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1-Carboxyallenyl Phosphate, an Allenic Analogue of Phosphoenolpyruvate†

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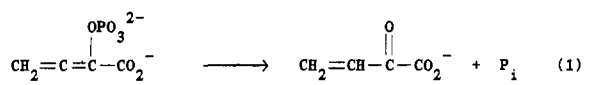
ABSTRACT: 1-Carboxyallenyl phosphate, the allenic homologue of phosphoenolpyruvate, has been synthesized in six steps. The key step in the synthesis is the isomerization of methyl 2-hydroxy-3-butynoate to the corresponding allenol and phosphorylation of this material. The allene is an excellent substrate 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,

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

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the cannula and ice bath were removed, and the mixture was stirred at room temperature at about 30 mmHg for 90 min, after which time the traps were removed and warmed. The traps contained two liquid layers. Solid NaCl (1 g) was added, and the lower aqueous layer was removed. The clear, almost colorless organic layer was dried with $MgSO_4$ and then filtered through glass wool. Clear, almost colorless propiolaldehyde was obtained (15.5 g, 29%). According to ^1H NMR, this material contained a small amount of water. Distillation resulted in substantial loss and was routinely omitted.

^1H NMR ($CDCl_3$): δ 3.50 (1 H, s), 8.40 (1 H, s).

(*b*) *Propiolaldehyde Cyanohydrin* (*Williams et al., 1979*). A 250-mL, three-neck, round-bottom flask containing a magnetic stirring bar was charged with dry, powdered NaCN (0.163 mol, 8.0 g) and 125 mL of dry ether. The suspension was cooled in an ice bath, and 1 mL of glacial acetic acid was added. Propiolaldehyde (0.10 mol, 5.4 g) in 10 mL of ether was added in portions alternately with glacial acetic acid (8.0 mL) over about 20 min. The bath was then removed and the suspension stirred at room temperature for 5 h. During this time, the suspension turned brown. The suspension was then filtered into a separatory funnel and the solid washed with 50 mL of ether. The yellow solution was then washed with water (4 × 20 mL) and brine (1 × 20 mL). After the solution was dried with $MgSO_4$ and filtered, the ether was removed on a rotary evaporator. A yellow-brown liquid remained (5.95 g, 73%), which was the cyanohydrin containing some acetic acid (thorough washing with water removed 95% of the acetic acid). This material was not purified. It could be stored dry for months at −20 °C.

^1H NMR ($CDCl_3$): δ 2.78 (1 H, d, $^4J_{HH}$ = 2.40 Hz), 5.20 (1 H, d, $^4J_{HH}$ = 2.40 Hz). IR ($CDCl_3$): 3570 (m), 3300 (s), 2250 (m), 2140 (m), 1060 (s) cm^{-1}.

(*c*) *Methyl 2-Hydroxy-3-butynoate* (*Palm et al., 1966*). A 50-mL round-bottom flask containing a magnetic stirring bar was charged with propiolaldehyde cyanohydrin (0.025 mol, 2.0 g) and 12 mL of anhydrous CH_3OH. The flask was fitted with a reflux condenser and warmed to 65 °C in an oil bath. A solution of 2.2 mL of concentrated HCl in 12 mL of HCl-saturated CH_3OH was then added dropwise until a white precipitate formed and refluxing occurred. The bath was then removed and the remainder of the HCl solution added such as to maintain a gentle reflux. The mixture was heated at 65 °C for 1 h. After cooling to room temperature, the solution was decanted into a separatory funnel containing 20 mL of ether (additional ammonium chloride precipitated) and the ammonium chloride washed with 75 mL of ether. The organic phase was then washed with water (3 × 20 mL), 5% $NaHCO_3$ (3 × 20 mL), water (2 × 20 mL), and brine (1 × 20 mL). The combined aqueous layers were then back-extracted with ether (5 × 20 mL), which was then washed with brine (1 × 20 mL). After drying over $MgSO_4$ and filtration, the ether and other volatiles were thoroughly removed on a rotary evaporator, giving a dark yellow liquid (2.5 g, 90%). In most cases, this liquid crystallized within 2 days, and the solid was recrystallized several times from saturated solutions in ether at −20 °C. Each time, the mother liquor was removed and evaporated and the crystals obtained were recrystallized. Ultimately, an off-white crystalline solid was obtained (0.830 g, 30%).

