Mode of Action of 4-Hydroxyphenylpyruvate Dioxygenase Inhibition by Triketone-type Inhibitors

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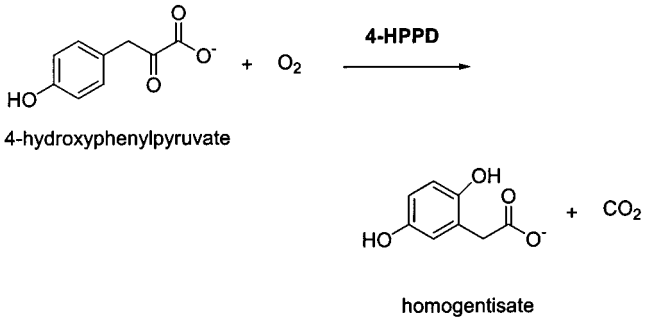
Received December 17, 2001

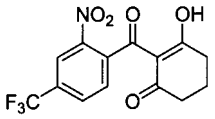
A series of 2-(2-nitrobenzoyl)cyclohexane-1,3-dione analogues (**1**−**9**) were designed, synthesized, and evaluated for inhibition of 4-hydroxyphenylpyruvate dioxygenase (4-HPPD), a key enzyme involved in the catabolism of tyrosine which catalyzes the conversion of 4-hydroxyphenylpyruvate to homogentisate. The correlations between the results of enzyme inhibition, ferric chloride tests, and the conformational analysis suggested that the tight binding between triketone-type inhibitors and 4-HPPD is likely due to chelation of the enzyme-bound ferric iron with the enol tautomer of 1,3-diketone moiety of the triketones. The presence of a 2-carbonyl group in the triketone is an essential structural feature for potent 4-HPPD inhibition. Modification of the 3-carbonyl group of triketone moiety to other functionality will reduce the overall planarity and thus prevent keto−enol tautomerization, resulting in a decrease or lack of inhibition activity.

Introduction

4-Hydroxyphenylpyruvate dioxygenase (4-HPPD, EC 1.13.11.27) is an important enzyme involved in the catabolism of tyrosine in most organisms.[1] It catalyzes the conversion of 4-hydroxyphenylpyruvate to homogentisate and carbon dioxide in the presence of oxygen and ferrous ion as shown in Scheme 1. The biotransformations catalyzed by 4-HPPD require an oxidative decarboxylation of the 2-oxoacid side chain of the substrate with an accompanying hydroxylation of the aromatic ring and a 1,2-migration of the carboxymethyl group.[2] Clinically, a hereditary disease called tyrosinemia type I[3] is caused by the deficiency of fumarylacetoacetate hydrolase activity which converts fumarylacetoacetate to fumarate and acetoacetate in the tyrosine degradation pathway. Inhibition of 4-HPPD activity, an enzyme preceding the furmarylacetoacetate hydrolase in the same pathway, will provide an alternate treatment for this fatal disease. Although the first effective drug therapy for tyrosinemia has been discovered, that is, 2-(2-nitro-4-trifluoromethylbenzoyl)cyclohexane-1,3-dione[4] (NTBC, a triketone-type compound as shown below), the mode of action for this inhibition remains unclear. Since NTBC is a strong competitive inhibitor of 4-HPPD and the enzyme activity may be partly reversed by ascorbate, it is reasonable to assume that the favored keto−enol form mimics the ketoacid functionality present in the substrate and is capable of binding strongly to the ferric ion in the active site.[5] Here we report the synthesis, characterization, and structure−activity relationships (SAR) of 2-(2-nitrobenzoyl)cyclohexane-1,3-dione derivatives as inhibitors of 4-HPPD in an effort to provide insights into its mode of action at the molecular level.

Scheme 1. Reaction Catalyzed by 4-HPPD





NTBC

Chemistry

The compounds designed and synthesized for SAR are compounds **1**−**9** as shown in Figure 1. Since our previous SAR studies[6] of 2-*o*-substituted-benzoyl- and 2-alkanoylcyclohexane-1,3-dione analogues have demonstrated that both the nitro group on benzene moiety and the benzene ring of NTBC are critical on 4-HPPD inhibition activity, the main objective of the present study is to investigate the crucial role of triketone functionality in NTBC at the molecular level. While keeping both the 2-nitrobenzoyl moiety and the six-membered ring intact, one of the carbonyl groups present in the triketone-type inhibitors was either selectively removed (compounds **2**, **8**, and **9**) or modified

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10.1021/jm010568y CCC: $22.00
Published on Web 04/27/2002

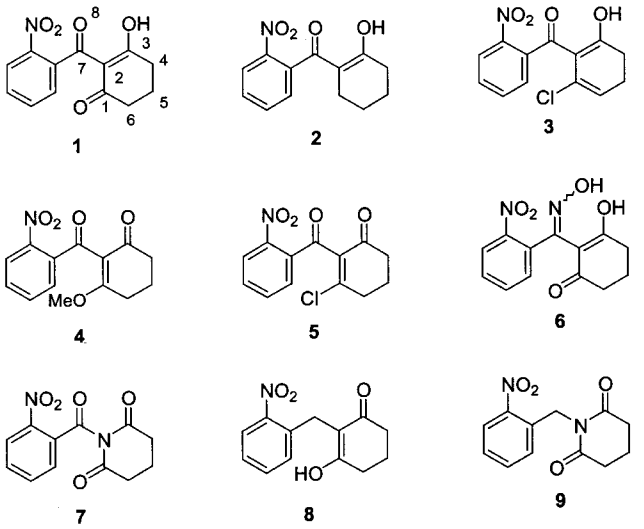


Figure 1. Compounds synthesized for SAR studies.

