Mechanistic and Stereochemical Study of Phenylpyruvate Tautomerase

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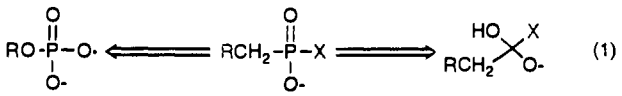
Received December 30, 1992

Abstract: A variety of substrates and potential enol/enolate mimics for the product/transition state of the enzyme phenylpyruvate tautomerase (E.C. 5.3.2.1) have been prepared and studied. Their stereostructures have been secured by a combination of NMR spectroscopy based on vicinal H–F and H–C coupling constants and X-ray crystallography. On the basis of the inhibition by stereoisomeric substituted cinnamates, it has been concluded that the enzyme produces the thermodynamically less stable (*E*) enol via a *syn* tautomerization transition state. Free energy profiles for the reaction suggest that vinyl fluorides act as product analogues. Because amide and dicarboxylate enolate mimics are relatively poor inhibitors of the enzyme, it is believed that an enolate is not involved in the tautomerization process.

Molecules that possess specific functions (binding to an enzyme, receptor, nucleic acid, or organelle; crystallization in a particular space group; self-replication) are desired throughout Science and Technology today, and the design and preparation of such molecules is a unique contribution to be made by Chemistry. Intrinsic to achieving such goals is an understanding of molecular recognition. Classical studies in this area,[1] such as metal ion binding by crown ethers, hydrophobic binding by cavity-shaped molecules, hydrogen bonding by small molecular arrays, and even antibody–hapten binding (and catalysis), have focused on preparing hosts for specific guests. The converse, the design of specific *micromolecules* to interact with macromolecules, is one of the most challenging problems facing organic and pharmaceutical chemists. The number of successes that can be pointed to is significant and increasing, but all are based on detailed knowledge of the host. The helical structure of DNA has made it a natural target for the design of specific binding molecules, yet many of the known DNA-targeted agents were discovered on the basis of their biological activity.[2] An oft-quoted example of success in rational drug design is captopril, an angiotensin-converting enzyme (ACE) inhibitor.[3] The construction of a chimeric ACE active site based on analogies to other zinc proteases, its known sequence selectivity, and the clever insight of providing a ligand for the zinc (in the form of a thiol) led to the discovery of this antihypertensive. However, the number of potentially-interesting target proteins exceeds the number for which even this level of information is available. It greatly exceeds those for which quality X-ray data are available for use in structure-based design.

For many problems in micromolecular design, the designer may have only knowledge of the "natural" ligand and some mechanistic information, obtained directly or reasoned by analogy to other enzymes or organic chemistry, as a starting point. The design process can then involve an atom replacement, producing a *molecular metaphor* of the natural ligand. A replacement that leads to a molecule closely approximating the transition state[4] should lead to tight binding, since theories of catalysis require that the transition state has highest affinity for the enzyme. Because the binding energy used by the substrate for catalysis is still available to a molecule unreactive for mechanistic reasons, substitutions that prevent catalysis should lead to strong inhibition.[5] Phosphonates are functional groups that have been used in both contexts (eq 1). Transition-state analogy using P for C



(1)

substitution has been well-demonstrated for enzymes conducting acyl-transfer reactions.[6] Development of inhibitors by stabilization of phosphate esters using C for O substitution has had mixed results.[7] Molecular metaphors might be used to infer characteristics of an unknown enzyme if a correlation between the properties of a compound with a particular atom replacement and active-site residues, cofactors, metal ions, etc. could be discovered. Much larger data bases of atom replacements and macromolecular structures will be required to develop such a general tool.

Functional information can flow in other ways into inhibitor design. Knowledge of, for example, paired stereochemical events gives insight into the orientation of active-site residues that can be used to arrange a complementary orientation in an analogue. The study of cryptic stereochemistry for enzymatic reactions[8] is therefore justified independent of issues of "enzyme perfection"[9] and macromolecular evolution.[10]

A need clearly exists to develop new functional group equivalents (*molecular metaphors*) for incorporation into ligands for enzymes to produce inhibitors.[11] Because of the pervasive

(1) Breslow, R. *Science* **1982**, *218*, 532. Lehn, J. M. *Ibid.* **1985**, *227*, 849. Cram, D. J. *Ibid.* **1983**, *219*, 1177. Rebek, J., Jr. *Ibid.* **1987**, *235*, 1478. Schultz, P. G. *Ibid.* **1988**, *240*, 426.

(2) Dervan, P. B. *Science* **1986**, *232*, 464.

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(5) Jencks, W. P. In *Stereochemistry of Enzymatic Reactions*; Frey, P. A., Ed.; New York, 1984; p 59.

(6) Bartlett, P. A.; Marlowe, C. K. *Biochemistry* **1983**, *22*, 4618.

(7) Norbeck, D. W.; Kramer, J. B.; Lartey, P. A. *J. Org. Chem.* **1987**, *52*, 2174 and extensive references therein. Engel, R. *Chem. Rev.* **1977**, *77*, 349–367. Mazur, A.; Tropp, B. E.; Engel, R. *Tetrahedron* **1984**, *40*, 3949–3956.

(8) Benner, S. A.; Glasfeld, A.; Picirilli, J. *Topics in Stereochemistry*; Academic Press: New York, 1988; Vol. 9. pp 127–207. Hansen, K. R.; Rose, I. A. *Acc. Chem. Res.* **1974**, *8*, 1.

(9) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5627, 5631.

(10) Benner, S. A. *Experientia* **1982**, *38*, 633. Nambiar, K. P.; Stauffer, D. M.; Kolodziej, P. A.; Benner, S. A. *J. Am. Chem. Soc.* **1983**, *105*, 5886. Stackhouse, J.; Nambiar, K. P.; Burbaum, J. J.; Stauffer, D. M.; Benner, S. A. *J. Am. Chem. Soc.* **1985**, *107*, 2757. Benner, S. A. *Chem. Rev.* **1989**, *89*, 789–806. Benner, S. A.; Ellington, A. D. *CRC Crit. Rev. Biochem.* **1988**, *23*, 369–426.

(11) Lipinski, C. A. *Annu. Rep. Med. Chem.* **1986**, *21*, 283. Thornber, C. W. *Chem. Soc. Rev.* **1979**, *8*, 563.

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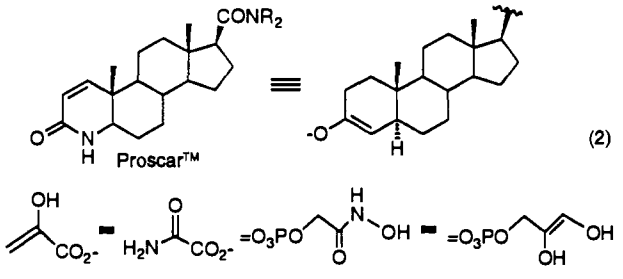
Table I. MNDO Comparison of Vinyl Fluoride and Vinyl Alcohol

		
r_{C-C} (Å)	1.35	1.35
r_{C-X} (Å)	1.36	1.325
E_{HOMO} (eV)	-9.3	-10.2
μ (D)	1.72	1.702

presence of enols[12] and enolates in biochemical transformations,[13] we have undertaken development of novel enol/enolate metaphors. Amides[14] (eq 2) and carboxylates[15] (eq 3) have previously been



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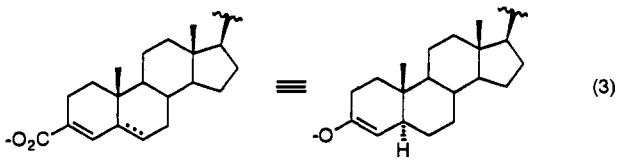

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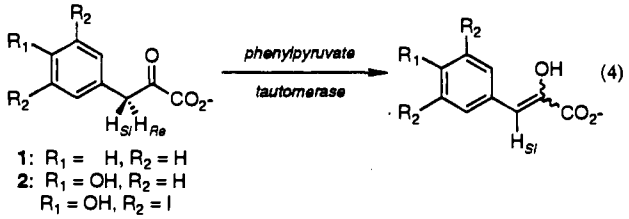


