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The Synthesis and Enzyme-Catalyzed One-Step *cis–trans* Double Isomerization of Monomethyl 5-Oxo-1,3-hexadien-1-ylphosphonate, an Analogue of Maleylacetone[1]

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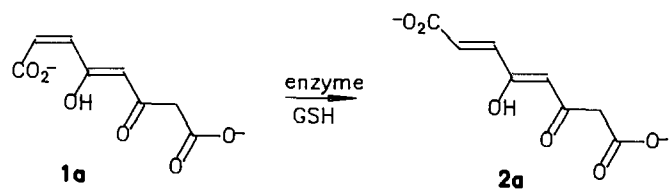
Phosphonate substrate analogues for maleylacetoacetate *cis–trans*-isomerase have been synthesized and shown to be processed by the enzyme. Thus the 1(*Z*),3(*E*)-, 1(*E*),3(*Z*)-, and 1(*E*),3(*E*)-isomers of monomethyl 5-oxo-1,3-hexadien-1-ylphosphonate monoanion have been prepared and characterized. The enzyme together with the coenzyme glutathione catalyzes one-step double *cis–trans* isomerization between the 1(*E*),3(*Z*)- and the 1(*Z*),3(*E*)-isomers in either direction in addition to catalyzing isomerization about one double bond in these substrates.

Maleylacetoacetate *cis–trans* isomerase, one of the enzymes found in mammalian liver and in some bacteria, is required for the stepwise metabolism of aromatic amino acids (*1*). It, together with the coenzyme glutathione (GSH), catalyzes the conversion of maleylacetoacetate (**1a**) to fumarylacetoacetate (**2a**, Eq. [1]),



1a **2a** [1]

which in turn is converted to fumarate and acetoacetate. Maleylacetoacetate itself is the product of enzymatic oxidation of homogentisate (2′,5′-dihydroxyphenylacetate), an earlier intermediate in the metabolism of phenylalanine and tyrosine. Previous studies utilizing maleylacetone (**1b**) as an analogue have shown that both substrate and coenzyme bind to the enzyme where the glutathiyl group adds to C2 of the substrate to form a diendiol intermediate followed by internal rotation (Eq. [2]) and then expulsion of GSH to yield the *trans*-isomer (*2*).

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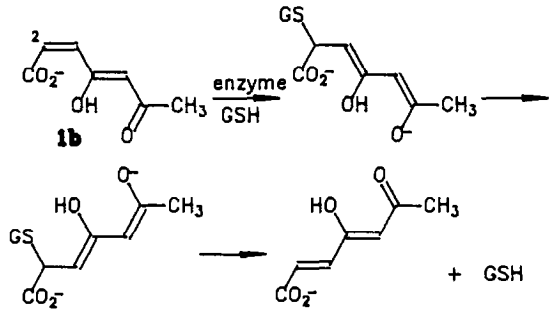
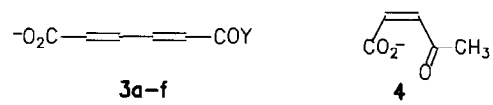


[2]

Other analogues which undergo *cis–trans* isomerization catalyzed by this enzyme are represented by **3a–3f** and **4** (*3–5*).