^1H NMR ($CDCl_3$): δ 2.54 (1 H, d, $^4J_{HH}$ = 2.43 Hz), 3.07 (1 H, d, $^3J_{HH}$ = 7.27 Hz), 3.90 (3 H, s), 4.88 (1 H, dd, $^4J_{HH}$ = 2.43, $^3J_{HH}$ = 7.27 Hz). ^1H NMR (benzene-d_6): δ 1.98 (1 H, d, $^4J_{HH}$ = 2.42 Hz), 3.14 (1 H, d, $^3J_{HH}$ = 7.27 Hz), 3.16 (3 H, s), 4.60 (1 H, dd, $^4J_{HH}$ = 2.42, $^3J_{HH}$ = 7.27 Hz). IR ($CDCl_3$): 3530 (m), 3310 (m), 2140 (w), 1755 (s), 1450 (m), 1100 (s) cm^{-1}. MS calculated for $C_5H_6O_3$, 114.0315; found, 114.0316. mp 57–59 °C.

(*d*) *Dimethyl 1-Carbomethoxyallenyl Phosphate.* A 25-mL conical flask was charged with diisopropylamine (1.48 mmol, 150 mg) and 18 mL of dry tetrahydrofuran. The solution was cooled to −78 °C in a dry ice–acetone bath, and then 1.03 mL of 1.50 M *n*-butyllithium in hexane was added. The solution was mixed by swirling, and then the flask was warmed at 0 °C for 20 min.

A 50-mL round-bottom flask containing a magnetic stirring bar was charged with methyl 2-hydroxy-3-butynoate (1.43 mmol, 163 mg) in a glovebag, and then 9 mL of dry tetrahydrofuran was added. This solution was cooled to −78 °C in a dry ice–acetone bath, and then dimethyl chlorophosphate (1.79 mmol, 258 mg) was added.

The above lithium diisopropylamide solution, at 0 °C, was then added slowly in 3-mL portions to this solution at −78 °C. After the addition, a translucent, light yellow solution was present. The bath was removed and the solution stirred at room temperature for 1 h. During this time, lithium chloride precipitated and the mixture became slightly more yellow.

A 10-mL portion of dry ether was then added, and the lithium chloride was allowed to settle. The solution was then removed by pipet and flash-eluted (using nitrogen gas) through a 3.5 × 0.7 cm column of flash silica gel that had been packed by using ether. One column volume of ether was then used for a final elution. The solvent was removed at 40 °C by using a stream of nitrogen. An amber-colored liquid remained (284 mg, 90%). This material was generally taken on immediately.

^1H NMR ($CDCl_3$): δ 3.83 (3 H, s), 3.87 (6 H, d, $^3J_{PH}$ = 11.46 Hz), 5.84 (2 H, d, $^5J_{PH}$ = 5.73 Hz). IR ($CDCl_3$): 3000 (w), 2980 (m), 2860 (w), 1975 (w), 1940 (w), 1740 (s), 1445 (m), 1430 (m), 1300 (s), 1250 (m), 1200 (m), 1060 (s), 865 (m) cm^{-1}. MS: calculated for $C_7H_{11}O_6P$, 222.02896; found, 222.0287. ^{13}C NMR ($CDCl_3$): δ 52.7, 54.9 (d, $^2J_{PC}$ = 6.13 Hz), 91.7 (d, $^4J_{PC}$ = 2.15 Hz), 116.9 (d, $^2J_{PC}$ = 8.24 Hz), 162.8 (d, $^3J_{PC}$ = 5.31 Hz), 206.1 (d, $^3J_{PC}$ = 4.81 Hz).

(*e*) *Bis(cyclohexylammonium) 1-Carbomethoxyallenyl Phosphate.* The deesterification of dimethyl 1-carbomethoxyallenyl phosphate presented difficulties. The protocol ultimately required (1) HBr-free bromotrimethylsilane, (2) predried reaction solutions, (3) 2 equiv (no excess) of bromotrimethylsilane, and (4) the shortest possible reaction time (20 min).