(compounds **3**−**6**) in order to eliminate the possibility of keto−enol tautomerization. In addition, the central carbon atom of the triketone moiety was replaced by a nitrogen atom (compound **7**) to force the 1,3-diketo group into a more rigid as well as nonplanar conformation. The inhibition results of these compounds with 4-HPPD will identify the essential structural elements required for the inhibition activity.

The synthetic route used to prepare compounds **1** and **3**−**7** is outlined in Scheme 2. The preparation of **1** started with the *O*-acylation of cyclohexane-1,3-dione by 2-nitrobenzoyl chloride to give enol ester **10**, which on treatment with cyanide ion and triethylamine rearranged to the corresponding *C*-acyl isomer **1**, according to the published procedure.[7] Methylation of **1** with diazomethane in ethyl acetate at 0 °C afforded **4**. Compound **1** was converted quantitatively to vinyl chloride **5** by treatment with an excess of oxalyl chloride at room temperature.[8] Base-promoted isomerization of diketovinyl chloride **5** to ketodienol form **3** was performed by treatment of **5** with potassium carbonate and a catalytic amount of tetra-*n*-butylammonium iodide in acetone under room temperature for 2 days with a 32% yield. Selective reaction of the carbonyl group adjacent to the nitrophenyl ring of **1** with hydroxylamine hydrochloride in the presence of sodium methoxide afforded oxime **6** as reported in the literature.[9] 2-Acyl imide **7** was prepared by a coupling reaction of glutarimide with 2-nitrobenzoyl chloride in the presence of triethylamine as a base in methylene chloride under reflux conditions for 3 h with a 50% yield.

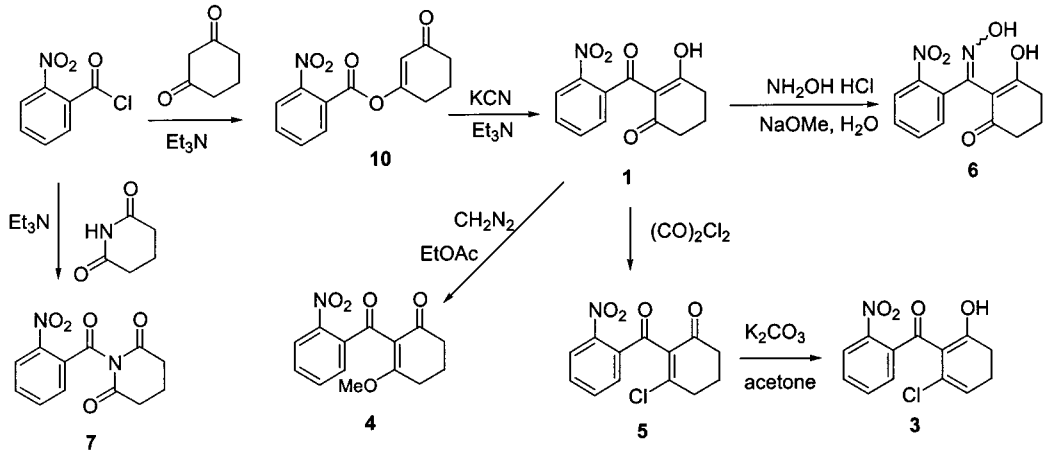
Next, compound **2**, as shown in Scheme 3, was generated by reacting 1-morpholinecyclohexene (prepared by dehydration of cyclohexanone with morpholine)[10] with 2-nitrobenzoyl chloride under basic conditions followed by refluxing in an aqueous acid for 5 h.[11] The ratio of enol/keto isomers, determined by ^{1}H NMR spectra, was found to be 97/3. Unfortunately but not surprisingly, reacting 2-nitrobenzyl bromide with cyclohexane-1,3-dione in triethylamine in CH_2Cl_2 gave the major undesired vinyl ether **12** and minor desired compound **8** in the ratio of 2/1. A similar coupling reaction was used to generate **9** except glutarimide and potassium carbonate were used instead of cyclohexane-1,3-dione and triethylamine, as depicted in Scheme 4.

Results and Discussion

With these potential 4-HPPD inhibitors available, incubation experiments were conducted to determine inhibition parameters of **1**−**9** by the spectrophotometric enol borate method.[12] The inhibition data for the reactions of these compounds with pig liver 4-HPPD are summarized in Table 1. All the synthesized compounds were also tested with ferric chloride to observe any colorimetric change. Only NTBC and **1**−**3** gave a positive characteristic purple color, while the rest of the compounds gave negative results, as shown in Table 1.

According to the inhibition results, the potent inhibitors included NTBC and **1**−**3** with an IC_{50} less than 1 μM. These four inhibitors also gave a positive test with ferric chloride assay, which suggests they all can bind tightly with enzyme-bound ferric ion via the enol tautomer of the triketones. The rest of the synthesized compounds were not only poor 4-HPPD inhibitors but also gave negative ferric chloride tests, indicating the lack of the critical triketone enol tautomer. These results prompted us to consider whether deprotonation of the enolic hydrogen at the C-3 position in the triketone system could alter the molecular three-dimensional orientation, thereby directly affecting the molecular affinity with the target enzyme and resulting in different inhibition activity. Thus, the structures of **1** and **4** were established by X-ray diffraction analysis. From the

