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Design, Synthesis, and Evaluation of Postulated Transient Intermediate and Substrate Analogues as Inhibitors of 4-Hydroxyphenylpyruvate Dioxygenase

Yun-Loung Lin, Jian-Lin Huang, Chung-Shieh Wu, Hung-Ge Liu and Ding-Yah Yang*

Department of Chemistry, Tunghai University, 181, Taichung-Kang Rd. Sec. 3, Taichung, Taiwan 40704, Republic of China

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Abstract—An epoxybenzoquinone, 4-hydroxyphenoxypropionic acid, and 2-hydroxy-3-phenyl-3-butenoic acid derivatives have been designed, synthesized, and evaluated for in vitro inhibition activity against 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) from pig liver by the spectrophotometric enol-borate method. The biological data demonstrated that neither epoxybenzoquinone ester nor 2-hydroxy-3-phenyl-3-butenoic acid is an inhibitor of 4-HPPD. The most potent 4-HPPD inhibitor tested was 3-hydroxy-4-phenyl-2(5H)-furanone with an IC_{50} value of 0.5 μM, which may serve as a lead compound for further design of more potent 4-HPPD inhibitors.

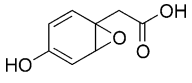
4-Hydroxyphenylpyruvate dioxygenase (4-HPPD, EC 1.13.11.27)[1] is a non-heme Fe(II)-dependent enzyme involved in the catabolism of tyrosine in most organisms[2] as well as the biosynthesis of plastoquinones and tocopherols in plants.[3] It catalyzes the conversion of 4-hydroxyphenylpyruvate (4-HPP, **1**) and molecular oxygen to homogentisate **2** and carbon dioxide, as shown in Scheme 1.

Inhibition of 4-HPPD has recently become the focus of considerable research interest because potent 4-HPPD inhibitors could provide an alternative treatment for life-threatening tyrosinaemia type I disease[4] and also have the potential to serve as a new class of bleaching herbicides for control of grass and broadleaf weeds.[5] For example, 2-benzoylcyclohexane-1,3-dione derivatives, referred to as triketones,[6,7] have been found to be competitive inhibitors of 4-HPPD with IC_{50} values as low as 40 nM. Recently, we discovered a new family of compounds, alkanoic acid 3-oxo-cyclohex-1-enyl esters,[8] which appear to be non-triketone type 4-HPPD inhibitors. Evidence suggests that these 4-HPPD inhibitors act as analogues[9] to the substrate 4-HPP. No transient intermediate 4-HPPD inhibitors have been reported so far.

1 2

Scheme 1.

Although the mechanistic details of this enzyme catalyzed reaction remain unclear, several lines of evidence have been reported to support the involvement of the arene oxide intermediate shown below.



Arene oxide

For example, studies involving the incubation of the unnatural substrate 3-thienylpyruvate with 4-HPPD resulted in formation of the product 3-carboxymethyl-3-thiolene-2-one,[10] which is best accounted for by the proposal of an arene oxide intermediate. Furthermore, excretion of the unusual amino acid hawkinsin[11] by patients suffering from Hawkinsinuria, a genetic disease attributed to production of defective 4-HPPD, also supports the formation of a transient arene oxide intermediate. Here, we report studies of the reactions of a chemically synthesized putative transient intermediate analogue **11** and series of substrate analogues **15a**–**b**, **27** with 4-hydroxyphenylpyruvate dioxygenase from pig

*Corresponding author. Tel.: +886-4-2359-7613; fax: +886-4-2359-0426; e-mail: yang@mail.thu.edu.tw