A solution of dimethyl 1-carbomethoxyallenyl phosphate (1.28 mmol, 284 mg) in 12 mL of CH_2Cl_2 in a 50-mL conical flask was dried by adding CaH_2. After 3 h, the yellow solution was transferred to a 25-mL flask. To this solution was added, with swirling, bromotrimethylsilane (2.56 mmol, 391 mg). After 20 min, the solvent and other volatiles were thoroughly removed at 40 °C by using a stream of nitrogen. A semitranslucent, reddish brown oil remained. This impure bis(trimethylsilyl) ester was diluted with 12 mL of dry ether, which caused precipitation of a yellow, gummy, resinous material. This was briefly allowed to settle, and then the supernatant was filtered through a plug of glass wool. The light yellow ethereal solution was cooled in ice, and then a solution of cyclohexylamine (1.04 mmol, 104 mg) in anhydrous methanol (7.7 mmol, 245 mg) was injected beneath the surface of the solution with stirring. A white precipitate developed. This material was taken through three centrifugation–ether washing cycles. After the material was dried with a gentle stream of nitrogen at 40 °C, a light yellow solid remained. The solid was recrystallized from ether–methanol. After a cen-

trifugation–ether wash cycle and drying, a white, powdery solid was obtained (102 mg, 20%).

^{1}H NMR (D_2O): δ 1.1–2.2 (20 H, m), 3.15 (2 H, br s), 3.81 (3 H, s), 5.81 (2 H, d, $^5J_{PH}$ = 3.87 Hz). IR (KBr): 2920 (br s), 2200 (w), 1945 (w), 1730 (s), 1630 (m), 1550 (m), 1450 (m), 1260 (s), 1120 (s), 865 (w) cm^{-1}. mp 135–140 °C dec.

(f) Monocyclohexylammonium 1-Carboxyallenyl Phosphate. A 20-mL screw-cap vial was charged with bis(cyclohexylammonium) 1-carbomethoxyallenyl phosphate (0.127 mmol, 50 mg) that was then dissolved in 2 mL of water. This solution (pH 5–6) was cooled in an ice bath, and Dowex 50 (H^+) was added until the pH was less than 2. After filtration through a plug of glass wool, the solution was cooled again in an ice bath and 0.83 mL of 1.5 M KOH (1.24 mmol) was added dropwise with swirling. The clear, colorless solution was then left at room temperature for 45 min. After this time, 8 mL of water was added, and the solution was left for another 15 min, after which it was cooled in an ice bath. Dowex 50 (H^+) was then added until the pH was about 2. The solution was filtered as above, diluted with 5 mL of water, and cooled in an ice bath. To the ice-cold solution was added cyclohexylamine (0.114 mmol, 11.4 mg). The clear, colorless solution was then lyophilized, leaving a white, fluffy, slightly hygroscopic solid (35 mg, 95%). It could be recrystallized from methanol–ether. This material could be stored dry for several months at −20 °C.

^{1}H NMR (D_2O): δ 1.1–2.15 (10 H, m), 3.15 (1 H, br s), 5.87 (2 H, d, $^5J_{PH}$ = 4.43 Hz). IR (KBr): 2920 (br s), 2650 (br), 2060 (w), 1950 (w), 1710 (s), 1620 (w), 1510 (m), 1450 (m), 1260 (s), 1150 (s), 870 (s) cm^{-1}. ^{13}C NMR (D_2O): δ 91.6, 118.0 (d, $^2J_{PC}$ = 6.0 Hz), 167.1 (d, $^3J_{PC}$ = 4.9 Hz), 205.9 (d, $^3J_{PC}$ = 5.0 Hz), cyclohexylammonium ion 23.8, 24.3, 30.3, 50.3. mp 75–80 °C dec. UV (H_2O): 240 nm (ϵ_{240} = 1.59 × 10^3 M^{-1} cm^{-1}).

Tris(cyclohexylammonium) salt ^{1}H NMR (D_2O): 1.1–2.2 (30 H, m), 3.15 (3 H, br s), 5.59 (2 H, d, $^5J_{PH}$ = 2.00 Hz). IR (KBr): 2950 (br s), 2230 (m), 1940 (w), 1640 (s), 1550 (s), 1470 (m), 1360 (s), 1280 (m), 1100 (s), 990 (s), 870 (m) cm^{-1}.

Kinetics. Enzymes were assayed at pH 8 as previously described (Wirsching & O'Leary, 1988). The reactions with alleno-PEP were also monitored by observing the increase in absorbance at 260 nm due to formation of 2-oxo-3-butenoate in solutions lacking nucleotide and dehydrogenase.