Scheme 2. Synthesis of Compounds **1** and **3**−**7**



Scheme 3. Preparation of Compound **2**

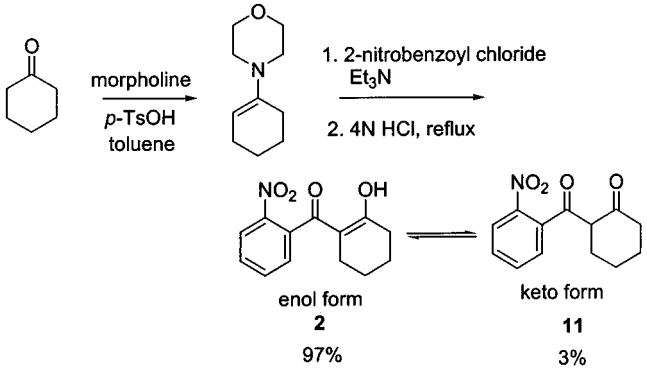


Table 1. Inhibition Constant for Reactions of **1**−**9** with 4-HPPD, Results of Ferric Chloride Tests, and Predicted Torsional Angles

compd	IC_{50} (μM)[a]	**FeCl**$_3$ test[b]	torsional anglec (deg)
NTBC	0.04 ± 0.01	+	0.3
1	0.16 ± 0.06	+	1.4 (1.6)
2	0.70 ± 0.15	++	0.5
3	0.53 ± 0.10	+	0.5
4	52 ± 2	−	59.8 (58.2)
5	64 ± 9	−	61.2
6	50 ± 4	−	11.1
7	60 ± 7	−	29.2
8	126 ± 12	−	−
9	250 ± 28	−	−

[a] All data presented are averages of at least two separate experiments. [b] Degree of colorimetric change (high, ++; moderate, +; no change, −). [c] Predicted torsional angle of O(8)−C(7)−C(2)−C(3) as labeled in compound 1. [d]Values in the parentheses are obtained from X-ray structures.

X-ray crystallographic analysis shown in Figure 2, compound **1** in the crystal exists only as the cis−enol tautomer with an intramolecular hydrogen bond. In this tautomer, the enol hydrogen is covalently bound to the oxygen atom that is remote from the phenyl group, which is similar to the structure of benzoyldimedone reported recently by Borisov.[13] It is clear that the 1,3-diketone and the 2-carbonyl moiety of **1** are coplanar and conjugated by hydrogen bonding of the C-3 enolic hydrogen to the oxygen atom of the C-2 carbonyl group with a torsional angle of 1.6°. If one of the carbonyl groups in the triketone is modified, however, the inhibition potency of the resulting compound decreases substantially. For example, when the C-3 enolic oxygen of

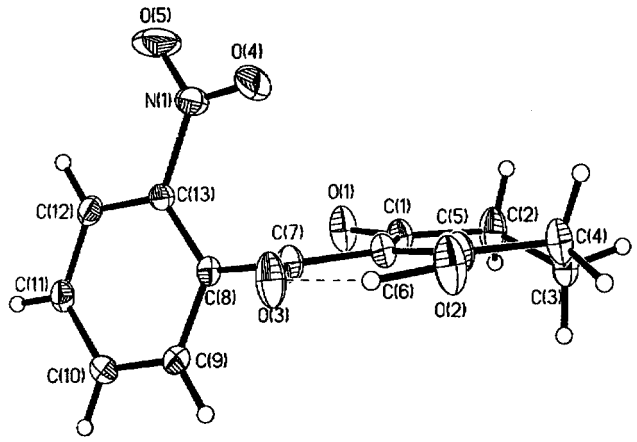


Figure 2. X-ray structure of compound **1**. ORTEP diagram showing the crystallographic atom-numbering scheme. Torsional angle of O(3)−C(7)−C(6)−C(5) = 1.60°.

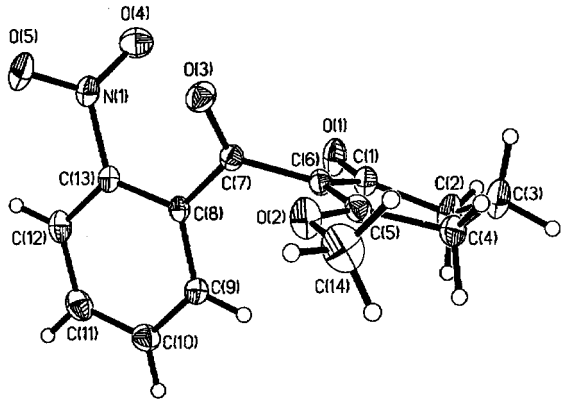
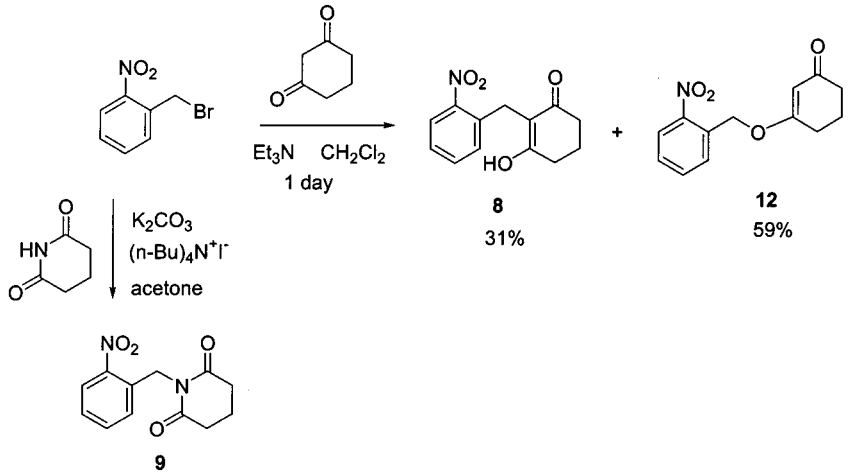


Figure 3. X-ray structure of compound **4**. ORTEP diagram showing the crystallographic atom-numbering scheme. Torsional angle of O(3)−C(7)−C(6)−C(5) = 58.2°.