(4)

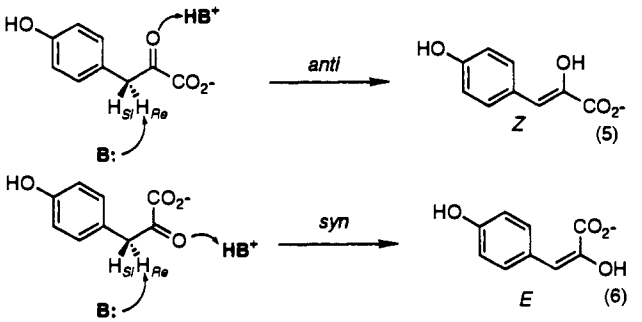
1: R_1 = H, R_2 = H
2: R_1 = OH, R_2 = H
R_1 = OH, R_2 = I

used as metaphors for the enol/enolate functionality and, in some cases, have resulted in powerful inhibitors. A pharmaceutical recently marketed for the treatment of prostatic hyperplasia, Proscar (Merck), inhibits a steroid 5α-reductase that likely generates an enolate intermediate. One limitation of the amide metaphor is the low rotational barrier that prevents study of individual stereoisomers. Because many of the enols in our studies are derived from α-keto acids, their carboxylate metaphors have *no* stereochemistry. We previously reported the synthesis and properties of vinyl fluoride analogs of the enols produced by α,β-dihydroxy-acid dehydratase, an enzyme involved in valine and isoleucine biosyntheses.[16] Vinyl fluorides were chosen on the basis of the similarity of the polarity of the C–O and C–F bonds and the small van der Waals radius of F (Table I; derived by semiempirical MO calculations using MNDO). Vinyl fluorides, as chemically stable enol mimics, are ideal for answering stereochemical questions. We report here on the use of vinyl fluorides as stereochemical probes and inhibitors for phenylpyruvate tautomerase. They can be "plugged in" to substrates to generate inhibitors for other enol-utilizing enzymes.

The highly stable enzyme phenylpyruvate tautomerase (E.C. 5.3.2.1) conducts the straightforward transformation depicted in eq 4.[17] The porcine thyroid enzyme has been purified to homogeneity[18] and has a molecular mass of 44 kDa. Its substrates (*p*-hydroxyphenyl)pyruvate, (diiodohydroxyphenyl)pyruvate, and phenylpyruvate have K_m's of 1.2, 1.2, and 8 mM, respectively. There is no evidence for a required metal ion or cofactor, and the optimum pH is 6.2, at which buffer-catalyzed tautomerization is slow. The kidney enzyme is known to exchange specifically the *pro-R* proton of phenylpyruvate with solvent.[19] It is used in an important clinical application, i.e. assays of phenylpyruvate and (*p*-hydroxyphenyl)pyruvate as their enols. These keto acids are degradation products of tyrosine and phenylalanine produced in inborn errors of metabolism such as phenylketonuria and general aminoaciduria.

It has been stated that tautomerase works by perturbing the equilibrium in favor of the enol form.[17] While impossible as a basis for any type of enzymatic or nonenzymatic catalysis, this statement does raise the issue of its mechanism. Generally, the mechanism of enzyme-catalyzed enolization reactions is an important issue because of the involvement of enol or enolate intermediates in a host of biochemical transformations, particularly those involving C–C bond formation. It has been suggested that, in the key initial step in enzyme-catalyzed β-elimination reactions, concerted general acid–general base catalysis generates the enol.[20] It is reasonable that some pairing of acidic and basic functionalities in the active site of tautomerase provides both an electrophile for the carbonyl oxygen and a base to remove the α-proton.

It was recognized in the earliest reports of tautomerase activity[21] that two configurations of the enol are possible and that the enzyme might produce an enol different from that generated by buffer-catalyzed tautomerization. Because tautomerase conducts stereospecific exchange reactions, a single (unknown) isomer of the enol must be generated. We assert that its stereochemistry can be assigned on the basis of differential inhibition data for stereoisomeric vinyl fluoride inhibitors. With this assignment in hand, the conformation of bound substrate can be inferred, as well as the relative orientation of the essential catalytic residues in the active site (eqs 5 and 6). To gain access to both vinyl



fluoride isomers, cinnamate esters were photochemically equilibrated, separated, and saponified. A free energy profile for the enzymatic reaction was developed on the basis of the K_i's and the Michaelis constants of the arylpyruvates and inferences about kinetic constants for the reverse reaction. The contributions of

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(13) Richard, J. P. In *The Chemistry of Enols*; Rappoport, Z., Ed.; Wiley-Interscience: New York, 1990; Chapter 11.
(14) Novoa, W. B.; Schwert, G. W. *J. Biol. Chem.* **1961**, *236*, 2150. Rasmusson, G. H.; Liang, T.; Brooks, J. R. In *Gene Regulation by Steroid Hormones 2*; Roy, A., Clar, J. H., Eds.; Springer: New York, 1983; pp 311–334. Bertics, P. J.; Edman, C. F.; Karavolas, H. J. *Endocrinology (Baltimore)* **1984**, *114*, 63. Northrop, D. B.; Cleland, W. W. *J. Biol. Chem.* **1974**, *249*, 2928–2931.
(15) Holt, D. A.; Levy, M. A.; Yen, H.-K.; Oh, H.-J.; Metcalf, B. W.; Wier, P. J. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 27–31.
(16) Pirrung, M. C.; Ha, H.-J.; Holmes, C. P. *J. Org. Chem.* **1989**, *54*, 1543. Pirrung, M. C.; Holmes, C. P.; Horowitz, D. M.; Nunn, D. S. *J. Am. Chem. Soc.* **1991**, *113*, 1020.
(17) Nishinaga, A.; Cahnmann, H. J.; Kon, H.; Matsuura, T. *Biochemistry* **1968**, *7*, 388. Blasi, F.; Fragomele, F.; Covelli, I. *Eur. J. Biochem.* **1968**, *5*, 215.
(18) Blasi, F.; Fragomele, F.; Covelli, I. *J. Biol. Chem.* **1969**, *244*, 4864.
(19) Retey, J.; Bartl, K.; Ripp, E.; Hull, W. E. *Eur. J. Biochem.* **1977**, *72*, 251.
(20) Gerlt, P. A.; Gassman, P. A. *J. Am. Chem. Soc.* **1992**, *114*, 5928–5934.
(21) Knox, W. E. *Methods Enzymol.* **1955**, *2*, 297–300.

Table II. Purification of Phenylpyruvate Tautomerase from Porcine Kidney

step	amt of protein (mg)	activity (units)	specific activity (units/mg)
Sigma	176	17425	99
Sephadex G-100	4.67	9103	1949
Sephadex CM C-50	2.42[a]	7900	3264
Sephadex G-50	0.585[b]	4555	7786

[a] Determined by the method of Bradford.[30] [b] Determined by the method of Warburg and Christian.[31]

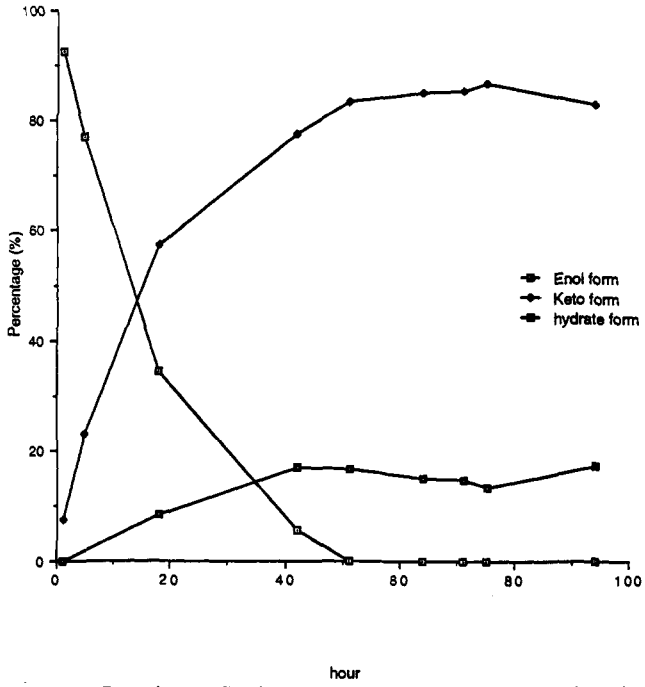


Figure 1. Reaction profile for the tautomerization and hydration of **3** in pH 6.0 acetate buffer.

the substituents in the substrates/inhibitors to the binding energy have been evaluated.

