are reported in Table I. Both these new compounds are excellent substrates for lactate dehydrogenase.

Reactions of Phosphoenolpyruvate Carboxylase. The enzyme phosphoenolpyruvate carboxylase lost activity at pH 8.0, 25 °C, upon addition of FMe-PEP, 4-fluoro-2-oxobutanoate, or 2-oxo-3-butenoate in the presence of Mn^{2+} (Figure 1). All three compounds gave similar kinetics for the inactivation, presumably because the first two are rapidly converted into 2-oxo-3-butenoate. The kinetics of inactivation were not first order, presumably because of concurrent decomposition of 2-oxo-3-butenoate.

Phospholactate and epoxymaleate are competitive inhibitors of PEP carboxylase and therefore presumably bind at the active site (O'Leary, 1981). The inactivation of PEP carboxylase by FMe-PEP proceeded at identical rates in the presence and absence of 0.5 mM phospholactate or 0.7 mM epoxymaleate (Figure 2).

Inactivation of PEP carboxylase by FMe-PEP was completely prevented by the presence of 2.3 mM dithiothreitol (Figure 2). If DTT was added during the inactivation, further inactivation was prevented, and the enzyme activity remained stable at the level it was when DTT was added (data not shown).

Pyruvate Kinase. Pyruvate kinase lost activity, at pH 7.8, 25 °C, using Mn^{2+} upon the addition of FMe-PEP (Figure 3). The kinetics were not first order, presumably because of decomposition of 2-oxo-3-butenoate during the inactivation. The presence of 1 mM phospholactate had no effect on the inactivation rate (data not shown). Inactivation was prevented by the presence of dithiothreitol.

Discussion

Stereochemistry of PEP Analogues. We have synthesized FMe-PEP and have assigned the *Z* configuration to the principal isomer obtained from our synthesis. The assignment of *E* and *Z* configurations to 3-substituted PEP derivatives is problematical because no single method for assigning configurations is totally unambiguous and the literature is not self-consistent. Several approaches have been taken: (1) The

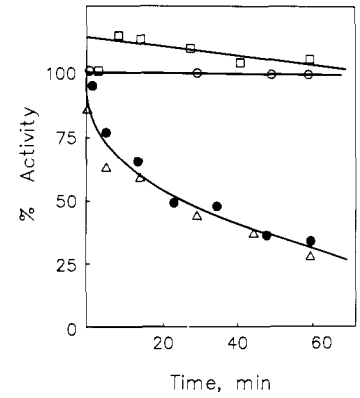


FIGURE 2: Inactivation of PEP carboxylase by 3.5 mM FMe-PEP at pH 8.0 in the presence of 5 mM $NaHCO_3$, 1 mM $MnCl_2$, and 0.05 mM EDTA: (○) control; (●) FMe-PEP and 0.5 mM phospholactate; (△) FMe-PEP and 0.7 mM epoxymaleate; (□) FMe-PEP and 2.3 mM DTT.

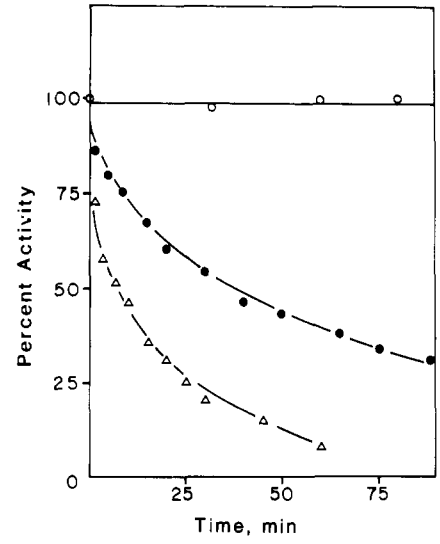


FIGURE 3: Inactivation of pyruvate kinase by 2.0 mM FMe-PEP at pH 7.5 in the presence of 0.58 mM ADP, 58 mM KCl, and 0.05 mM EDTA: (△) in the presence of 3.5 mM $MgCl_2$; (●) in the presence of 1.75 mM $MnCl_2$; (○) control.

geometries have been assigned on the basis of four-bond phosphorus–hydrogen coupling, assuming that the trans coupling is larger than the cis coupling (Duffy et al., 1982). (2) The geometries have been assigned on the basis of three-bond C–H coupling constants, assuming that the trans coupling is larger than the cis coupling. (3) The geometries have been assigned by using the chemical shift of the vinylic proton, assuming that the vinylic proton of the *Z* isomer is downfield of that of the *E* isomer (Stubbe & Kenyon, 1971). (4) The geometries have been assigned on the assumption that Perkow reactions of pyruvate derivatives give principally *Z* isomers as products (Stubbe & Kenyon, 1971). Unfortunately, these approaches do not give consistent assignments.