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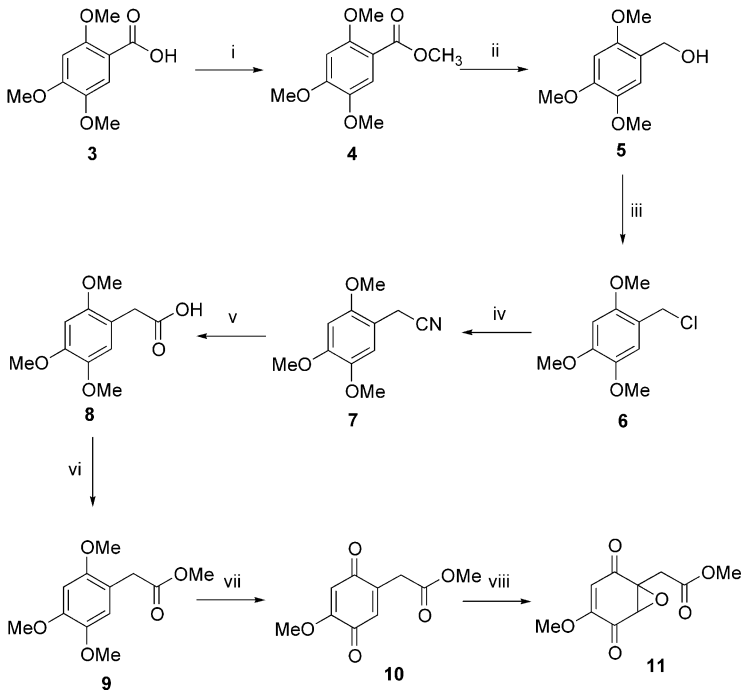
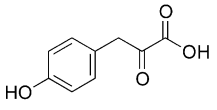
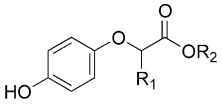


Scheme 2. Preparation of compound **11**: (i) $SOCl_2$, CH_3OH; (ii) $LiAlH_4$, Et_2O; (iii) $SOCl_2$, $CHCl_3$; (iv) NaCN, DMSO; (v) 10% NaOH, EtOH; (vi) CH_2N_2, Et_2O; (vii) AgO, 6 N HNO_3, acetone; (viii) H_2O_2, $NaHCO_3$, CH_3OH.

liver as part of our continuing effort to develop potent inhibitors for this enzyme. The aim of incorporation of a 1,4-diketo moiety on the ring system of epoxybenzoquinone ester **11** is to stabilize the arene oxide structure and to keep the six-membered ring nearly planar as it is in the proposed arene oxide intermediate. In the case of 4-hydroxyphenoxypropionic acid derivatives **15a**–**b**, the β-carbon atom of the natural substrate 4-HPP was replaced by oxygen and the α-keto fuctionality was modified to either a hydrogen atom or methyl group. We expected the resulting analogues, shown below, although structurally similar to 4-HPP, would not be able to serve as substrates for 4-HPPD.



4-HPP



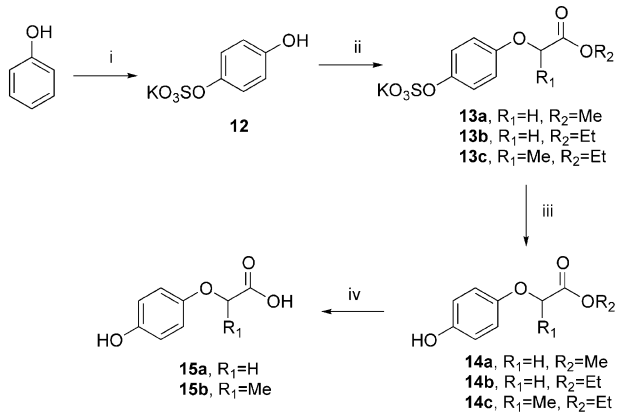
4-Hydroxyphenoxyacetic acid derivatives

As for the 3-phenyl-2-oxo-3-butenoic acid **27**, we introduced a methylene group at the C-3 position of 4-HPP. The design of this analogue took into account the fact that interaction of the *ortho* substituent of 4-HPP analogue with some active site groups may result in rotation of the aromatic ring away from the enzymatic oxygenating agent to prevent catalysis.[12] Similarly, incorporation of an extra methylene group on the side chain of 4-HPP may alter its molecular geometry. Thus, this substrate modification is expected to have a major effect on binding when incubated with the enzyme 4-HPPD.