compound **1** was methylated to vinyl ether **4** or chlorinated to vinyl chloride **5**, their IC_{50} values increased to 52 and 64 μM, respectively. The solid-state conformation of **4** from an X-ray diffraction study indicates that the orientation of the 2-aroyl group is forced out of the plane of the 1,3-diketone system with a torsional angle of 58.2°, as shown in Figure 3. This deformation from planarity is due to the dipolar repulsions between the C-2 carbonyl group and the C-1 carbonyl group as well as the C-3 oxygen atom. Similar observations have been

Scheme 4. Synthesis of Compounds **8** and **9**



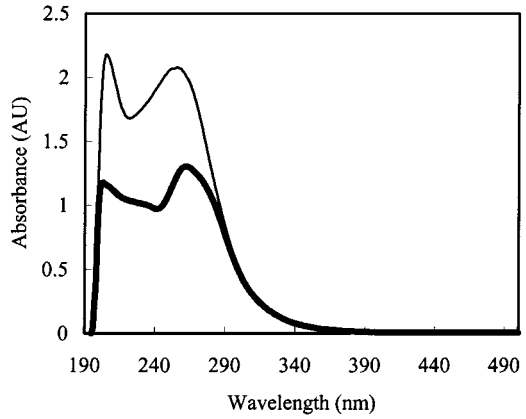


Figure 4. UV spectra of compounds **1** and **4**. Compound **1**: heavy line, $\lambda_{max} = 262$ nm. Compound **4**: light line, $\lambda_{max} = 255$ nm.

reported in the literature.[14] Additionally, the bond length of 1.445 Å between the C-2 and the 2-carbonyl carbon in **1** indicates extensive conjugation in the triketone system, while that in **4** is shown to be 1.487 Å, suggesting its single bond character. This observation implies that the conformation of triketone-type compounds can be controlled or regulated by protonation or deprotonation of the enolic hydrogen at the C-3 position. Furthermore, the UV spectral data of **1** and **4**, shown in Figure 4, also supported their observed conformational differences. The longest wavelength absorption band for **1** was at 262 nm, indicating full conjugation in the triketone system. With **4**, the corresponding absorption bands shifted to 255 nm. This blue shift of the absorption band indicates a loss of conjugation because of the dipolar repulsions between the C-2 carbonyl group and the C-1 carbonyl group as well as the C-3 oxygen atom. Moreover, the distinctly different polarity of **1** ($R_f = 0.59$, 60% EtOAc/hexanes) and **4** ($R_f = 0.17$, 60% EtOAc/hexanes) also resulted possibly from their conformational differences.

To obtain a further understanding of the SARs of triketone analogues, we performed conformational searches for compounds **1**−**7** using Insight II software.[15] The values of the resulting torsional angles [O(8)−C(7)−C(2)−C(3)] of stable three-dimensional structures are shown in Table 1. Clearly, in the cases in which the 3-hydroxyl groups of the triketones were replaced or modified, the resulting compounds (**3**−**5** and **7**) tend to have higher torsional angles values ranging from 29 to 61° compared to those potent inhibitors (NTBC and **1**−**3**), which can undergo keto−enol tautomerization with torsional angles of 0−2°. These results are consistent with the hypothesis that the dipolar repulsions between the C-2 carbonyl group and the other two oxygen atoms on the ring system cause deformation of the triketone from planarity and result in poor affinity to the enzyme 4-HPPD. Furthermore, the solid-state conformation and torsional angle data for compounds **1** and **4** from X-ray structure are similar to the computational data. The only exception is compound **6** with the predicted torsional angle of 11.1°. Although the conformation of **6** is relatively close to planarity based on the molecular modeling studies, we speculate that the presence of the oxime functionality prevents its chelating with the enzyme active site ferric ion, and therefore results in low affinity with 4-HPPD. The fact that compound **6** gave a negative ferric chloride test and was a poor 4-HPPD inhibitor with $IC_{50} = 50$ μM supported our assumption. The foregoing studies indicated that the absence of the C-3 enolic oxygen of 1,3-triketone results in dipolar repulsion, which is relieved in part by the out-of-plane orientation, thereby disturbing the overall planarity of the triketone system. Accordingly, the dipolar repulsion and consequent skeletal deformation might be responsible for the lack of 4-HPPD inhibition in **4** and obvious decrease in **5**.

When vinyl chloride **5** was treated with potassium carbonate in acetone under room temperature, it isomerized to ketodienol **3** with $IC_{50} = 0.53$ μM. The presence of the typical signal of the enolic hydrogen absorption peak at 16.43 ppm in ^{1}H NMR spectra as well as a positive test with ferric chloride provided evidence of **3** in the enolized form. The fact that the inhibition potency of **3** increased 120-fold relative to **5** and there was only a 4-fold decrease in the inhibition potency of **2** compared to **1** suggest that the 3-carbonyl group of the triketone type inhibitors may not play a significant role in 4-HPPD inhibition. On the other hand, when the 2-carbonyl group was removed to give **8**, **9** or modified to obtain oxime **6**, none of them showed potent 4-HPPD inhibition activity. These results can be easily explained since no ligands were available in these compounds to interact with the ferric ion in the active site and thus resulted in a weak affinity with the enzyme. Taken together, these results demonstrate that the presence of the 2-carbonyl group, but not the 3-carbonyl group, is crucial for potent 4-HPPD inhibition, presumably by chelation with the enzyme active site ferric iron. Finally, the failure of **7** as a 4-HPPD inhibitor is consistent with the results that the enol form as well as the coplanar structure of the triketone is required for potent 4-HPPD inhibition, even though compound **7** also contains the same triketone functionality as NTBC.