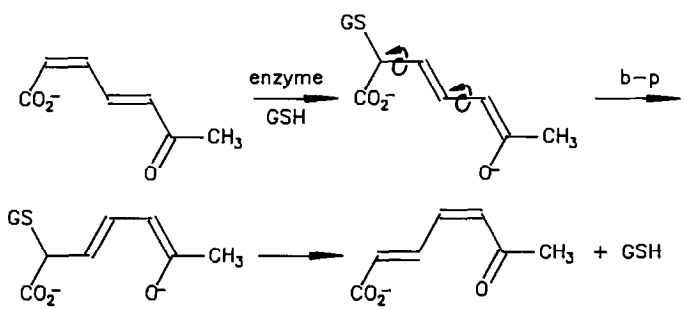
3a–f **4**

a: *cis, trans;* Y=CH_3, **d:** *cis, trans;* Y=OCH_3

b: *trans, cis;* Y=CH_3, **e:** *trans, cis;* Y=OCH_3

c: *cis, cis;* Y=OCH_3, **f:** *cis, cis;* Y=O^-

Compared to **1b, 3a–3e** lack hydroxyl groups at C4 and consequently provide greater configurational stability about the C4–C5 double bond in the absence of the isomerase. This absence of lability has led to the discovery of enzyme-catalyzed one-step *cis–trans* isomerization about both carbon–carbon double bonds in **3a–3e** (*3, 4*). The mechanism follows from that shown in Eq. [2]. One-step double isomerization is readily rationalized by GS-addition to C2 followed by bicycle-pedal (b-p) rotation and then expulsion of GSH (Eq. [3]).



[3]

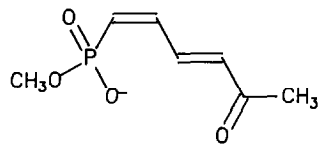
The rate of enzyme-catalyzed reaction decreases in the order **3a** > **3d** > **3f**, suggesting that nucleophilic addition of GSH at C2 is rate controlling in the enzymatic reaction (*4*).

Isomerization of two double bonds in concert by bicycle-pedal rotation results in only a little translational movement of the diene's terminal groups from their

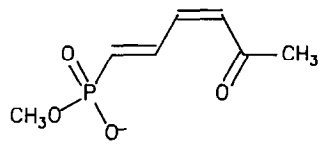
initial positions (*6*). Isomerization of one double bond or two double bonds in stepwise fashion, however, results in substantial movement of end groups during reaction. Consequently, single or stepwise isomerization in solution or on the surface of an enzyme requires energy to move solvent molecules and/or to stretch substrate–enzyme interactions. The net effect is to raise the energy for single isomerization with respect to the concerted double isomerization making the latter process more competitive with the former (*7*).

Acceptance of a stepwise mechanism for double isomerization is dissuaded by energy considerations. If for example, an *E,Z*-dienyl system were to undergo stepwise double isomerization to the corresponding *Z,E*-system two paths are available. The first is $E,Z \rightarrow Z,Z \rightarrow Z,E$ and the second is $E,Z \rightarrow E,E \rightarrow Z,E$. One step in each path requires conversion of a more stable to a less stable isomer, an unlikely path to funnel reactant through at a relatively fast rate. Moreover, we find a one-step double isomerization mechanism in the most likely place to find the stepwise mechanism. In the conversion of a *Z,Z*- to an *E,E*-dienyl system both steps in a stepwise double isomerization are downhill. Using ^{14}C-labeled **3c**, however, a direct one-step path to the *trans,trans*-isomer is found (*4*). Evidence for whether this is concerted, i.e., by an apparent bicycle-pedal rotation, is lacking. More information is desirable.

If both double bonds were in a medium-sized ring in the ground state, isomerization about one double bond or stepwise isomerization about both double bonds would require very high activation energies because of the ring strain encountered in these reactions. Concerted bicycle-pedal double isomerization is likely to require less energy because of the smaller displacement of the diene's terminii as the reactants proceed along the reaction coordinate. Previously studied substrate analogues are all conjugated unsaturated carboxylate anions. The negative charge is undoubtedly important for binding of substrate to the enzyme. Were derivatives of these compounds lactonized to place the diene in a medium sized ring, the negative charge would be lost. A negatively charged cyclic substrate analogue could be achieved, however, by replacing the carboxylate with a phosphonate group. Substrate analogues of glycolytic intermediates, containing phosphonomethyl groups instead of phosphate groups, have been found in many but not all cases to be successfully processed enzymatically. For those that are processed the phosphate group is presumably used primarily for binding (*8*). Substrate analogues with phosphonate in place of carboxylate, or phosphonamide instead of carboxyamide groups, however, are generally protease inhibitors, suggesting they are transition state analogues (*9*). (1-Aminoethyl)phosphonate, on the other hand, appears to inhibit alanine racemase by inducing a conformational change leading to tighter binding and reduced access to the enzyme–inhibitor complex (*10*). Phosphonates are known in Nature; in fact a substituted vinylphosphonate is a naturally occurring antibiotic (*11*). Whether maleylacetoacetate *cis–trans*-isomerase, however, will process a phosphonate substitute for a carboxylate substrate needs first to be addressed. This study is concerned with the synthesis of an acyclic phosphonate monoester anion analogue (**5ZE**), its phototransformation to **5EZ**, and their reactions with the enzyme system.