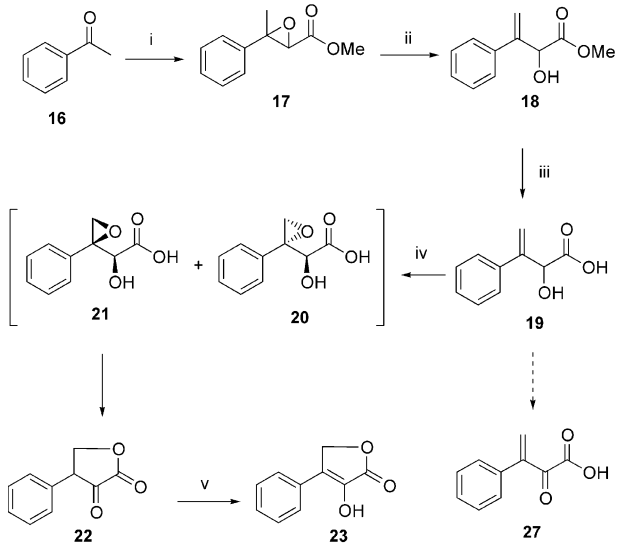
The synthetic route used to prepare compound **11** is outlined in Scheme 2. Commercially available trimethoxybenzoic acid **3** was first methylated with thionyl chloride in methanol, then reduced by $LiAlH_4$ to give the primary alcohol **5** with high yield. The alcohol **5** was converted to the corresponding chloride **6**, followed by reacting with sodium cyanide in DMSO. The resulting nitrile **7** was hydrolyzed under basic conditions to produce acid **8** with the desired carbon skeleton. Direct oxidation of **8** with silver oxide resulted in decomposition of the starting material. This observation prompted us to protect the acid functionality of **8** with diazomethane to furnish the methyl ester **9**. Subsequent oxidation[13] of the benzene moiety of **9** with silver(II) oxide and 6 N nitric acid to benzoquione derivative **10**, followed by selective epoxidation with hydrogen peroxide in the presence of sodium bicarbonate afforded the target epoxybenzoquinone ester **11** with an overall yield of 15%.

An effective route for preparation of 4-hydroxyphenoxyacetic acid **15a** and 2-(4-hydroxyphenoxy)-propionic acid **15b** is depicted in Scheme 3. It commenced with Elbs persulfate oxidation[14] of phenol with potassium persulfate in aqueous solution to 4-hydroxyphenyl sulfate salt **12**. The sulfate salt **12** was then treated with proper 2-chloroacid ester in the presence of potassium carbonate in DMF/xylene to give the 4-substituted phenyl sulfate salts **13a**–**c**.[15] Further removal of the sulfate group by boiling **13a**–**c** with acetic acid afforded 4-hydroxyphenoxyacid esters **14a**–**c** with a 50–55% combined yield. A final alkaline hydrolysis[16] of esters **14a**–**c** in 10% NaOH solution yielded **15a** and **15b**, respectively.

An attempt to prepare 3-phenyl-2-oxo-3-butenoic acid **27** is outlined in Scheme 4. According to this strategy, epoxy ester **17** was obtained by Darzens[17] glycidic ester condensation of acetophenone **16** with methyl chloroacetate in the presence of sodium methoxide. Acid-catalyzed ring opening of **17** in benzene produced the

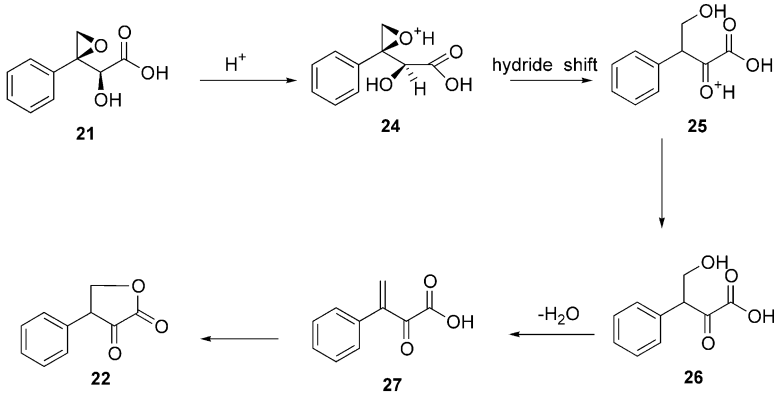


Scheme 3. Preparation of compounds **15a** and **15b**: (i) $K_2S_2O_8$, H_2O; (ii) $R_1CHClCO_2R_2$, K_2CO_3, DMF/xylene; (iii) AcOH, reflux; (iv) 10% NaOH, reflux.



Scheme 4. Preparation of compound **23**: (i) NaOMe, methyl chloroacetate, MeOH; (ii) *p*-TsOH, benzene; (iii) 2 M NaOH; (iv) mCPBA, CH_2Cl_2; (v) H_2O.