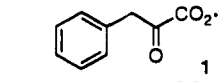
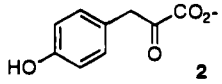
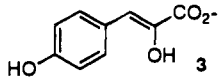
Results

A commercial tautomerase preparation from porcine kidney that had been subjected only to ammonium sulfate precipitation is a good source of crude enzyme. Chromatographic purification using Sephadex G-100, CM-Sephadex C-50, and Sephadex G-50 columns, along the lines used to purify the thyroid enzyme,[21,22] provided the tautomerase that was used for all of the studies reported herein (Table II). SDS-PAGE analysis (data not shown) indicates that this protocol provides a major improvement in purity, but the preparation is not homogeneous.

The substrate phenylpyruvate (**1**) is available commercially. (*p*-Hydroxyphenyl)pyruvate (**2**) is prepared by hydrolysis of (*p*-hydroxybenzylidene)hydantoin, which provides initially the crystalline enol form (2,*p*-dihydroxycinnamate, **3**) of the keto acid. When solutions of it are prepared in pH 6 acetate buffer,[21] the rate of tautomerization to the keto form is quite slow, as shown in Figure 1. Solutions were permitted to stand for 72 h before use. It is important to exclude oxygen during this stage, as it converts the enol to oxalate and *p*-hydroxybenzaldehyde. The half-time for equilibration under these conditions is 12.5 h. Earlier workers had reported that equilibration is complete within 30 min at pH 9.5.[22] Tautomerization in acetate buffer reached equilibrium after about 70 h, as shown by ^{1}H NMR spectroscopy. The olefinic proton signal of the enol form disappeared and two new peaks appeared corresponding to the methylene groups of

(22) Knox, W. E.; Pitt, B. M. *J. Biol. Chem.* **1957**, *225*, 675–688. Constantsas, W. S.; Knox, W. E. *Arch. Biochem. Biophys.* **1966**, *117*, 54–64.

Table III. Kinetic Data for Substrates

	K_m (mM)	k_{cat} (s^{-1} mg^{-1})	k_{cat} (rel)	k_{cat}/K_m (mM^{-1} s^{-1} mg^{-1})	k_{cat}/K_m (rel)
 1	6.1	0.45	1	0.074	1
 2	2.2	1.89	4.2	0.86	12
 3	0.072	0.052	0.12	0.72	9.7
for reverse reaction					

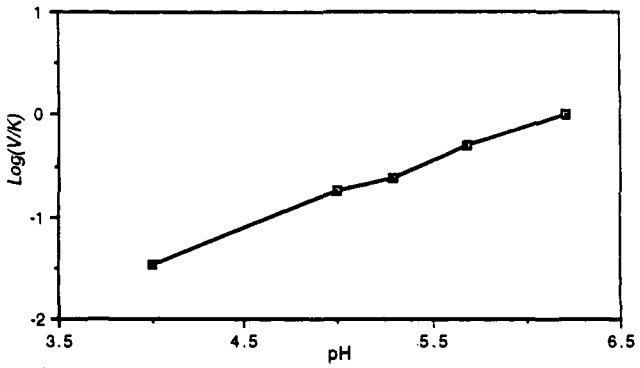
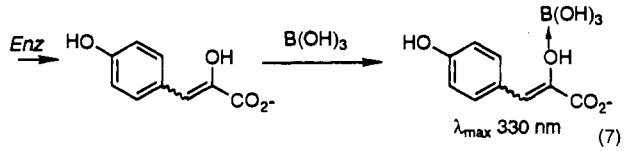


Figure 2. pH dependence of k_{cat}/K_m for tautomerase with (*p*-hydroxyphenyl)pyruvate as substrate.

the keto (85%) and hydrate (15%) forms. For studies of the enol, buffer solutions were prepared within 5 min of use by dissolution of crystalline 2,*p*-dihydroxycinnamic acid.

An optical assay based on a chromophoric enol–borate complex was used (eq 7). Enzymatic tautomerization of the keto form



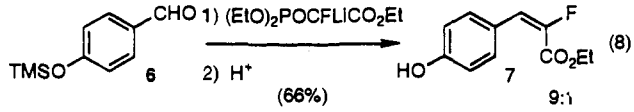
(7)

produces the enol which is trapped by complexation; the latter process is followed by the increase of the absorbance at 330 nm. It is therefore important to completely equilibrate the substrate since residual enol raises the background absorbance. The kinetic parameters for the two substrates closely parallel those for the thyroidal enzyme and are summarized in Table III. Values for k_{cat} and k_{cat}/K_m are also given. Enzyme activity was decreased by concentrations of (*p*-hydroxyphenyl)pyruvate above 4 mM and of phenylpyruvate above 6 mM. The conversion of the (*Z*) enol to the keto form in the reverse reaction was followed by the loss of the 330-nm absorption. Its kinetic parameters are also summarized in Table III. As will be shown below, it is likely that this does not represent a "natural" reaction of the enzyme and that the natural reverse reaction likely has a much lower K_m.

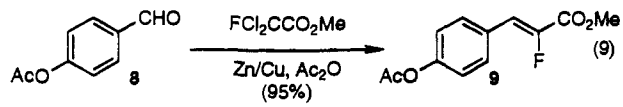
The pH–rate profile that was obtained for tautomerase is shown in Figure 2. The substrate K_m is hardly affected by pH; k_{cat} strongly influences their ratio, which shows nearly a 2-decade drop as the pH is lowered to 4 from 6.2. The loss of catalytic activity in this pH range suggests that histidine may be the titratable group, which would serve as the *base* to remove the α-proton. No inference about the acid that serves to protonate the oxygen can be gained from these data. It is also possible that the carboxylate of the substrate is titrated at lower pH. It is not clear that this should slow the rate, however, since the product

should have the higher pK_a due to its long, electron-donating, conjugated system.

It was desirable that stereochemically-defined analogues bearing the functionalities thought to provide enzymatic recognition, the enol/vinyl fluoride and the *p*-hydroxyl, be prepared for analysis with tautomerase. The synthetic targets therefore include both stereoisomers of the four compounds cinnamate, *p*-hydroxycinnamate, α-fluorocinnamate, and *p*-hydroxy-α-fluorocinnamate. On the basis of the K_i's for these eight compounds and the K_m's for the two substrates, the stereochemistry could be assigned. The desired vinyl fluorides were prepared by three routes. α-Fluorocinnamate is commercially-available as the (*Z*) isomer (**4**). It was converted to the methyl ester and equilibrated photochemically to a 1:3 (*Z*):(*E*) mixture by irradiation at 254 nm. These were separated by careful, repeated chromatography, and the (*E*) isomer was hydrolyzed to **5**. The hydroxylated vinyl fluorides were prepared by stereospecific synthesis. The trimethylsilyl ether of *p*-hydroxybenzaldehyde was converted to the (*E*) isomer of the ethyl ester using a fluorinated phosphonate (eq 8).[23] Acidic workup of the phosphonate reaction cleaves the silyl



ether, and the ester is hydrolyzed in base to **11**. The acetate of *p*-hydroxybenzaldehyde is used with a fluorinated Reformatsky reagent[24] to produce specifically the (*Z*) isomer **9** (eq 9) which



is hydrolyzed to **10**. Finally, commercially-available (*E*)-*p*-hydroxycinnamate (**12**) was converted to the methyl ester and photochemically equilibrated to a 7:3 (*Z*):(*E*) mixture by irradiation at 300 nm. The mixture was *tert*-butyldimethylsilylated, the isomers were separated by chromatography, and the (*Z*) isomer was hydrolyzed with LiOH to **13**.