5ZE



5EZ

METHODS

General

THF was distilled from $LiAlH_4$ or sodium benzophenoneketyl. ^1H NMR spectra were taken on a Bruker AM-300 or a GE QE-300 using internal TMS as a standard. ^{31}P NMR spectra were taken on a Bruker AM-300 or a GE/Nicolet NT-300 using 85% H_3PO_4 as a standard. In certain ^1H NMR spectra specified below with PANIC, the resonances of the vinyl regions were fitted for chemical shifts and coupling constants using the program PANIC (Bruker Instruments). $CDCl_3$ was used for NMR determinations except where noted otherwise. GC analyses were carried out on a Hewlett–Packard 5890A chromatograph equipped with an HP-1 column (30 m × 0.53 mm) and quantitated with a 3396A integrator. Mass spectra were recorded on either a Finnigan 5100 GC/MS (low resolution) using a DB-5 column (30 m × 0.25 mm) or a Kratos MS90 (high resolution) using a DS90 data system. Elemental analyses were performed by Schwarzkopf Analytical Laboratory (Woodside, NY).

Dimethyl Chlorophosphate

This was prepared by the method of Sosnovsky and Zaret (*12*). Yield: 74%, b.p. 91–93°C/27 mm. ^1H NMR δ 3.90. ^{31}P NMR δ 8.04.

1,1-Diethoxy-2-propyne (**6**)

This was prepared according to the method of Ward and Van Dorp (*13*). 2,3-Dibromopropanal was obtained in 77% yield; b.p. 74–75°C/12 mm. ^1H NMR δ 3.66 (m, 1H), 3.79 (m, 1H), 4.45 (m, 1H), 9.29 (s, 1H). Treatment with triethyl orthoformate provided 2,3-dibromo-1,1-diethoxypropane, b.p. 109–110°C/12 mm, 81%. ^1H NMR δ 1.17 (t, 6H), 3.56 (m, 6H), 4.10 (m, 1H), 4.61 (d, 1H). Treatment with $NaNH_2/NH_3$(l) yielded the propyne; b.p. 139–140°C, 72%. ^1H NMR δ 1.16 (t, 6H), 2.96 (s, 1H), 3.58 (m, 4H), 5.20 (s, 1H).

Dimethyl 3,3-Diethoxy-1-propyn-1-ylphosphonate (**7**)

To a solution of 1,1-diethoxy-2-propyne (2.5 g, 19.5 mmol) in 15 ml of THF, at −70°C, was added dropwise, 8 ml of *n*-BuLi (2.5 M in hexane) while the temperature was maintained at −65°C. Dimethyl chlorophosphate (2.90 g, 20 mmol) was

added dropwise and the mixture was stirred for 8 h at −65°C after which the temperature was allowed to rise to −30°C, whereupon the reaction was quenched with 10 ml of water. The aqueous layer was extracted with ether (3 × 20 ml) and the combined organic layer was dried with $MgSO_4$. The crude product was flash chromatographed twice (silica gel, ether/hexane, 3/1) to provide >99% pure product as determined by GC; yield 35%. ^{1}H NMR δ 1.17 (t, 6H), 3.68 (m, 10H), 5.28 (d, 1H), ^{31}P NMR δ 4.09. *Anal.* Calcd for $C_9H_{17}O_5P$: C, 45.76; H, 7.26. Found: C, 44.86; H, 7.55.