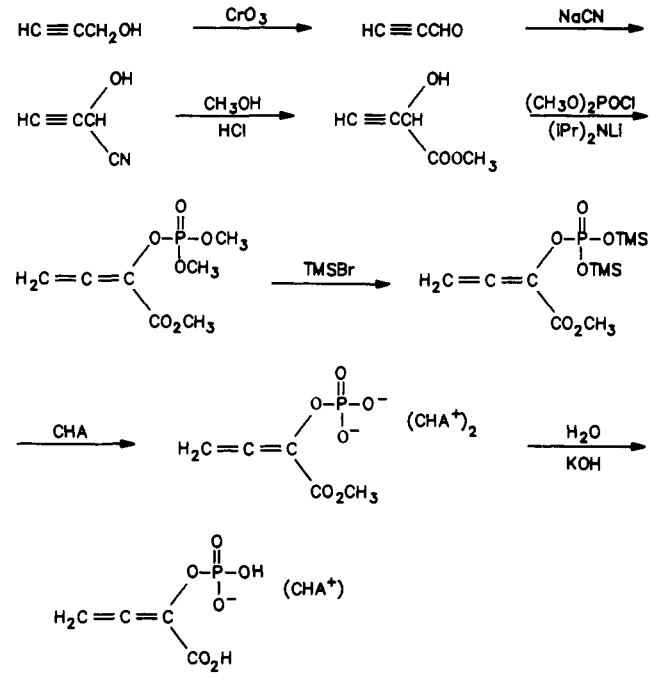
Results

Synthesis. The monocyclohexylammonium salt of 1-carboxyallenyl phosphate (alleno-PEP) was obtained in six chemical steps starting from propargyl alcohol (Scheme I).

Propiolaldehyde cyanohydrin was prepared from propiolaldehyde and NaCN (Sauer, 1963; Williams et al., 1979; Rambaud, 1934; Glattfeld & Hoen, 1935), but this compound could not be hydrolyzed to the hydroxy acid. Instead, a procedure analogous to one used to prepare methyl 2-hydroxy-3-butenoate (Palm et al., 1966) was used to prepare the crystalline alkyne. The corresponding ethyl ester of this material could be generated similarly by using ethanol, but in lower yield.

Surprisingly, all attempts to generate methyl 2-dimethylphosphinyloxy-3-butynoate, the expected precursor of the desired allenic substrate, were unsuccessful. Instead, it was fortunate that reaction of methyl 2-hydroxy-3-butynoate with 1 equiv of lithium diisopropylamide and 1.2 equiv of dimethyl chlorophosphate resulted in the formation of the desired allenyl framework, apparently through transient formation of 2-dimethylphosphinyloxy-3-butynoate. The use of 1 equiv of

Scheme I



tert-butyllithium as the base instead of lithium diisopropylamide gave a similar result. This compound, though pure by ^{1}H NMR, was an amber-colored liquid. The color could not be removed by any of a variety of procedures.

Deprotection of the trialkyl ester was accomplished by use of bromotrimethylsilane to remove the phosphate esters, followed by conversion to the bis(cyclohexylammonium) salt and hydrolysis with aqueous KOH to give the unisolated tripotassium salt. Exchange of potassium ions for protons followed by the addition of 1 equiv of cyclohexylamine gave, after lyophilization, the desired pure monocyclohexylammonium 1-carboxyallenyl phosphate.

Stability of Alleno-PEP. Alleno-PEP was completely stable for at least several hours at 25 °C, pH 7–8. The compound did not react with dithiothreitol under these conditions.

Reaction with PEP Carboxylase. In a solution containing alleno-PEP, PEP carboxylase, lactate dehydrogenase, Mg^{2+} or Mn^{2+}, and HCO_3^- at pH 8, a decrease in absorbance at 340 nm was observed due to the disappearance of NADH. Alleno-PEP is presumably being converted to 2-oxo-3-butenoate, which is a substrate for lactate dehydrogenase (Wirsching & O'Leary, 1988). In the absence of PEP carboxylase, there was no decrease in absorbance, indicating that there was no detectable spontaneous hydrolysis of alleno-PEP. The rates of both the alleno-PEP and PEP reactions were reduced in solutions of low bicarbonate concentration.