The stereostructures of these compounds were assigned using NMR spectroscopy, and complete spectral data are recorded in the Experimental Section. The stereochemistries of the vinyl fluorides are obvious on the basis of vicinal H–F coupling constants. Thus, another advantage of the vinyl fluoride/enol metaphor is the magnetic moment of the ^{19}F nucleus that permits such spectroscopic determinations of structure. For the enols, this magnetic interaction is absent, so further correlations were made with vicinal C–H coupling constants ($^3J_{C-H}$) between the vinyl hydrogen and the carboxyl carbon.[25] As summarized in Table IV, which includes known compounds, small coupling constants reflect (*Z*) stereochemistry and large coupling constants indicate (*E*). For further confirmation of this assignment, 2,*p*-dihydroxycinnamic acid (**3**) was subjected to an X-ray crystallographic analysis. As shown in the ORTEP diagram in the supplementary material, the compound has the expected, thermodynamically more stable, (*Z*) stereochemistry.

For comparison to some of the other functional groups that have been used as enol metaphors, *p*-hydroxyoxanilic acid (**17**) was prepared by literature methods[26] and (*p*-hydroxybenzylidene)-malonate (**16**) was prepared by Knoevenagel condensation of dimethyl malonate with *p*-hydroxybenzaldehyde, silylation, and hydrolysis.

(23) Etemad-Moghadan, G.; Seyden-Penne, J. *Bull. Soc. Chim. Fr.* **1985**, *3*, 448.
(24) Ishihara, T.; Kurobashi, M. *Chem. Lett.* **1987**, 1145.
(25) Vogeli, U.; von Philipsborn, W. *Org. Magn. Reson.* **1975**, *7*, 617.
(26) Pascal, R. A., Jr.; Oliver, M. A.; Chen, Y.-C., J. *Biochemistry* **1985**, *24*, 3158–3165.

Table IV. $^3J_{C-H}$ NMR Data for Substrates/Inhibitors and Model Compounds

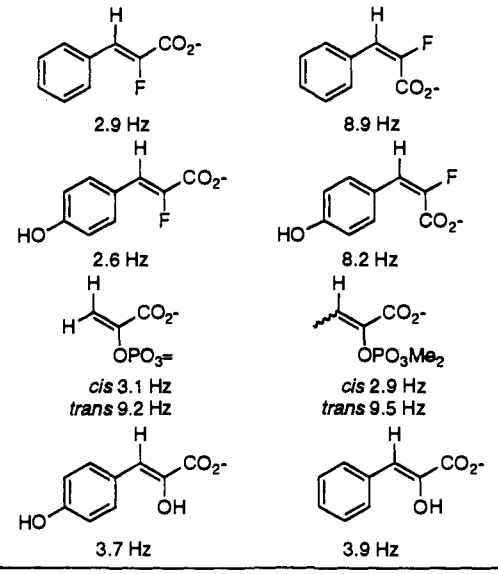
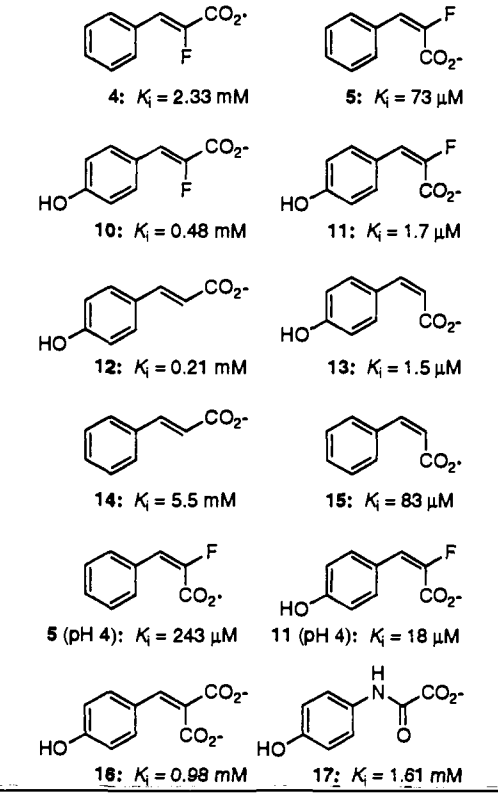


Table V. Inhibition Data for Enol Analogues



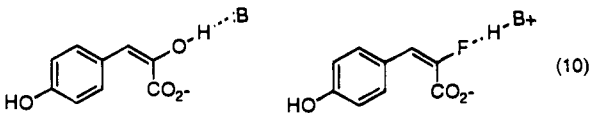
All of the studies of the designed inhibitors were performed with (*p*-hydroxyphenyl)pyruvate as the substrate. The data are summarized in Table V. All inhibition was competitive, as demonstrated by graphical analysis of the data plotted in Lineweaver–Burk double-reciprocal form. The data show that all of the (*E*) stereoisomers (**5**, **11**, **13**, and **15**) are far better inhibitors of tautomerase activity than corresponding (*Z*) stereoisomers, thereby implying that the product of the tautomerase reaction has (*E*) stereochemistry. This enol is unknown, but is corresponding *O*-glycoside has been identified as a leaf-opening substance in nyctinastic plants.[27]

The study of *p*-hydroxyoxanilic acid was conducted because amides have previously been used as enol analogues on the basis

(27) Shigemori, H.; Sakai, N.; Miyoshi, E.; Shizuri, Y.; Yamamura, S. *Tetrahedron* **1990**, *46*, 383–390.

of their restricted rotation and their enol-like canonical structure. In this case, it is likely that the preferred conformation of the amide is more like a (*Z*) enol than an (*E*) enol, and we therefore expect it to be comparable to the (*Z*) inhibitors. The study of (*p*-hydroxybenzylidene)malonate was performed because of recent work in our and other laboratories that has suggested that carboxylates can serve as molecular metaphors for *enolates*. Since an *enol*, not an enolate, is the product of the tautomerase reaction, we expect that the malonate would be a poor inhibitor. This compound is also similar to the poor inhibitors **4**, **10**, **12**, and **14**, all of which have an (*E*) carboxylate group. The K_i's observed for **16** and **17** are in concert with the above predictions.[28]

The comparable K_i's of the pairs of compounds **11**/**13** and **5**/**15** suggest that under these assay conditions the vinyl fluoride is contributing little to the binding interaction between inhibitors and the enzyme. A simple idea to explain this observation is shown in (10). When the enol is generated in the course of



(10)

catalysis, it may be hydrogen bonded to the enzymatic group that has delivered the proton to the oxygen. When the vinyl fluoride is present, however, this proton may not be, depending on the pK_a of the basic group on the enzyme and the pH. In order to explore this point, we examined the pH dependence of the K_i's for compounds **5** and **11**. They are 3–10-fold *poorer* inhibitors at pH 4. It is difficult to determine the influence of protonation state on the interactions between the macromolecule and the vinyl fluoride, since this is about the same pH effect that is seen for the catalytic process.

Discussion

The inhibition data for the compounds prepared in this study unambiguously show that the strongly preferred stereochemistry is (*E*). These data permit the probative assignment of the stereochemistry of the enol produced by tautomerase as (*E*).[29] This assignment relies on the assumption that the carboxylates of the inhibitors are fulfilling the "carboxylate" binding site on the enzyme. If their carboxylates were instead fulfilling the enol/enolate binding site, one would expect that (*p*-hydroxybenzylidene)malonate, satisfying both carboxylate and enol/enolate binding sites, would be an excellent inhibitor. Because this is not observed, the assumption seems a safe one. Troubling aspects of the data are the nearly identical second-order rate constants for the forward reaction of **2** and the reverse reaction of **3**, which has the "wrong" double-bond geometry. However, one would expect, on the basis of the K_i's for **11** and **13** and the notion that they are product analogues, that the (*E*) enol would have about a 1 μM Michaelis constant. The observed K_m of $\sim$70 μM suggests that a few percent of contamination by the (*E*) isomer in solutions prepared from the crystalline (*Z*) isomer would account for the kinetics. On the basis of the known stereochemistry of the hydrogen-exchange reaction and the assignment of enolate stereochemistry made above, the enolization process proceeds through the *syn* transition state (eq 6).