Dimethyl 3,3-Diethoxy-cis-1-propen-1-ylphosphonate (**8**)

A suspension of 20.8 mg of 5% $Pd/CaCO_3$ in 26.5 ml of methanol containing 5.8 mg of quinoline was equilibrated with H_2 at atmospheric pressure. Dimethyl 3,3-diethoxy-1-propyn-1-ylphosphonate (1.56 g, 6.61 mmol) was added and the mixture was stirred until 1 eq of H_2 was consumed. After filtration, GC analysis indicated the presence of two compounds: the desired compound and the saturated phosphonate in the ratio of 8:1. Flash chromatography (silica gel, ether/hexane, 2/1) gave 0.92 g (59%) of pure dimethyl 3,3-diethoxy-*cis*-1-propen-1-ylphosphonate. ^{1}H NMR (PANIC) δ 1.88 (t, 6H), 3.63 (m, 10H), 5.68 (m, H-3, J = 7.69 Hz), 5.69 (m, H-1, $J_{1,2}$ = 13.3, $J_{H\text{-}1,P}$ = 16.9), 6.39 (dq, H-2, $J_{1,2}$ = 13.3, $J_{H\text{-}2,P}$ = 52.0). ^{31}P NMR δ 18.25. *Anal.* Calcd for $C_9H_{19}O_5P$: C, 45.37; H, 8.04. Found: C, 44.89; H, 8.46.

Dimethyl 2-Formylvinylphosphonate (**9**)

Dimethyl 3,3-diethoxy-*cis*-1-propen-1-ylphosphonate (0.516 g, 2.15 mmol) was allowed to react with 5 ml of 97% formic acid at ambient temperature for 15 min, after which the formic acid was removed by lyophilization. The ^{1}H NMR spectrum showed a small amount of formic acid remaining but because of the lability of the product the crude material was used in the next step. ^{1}H NMR δ 3.79 (d, 6H), 6.57 (m, 2H), 10.41 (d, 1H, $J_{2,3}$ = 7.4). A small amount of the *trans*-aldehyde (δ 9.6) was also present.

Dimethyl 5-Oxo-1(Z),3(E)-hexadien-1-ylphosphonate (**10**)

The crude dimethyl 2-formylvinylphosphonate was immediately mixed with 0.898 g (2.82 mmol) of triphenylphosphoranylidene 2-propanone in 10 ml of benzene. After 2 h at ambient temperature the benzene was removed and the red-brown residue was flash-chromatographed (silica gel, ethyl acetate/hexane, 3/1) and then further purified by preparative TLC (2 mm silica gel 60 F_{254}, ethyl acetate/hexane, 3/1) to give 99% pure product as determined by GC. Yield, 15%. ^{1}H NMR (PANIC) δ 2.26 (s, 3H), 3.44 (d, 6H, $J_{H,P}$ = 10.9), 5.91 (m, H-1, $J_{1,2}$ = 12.9, $J_{H\text{-}1,P}$ = 15.5), 6.24 (d, H-4, $J_{3,4}$ = 15.9), 7.01 (m, H-2, $J_{1,2}$ = 12.9, $J_{H\text{-}2,P}$ = 50.1, $J_{2,3}$ = 11.3), 8.11 (m, H-3, $J_{3,4}$ = 15.9, $J_{2,3}$ = 11.3). ^{31}P NMR δ17.7. MS *m/e* 204 (15, M+), 189 (27), 161 (100), 129 (19), 115 (6), 109 (41), 95 (42), 79 (31), 52 (11). HRMS. *Anal.* Calcd for $C_8H_{13}PO_4$ 204.0551. Found: 204.0545.