In summary, we have designed and prepared a series of 2-(2-nitrobenzoyl)cyclohexane-1,3-dione (**1**) derivatives. SAR studies within this series indicate that the enol form and coplanar structure of the triketone are absolutely required in order to chelate with the enzyme-bound ferric iron. The correlations between the results of enzyme inhibition, ferric chloride tests, and conformational analysis suggest that the tight binding between triketone-type inhibitors and 4-HPPD is likely due to chelation of the enzyme-bound ferric iron with the enol tautomer of 1,3-diketone moiety of the triketones (Figure 5). The presence of the 2-carbonyl group in the triketone is an essential feature for potent 4-HPPD inhibition, whereas the 3-carbonyl group is not. Modification of the 3-carbonyl group of the triketone moiety to another functionality will reduce the overall planarity and thus prevent keto−enol tautomerization, resulting in decrease or loss of inhibition activity. Furthermore, the ferric chloride assay may serve as a simple and easy screening test for further development of triketone type 4-HPPD inhibitors. Understanding the mode of action of triketone type 4-HPPD inhibitors will certainly aid the design of new 4-HPPD inhibitors for use in tyrosinemia treatment.

Experimental Section

Chemistry. Melting points were determined on a Mel-Temp melting point apparatus in open capillaries and are uncor-

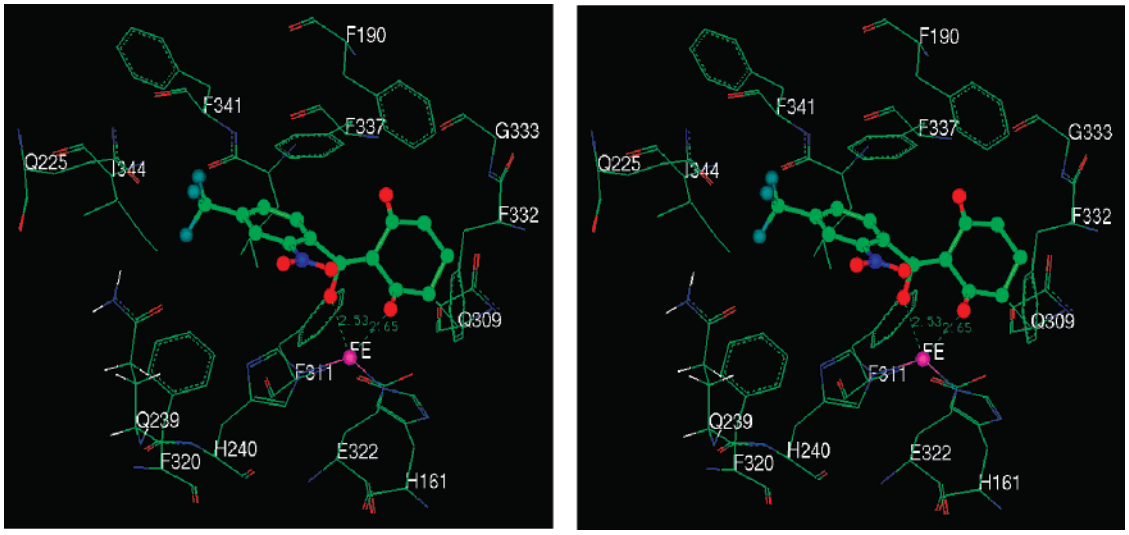


Figure 5. Model predicting the binding mode of NTBC to 4-HPPD. The chelation of the enzyme bound iron (purple) with enol tautomer of 1,3-tiketone is indicated with a dashed line. See Experimental Section for modeling detail.

rected. Elemental analysis was performed using a Heraeus CHN−OS rapid element analyzer. Ultraviolet−visible spectroscopy was performed on a Hewlett-Packard 8453 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz on a Varian VXR300 spectrometer. Chemical shifts were reported in parts per million on the δ scale relative to an internal standard (tetramethylsilane, or appropriate solvent peaks) with coupling constants given in hertz. ^{1}H NMR multiplicity data are denoted by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and b (broad). Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60G-254 plates (25 mm) and developed with the solvents mentioned. Flash chromatography was performed in columns of various diameters with Merck silica gel (230−400 mesh ASTM 9385 kieselgel 60H) by elution with the solvent systems indicated in the parentheses after the R_f values. Solvents, unless otherwise specified, were reagent grade and distilled once prior to use. All new compounds exhibited satisfactory spectroscopic and analytical data.

Enzyme Purification and Assay. 4-HPPD was purified from pig liver by the method of Hamilton[16] with a specific activity of 0.1 μmol min^{-1} mg^{-1}. The protein concentration was determined by the BCA method. Routine assay of the enzyme utilized the spectrophotometric enol borate method of Lindstedt and Rundgren.[12] The typical assay mixtures contained 0.85 mL of potassium phosphate/borate buffer (prepared by adjusting the pH of 0.42 M H_3BO_3 to 6.2 with a 0.17 M Na_3PO_4 solution), 0.06 mL of 4-hydroxyphenylpyruvic acid (1.8 mM, in 0.2 M Na_3PO_4 buffer), 0.03 mL of dichlorophenolindophenol (reduced form, prepared by mixing 1 mL of 3.3 mM sodium dichlorophenolindophenol in H_2O and 0.16 M glutathione in 0.2 M sodium phosphate buffer), and 0.01 mL of phenylpyruvate tautomerase (10U/mL, Sigma). The above solution was equilibrated for about 15 min (monitored at 308 nm); then the 4-HPPD to be tested was added (0.05 mL solution). For calculation of dioxygenase activity the change in absorbance between the 8th and 10th min was used. The inhibition reaction of NTBC and **1**−**9** with the enzyme 4-HPPD was evaluated by measuring the decrease in absorbance at 308 nm over a 15 min period following coadministration of varying concentrations of the inhibitors and 4-HPPD. The IC_{50} values were determined by fitting the data to the equation $v_i = v_0/(1 + [\text{inhibitor}]/IC_{50})$, where v_i is the rate of absorbance change at a given inhibitor concentration and v_0 the rate of absorbance change in the absence of inhibitor.