The preceding experiments were conducted with lactate dehydrogenase as the reducing agent. Corresponding experiments using malate dehydrogenase produced no decrease in absorbance at 340 nm. However, it is not known whether the putative carboxylation product from alleno-PEP, 3-methyleneoxalacetate, is a substrate for malate dehydrogenase. In order to determine independently whether this compound might be formed, the reaction of alleno-PEP with PEP carboxylase was conducted in the presence of $H^{13}CO_3^-$. ^{13}C NMR indicated that no ^{13}C-containing organic compound was produced (the detection limit was about 1% of the initial quantity of substrate). It is unlikely that the putative carboxylation product would be completely decarboxylated under these conditions. Thus, we conclude that alleno-PEP is converted by PEP carboxylase into 2-oxo-3-butenoate (or its enolate)

Table I: Kinetic Parameters for Reactions of Alleno-PEP and PEP with PEP Carboxylase[a]

metal	substrate	V_{max} (μM/min)	$V_{max}/K_m \times 10^2$ (min^{-1})
Mg^{2+}	PEP	14.7	1.11
	alleno-PEP	13.1	0.51
Mn^{2+}	PEP	21.1	3.1
	alleno-PEP	9.1	1.4

[a] At pH 8.0, 25 °C. Reactions with PEP were coupled to malate dehydrogenase; those with alleno-PEP were coupled to lactate dehydrogenase.

Table II: Kinetic Parameters for Reactions of Alleno-PEP and PEP with Pyruvate Kinase[a]

metal	substrate	V_{max} (μM/min)	$V_{max}/K_m \times 10^2$ (min^{-1})
Mg^{2+}	PEP	14.0	28.0
	alleno-PEP	10.0	1.4
Mn^{2+}	PEP	5.3	28.0
	alleno-PEP	6.0	5.7

[a] At pH 8.0, 25 °C. Activities were measured by observing ATP formation using hexokinase and glucose-6-phosphate dehydrogenase.

and not into 3-methyleneoxalacetate.

Conversion of alleno-PEP into 2-oxo-3-butenoate followed Michaelis–Menten kinetics in the presence of either Mg^{2+} or Mn^{2+}. Kinetic properties for PEP and alleno-PEP are summarized in Table I. Reactivity of alleno-PEP could also be measured by observing the increase in absorbance at 260 nm due to the appearance of 2-oxo-butenoate. The rates compared well with the rates obtained by using the enzyme coupled assay (Table I).

Reaction with Pyruvate Kinase. When alleno-PEP was treated with pyruvate kinase in the presence of ADP, lactate dehydrogenase, NADH, and Mg^{2+} or Mn^{2+} at pH 7.5, 25 °C, a decrease in absorbance at 340 nm was observed due to conversion of NADH to NAD^+. Alleno-PEP is presumably serving as a substrate for pyruvate kinase and is being converted into 2-oxo-3-butenoate. In order to confirm that ATP was also produced, the reaction was followed by coupling the enzyme to hexokinase and glucose-6-phosphate dehydrogenase. It was found that ATP was produced and that the kinetic parameters obtained when this assay (Table II) was used were similar to those obtained when the lactate dehydrogenase method was used. In both cases, the reaction with the natural substrate, PEP, was run in parallel under the same conditions to allow comparison between the two compounds.

Inactivation of PEP Carboxylase. PEP carboxylase showed time-dependent inactivation by alleno-PEP in the presence of HCO_3^- and either Mg^{2+} or Mn^{2+} (Figure 1). Kinetics were not first order, presumably because the actual inactivator, 2-oxo-3-butenoate, decomposes during the reaction (Wirsching & O'Leary, 1988). Similar rates were observed with Mg^{2+} and with Mn^{2+}. With 2 mM inhibitor, complete inactivation of the enzyme was not obtained. If dithiothreitol was present during the incubation, no loss in activity occurred.

Inactivation of Pyruvate Kinase. Pyruvate kinase was inactivated at pH 7.8 with either Mg^{2+} or Mn^{2+} as the metal ion cofactor in presence of ADP (Figure 2). There was an initial, rapid drop in activity, followed by a slower loss in activity lasting for an hour or more. The final activity did not approach zero even in the presence of 2 mM alleno-PEP. The extent of inactivation increased with increasing ADP concentration; 1 mM DTT completely prevented inactivation. Phospholactate was ineffective in preventing the inactivation.