Monomethyl 5-Oxo-1(Z),3(E)-hexadien-1-ylphosphonate Anion (**5ZE**)

Dimethyl 5-oxo-1(*Z*),3(*E*)-hexadien-1-ylphosphonate (10 mg, 49 μmol) was added to 1 ml of CD_3COCD_3 containing 8.76 mg (58 μmol) of NaI. The reaction mixture was kept at 48°C for 96 h while it was monitored periodically by ^1H NMR. The acetone was evaporated, leaving the anion together with a small amount of NaI suitable for enzyme studies. ^1H NMR (PANIC) (D_2O) δ 2.23 (s, 3H), 3.71 (d, 3H, $J_{H,P}$ = 11.2), 5.94 (dd, H-1, $J_{1,2}$ = 12.7, $J_{H\text{-}1,P}$ = 15.0), 6.11 (d, H-4, $J_{3,4}$ = 15.7), 6.71 (dt, H-2, $J_{1,2}$ = 12.7, $J_{H\text{-}2,P}$ = 44.15, $J_{2,3}$ = 11.9), 7.83 (dd, H-3, $J_{3,4}$ = 15.7, $J_{2,3}$ = 11.9). ^{31}P NMR (D_2O) δ13.23. UV (H_2O) λ_{max} 269 nm (log ε 4.18).

Thermal Isomerization of **5ZE**

A solution of **5ZE** (≈0.08 M) in D_2O containing KSCN (0.6 M) was heated at 85°C for 62.5 h. HPLC showed it to contain only **5EE**, the earliest eluted isomer (vide infra).

Photoisomerization of Monomethyl 5-oxo-1(Z),3(E)-hexadien-1-ylphosphonate Anion

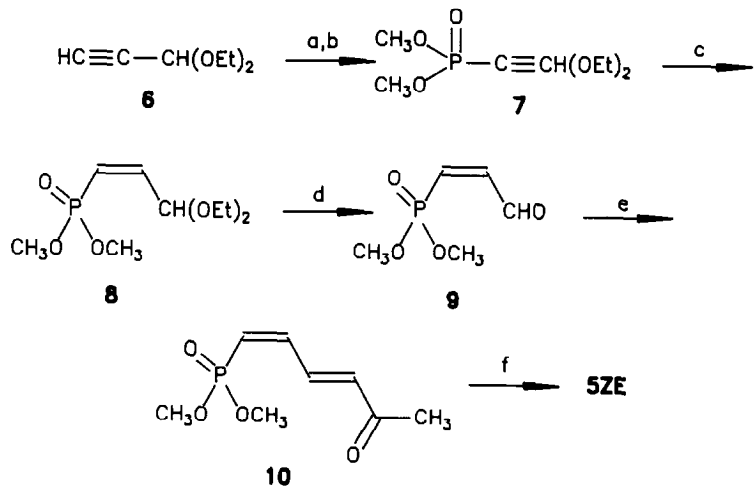
The 1(*Z*),3(*E*)-isomer (0.086 M in water) in a 1-mm-path cuvette was irradiated with three 254-nm lamps in a Rayonet Mini-Photochemical Reactor. After 40 min the composition as determined by HPLC (C18 column, 7% CH_3CN/H_2O, 5 mM tetra-*n*-butylammonium phosphate (TBAP), pH 7, 254-nm detection) was 49% 1(*E*),3(*E*), 15% 1(*Z*),3(*E*), 32% 1(*E*),3(*Z*), and 3% unknown. Irradiation was continued for another 30 min after which the mixture was separated into its components by HPLC (same conditions except 1 mM TBAP). Monomethyl 5-oxo-1-(*E*),3(*Z*)-hexadien-1-ylphosphonate anion. ^1H NMR (PANIC) (D_2O, TBAP int std) δ 2.05 (s, 3H), 3.19 (d, 3H), 6.20 (t, H-1, $J_{1,2}$ = 17.5, $J_{H\text{-}1,P}$ = 17.4), 6.37 (dd, H-4, $J_{3,4}$ = 11.2), 6.62 (dt, H-3, $J_{3,4}$ = 11.2, $J_{2,3}$ = 11.2), 7.66 (dt, H-2, $J_{1,2}$ = 17.5, $J_{H\text{-}2,P}$ = 17.6). Monomethyl 5-oxo-1(*E*),3(*E*)-hexadien-1-ylphosphonate anion. ^1H NMR (PANIC) (D_2O) δ 2.35 (s, 3H), 3.55 (d, 3H, $J_{H,P}$ = 10.9), 6.33 (t, H-1, $J_{1,2}$ = 17.1, $J_{H\text{-}1,P}$ = 17.2), 6.39 (d, H-4, $J_{3,4}$ = 15.9), 6.98 (m, H-2, $J_{1,2}$ = 17.1, $J_{H\text{-}2,P}$ = 18.6, $J_{2,3}$ = 10.6), 7.34 (dd, H-3, $J_{2,3}$ = 10.6, $J_{3,4}$ = 15.9). UV (H_2O) λ_{max} 267 nm.