Molecular Modeling. The atomic coordinates of 4-HPPD were obtained from the Protein Data Bank maintained by the Research Collaboratory for Structural Bioinformatics (RCSB) under the accession code 1cjx. The construction of molecular models, structure optimization, and conformational analysis were performed by means of Insight II software using the CFF91 force field. Modeling to predict the binding mode of NTBC to 4-HPPD (Figure 5) was carried out on a SGI R5000 workstation. Building and calculations were done with Insight II software according to the following protocol. First, the hydrogen atoms were added to the structure of one monomer. Second, the energy minimization via Discover took place with the following specifications: consistent valence force field (CVFF),[17] 10 Å cutoff distance, implicit solvent, and distance-dependent dielectric constant $\epsilon = 4r$. Third, the acetate molecule and three water molecules (wat5, wat25, and wat88) bound in the active site were removed and one NTBC molecule was included. Fourth, the ligand and the protein side chains in a spherical domain centered on the iron atom and defined by a 10 Å radius were submitted to a 1000 K simulated annealing procedure; five constraints corresponding to the iron coordination bonds with His161, His240, Glu322, and the two oxygen atoms of the 1,3-diketone moiety of the NTBC molecule were set in the range 1.8−2.3 Å. Finally, the best energy structure was energy minimized without any aggregate and without constraints.

3-Hydroxy-2-(2-nitrobenzoyl)cyclohex-2-en-1-one (1). This compound was prepared by the literature procedure[6] to give a light yellow solid with a yield of 85%; mp 135−137 °C. R_f = 0.53 (25% EtOAc/hexanes). ^{1}H NMR ($CDCl_3$): δ 16.61 (bs, 1H, OH), 8.19 (dd, J = 8.4, 1.2 Hz, 1H, Ar H), 7.69 (td, J = 7.5, 1.2 Hz, 1H, Ar H), 7.56 (td, J = 8.4, 1.5 Hz, 1H, Ar H), 7.23 (dd, J = 7.2, 1.5 Hz, 1H, Ar H), 2.77 (t, J = 6.3 Hz, 2H, 6-CH_2), 2,34 (t, J = 6.3 Hz, 2H, 4-CH_2), 2.00 (quintet, J = 6.3 Hz, 2H, 5-CH_2). ^{13}C NMR ($CDCl_3$): δ 197.6 (C-1), 195.5 (C-3), 193.8 (C=O), 145.4, 136.4, 134.0, 129.5, 126.6, 123.5 (Ar C's), 112.7 (C-2), 37.4 (C-6), 31.7 (C-4), 19.0 (C-5). IR (KBr): ν 3200−2800 (OH), 1666 (C=O), 1581 (C=O), 1562, 1345 (NO_2) cm^{-1}. Anal. ($C_{13}H_{11}NO_5$) C, H, N.

2-(2-Nitrobenzoyl)cyclohex-1-enol (2). A solution of 1-morpholinocyclohexene (1.0 g, 5.98 mmol, prepared by the reported literature procedure[10]) and triethylamine (0.83 mL, 5.98 mmol) in chloroform (25 mL) was cooled to −10 °C, and a solution of 2-nitrobenzoyl chloride (1.1 g, 5.98 mmol) in 10 mL of chloroform was added dropwise. The mixture was stirred at room temperature overnight. Then, 2.0 mL of 4 N hydrochloric acid was, added and the solution was refluxed for 5 h. The organic layer was separated and washed with water (to pH 5). The aqueous layer was neutralized and extracted with chloroform. The combined organic extracts were dried, concentrated, and purified by column chromatography to give a white solid with a yield of 65%; mp 90−92 °C. R_f = 0.59 (20% EtOAc/hexanes). ^{1}H NMR ($CDCl_3$): δ 15.05 (s, 1H, OH), 8.19 (dd, J = 8.1, 1.2 Hz, 1H, Ar H), 7.74 (td, J = 8.4, 1.2 Hz, 1H, Ar H), 7.59 (td, J = 8.1, 1.5 Hz, 1H, Ar H), 7.36 (dd, J = 8.4,

1.5 Hz, 1H, Ar H), 2.46 (t, J = 6.6 Hz, 2H, 6-CH_2), 1.94 (t, J = 5.7 Hz, 2H, 3-CH_2), 1.75−1.68 (m, 2H, 5-CH_2), 1.60−1.56 (m, 2H, 4-CH_2). ^{13}C NMR ($CDCl_3$): δ 195.0 (C═O), 182.0 (C-1), 145.4, 134.7, 134.2, 129.9, 127.9, 124.3 (Ar C's), 106.7 (C-2), 30.6 (C-6), 24.4 (C-3), 22.5 (C-4), 21.3 (C-5). β-Diketone:enol ratio: 97:3. IR (KBr): ν 3200−2800 (OH), 1620 (C═O), 1574, 1344 (NO_2) cm^{-1}. Anal. ($C_{13}H_{13}NO_4$) C, H, N.