α-hydroxy ester **18**, which was then hydrolyzed, under basic conditions, to give α-hydroxy acid **19** with a 32% combined yield. Subsequent oxidation of the α-hydroxy group to the corresponding keto functionality proved to be problematic. All attempts to effect oxidation, employing a variety of oxidizing reagents, failed to produce the desired α-keto acid **27**; presumably due to the propensity of the alkenylbenzene moiety to undergo side chain oxidation. Thus, a different methodology was applied to synthesize the compound. Epoxidation of α-hydroxy acid **19** with *m*-chloroperoxybenzoic acid in methylene chloride under room temperature generated diastereomeric epoxy alcohols **20** and **21**, respectively. Under acidic conditions, epoxy alcohol **21** with the α-hydrogen atom *anti* to the epoxy oxygen rearranged to the more stable α-keto lactone **22** via a transient intermediate **27**, which then tautomerized to enol **23** in an aqueous solution. The proposed mechanism for the formation of α-keto lactone **22** from epoxy alcohol **21** is depicted in Scheme 5. It started with an acid-catalyzed ring opening of the protonated epoxy alcohol **24** via a hydride shift to α-keto lactone **26**. Subsequent dehydration of **26** yielded the desired 3-phenyl-2-oxo-3-butenoic acid **27**, which was highly accessible to the intramolecular 1,4-addition reaction, giving rise to the cyclized α-keto lactone **22**. Similar intramolecular cyclization reactions have been reported in the literature.[18] Evidence supporting this proposed mechanism is provided by monitoring the epoxidation reaction with proton NMR spectra as shown in Figure 1. Spectrum (a) is the partial 300 MHz H NMR spectrum of **19** after treatment with 0.5 equiv of mCPBA in $CDCl_3$ for 2 h. The three peaks at 5.57, 5.53, and 5.18 ppm are the absorption of the methylene and α-hydrogens of starting material **19**. The doublets at 3.42, 2.86 ppm and 3.28, 2.94 ppm indicate formation of the expected epoxides **20** and **21**, respectively. The two other downfield peaks at 6.65 and 6.20 ppm are the distinct absorption peaks for the methylene hydrogens of compound **27**, which is apparently generated from **21**. Spectrum (b) shown in Figure 1 is the partial H NMR spectrum of **19** after treatment with 0.5 equiv of mCPBA in $CDCl_3$ for 12 h. While the intensity of the absorption peaks at 3.42 and 2.86 ppm remains unchanged, the near disappearance of absorption peaks at 3.28 and 2.94 ppm suggests that most of **21** has rearranged to **27** under these reaction conditions.



Scheme 5. Proposed mechanism for the formation **22** from **21**.