Maleylacetoacetate *cis–trans*-isomerase was isolated from *Vibrio O1* and purified according to previously published procedures (*14*). The enzyme was purified up to and including the affinity column step. Assays to determine enzyme activity were carried out with maleylacetone as reported previously.

HPLC analysis of enzyme-phosphonate analogue reactions were carried out by injecting aliquots of the reaction mixtures into an HPLC system with a C18 column (Hypersil, 3 μm, 30 × 0.46 cm) eluted with 8% CH_3CN/H_2O containing 5 mM tetra-*n*-butylammonium phosphate. Detection was by UV-monitor at 254 nm. Peak areas were quantitated with a Hewlett Packard integrator (3392A). The order of elution was **5EE**, **5ZE**, and **5EZ** last.

Monitoring of Enzyme-Catalyzed cis–trans Isomerization of **5ZE**

(*A*) By HPLC. In a typical run 5 μl of 39.4 mM GSH was added to 130 μl of 0.01 M phosphate buffer, pH 7.4, in an Eppendorf microcentrifuge tube followed by 5 μl of enzyme containing 0.1% mercaptoethanol and then 5 μl of **5ZE** (86 mM).



SCHEME 1. (a) *n*-BuLi/THF, −70°C; (b) $(CH_3O)_2POCl$; (c) $H_2/Pd/CaCO_3$, quinoline; (d) 97% HCO_2H; (e) $\phi_3P{=}CHCOCH_3$; (f) NaI/acetone.

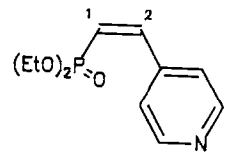
Aliquots (1–2 μl) were injected into the HPLC periodically to determine the extent of isomerization. Equal response factors were assumed.

(*B*) By spectrophotometry. Using similar concentrations as above the change in absorbance at 303 nm was monitored automatically (*15*). Inhibition runs with 57 μM **1b**, 1 mM GSH, 0–8 mM **5ZE**, and a suitable amount of enzyme were carried out by automatically measuring the optical density at 345 nm.