Discussion

Alleno-PEP has been synthesized by a route involving isomerization of an acetylenic precursor. This isomerization

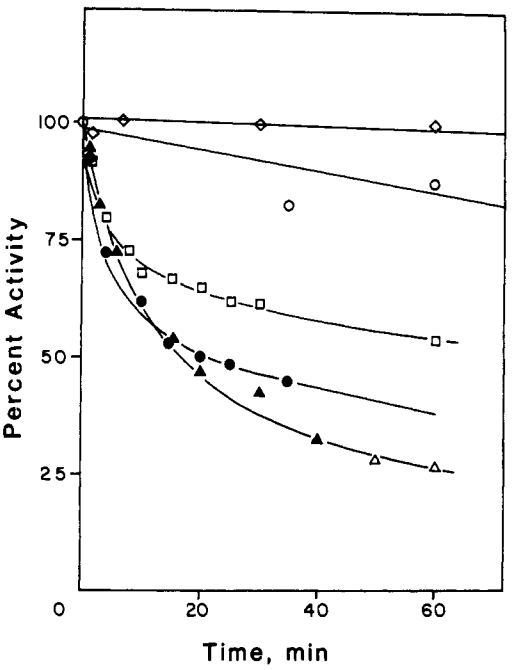


FIGURE 1: Inactivation of PEP carboxylase by alleno-PEP at pH 8, 25 °C, in the presence of 5 mM $NaHCO_3$ and 5 mM $MgCl_2$: (○) control; (●) 2.0 mM alleno-PEP and 5 mM $MgCl_2$; (▲) 7.5 mM alleno-PEP and 5 mM $MgCl_2$; (□) 2.0 mM alleno-PEP and 1 mM $MnCl_2$; (◇) 7.5 mM alleno-PEP, 5 mM $MgCl_2$, and 8.0 mM dithiothreitol.

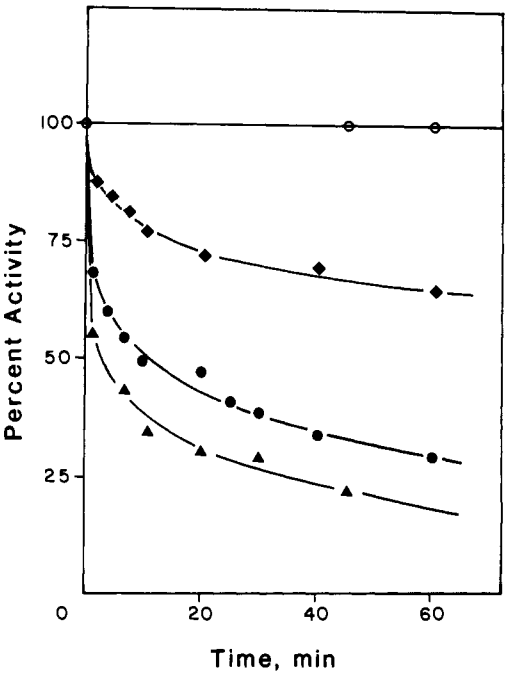


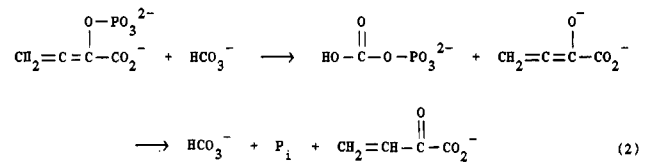
FIGURE 2: Inactivation of pyruvate kinase by alleno-PEP at pH 7.8, 25 °C, in the presence of 58 mM KCl and 0.05 mM EDTA: (○) control; (◆) 2.0 mM alleno-PEP, 1.75 mM $MnCl_2$, and 0.58 mM ADP; (●) 2.0 mM alleno-PEP, 2.3 mM ADP, and 3.5 mM $MgCl_2$; (▲) same, plus 1.0 mM phospholactate.

was not expected, and a number of variations were tried before a satisfactory procedure was found, but the procedure as presented here provides a satisfactory yield of the final product. Alleno-PEP is stable in aqueous solution and does not react at an appreciable rate with nucleophiles.

PEP Carboxylase. In the presence of HCO_3^- and a divalent metal ion, PEP carboxylase catalyzes the hydrolysis of alleno-PEP, forming P_i and 2-oxo-3-butenoate (or the enolate of this compound). The properties of 2-oxo-3-butenoate, in-

cluding its reduction by lactate dehydrogenase, its rapid reaction with DTT, and its absorption spectrum, are known from previous studies of (*Z*)-3-(fluoromethyl)-PEP (Wirsching & O'Leary, 1988) and are fully consistent with this reaction pathway.