The claim that the trans four-bond phosphorus–hydrogen coupling (for the *Z* isomer) is greater than the cis coupling (for the *E* isomer) in PEP derivatives is modeled on the proven three-bond proton–proton coupling in olefinic systems (Borowitz et al., 1971), but it is not borne out by experiment in phosphorus–hydrogen systems, probably because of conformational variations in the latter systems.

The C–H coupling argument has many analogies in a variety of compounds. This approach can be safely used, but to date it has only been used for one compound in the phosphoenolpyruvate series. Stubbe and Kenyon (1971) labeled

phosphoenol-α-oxobutanoate with carbon-13 in the carboxyl carbon and then used the three-bond carbon–hydrogen coupling assuming that, as in olefins, trans coupling is larger than cis coupling.

The chemical shift argument is based on experiment and subsequent analogies. In the case of phosphoenol-α-oxobutanoate (where the *E*/*Z* assignment is least ambiguous), the *Z* isomer is downfield of the *E* isomer. However, the four-bond P–H coupling is smaller for the *Z* isomer than for the *E* isomer, which is inconsistent with the previous argument. Unfortunately, the ^{13}C labeling experiment has only been done in one case, and we are left to extrapolate from this single example.

If this chemical shift argument is correct, then it appears that, at least for PEP analogues, Perkow reactions always lead principally to the *Z* isomer (Stubbe & Kenyon, 1971). We have prepared several esters and salts of the Perkow reaction products phosphoenol-α-oxobutanoate, 3-chloro-PEP, and 3-bromo-PEP, and in all cases, the major isomer (believed to be *Z*) is downfield of the minor isomer (believed to be *E*). However, these assignments should all be considered tentative, and further NMR or X-ray studies are needed to resolve this question.

Thus, we tentatively conclude that arguments 2–4 above are correct, but (1) is uncertain. By the same token, we believe that our compounds are all *Z* isomers.

Decomposition of FMe-PEP. FMe-PEP is stable in aqueous solution at pH values below 3, but at neutral pH, the compound decomposes in a few seconds. Although detailed kinetic studies have not been done, this reactivity appears to be associated with conversion of the phosphate group to the dianion form. By comparison, both PEP and 3-fluoro-PEP are stable for at least several days at neutral pH (Stubbe & Kenyon, 1971; Benkovic & Schray, 1968).

The instability of FMe-PEP is probably due to concerted decomposition of the compound to form fluoride, metaphosphate, and 2-oxo-3-butenoate, rather than to stepwise loss of phosphate and fluoride. We were unsuccessful in trapping 4-fluoro-2-oxobutanoic acid with lactate dehydrogenase and NADH during the decomposition of FMe-PEP, even though we know that this compound has a significant lifetime and is a good substrate for lactate dehydrogenase.

The instability of 4-fluoro-2-oxobutanoate is probably due to the ready enolization of this compound, which then rapidly loses fluoride ion, to form 2-oxo-3-butenoate.

2-Oxo-3-butenoate is formed by the spontaneous decomposition of both FMe-PEP and 4-fluoro-2-oxobutanoate. NMR spectra are consistent with this structure, and our data are consistent with previous observations of this compound. The compound persists in aqueous solution at neutral pH for at least a few hours. On the other hand, it reacts within seconds with a variety of thiols when their concentrations are near 1 mM. The products of some of these thiol reactions have been characterized.

Inactivation of PEP Carboxylase by FMe-PEP. When a solution of FMe-PEP at low pH is added to a solution of PEP carboxylase at pH 8, the enzyme is gradually inactivated over a period of more than an hour. FMe-PEP is converted within seconds into 2-oxo-3-butenoate under these conditions, and we believe that the actual inactivation results from reaction of this compound with the enzyme. Consistent with this view is the observation that the enzyme is inactivated at essentially the same rate by 4-fluoro-2-oxobutanoate, which is also rapidly converted into 2-oxo-3-butenoate. Similarly, if 2-oxo-3-butenoate is pregenerated by hydrolysis of FMe-PEP at pH 8, inactivation of the enzyme occurs in a similar manner. Also consistent with the key role of 2-oxo-3-butenoate is the ability of dithiothreitol to protect the enzyme against inactivation. As we noted above, dithiothreitol reacts very rapidly with 2-oxo-3-butenoate.