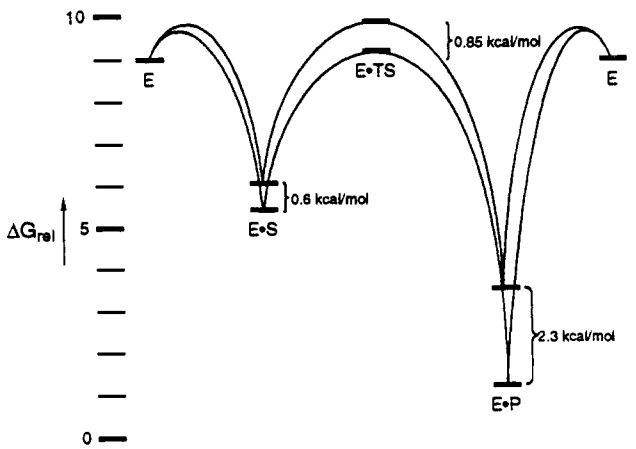


Figure 3. Free energy profile for the tautomerization of phenylpyruvate (higher energy) and (*p*-hydroxyphenyl)pyruvate (lower energy) by tautomerase. The binding of product to the enzyme is inferred on the basis of the inhibition by (*E*) product analogues. The absolute energy of E·TS is arbitrary because absolute k_{cat}'s were not determined.

Analysis of the kinetic and inhibition data permits the determination of the thermodynamic contributions of various groups in the substrates/inhibitors to binding and catalysis. Pairwise comparisons of **11**/**13** and **5**/**15** suggest that the vinyl fluoride contributes only a small factor to the overall binding interaction of (*E*) analogues with tautomerase. The *para* hydroxyl of the substrate is worth $\sim$0.6 kcal/mol in binding energy (Figure 3). The difference in k_{cat}'s for **1** and **2** assigns to this hydroxyl an 0.8 kcal/mol binding energy in the transition state of the catalytic step. A linear free energy analysis of the binding of the (*E*) inhibitors, by making pairwise comparisons of **5**/**11** and **13**/**15**, reflects a 2.3 kcal/mol contribution of the phenolic hydroxyl in the product. Overall, these data provide direct evidence for recognition of the phenolic hydroxyl by a hydrogen-bond acceptor in tautomerase. Binding increases as the phenol becomes a better hydrogen-bond donor during the conversion of starting material to product. This is a disadvantage for turnover, as is evidenced in Figure 3, since E·P has a large well to escape to release the product. For the reverse reaction, while a sub-millimolar Michaelis constant was determined for the (*Z*) stereoisomer **3**, the micromolar inhibition potency of the (*E*) stereoisomers **11** and **13** suggests that, were the (*E*) stereoisomer of the enol (which we infer to be produced) available for independent analysis, it would have a much higher affinity for tautomerase. This assertion suggests the value of the preparation of specifically the (*E*) enol for evaluation of its kinetic properties.

Several functional groups have been suggested as metaphors for enols/enolates, including amides, carboxylates, and vinyl fluorides. There is no major benefit to be gained from vinyl fluorides as compared to unfunctionalized vinyl derivatives with tautomerase, whereas with dihydroxy-acid dehydrase, the former are far superior equivalents for enols. Vinyl fluorides may be the most potent inhibitors when specific catalytic residues are present in the active site. The possibility has been raised that a cysteine thiolate is the base in dihydroxy-acid dehydrase, while the evidence

(28) A reviewer has suggested that the carboxylates in all of the inhibitors may fulfill the "enol" binding site on the enzyme rather than the "carboxylate" binding site. If true, **16** should be the most powerful inhibitor, since it possesses both the natural carboxylate and the enol metaphor. An explanation for its poor inhibition could lie in its protonation state, since it should have a second pK_a around 6, approximately the assay pH. This possibility was addressed through a limited study of the pH dependence of the K_i. These were difficult experiments because the absorbance of **16** at 330 nm increases at lower pH, and the rate at pH >7 of buffer-catalyzed tautomerization of substrate at the low concentrations required by its high background absorbance exceeds the rate of enzyme-catalyzed tautomerization. Using single-line K_i determinations at a substrate concentration of 1.4 μM, it was shown that the inhibition by **16** *increases* at lower pH (figure in the supplementary material). At pH 7.0, **16** at 1 mM shows *no* inhibition. This crude profile suggests that monoprotonated **16** is the inhibitory species.

(29) A reviewer has argued that the true, natural substrate for tautomerase is unknown because it is somewhat promiscuous. It was further argued that enzymes do not have stereochemical imperatives for unnatural substrates, so there would be no reason to expect stereospecific exchange with substrates other than phenylpyruvate (where it has been demonstrated) and there would be no basis for the assertion that stereospecific exchange implies the formation of a single enol geometry. While it is true that *absolute* stereochemical requirements for natural substrates evolve under selective pressure, this is not true for "cryptic" stereochemistry[8] (as studied here), which is related only to the three-dimensional arrangement of catalytic groups in the active site. Cryptic stereochemistry may be the result of happenstance. The processing of a substrate and its analogues probes only this three-dimensional arrangement.

for tautomerase points to histidine. A larger data base of inhibitors, enzymes, and active-site residues will be necessary to formulate the rules that dictate when vinyl fluorides serve as the best metaphors for enols.

Experimental Section

General Details. α-Fluorocinnamate and phenylpyruvate were obtained from Sigma. (*E*)-Cinnamic acid was purchased from Aldrich. Irradiations were conducted in a Rayonet photochemical reactor. NMR spectra were recorded at 300 and 75 MHz for ^1H and ^{13}C, respectively, on a Varian XL-300 spectrometer.

Protein Purification. The crude tautomerase (2 mL, 88 mg/mL, Grade II from porcine kidney, Sigma) was loaded onto a Sephadex G-100 column (1 × 120 cm), which had been equilibrated with 0.1 M potassium phosphate, pH 7.4, containing 0.14 M KCl and 1 mM sodium azide, and eluted with the same buffer. The fractions containing activity were pooled and subjected to overnight dialysis against 0.01 M potassium phosphate, pH 6.2, containing 1 mM sodium azide. The dialyzed enzyme solution was loaded onto a CM Sephadex C-50 column (1 × 18 cm), which had been equilibrated with the dialysis buffer. The C-50 column was washed with three volumes of buffer and then eluted with 200 mL of a 0–1 M KCl gradient. The fractions containing tautomerase activity were collected and dialyzed overnight against 0.1 M potassium phosphate buffer, pH 7.4 (with addition of 1.4 M KCl). This concentrated enzyme preparation was subjected to a Sephadex G-50 column, which had been equilibrated with 0.1 M potassium phosphate buffer, pH 7.4, containing 0.14 M KCl and 1 mM sodium azide, and eluted with the same buffer. The fractions containing tautomerase activity were pooled and concentrated via first a Millipore Immersible CX-10 and then an Amicon Centricon 30 microconcentrator. The protein concentration was determined either by the Coomassie brilliant blue binding assay (Bio-Rad) of Bradford[30] or by UV absorption (A_{280}/A_{260}) according to the treatment of Warburg and Christian.[31]

Enzyme Assays. The keto substrate solution was prepared by dissolving 2,*p*-dihydroxycinnamic acid (crystalline free acid) in 0.05 M acetate buffer, pH 6.0, and left at room temperature for at least 72 h to tautomerize before use. The sodium salt of phenylpyruvate exists as a keto form which was used as the keto substrate. Tautomerase activity was determined by following the assay procedure of Blasi.[18] The assay solution contained 1.25 mmol of (*p*-hydroxyphenyl)pyruvic acid, 0.010 mmmol of acetate, 1.5 mmol of boric acid, pH 6.2, and an appropriate amount of phenylpyruvate tautomerase in a total volume of 3.3 mL. The enzymatic reaction was monitored by the absorbance increase at 330 nm for the first 5 min after it was initiated by the addition of the enzyme. The increase of the absorbance at 330 nm reflects formation of the borate complex of 2,*p*-dihydroxycinnamic acid. The extinction coefficient for the enol–borate complex of 6330 M^{-1} cm^{-1} at 330 nm was used. A blank was prepared in the same way except that enzyme was omitted. All enzymatic reactions were carried out at 25 °C unless specified otherwise. One unit of activity is defined as the amount of enzyme required to catalyze the keto–enol transformation of 1 nmol of substrate/min. The enol substrate solution was prepared by dissolving the crystalline 2,*p*-dihydroxycinnamic acid in 0.05 M acetate buffer, pH 6.0, immediately before the assay.