RESULTS AND DISCUSSION

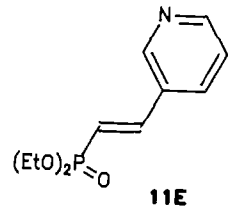
Attempts to synthesize the diester of the target compound by a Peterson olefination reaction (*16*) of the anion of dimethyl trimethylsilylmethylphosphonate with 4-oxo-2-pentenal in THF were unsuccessful, requiring synthesis by a longer route. Monomethyl 5-oxo-1(*Z*),3(*E*)-hexadien-1-ylphosphonate monoanion (**5ZE**) was obtained by the reactions shown in Scheme 1. 1,1-Diethoxy-2-propyne was obtained according to published procedures and treated with dimethyl chlorophosphate in the presence of *n*-BuLi to obtain dimethyl 3,3-diethoxy-1-propyn-1-ylphosphonate. Addition of 1 eq. of hydrogen provided, after flash chromatography, pure dimethyl 3,3-diethoxy-1(*Z*)-propen-1-ylphosphonate. That this is the *cis*-isomer was shown by its ^1H NMR spectrum. The $J_{1,2}$ = 13.3 Hz while $J_{H\text{-}1,P}$ = 16.9 and $J_{H\text{-}2,P}$ = 52.0. Reported coupling constants for diethyl *cis*-(**11Z**) and *trans*-2-(3′-pyridyl)-vinylphosphonate (**11E**) support the assignment of dimethyl 3,3-diethoxy-1(*Z*)-propen-1-ylphosphonate (**8**) as the *cis*-isomer (*17*). Deacetalization was accomplished with 97% formic acid followed by lyophilization to rapidly remove the formic acid; a small amount of formic acid remained but subsequent washing with bicarbonate led to further decomposition. Other methods of deacetal-

ization such as treatment with an acidic ion-exchange resin also led to decomposition (*18*). The product, dimethyl *cis*-2-formylvinylphophonate (**9**), still containing a fraction of an equivalent of formic acid and a minor amount of the *trans*-isomer,



11Z

$J_{H-1,P}$=14·3
$J_{H-2,P}$=49·7
$J_{H-1,H-2}$=14·3



11E

$J_{H-1,P}$=16·0
$J_{H-2,P}$=22·2
$J_{H-1,H-2}$=17·8

was treated rapidly with an excess of triphenylphosphoranylidene 2-propanone. Preparative TLC furnished pure dimethyl 5-oxo-1(*Z*),3(*E*)-hexadien-1-ylphosphonate (**10**). Again analysis of the ^{1}H NMR spectrum provides the following constants: $J_{1,2}$ = 12.8, $J_{H\text{-}1,P}$ = 15.3, $J_{H\text{-}2,P}$ = 50.0, and $J_{3,4}$ = 15.9, indicating a *cis*-1,2 and a *trans*-3,4 geometry. Finally, nucleophilic substitution by iodide in acetone cleaves one of the ester groups to provide the monoester anion (**5ZE**) without *cis–trans* isomerization. The pertinent coupling constants, $J_{1,2}$ = 12.7, $J_{H\text{-}1,P}$ = 15.0, $J_{H\text{-}2,P}$ = 44.2, and $J_{3,4}$ = 15.7, show that the *cis,trans* geometry is retained during monodemethylation.

5ZE, having a strong absorption at 269 nm, was irradiated at 254 nm to form other isomers. The steady state composition, in the order of elution by HPLC, contained 49% **5EE**, 15% **5ZE**, 32% **5EZ**, and 3% of an unknown but apparently related material. Separation by HPLC yielded highly enriched samples of **5EZ** (88%) and **5EE** (72%) and these were used for ^{1}H NMR analysis. The pertinent proton coupling constants are shown in Table 1 for the monoanion isomers. $J_{1,2}$ for **5EZ** and **5EE** are >17 while that for **5ZE** is equal to 12.7. $J_{3,4}$ for **5ZE** and **5EE** are 15.7 and 15.9, respectively, while that for **5EZ** is equal to 11.2. In addition $J_{H\text{-}2,P}$ is also an important reporter of geometry; values for these are 17.6 and 18.6 for **5EZ** and **5EE**, respectively, and 44.2 for **5ZE**, supporting the structures as assigned. The small amount of an unknown related compound noted above sug-

TABLE 1

Proton–Proton and Proton–Phosphorus Vinyl Coupling Constants for **5ZE, 5EZ** and **5EE**