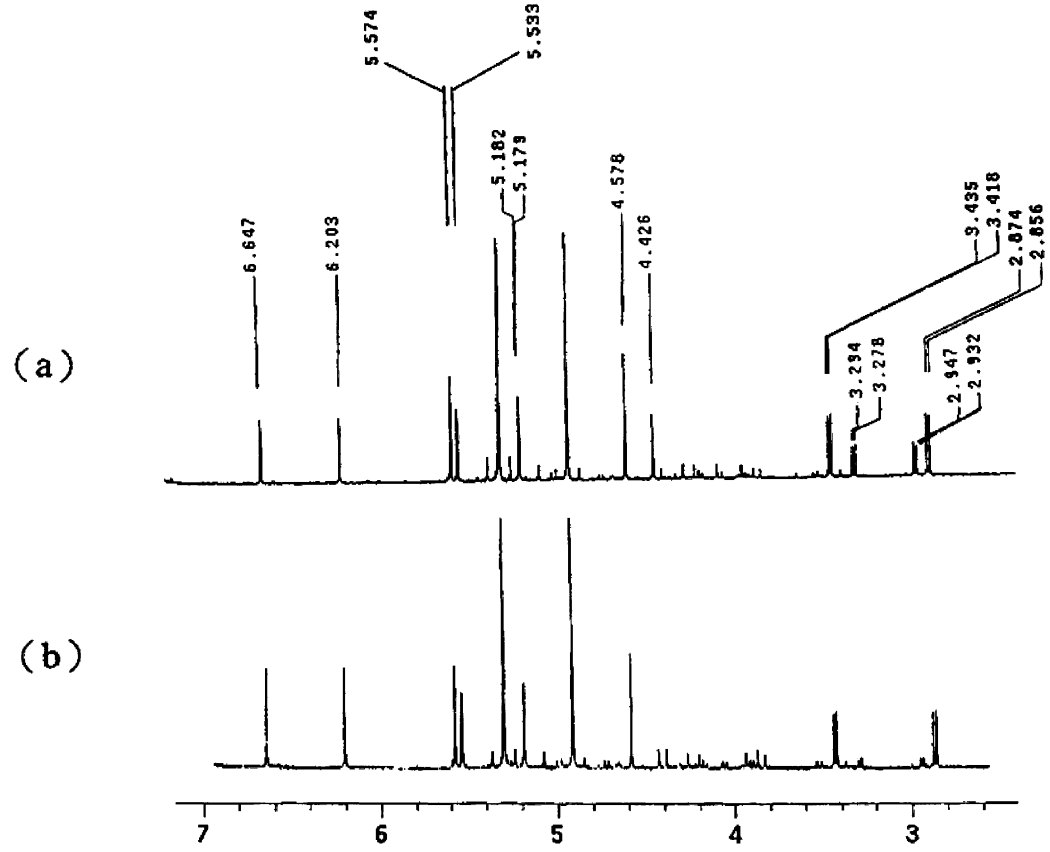


Figure 1. ^{1}H NMR spectra (300 MHz) of **19** after treatment with 0.5 equiv of mCPBA in $CDCl_3$ for (a) 2 h, (b) 12 h. The doublets at 3.42, 2.86 ppm and 3.28, 2.94 ppm in (a) indicate formation of the epoxides **20** and **21**. The decreased intensity at 3.28 and 2.94 ppm and increased intensity at 6.65 and 6.20 ppm in (b) indicated that **21** has rearranged to **27**.

With these potential 4-HPPD inhibitors available, incubation experiments were conducted to determine inhibition parameters of **10**, **11**, **14a–c**, **15a–b**, **17–19** and **23** by the spectrophotometric enol borate method.[19] The inhibition data for the reactions of these compounds with pig liver 4-HPPD are listed in Table 1. Unfortunately, both epoxybenzoquinone ester **11** and benzoquinone ester **10** were found to be poor 4-HPPD inhibitors with IC_{50} of 500 and 150 μM, respectively. This result implied that the presence of the two oxo groups in the ring system is detrimental to the inhibition potency. Further efforts to develop transient arene oxide mimics as potential 4-HPPD inhibitors should try to avoid the benzoquinone functionality. In the case of 4-hydroxyphenoxypropionic acid derivatives, the best synthesized 4-HPP analogue tested was compound **15a** with an IC_{50} of 6 μM. The fact that compound **15b** decreased inhibition activity up to 15-fold less relative to **15a** suggested that this type of 4-HPPD inhibitor appears to have higher functional or structural demands on the α-substituent of the 4-hydroxyphenoxyacetic acid. Additionally, more than a 12-fold decrease in potency by introducing the ester functionality on **14a–b** relative to **15a** indicated that the terminal carboxylate group is crucial for binding, presumably by chelating with ferrous ion in the enzyme active site. For compounds **18–19**, no inhibition activity was observed up to a concentration of 0.9 mM. This result suggests the 2-keto functionality of 4-HPP is crucial for chelating with the enzyme active site iron. Finally, compound **23** was found to be a potent 4-HPPD inhibitor, with an IC_{50} value of 0.5 μM. It is likely that this conformation-restricted substrate analogue possesses a α-hydroxyl lactone functionality which can chelate strongly with the enzyme active site iron. In fact, compound **23** did give a positive ferric chloride test. Thus, compound **23** may share a similar mode of action with the triketone type 4-HPPD inhibitors,[20] although further studies are needed to elucidate this point.

Table 1. Inhibition constants for reactions of **10**, **11**, **14a–c**, **15a–b**, **17–19** and **23** with 4-HPPD from pig liver by the enol borate method

Compd	R_1	R_2	IC_{50} (μM)[a]
10	—	—	150
11	—	—	500
14a	H	Me	223
14b	H	Et	73
14c	Me	Et	588
15a	H	—	6
15b	Me	—	93
17	—	—	120
18	—	—	>900
19	—	—	>900
23	—	—	0.5

[a]Mean of two determinations.

In summary, a postulated reactive intermediate analogue **11** was designed and synthesized as a potential

4-HPPD inhibitor. The biological data demonstrated that epoxybenzoquinone **11** is not a 4-HPPD inhibitor, with an IC_{50} of more than 500 μM. Several 4-hydroxyphenoxypropionic acid and 2-hydroxy-3-phenyl-3-butenoic acid derivatives were also prepared as structure-based 4-HPPD inhibitors. The inhibition results suggest 3-hydroxy-4-phenyl-2(5H)-furanone **23** is the best inhibitor tested, with an IC_{50} value of 0.5 μM. Further investigation toward development of more potent 4-HPPD inhibitors as a new class of bleaching herbicides based on the 3-hydroxy-4-phenyl-2(5H)-furanone template are currently underway in our laboratory.

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