All kinetic inhibition studies were conducted in an assay mixture containing 4.2 mM acetate, 0.42 M boric acid, pH 6.2, and phenylpyruvate tautomerase in a total volume of 3.3 mL. Three different inhibitor concentrations and five concentrations of substrate were used. The kinetic properties of the two substrates were determined over substrate concentrations from 0.36 to 2.34 mM. Data were fit to the parabolic form of the Michaelis–Menten equation. For determinations of K_m and K_i, the data were fit to the Michaelis–Menten equation by the nonlinear regression program Enzfitter.

pH Studies. The K_m of (*p*-hydroxyphenyl)pyruvate and K_i of (*E*)-*p*-hydroxy-2-fluorocinnamic acid were determined at 25 °C over the pH range of 4.0–6.2. The acetate/boric acid buffer was used for this pH range. The amounts of enzyme used per assay were 1.5 μg for pH 6.2, 5.68, and 5.30, 3.0 μg for pH 5.0, and 6.75 μg for pH 4.0. The concentrations of *p*-hydroxy-2-fluorocinnamic acid were 0, 0.76, 1.36, and 2.27 μM at all pH's except 4.0, where 0, 7, and 23 μM were used.

(*Z*)-2,*p*-Dihydroxycinnamic Acid (3). According to the method of Billek,[32] a mixture of *p*-hydroxybenzaldehyde (6.11 g), hydantoin (5.5 g), and morpholine (10 mL) was slowly heated to 130 °C and kept at this temperature for 30 min. The reaction mixture was cooled, and 200 mL of water at about 60 °C was added. The resulting solution was filtered if any undissolved material was present. The solution was cooled to room temperature and acidified by dropwise addition of 20 mL of concentrated HCl. The mixture was allowed to stand at room temperature for a few hours, and the yellow precipitate of 5-(*p*-hydroxybenzylidene)-hydantoin was collected on a Buchner funnel, washed thoroughly with cold water, and dried in a vacuum desiccator over KOH overnight (0.89 g).

5-(*p*-Hydroxybenzylidene)hydantoin (1.7 g) was dissolved in 48 mL of 20% NaOH solution under nitrogen made oxygen-free by passing it through 50% NaOH solution (178 mL) containing pyrogallol (23.5 g). The temperature was raised to 176–178 °C, and the solution was refluxed for 3 h. The reaction was cooled in a water bath, and 20 mL of concentrated HCl was added dropwise, followed by 1 g of sodium bicarbonate. The resulting solution was continuously extracted with ether for 6 h. The ether layer was evaporated to dryness on a steam bath. The pale yellow residue (1.35 g) was dried in a vacuum desiccator over KOH overnight. The crude acid was suspended in 16.2 mL of water, and the suspension was placed in a 150 °C oil bath for 10 min. When the solution was a clear orange-brown, it was filtered through a Buchner funnel. When the filtrate began to crystallize, 1.6 mL of concentrated HCl was added. The resulting solution was cooled to room temperature and placed in the refrigerator overnight to produce tan crystals (0.4 g): ^1H NMR (DMSO-d_6) δ 6.35 (s, 1H), 6.75 (d, J = 18.7 Hz, 2H), 7.61 (d, J = 18.7 Hz, 2H), 8.82 (br s, 1H), 9.6 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 110.6, 115.4, 126.1, 131.0, 139.4, 156.9, 166.8.

Crystals suitable for X-ray analysis were obtained by slow recrystallization at room temperature from a solution of diethyl ether and pentane. Crystal data: $C_9H_8O_4$, M = 180.16, monoclinic, space group $P2_1/c$, a = 19.573(7) Å, b = 5.457(1) Å, c = 7.394(3) Å, β = 90.87(2)° (from 25 accurately-centered reflections, 35° < θ < 40°, widely separated in reciprocal space), V = 789.7(8) Å^3, Z = 4, D_{calcd} = 1.515 g cm^{-3}, μ(Cu Kα radiation) = 9.8 cm^{-1}; crystal dimensions 0.07 × 0.26 × 0.30 mm. Intensity data ($+h,+k,\pm l$, 1627 nonequivalent reflections) were recorded on an Enraf-Nonius CAD-4 diffractometer [Cu Kα radiation, graphite monochromator; λ = 1.5418 Å; ω–2θ scans, scan width (1.40 + 0.14 tan θ)°]. The intensities of four reference reflections, remeasured every 2 h, showed no significant variation (<1%). In addition to the usual Lorentz and polarization corrections, a φ-scan-derived empirical absorption correction (T_{max}:T_{min} = 1.00:0.80) was also applied to the intensity data. The crystal structure was solved by direct methods (MULTAN11/82). Approximate coordinates for all non-hydrogen atoms were obtained from an E map. Following several rounds of full-matrix least-squares adjustment {minimizing $\sum w\delta^2$ [$w = 1/\sigma^2(|F_o|)$, $\Delta = (|F_o| - |F_c|)$}] of positional and thermal parameters of these atoms (at first isotropic, then anisotropic), a difference Fourier synthesis yielded hydrogen atom positions. In the subsequent least-squares iterations, hydrogen atom positional and isotropic thermal parameters were included as variables. The parameter refinement converged (max shift:esd = 0.02) at R = 0.057 (R_w = 0.080, GOF = 2.08) over 1073 reflections with $I > 3.0\sigma(I)$. A final difference Fourier synthesis contained no usual features [$\Delta\rho$ (e/Å^3): max, 0.26; min, –0.20]. Crystallographic calculations were performed on PDP11/44 and MicroVAX computers by use of the Enraf-Nonius Structure Determination Package (SDP). For all structure factor calculations, neutral-atom scattering factors and their anomalous dispersion corrections were taken from ref 33.

(*Z*)-Cinnamic Acid. (*Z*)-Cinnamic acid was prepared by the procedure of Bloomfield and Fuchs.[34] Ethyl phenylpropiolate (1.19 g) was hydrogenated in diethyl ether (10 mL) containing $Pd/CaCO_3$ (Pb poisoned) (0.28 g) and distilled quinoline (0.2 mL). The catalyst was removed by filtration and washed with diethyl ether. The combined filtrates were washed with cold 3 N HCl (10 mL) and with cold 10% NaOH (10 mL) and dried. The residue after evaporation of solvent was saponified to give (*Z*)-cinnamic acid quantitatively: ^1H NMR ($CDCl_3$) δ 5.99 (d, J = 12.7 Hz, 1H), 7.08 (d, J = 12.7 Hz, 1H), 7.37 (m, 3H), 7.60 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 118.7, 128.0, 129.3, 129.9, 134.3, 145.9, 171.7.

(*Z*)-2-Hydroxycinnamic Acid. This enol tautomer of phenylpyruvic

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(31) Warburg, O.; Christian, W. *Biochem. Z.* **1941**, *310*, 384.
(32) Billek, G. *Organic Synthesis*; Wiley: New York, 1973; Collect. Vol. V, p 627.
(33) *International Tables for X-Ray Crystallography*; Kynoch Press: Birmingham, U.K., 1974; Vol. IV.
(34) Bloomfield, J. J.; Fuchs, R. *J. Org. Chem.* **1961**, *26*, 2991.

acid was prepared from sodium phenylpyruvate by acidification of the sodium salt with 1 N HCl and crystallization of phenylpyruvic acid ((*Z*) enol form) from the aqueous HCl solution: ^{1}H NMR (DMSO-d_6) δ 6.04 (s, 1H), 7.23 (d, J = 8.06 Hz, 1H), 7.34 (m, 5H), 7.76 (d, J = 7.62 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 109.6, 127.2, 128.4, 129.3, 135.0, 141.9, 166.4.