	$J_{1,2}$	$J_{3,4}$	$J_{2,3}$	$J_{H\text{-}1,P}$	$J_{H\text{-}2,P}$
5ZE	12.7	15.7	11.9	15.0	44.2
5EZ	17.5	11.2	11.2	17.4	17.6
5EE	17.1	15.9	10.6	17.2	18.6

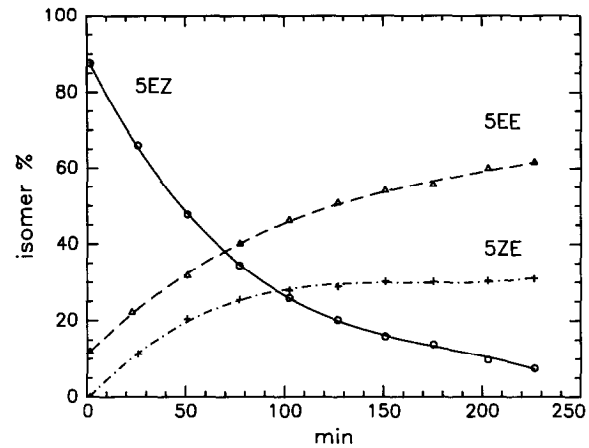


FIG. 1. The course of the enzyme-catalyzed *cis–trans* isomerization of **5EZ** as determined by HPLC. The initial substrate was a **5EZ/5EE** (88/12) mixture. Equal response factors are assumed.

gests that it may be **5ZZ**, the fourth isomer of this series. Assignment of structure for this compound is precluded by the limited amount of this material.

Enzyme-Catalyzed Isomerization

The isomerase together with GSH catalyzes *cis–trans* isomerization. HPLC analyses of the enzyme/**5ZE** reaction mixture with time exhibit a decrease in **5ZE** concentration with a concomitant increase of **5EE**. The *trans,trans*-isomer is also formed through nucleophilic-catalyzed *cis–trans* isomerization of **5ZE** by thiocyanate ion. Extended heating with SCN^- leads to only one stable isomer (**5EE**), which is the same as that produced in greatest amount from enzyme-catalyzed isomerization of **5ZE**. In addition to the formation of **5EE** a new small peak is observed in the HPLC trace of the enzymatic reaction which reaches about 6% of the total at its maximum. The new peak does not appear in nonenzymatic chemically catalyzed isomerization of **5ZE**. The new peak was shown to be due to **5EZ** obtained from photolysis experiments and most likely produced in the enzymatic reaction through a one-step double isomerization by a bicycle-pedal mechanism. The K_M for disappearance of **5ZE** is 2.7×10^{-3} M, which is about twice as large as that for maleylacetone (**1b**). The V_{max}, however, is about 1/10 that for **1b**. Studies with a constant concentration of **1b** and varying concentrations of **5ZE** were carried out. Isomerization of maleylacetone is indeed inhibited by increasing concentrations of **5ZE**, indicating that the same active site is used by both substrate analogues.

5EZ isolated from the photolysate of **5ZE** was also subjected to enzyme-catalyzed isomerization. A plot of the composition vs time, through HPLC analysis, is shown in Fig. 1. Here too, the enzyme apparently catalyzes both single and one-step double isomerization. From this run and others, the ratio of the concentrations

of **5ZE** to **5EZ** at long reaction times approaches a value of about 3.5, which we take to mean that the former is more stable than the latter by about 0.7 kcal/mol.

In conclusion, the isomerase has been shown to catalyze one-step interconversion between **5EZ** and **5ZE**, monoanion phosphonate analogues of unsaturated ketocarboxylates, **3b** and **3a**, respectively, which were also previously shown to undergo one-step catalyzed interconversion. These observations are in agreement with the proposition that the anionic carboxylate group of maleylacetone is used for binding to the enzyme. These results offer encouragement for studying the interaction of the isomerase with cyclic conjugated oxodienylphosphonate monoanions as a way to probe the mechanism of double *cis–trans* isomerization.

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