Interestingly, the inactivation of PEP carboxylase appears not to occur at the active site. Neither phospholactate nor epoxymaleate protects the enzyme against inactivation. Previous studies have shown that PEP carboxylase can be inactivated by a variety of sulfhydryl reagents, including bromopyruvate (Gonzalez et al., 1986; J. O'Laughlin and M. H. O'Leary, unpublished results) and *p*-(chloromercuri)-benzoate (Stiborova & Leblova, 1986). In the case of bromopyruvate, inactivation shows saturation kinetics and the enzyme can be protected by substrates and substrate analogues. The fact that similar protection cannot be observed in the present case indicates that 2-oxo-3-butenoate reacts with a different sulfhydryl group than is reactive toward bromopyruvate.

FMe-PEP persists for at least a few seconds in aqueous solution at pH 8, so we added this compound directly (with rapid stirring) to enzyme solutions at pH 8 in an attempt to see mechanism-based inactivation (cf. Scheme I). However, the only inactivation seen was the same as that seen with preformed 2-oxo-3-butenoate. No evidence for direct suicide inactivation was ever seen. Consequently, either the compound is not a substrate or the product does not inactivate the enzyme before being released into solution.

Walsh has postulated that 2-oxo-3-butenoate is responsible for the inactivation of L-amino acid oxidase by vinylglycine and of L-α-hydroxy acid oxidase by 2-hydroxy-3-butenoate (Walsh & Cromartie, 1975; Walsh & Marcotte, 1976; Walsh et al., 1975). In the former case, the fact that the inactivator was inaccessible for scavenging by exogenous thiols indicated that the inactivation is active-site-directed. On the other hand, the hydroxy acid oxidase was alkylated randomly (30–40 times) before activity was lost and the enzyme could be completely protected by the addition of DTT.

Inactivation of Pyruvate Kinase by FMe-PEP. The inactivation of pyruvate kinase by FMe-PEP appears to be quite similar to that of PEP carboxylase. The rates of inactivation are similar, and inactivation is prevented by the presence of DTT. The reactive compound is apparently 2-oxo-3-butenoate, and the reaction does not occur at the active site. This inactivator appears to be relatively specific for sulfhydryl groups, and pyruvate kinase apparently has no sulfhydryl groups at the active site (Muirhead et al., 1986).

ACKNOWLEDGMENTS

We thank Selene Nikaido for her work on the early steps in this synthesis.

REFERENCES

Bartlett, P. A., & Chouinard, P. M. (1983) *J. Org. Chem. 48*, 3854.

Benkovic, S. J., & Schray, K. J. (1968) *Biochemistry 7*, 4090.

Borowitz, I. J., Firstenberg, S., Casper, E. W. R., & Crouch, R. K. (1971) *J. Org. Chem. 36*, 3282.

Corey, E. J., & Erickson, B. W. (1971) *J. Org. Chem. 36*, 3553.

Díaz, E., O'Laughlin, J. T., & O'Leary, M. H. (1988) *Biochemistry* (first of four papers in this issue).

Duffy, T. H., Saz, H. J., & Nowak, T. (1982) *Biochemistry 21*, 132.

Glattfeld, J. W. E., & Hoen, R. E. (1935) *J. Am. Chem. Soc. 57*, 1405.

Gonzalez, D. H., Iglesias, A. A., & Andreo, C. S. (1986) *Arch. Biochem. Biophys. 245*, 179.
Kollonitsch, J. (1982) in *Biomedical Aspects of Fluorine Chemistry* (Filler, R., & Kobayashi, Y., Eds.) pp 93–122, Elsevier, New York.
Meister, A., Stephani, R. A., & Cooper, A. J. L. (1976) *J. Biol. Chem. 251*, 6674.
Mosher, H. S., & Nimitz, J. S. (1981) *J. Org. Chem. 46*, 211.
Muirhead, H., Clayden, D. A., Barford, D., Lorimer, C. G., Fothergill-Gilmore, L. A., Schlitz, F., & Schmitt, W. (1986) *EMBO J. 5*, 475.
O'Leary, M. H. (1981) *Annu. Rev. Plant Physiol. 33*, 297.
O'Leary, M. H., Rife, J. E., & Slater, J. D. (1981) *Biochemistry 20*, 7308.
Rose, I. A. (1970) *J. Biol. Chem. 245*, 6052.
Sekine, M., Futatugi, T., Yamada, K., & Hata, T. (1982) *J. Chem. Soc., Perkin Trans. 1*, 2509.
Stiborova, M., & Leblova, S. (1986) *FEBS Lett. 205*, 32.
Stubbe, J., & Kenyon, G. (1971) *Biochemistry 10*, 2669.
Walsh, C. (1984) *Annu. Rev. Biochem. 53*, 493.
Walsh, C., & Cromartie, T. H. (1975) *Biochemistry 14*, 3482.
Walsh, C., & Marcotte, P. (1976) *Biochemistry 15*, 3070.
Walsh, C., Kaback, R. H., & Kaczorowski, G. (1975) *Biochemistry 14*, 3903.