Methyl (*Z*)-2-Fluorocinnamate. A mixture of (*Z*)-2-fluorocinnamic acid (1.00 g), sulfuric acid (0.24 g), and methanol (12 mL) was refluxed overnight. The reaction mixture was diluted with methylene chloride (30 mL) and washed with water (10 mL), 1% sodium bicarbonate (10 mL), and water (10 mL). The organic layer was dried over sodium sulfate and rotary-evaporated to give the ester (1.16 g, 100%), which was used without further purification: ^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H), 6.93 (d, J = 35.4 Hz, 1H), 7.39 (m, 3H), 7.64 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 52.6 (d, J = 20.2 Hz), 117.6 (d, J = 12.6 Hz), 128.4, 129.2, 129.9, 130.1, 146.7 (d, J = 267.1 Hz), 161.84 (d, J = 34.5 Hz).

Methyl (*E*)-2-Fluorocinnamate. The methyl ester of (*Z*)-2-fluorocinnamic acid (1.16 g) was dissolved in acetonitrile (150 mL) and irradiated under UV light (254 nm) for 12 h. Proton NMR showed 74% conversion to (*E*)-2-fluorocinnamic acid methyl ester. After removal of acetonitrile by rotary evaporation, 0.91 g of the *cis*/*trans* mixture was obtained. The isomers were separated by careful silica gel chromatography using toluene/hexanes (7:20, v/v); ^{1}H NMR ($CDCl_3$) δ 3.80 (s, 3H), 6.93 (d, J = 22.6 Hz, 1H), 7.35 (m, 3H), 7.45 (m, 2H).

(*Z*)-2-Fluorocinnamic acid (4): ^{1}H NMR (D_2O) δ 7.03 (d, J = 36.9 Hz, 1H), 7.43 (m, 3H), 7.68 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 116.5 (d, 5.8), 129.0, 129.7, 130.1 (d, 7.8), 131.0 (d, 3.9), 147.3 (d, 265.2), 162.0 (d, 34.4).

(*E*)-2-Fluorocinnamic Acid (5). The (*E*) methyl ester (0.26 g) was hydrolyzed by stirring with 0.25 N NaOH in water/THF (1:3, v/v) (10 mL) for 70 min. The reaction mixture was acidified with 1 N HCl (3 mL) and extracted twice with methylene chloride (20 mL × 2). The methylene chloride was washed with water (10 mL), 1% sodium bicarbonate (10 mL), and water (10 mL), dried over sodium sulfate, and rotary-evaporated. The yield of the (*E*) isomer in the hydrolysis step was almost quantitative (25% overall from methyl ester of *trans* isomer): ^{1}H NMR (D_2O) δ 7.05 (d, J = 22.35 Hz, 1H), 7.35 (m, 3H), 7.47 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 120.5 (d, 26.2), 128.0, 128.5, 129.8, 131.1 (d, 10.4), 147.1 (d, 252.9), 161.4 (d, 36.3).

Methyl (*E*)-*p*-Hydroxycinnamate. A mixture of *trans*-*p*-hydroxycinnamic acid (5.00 g), sulfuric acid (1.20 g), and methanol (60 mL) was refluxed for 7 h. The reaction mixture was diluted with methylene chloride (120 mL) and washed with water (60 mL), 1% sodium bicarbonate (60 mL), and water (60 mL). The organic layer was dried over sodium sulfate and rotary-evaporated to give the desired product (5.35 g, 99%): ^{1}H NMR ($CDCl_3$) δ 3.75 (s, 3H), 5.84 (d, J = 12.7 Hz, 1H), 6.80 (d, J = 8.7 Hz, 2H), 6.88 (d, J = 12.7 Hz, 1H), 7.63 (d, J = 8.7 Hz, 2H); ^{13}C NMR (DMSO-d_6) δ 113.9, 115.8, 125.1, 130.3, 144.8, 159.9, 167.1, 51.2.

Ethyl (*E*)-2-Fluoro-*p*-hydroxycinnamate (7). To a solution of triethyl 2-fluoro-2-phosphonoacetate (5.2 g, 21.5 mmol) in 40 mL of THF at −78 °C was added *n*-BuLi (1.6 M in hexane, 13.5 mL, 21.6 mmol). After 30 min of stirring, a solution of 4-(trimethylsiloxy)benzaldehyde (3.8 g, 19.6 mmol) in 10 mL of THF was added dropwise. The reaction mixture was allowed to warm to room temperature overnight and then was poured into 80 mL of 6 N HCl. The layers were separated, and the aqueous layer was extracted with four 50-mL portions of ether. The combined organic extracts were washed with brine and dried over $MgSO_4$. After evaporation of the solvent, flash chromatography (1:1 ether/petroleum ether) gave 3.75 g (91%) of product as a pale yellow oil: R_f 0.29 (1:1 ether/petroleum ether); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43 (d, J = 8.4 Hz, 2H), 6.86 (d, J = 23.7 Hz, 1H), 6.78 (d, J = 8.7 Hz, 2H), 6.02 (br s, 1H), 4.29 (q, J = 7.8 Hz, 2H), 1.30 (t, J = 7.5 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 161.24 (d, 63.4), 156.50 (145.59, d, 249.9), 131.75 (d, 6.8), 122.89, 122.44 (d, 26.3), 115.15, 61.82, 13.91; IR (neat) 3388, 1711, 1608 cm^{-1}.

Methyl (*Z*)-2-Fluoro-*p*-acetoxycinnamate (9). To a slurry of 3.9 g (59.7 mmol) of activated zinc powder, 0.63 g (6.4 mmol) of cuprous chloride, and 3.3 g of 3A molecular sieves in 30 mL of THF were added 3.4 g (20.7 mmol) of 4-acetoxybenzaldehyde and 2.2 mL (23.3 mmol) of acetic anhydride. The mixture was heated at 50 °C for 20 min, and then 3.0 g (18.6 mmol) of methyl dichlorofluoroacetate was added very slowly so as to keep the temperature below 65 °C. After 3.5 h at 50 °C, the mixture was cooled and filtered through Celite. The Celite was washed with ether, and the combined solutions were evaporated. Flash chromatography with 1:1 ether/petroleum ether gave 2.49 g (57%) of a white solid: R_f 0.44 (1:1 ether/petroleum ether); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.66 (d, J = 8.7 Hz, 2H), 7.14 (d, J = 8.7 Hz, 2H), 6.91 (d, J = 34.8 Hz, 1H), 3.89 (s, 3H), 2.31 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 169.05, 161.68 (d, 34.0), 151.41, 146.67 (d, 627.2), 131.47 (d, 12.6), 128.69 (d, 4.0), 122.05, 116.71, 52.63, 21.06; IR (KBr) 1763, 1727, 1659 cm^{-1}.

(*E*)-2-Fluoro-*p*-hydroxycinnamic Acid (11). A solution of 3.25 g (15.5 mmol) of ethyl (*E*)-2-fluoro-*p*-hydroxycinnamate (7), 25 mL of 1 M NaOH, and 5 mL of methanol was stirred at room temperature for 20 h. The reaction mixture was extracted with 50 mL of ether and then acidified with concentrated HCl. The acidic solution was extracted with four 50-mL portions of ethyl acetate. After the solution was dried over $MgSO_4$ and the solvent was evaporated, the crude solid was recrystallized from 20 mL of hot water. The crystals were dried under vacuum over P_2O_5 to give 2.01 g (72%) of a light yellow solid: mp 170.8–172.4 °C; ^{1}H NMR (300 MHz, 1:1 $CDCl_3$/DMSO-d_6) δ 12.98 (br s, 1H), 9.47 (br s, 1H), 7.42 (d, J = 8.4 Hz, 2H), 6.74 (d, J = 24.6 Hz, 1H), 6.69 (d, J = 8.4 Hz, 2H); ^{13}C NMR (1:1 $CDCl_3$/DMSO-d_6) δ 161.94 (d, 36.8), 158.17, 145.30 (d, 248.8), 131.90 (d, 6.7), 121.48 (d, 29.6), 121.15, 114.90; IR (KBr pellet) 3140, 1690, 1610 cm^{-1}. Anal. Calcd for $C_9H_7FO_3$: C, 59.35; H, 3.87. Found: C, 59.20; H, 3.88.