3-Chloro-2-(2-nitrobenzoyl)-1,3-cyclohexadien-1-ol (3). To a solution of vinyl chloride **5** (500 mg, 1.80 mmol) in acetone (10 mL) was added potassium carbonate (500 mg, 3.6 mmol) and a catalytic amount of tetra-*n*-butylammonium iodide (50 mg, 0.14 mmol) at room temperature. The resulting mixture was stirred at that temperature for 2 h. After acetone was removed under reduced pressure, the residue was dissolved in water. The aqueous layer was neutralized and extracted with ethyl acetate. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated, and the residue was purified by column chromatography to give a light brown solid with a yield of 32%; mp 80−82 °C (dec.). R_f = 0.81 (10% EtOAc/hexanes). ^{1}H NMR ($CDCl_3$): δ 16.43 (s, 1H, OH), 8.16 (d, J = 6.9 Hz, 1H, Ar H's), 7.70−7.58 (m, 2H, Ar H's), 7.42 (dd, J = 7.2, 1.5 Hz, 1H, Ar H), 5.84 (t, J = 5.4 Hz, 1H, 4-CH), 2.66 (t, J = 7.2 Hz, 2H, 6-CH_2), 2.40 (td, J = 7.2, 5.4 Hz, 2H, 5-CH_2). ^{13}C NMR ($CDCl_3$): δ 189.2 (C═O), 181.7 (C-1), 147.1, 136.8, 133.2, 131.3, 130.7, 124.4 (Ar C's), 128.6 (C-4), 124.9 (C-3), 107.3 (C-2), 33.1 (C-6), 27.0 (c-5). Anal. ($C_{13}H_{10}ClNO_4$) C, H, N.

3-Methoxy-2-(2-nitrobenzoyl)-2-cyclohexen-1-one (4). To a solution of **1** (1.0 g, 3.85 mmol) in ethyl acetate (25 mL) was added dropwise diazomethane (3.85 mmol, dissolved in ether, generated by Diazald) at 0 °C. The resulting solution was allowed to stand at that temperature until the precipitation of the product was complete. After filtration, the crude product was recrystalized in ethyl acetate to give a white solid with a yield of 89%; mp 137−138 °C. R_f = 0.38 (100% EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.85 (d, J = 8.1 Hz, 1H, Ar H), 7.65−7.53 (m, 3H, Ar H's), 3.91 (s, 3H, OMe), 2.70 (t, J = 6.3 Hz, 2H, 4-CH_2), 2.34 (t, J = 6.3 Hz, 2H, 6-CH_2), 2.03 (quintet, J = 6.3 Hz, 2H, 5-CH_2). ^{13}C NMR ($CDCl_3$): δ 195.2 (C═O), 190.4 (C═O), 180.4 (C-3), 147.1, 137.4, 133.0, 130.4, 129.7, 123.2 (Ar C's), 118.9 (C-2), 56.9 (OMe), 36.5 (C-6), 26.2 (C-4), 19.8 (C-5). Anal. ($C_{14}H_{13}NO_5$) C, H, N.

3-Chloro-2-(2-nitrobenzoyl)-2-cyclohexen-1-one (5). A solution of **1** (1.0 g, 3.84 mmol) in oxalyl chloride (5 mL) was stirred at room temperature for 3 h. The excess oxalyl chloride was removed at reduced pressure. The residue was dissolved in methylene chloride. The solution obtained was washed with saturated sodium bicarbonate solution, and then with water, and dried with magnesium sulfate. The solvent was removed on a rotary evaporator to obtain a crude product, which was then recrystallized in ethyl acetate to give quantitatively a yellow solid; mp 120−121 °C. R_f = 0.35 (40% EtOAc/hexanes). ^{1}H NMR ($CDCl_3$): δ 7.84 (d, J = 8.7 Hz, 1H, Ar H), 7.71−7.64 (m, 3H, Ar H's), 2.88 (t, J = 6.3 Hz, 2H, 4-CH_2), 2.50 (t, J = 6.3 Hz, 6-CH_2), 2.12 (quintet, J = 6.3 Hz, 2H, 5-CH_2). ^{13}C NMR ($CDCl_3$): δ 193.4 (C-1), 188.7 (C═O), 159.1 (Ar C), 148.2 (C-3), 136.3 (Ar C), 133.3 (C-2), 132.8, 132.4, 130.6, 123.7 (Ar C's), 36.8 (C-6), 35.4 (C-4), 21.3 (C-5). IR (KBr): ν 1687 (C═O), 1678 (C═O), 1536, 1366 (NO_2), 762 (C−Cl) cm^{-1}. Anal. ($C_{13}H_{10}ClNO_4$) C, H, N.

2-[(2-Nitrophenyl)-1-hydroxyiminomethyl)]-3-hydroxycyclohex-2-enone (6). To a solution of **1** (500 mg, 1.94 mmol) in water (30 mL) was added hydroxylamine hydrochloride (NH_2OH HCl, 272 mg, 3.91 mmol), and sodium methoxide (211 mg, 3.91 mmol) at 0 °C. The mixture was stirred at room temperature for 2 days. After completion of the reaction, the solution was neutralized to pH 7. The product was extracted twice with methylene chloride. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated. The resulting crude product was purified by column chromatography to give a white liquid with a yield of 75%; mp 113−115 °C. R_f = 0.48 (40% EtOAc/hexanes). ^{1}H NMR ($CDCl_3$): δ 8.17−8.14 (m, 1H, Ar H), 7.76−7.71 (m, 3H, Ar H's), 3.01 (t, J = 6.0 Hz, 2H, 4-CH_2), 2.53 (t, J = 6.0 Hz, 6-CH_2), 2.20 (quintet, J = 6.0 Hz, 2H, 5-CH_2). ^{13}C NMR ($CDCl_3$): δ192.3 (C-1), 166.8 (C═N), 164.1 (C-3), 148.1, 133.0, 132.0, 131.6, 124.9, 121.5 (Ar C's), 113.5 (C-2), 38.9 (C-6), 22.2 (C-4), 21.3 (C-5). IR (KBr): ν 3100−2900 (OH), 1669 (C═O), 1545, 1351 (NO_2) cm^{-1}. Anal. ($C_{13}H_{12}N_2O_5$) C, H, N.