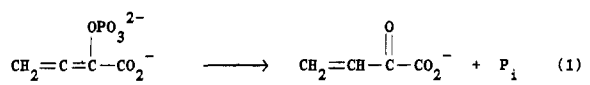
1-Carboxyallenyl Phosphate, an Allenic Analogue of Phosphoenolpyruvate†

Peter Wirsching and Marion H. O'Leary*
Departments of Chemistry and Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706
Received July 10, 1987; Revised Manuscript Received October 16, 1987

ABSTRACT: 1-Carboxyallenyl phosphate, the allenic homologue of phosphoenolpyruvate, has been synthesized in six steps. The key step in the synthesis is the isomerization of methyl 2-hydroxy-3-butynoate to the corresponding allenol and phosphorylation of this material. The allene is an excellent substrate for pyruvate kinase, undergoing reaction at more than half the rate of phosphoenolpyruvate. The allene is also a substrate for phosphoenolpyruvate carboxylase, being hydrolyzed by the enzyme rather than carboxylated. With both enzymes, the organic product is 2-oxo-3-butenoate, which gradually inactivates the enzymes by reaction with one or more sulfhydryl groups not at the active site.

Allenes have served as mechanism-based inhibitors of a number of enzymes. In the original work of Bloch et al. on β-hydroxydecanoyl thio ester dehydrase (Bloch, 1971), an allene was formed by rearrangement of an acetylenic substrate analogue, the *N*-acetylcysteamine thio ester of 3-decynoic acid. The elucidation and complete characterization of the inactivation mechanism has been accomplished only recently (Schwab et al., 1986). The time-dependent inactivation of Δ^5-3-ketosteroid isomerase via an allenic species has also been observed (Batzold & Robinson, 1975). In a somewhat different mode of inactivation, the allenic anions formed from propargyl amines inactivated flavin-linked monoamine oxidase by reaction with the flavin moiety (Maycock et al., 1976).

In all previous cases, inactivation has resulted from reaction of the allenic functionality with an enzyme cofactor or other nucleophile. We considered that the allenic analogue of PEP[1] might serve as a mechanism-based inhibitor of enzymes that metabolize PEP. Following phosphate transfer to a suitable acceptor, the resulting allenolate could be protonated, forming 2-oxo-3-butenoate (eq 1), which is known to be an inactivator



(1)

of a number of enzymes (Wirsching & O'Leary, 1988). We report here the synthesis of this compound and its reactions with PEP carboxylase and pyruvate kinase.

Experimental Procedures

Materials. Propargyl alcohol (Aldrich Chemical Co.), chromium trioxide (Baker Chemical Co.), glacial acetic acid (CCI), gaseous HCl (Airco), anhydrous ether (Fisher), *n*-butyllithium (Aldrich), and calcium hydride (Aldrich) were used without further purification. Sodium cyanide (Fisher) was dried under vacuum at 100 °C. Methanol (Fisher) was stored over 4A molecular sieves. Diisopropylamine was distilled from CaH_2. Tetrahydrofuran (Aldrich Gold Label) was distilled from sodium benzophenone ketyl. Dimethyl chlorophosphate was prepared as described by Muller (1964). Other reagents have been described previously (Wirsching & O'Leary, 1988).

Syntheses. General synthetic precautions and procedures are described in the preceding paper (Wirsching & O'Leary, 1988).

(*a*) *Propiolaldehyde [Modification of the Method of Sauer (1963)].* A 1000-mL three-neck, round-bottom flask fitted with a mechanical stirrer and septum was charged with 68 mL of concentrated H_2SO_4 in 100 mL of water. The flask was cooled to 0–5 °C, and propargyl alcohol (1.0 mol, 56 g) was added. The flask was then fitted with a stoppered, one-piece short-path distillation unit fitted with a 100-mL round-bottom flask, and the latter was immersed in a dry ice–acetone slurry. The entire system was then connected to an aspirator. A 500-mL round-bottom flask containing a solution of CrO_3 (1.05 mol, 105 g) in 68 mL concentrated H_2SO_4 and 160 mL of water was then connected via cannula to the reaction flask with the cannula still in the head space of the 500-mL flask. A vacuum of 40–60 mmHg was then obtained, and the CrO_3 solution was gradually transferred over 2 h. After the addition,

† This work was supported by Grant 83-CRCR-1-1297 from the Competitive Research Grants Office, U.S. Department of Agriculture.

[1] Abbreviations: PEP, phosphoenolpyruvate; DTT, dithiothreitol; alleno-PEP, 1-carboxyallenyl phosphate; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; P_i, inorganic phosphate.

0006-2960/88/0427-1355$01.50/0