(*Z*)-2-Fluoro-*p*-hydroxycinnamic Acid (10). A solution of 2.1 g (8.8 mmol) of methyl (*Z*)-2-fluoro-*p*-acetoxycinnamate (9), 25 mL of 1 M NaOH, and 15 mL of methanol was stirred at room temperature for 20 h. The reaction mixture was extracted with 50 mL of ether and then acidified with concentrated HCl. The acidic solution was extracted with three 50-mL portions of ethyl acetate. After the extract was dried over $MgSO_4$ and the solvent was evaporated, the crude solid was recrystallized from 30 mL of hot water. The crystals were dried under vacuum over P_2O_5 to give 1.11 g (69%) of a light tan solid: mp 241.2–242.6 °C; ^{1}H NMR (300 MHz, 1:1 $CDCl_3$/DMSO-d_6) δ 12.95 (br), 9.72 (br s), 7.43 (d, J = 8.7 Hz, 2H), 6.79 (d, J = 36.6 Hz, 1H), 6.76 (d, J = 8.7 Hz, 2H); ^{13}C NMR (1:1 $CDCl_3$/DMSO-d_6) δ 162.40 (d, 34.1), 158.82, 145.23 (d, 259.3), 131.75 (d, 7.3), 121.81 (d, 4.9), 116.88, 115.64. Anal. Calcd for $C_9H_7FO_3$: C, 59.35; H, 3.87. Found: C, 59.14; H, 3.92.

(*E*)-*p*-Hydroxycinnamic acid (12): ^{1}H NMR (D_2O) δ 6.3 (d, J = 16 Hz, 1H), 6.78 (d, J = 8.5 Hz, 2H), 7.50 (d, J = 16 Hz, 1H), 7.52 (d, J = 8.5 Hz, 2H); ^{13}C NMR (DMSO-d_6) δ 115.3, 115.8, 125.4, 130.2, 144.3, 159.7, 168.1.

(*Z*)-*p*-Hydroxycinnamic Acid (13). The methyl ester of *trans*-*p*-hydroxycinnamic acid (2.0 g) was dissolved in acetonitrile (225 mL) and irradiated under UV light (300 nm) for 18 h. After removal of acetonitrile by rotary evaporation, 1.95 g of a *trans*/*cis* mixture was obtained (>99%). Proton NMR showed 69% conversion to the *cis* isomer: ^{1}H NMR ($CDCl_3$) δ 3.75 (s, 3H), 5.84 (d, J = 12.7 Hz, 1H), 6.80 (d, J = 8.7 Hz, 2H), 6.88 (d, J = 12.7 Hz, 1H), 7.63 (d, J = 8.7 Hz, 2H); ^{13}C NMR (DMSO-d_6) δ 114.9, 115.1, 125.5, 132.6, 144.4, 158.6, 166.5, 51.1. A *trans*/*cis* mixture (3.00 g) was silylated by stirring with *tert*-butyldimethylsilyl chloride (3.57 g) and imidazole (2.90 g) in DMF (16 mL) at room temperature under nitrogen for 24 h. The separation of the *cis* isomer from *trans* was effected by silica gel chromatography using toluene. *cis*: R_f 0.57; ^{13}C NMR (DMSO-d_6) δ 116.7, 119.4, 127.8, 132.2, 142.6, 156.2, 166.2, 51.1, −8.0, 17.9, 25.5. *trans*: R_f 0.42; ^{13}C NMR (DMSO-d_6) δ 115.3, 120.5, 127.6, 129.7, 144.6, 157.8, 167.7, 51.5, −6.6, 12.2, 25.6. The methyl ester and *tert*-butyldimethylsilyl ether linkages of the *cis* isomer (0.36 g) were cleaved by refluxing with 10 mL of 1.4 N LiOH in methanol/water (8:2, v/v) for 3 h. The reaction mixture was diluted with 10 mL of water, and most of the methanol was removed under reduced pressure. The resulting solution was washed with ether (10 mL × 2) and acidified with 1 N HCl (9 mL). The acidic aqueous solution was extracted with chloroform (15 mL × 2), and the organic layers were dried over sodium sulfate and evaporated to give *cis*-*p*-hydroxycinnamic acid (57 mg, 17%): ^{1}H NMR (DMSO-d_6) δ 5.73 (d, J = 12.9 Hz, H), 6.75 (d, J = 8.4 Hz, H), 6.77 (d, J = 12.9 Hz, H), 7.64 (d, J = 8.4 Hz, H); ^{13}C NMR (DMSO-d_6) δ 114.8, 117.5, 125.7, 132.3, 141.7, 158.5, 167.7.

(*p*-Hydroxybenzylidene)malonate (16). A benzene solution (130 mL) of *p*-hydroxybenzaldehyde (6.10 g, 50 mmol), dimethyl malonate (6.60 g, 50 mmol), pyridine (1 mL), and glacial acetic acid (1 mL) was refluxed for 4 h in a Dean-Stark trap. The solvent was rotary-evaporated at elevated temperature under reduced pressure. The residue was dissolved in 50 mL of methylene chloride, and the solution was washed with an equal volume of water and dried over sodium sulfate. After removal of the solvent, a yellow solid (9.94 g) was obtained, which was used without further purification. The dimethyl (*p*-hydroxybenzylidene)malonate (1.95 g) was silylated by stirring with *tert*-butyldimethylsilyl chloride (1.85 g) and imidazole (1.41 g) in DMF (10 mL) for 20 h at room temperature. The reaction solution was diluted with methylene chloride, and the resulting

solution was washed with water. The separated organic layer was dried over sodium sulfate and rotary-evaporated. After silica gel chromatography (ether/petroleum ether (1:1, v/v), R_f 0.7), the desired product (2.36 g) was obtained: ^{1}H NMR ($CDCl_3$) δ 0.2 (s, 6H), 0.96 (s, 9H), 3.81 (s, 3H), 3.84 (s, 3H), 6.82 (d, J = 8.78 Hz, 2H), 7.32 (d, J = 8.78 Hz, 2H), 7.68 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 120.4, 122.7, 125.7, 131.4, 142.5, 158.2, 164.7, 167.5, 52.5, -7.7, 18.1, 25.5. The dimethyl ((*tert*-butyldimethylsiloxy)benzylidene)malonate (0.76 g) was hydrolyzed by stirring with 8 mL of 1 N NaOH in water/THF (2:6, v/v) for 24 h. The reaction mixture was diluted with 10 mL of water and continuously extracted with diethyl ether overnight. The separated aqueous layer was passed through a Dowex 50 resin (acidic form). The resin was washed with water until the washing was neutral. The collected aqueous solution was rotary-evaporated at 30–40 °C under reduced pressure to give 0.37 g of (*p*-hydroxybenzylidene)malonate (85%): ^{1}H NMR (D_2O) δ 6.87 (d, J = 8.7 Hz, 2H), 7.45 (d, J = 8.7 Hz, 2H), 7.53 (s, 1H); ^{13}C NMR (D_2O) δ 115.8, 123.6, 124.4, 131.7, 139.0, 159.9, 165.7, 168.5.

Acknowledgment. Financial support by NIH Grant GM-43816 is appreciated. The reviewers are thanked for their suggestions.

Supplementary Material Available: A figure showing the pH dependence of the inhibition of tautomerase by **16**, an ORTEP diagram of **3**, and tables of atomic positional and thermal parameters, bond lengths, bond angles, torsion angles, and hydrogen-bonding geometries for **3** (6 pages); a listing of observed and calculated structure amplitudes for **3** (8 pages). Ordering information is given on any current masthead page.