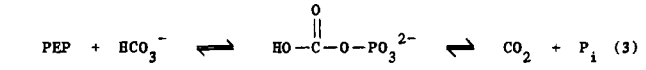
Interestingly, there is no evidence that alleno-PEP is ever carboxylated at the active site of PEP carboxylase. Instead, the substrate is hydrolyzed, giving a pyruvate derivative and P_i. A number of PEP analogues have been shown to undergo a similar hydrolysis, including (*Z*)-3-methyl-PEP (Gonzalez & Andreo, 1986; J. O'Laughlin and M. H. O'Leary, unpublished results), (*Z*)-3-bromo-PEP (Díaz et al., 1988), (*Z*)-3-chloro-PEP (J. Liu and M. H. O'Leary, unpublished results), (*Z*)-3-fluoro-PEP (Díaz et al., 1988), and phosphoenolthiopyruvate (Sikkema & O'Leary, 1988). In the cases that have been examined (3-bromo-PEP and 3-methyl-PEP), transfer of ^{18}O from HCO_3^- to P_i accompanies this process. This suggests that the first step in the reaction is the usual first step, namely, formation of the usual enzyme-bound carboxyphosphate and enolate (O'Leary, 1982, 1983). However, following this, the intermediate undergoes hydrolysis, rather than carboxylation, giving a substituted pyruvate. This reaction is illustrated for alleno-PEP in eq 2. However, the



(2)

structural features of the substrate that control the partitioning between carboxylation and hydrolysis are not known. Simple steric arguments are eliminated by the fact that both fluoro-PEP and alleno-PEP undergo principally hydrolysis, even though both analogues contain only very small substituents. Electronic arguments are rendered difficult by the fact that compounds having substituents which are electronically like the natural substrate (e.g., methyl-PEP) are hydrolyzed, as are compounds having very electronegative substituents (fluoro-PEP). In fact, the only compound that gives principally carboxylation is PEP itself. A similar reaction has been observed in the case of PEP carboxytransphosphorylase, which at low HCO_3^- concentrations catalyzes the formation of pyruvate and P_i from PEP (Lochmuller et al., 1966).

Diversion of the carboxyphosphate–enolate intermediate to the hydrolysis pathway might come about in any of several ways. The enolate intermediate might be released into solution, or else protonation of the enolate might occur on the surface of the enzyme. Alternatively, carboxyphosphate might be released into solution. One piece of evidence which suggests that the last possibility is correct is that both PEP carboxylase from *Escherichia coli* and PEP carboxylase from maize catalyze the incorporation of more than a single atom of oxygen from HCO_3^- into P_i (Fujita et al., 1984; J. O'Laughlin and M. H. O'Leary, unpublished results). This may occur as a result of reversible formation of CO_2 and P_i from carboxyphosphate, positional isotope exchange, and reversion of the intermediate to form starting material (eq 3).



(3)

PEP carboxylase is inactivated over the course of a few minutes by alleno-PEP in the presence of HCO_3^- and metal ion. As noted above, we believe that 2-oxo-3-butenoate is being formed under these conditions, and the nature of the inactivation is like that seen with 3-(fluoromethyl)-PEP (Wirsching & O'Leary, 1988). Inactivation is prevented by dithiothreitol, which results in the destruction of 2-oxo-3-butenoate. Interestingly, the inactivation is unaffected by either phospholactate or epoxymaleate, both of which are competitive inhibitors of the enzyme (O'Leary, 1982). Apparently, the inactivation does not occur at the active site of the enzyme. The chemical reactivity of 2-oxo-3-butenoate is such that the most likely candidate for reaction is a sulfhydryl group, as the compound is quite unreactive toward amines. Modification of sulfhydryl groups has previously been correlated with inactivation of PEP carboxylase (Stiborova & Leblova, 1983, 1986; Gonzalez et al., 1986). However, it appears that the sulfhydryl group being modified here is not the same as that seen in those studies.

Pyruvate Kinase. Alleno-PEP is a substrate for pyruvate kinase. Formation of 2-oxo-3-butenoate can be monitored by use of lactate dehydrogenase and NADH, and formation of ATP can be monitored by use of hexokinase and glucose-6-phosphate dehydrogenase. The reaction follows Michaelis–Menten kinetics, and the maximum velocities for PEP and alleno-PEP are within a factor of 2 of each other. In a comparison of V_{max}/K_m, alleno-PEP is slower by a factor of 5–20.