1-(2-Nitrobenzoyl)piperidine-2,6-dione (7). To a solution of glutarimide (500 mg, 4.42 mmol) in methylene chloride (20 mL) was added triethylamine (0.65 mL, 4.66 mmol), and then dropwise 2-nitrobenzoyl chloride (freshly prepared and dissolved in 10 mL of CH_2Cl_2, 820 mg, 4.42 mmol) at ice-bath temperature. The mixture was refluxed for 3 h, and the reaction was then quenched with water. The product was extracted twice with methylene chloride. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated. The resulting crude product was purified by column chromatography to give a white solid with a yield of 50%; mp 155−156 °C. R_f = 0.21 (50% EtOAc/hexanes). ^{1}H NMR ($CDCl_3$): δ 7.90−7.66 (m, 4H, Ar H's), 2.72 (t, J = 6.6 Hz, 4H, 3-CH_2, 5-CH_2), 2.04 (quintet, J = 6.6 Hz, 2H, 4-CH_2). ^{13}C NMR ($CDCl_3$): δ 171.1 (C═O), 166.6 (C═O), 148.2, 133.1, 132.9, 130.9, 129.3, 124.0 (Ar C's), 32.8 (C3, 5), 16.8 (C-4). IR (KBr): ν 1755 (C═O), 1695 (C═O), 1576, 1337 (NO_2) cm^{-1}. Anal. ($C_{12}H_{10}N_2O_5$) C, H, N.

2-(2-Nitrobenzyl)-3-hydroxycyclohex-2-enone (8). To a solution of 1,3-cyclohexadione (1.0 g, 8.93 mmol) in methylene chloride (30 mL) was added triethylamine (1.25 mL, 8.89 mmol) and 2-nitrobenzyl bromide (2.0 g, 9.26 mmol) at 0 °C. The resulting mixture was stirred at room temperature for 1 day. After the reaction was completed, the products were washed with water. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated, and the residue was purified by column chromatography to give a major white solid 3-(2-nitrobenzyloxy)-2-cyclohexen-1-one (**12**) with a yield of 59%; mp 71−72 °C. R_f = 0.68 (50% EtOAc/hexanes). ^{1}H NMR ($CDCl_3$): δ 8.16 (d, J = 7.8 Hz, 1H, Ar H), 7.70−7.66 (m, 2H, Ar H's), 7.55−7.20 (m, 1H, Ar H), 5.49 (s, 1H, 2-CH), 5.33 (s, 2H, benzylic H's), 2.53 (t, J = 6.3 Hz, 2H, 4-CH_2), 2.39 (t, J = 6.3 Hz, 2H, 6-CH_2), 2.04 (quintet, J = 6.3 Hz, 2H, 5-CH_2). A minor desired white solid **8** with a yield of 31%; mp 170−171 °C. R_f = 0.83 (50% EtOAc/hexanes). ^{1}H NMR (acetone-d_6): δ 7.80 (dd, J = 8.1, 1.5 Hz, 1H, Ar H), 7.51 (td, J = 7.5, 1.2 Hz, 1H, Ar H), 7.39−7.31 (m, 2H, Ar H's), 3.87 (s, 2H, benzylic H's), 2.46 (t, J = 6.3 Hz, 2H, 6-CH_2), 2.05 (t, J = 6.3 Hz, 2H, 4-CH_2), 1.98 (quintet, J = 6.3 Hz, 2H, 5-CH_2). ^{13}C NMR ($CDCl_3$): δ 199.3 (C═O), 176.5 (C-3), 146.8, 133.8, 131.2, 128.8, 128.5, 125.0 (Ar C's), 103.7 (C-2), 66.9 (benzylic C), 36.5 (C-6), 28.5 (C-4), 21.0 (C-5). IR (KBr): ν 2930−3065 (OH), 1723 (C═O), 1576, 1378 (NO_2) cm^{-1}. Anal. ($C_{13}H_{13}NO_4$) C, H, N.

1-(2-Nitrobenzyl)piperidine-2,6-dione (9). To a solution of glutarimide (500 mg, 4.42 mmol) in acetone (10 mL) was added 2-nitrobenzyl bromide (955 mg, 4.42 mmol), potassium carbonate (800 mg, 5.79 mmol), and a catalytic amount of tetra-*n*-butylammonium iodide (50 mg, 0.14 mmol). The resulting mixture was stirred at room temperature for 24 h. After acetone was removed under reduced pressure, the residue was dissolved in water. The aqueous layer was neutralized with saturated ammonium chloride solution and extracted with ethyl acetate. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography to give a white solid with a yield of 92%; mp 92−93 °C. R_f = 0.26 (50% EtOAc/hexanes). ^{1}H NMR ($CDCl_3$): δ 7.96 (dd, J = 8.4, 1.2 Hz, 1H, Ar H), 7.53 (td, J = 7.8, 1.2 Hz, 1H, Ar H), 7.39 (td, J = 8.4, 1.2 Hz, Ar H), 7.14 (dd, J = 7.8, 0.9 Hz, 1H, Ar H), 5.32 (s, 2H, N-CH_2), 2.74 (t, J = 6.6 Hz, 4H, 3, 5-CH_2), 2.04 (quintet, J = 6.6 Hz, 2H, 4-CH_2). ^{13}C NMR ($CDCl_3$): δ 172.3 (C-2), 148.8, 133.2, 132.3, 127.9, 127.7, 124.9 (Ar C's), 39.8 (benzylic C), 32.7 (C-3, 5), 17.0 (C-4). IR (KBr): ν 1731 (C═O), 1578, 1378 (NO_2) cm^{-1}. Anal. ($C_{12}H_{12}N_2O_4$) C, H, N.

Acknowledgment. This work was supported by the National Science Council of the Republic of China.

Supporting Information Available: X-ray crystallographic data and structure refinement and bond lengths and

angles of compounds **1** and **4** (Tables 1−4). This material is available free of charge via the Internet at http://pubs.acs.org.

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JM010568Y