In the absence of lactate dehydrogenase, pyruvate kinase is gradually inactivated by reaction with alleno-PEP. This inactivation could also be prevented by the presence of DTT. Thus, the inactivation does not occur directly at the active site of the enzyme but rather takes place following release of 2-oxo-3-butenoate into solution, as is observed with PEP carboxylase and as is observed during inactivation of pyruvate kinase by 3-(fluoromethyl)-PEP (Wirsching & O'Leary, 1988). The kinetics of the inactivation are not first order because of the contributions of a variety of processes. Phospholactate was not effective in protecting pyruvate kinase against inactivation.

It is interesting that 2-oxo-3-butenoate, though very reactive, does not appear to react at the active site of pyruvate kinase. The initially formed enolate is presumably protonated at the active site of the enzyme, and thus 2-oxo-3-butenoate is formed within the active site. However, the acid–base group at the active site of pyruvate kinase is believed to be a lysine residue (Dougherty & Cleland, 1985; Muirhead et al., 1986), and amino groups are inert toward 2-oxo-3-butenoate. Pyruvate kinase reacts with standard sulfhydryl reagents, but the thiol groups are not unusually reactive and there is no direct 1:1 stoichiometry between thiol groups reacted and inhibition of catalysis (Mildvan & Cohn, 1966). The enzyme from rabbit muscle contains 34 cysteines (Cottam et al., 1969), and reaction of 4 of these with 5,5′-dithiobis(2-nitrobenzoic acid) caused no inhibition (Bonar & Pon, 1968; Bonar & Suelter, 1971). Apparently, the sulfhydryl groups of pyruvate kinase are not particularly reactive, and limited sulfhydryl modification may have only a limited effect on pyruvate kinase activity.

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Benzylic Monooxygenation Catalyzed by Toluene Dioxygenase from *Pseudomonas putida*†

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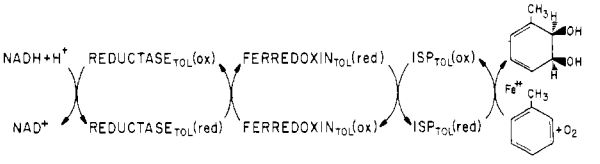
ABSTRACT: Toluene dioxygenase, a multicomponent enzyme system known to oxidize mononuclear aromatic hydrocarbons to *cis*-dihydrodiols, oxidized indene and indan to 1-indenol and 1-indanol, respectively. In addition, the enzyme catalyzed dioxygen addition to the nonaromatic double bond of indene to form *cis*-1,2-indandiol. The oxygen atoms in 1-indenol and *cis*-1,2-indandiol were shown to be derived from molecular oxygen, whereas 70% of the oxygen in 1-indanol was derived from water. All of the isolated products were optically active as demonstrated by ^{19}F NMR and HPLC discrimination of diastereomeric esters and by chiroptic methods. The high optical purity of (−)-(1*R*)-indanol (84% enantiomeric excess) and the failure of scavengers of reactive oxygen species to inhibit the monooxygenation reaction supported the contention that the monooxygen insertion is mediated by an active-site process. Experiments with 3-[^2H]indene indicated that equilibration between C-1 and C-3 occurred prior to the formation of the carbon–oxygen bond to yield 1-indenol. Naphthalene dioxygenase also oxidized indan to 1-indanol, which suggested that benzylic monoxygenation may be typical of this group of dioxygenases.

Oxygenases are enzymes that incorporate molecular oxygen into organic compounds (Hayaishi et al., 1955). The importance of these enzymes in mammalian and microbial metabolism has been well documented (Hayaishi, 1982; Dagley, 1986).

In the case of unsubstituted aromatic hydrocarbons, different reaction mechanisms are used by mammals and bacteria to initiate oxygenation of the benzenoid nucleus. For example, mammals utilize a monooxygenase enzyme system to incorporate one atom of molecular oxygen into the aromatic ring. The initial reaction products are arene oxides that can react with cellular nucleophiles, isomerize to form phenols, or undergo enzymatic hydration to form *trans*-dihydrodiols (Daly et al., 1972). In contrast, bacteria utilize a dioxygenase enzyme system to initiate the oxidation of the aromatic nucleus. Both atoms of molecular oxygen are incorporated into the substrate, and the first detectable products are dihydrodiols in which the hydroxyl groups have a cis-relative stereochemistry (Gibson & Subramanian, 1984).

Scheme I



Toluene dioxygenase oxidizes toluene to (+)-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol; Gibson et al., 1970; Kobal et al., 1973; Ziffer et al., 1973).

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