Alternate Substrates and Inhibitors of Bacterial 4-Hydroxyphenylpyruvate Dioxygenase†

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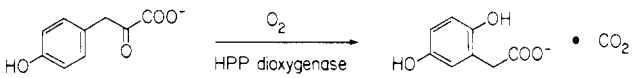
ABSTRACT: A variety of analogues of (4-hydroxyphenyl)pyruvic acid were synthesized, and the reactions of these compounds with the 4-hydroxyphenylpyruvate dioxygenase from *Pseudomonas* sp. P.J. 874 were examined. Several of the ring-substituted substrate analogues are reversible inhibitors of the enzyme, the most potent being the competitive inhibitor (2,6-difluoro-4-hydroxyphenyl)pyruvate (K_i = 1.3 μM). Two substrate analogues (2-fluoro-4-hydroxyphenyl)pyruvate and [(4-hydroxyphenyl)thio]pyruvate proved to be alternate substrates for the enzyme. The former compound is converted to (3-fluoro-2,5-dihydroxyphenyl)acetate in an essentially normal catalytic sequence including oxidative decarboxylation, ring hydroxylation, and side-chain migration. The latter compound, however, undergoes oxidative decarboxylation and sulfoxidation to give [(4-hydroxyphenyl)sulfinyl]acetate; ring oxidation is not observed. The implications of these results with regard to the catalytic mechanism of 4-hydroxyphenylpyruvate dioxygenase are discussed.

A key enzymatic reaction in the tyrosine catabolic pathway is the conversion of (4-hydroxyphenyl)pyruvate (HPP; **1**)[1] to homogentisate (**2**) by 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27). The enzyme has been isolated from avian liver (Wada et al., 1975), mammalian liver (Nakai et al., 1975; Lindblad et al., 1977; Leinberger et al., 1981; Roche et al., 1982) and from a pseudomonad (Lindstedt et al., 1977), and most of these purified proteins have been shown to contain non-heme iron as a cofactor essential for catalytic activity. The reaction carried out by HPP dioxygenase is thought to be



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related to the processes catalyzed by the α-ketoglutarate-dependent dioxygenases (Abbott & Udenfriend, 1974), and while several possible mechanisms have been discussed for this reaction (Goodwin & Witkop, 1957; Hamilton, 1971; Jefford & Cadby, 1981a), mechanistic studies have been relatively few. However, any proposal must account for the incorporation of one atom each from molecular oxygen into the newly formed carboxyl and hydroxyl groups of the product homogentisate (Lindblad et al., 1970). Thorough steady-state kinetic studies of the enzymes from human liver and *Pseudomonas* have indicated that the binding of substrates is ordered with oxygen as the second substrate (Rundgren, 1977, 1983). Furthermore, the side-chain migration is known to proceed with retention of configuration at the β-carbon (Leinberger et al., 1981), and participation of the enol form of HPP now appears to be ruled out (Rundgren, 1982). Nevertheless, none of the proposed enzyme-bound intermediates in the reaction have been observed or trapped, and no enzymatic processing of synthetic samples of putative intermediates has been detected (Nakai et al., 1975; Jefford & Cadby, 1981b). Thus, the sequence of events in the enzyme active site leading to decarboxylation, double oxygenation, and rearrangement of HPP remains unclear.

In this paper, we report studies of the reactions of various analogues of HPP with the HPP dioxygenase from *Pseudomonas* sp. P.J. 874 in our initial effort to elucidate the pathway for this interesting enzymatic transformation.

Materials and Methods

General. Unless otherwise stated, all solvents, organic chemicals, and inorganic chemicals were of analytical reagent grade or the highest purity commercially available. Melting points were recorded on an Electrothermal apparatus and are uncorrected. Ultraviolet–visible spectroscopy was performed on Hewlett-Packard 8450A or Pye-Unicam 6-550 spectrophotometers. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on IBM NR-80 and Bruker WM-250 Fourier-transform spectrometers operating at 80 and 250 MHz, respectively. ^{13}C NMR spectra were obtained on the latter instrument operating at 62.9 MHz. Chemical shifts are reported as parts per million downfield (δ) from tetramethylsilane (Me_4Si) or sodium 4,4-dimethyl-4-silapentanesulfonate (DSS). ^{19}F NMR spectra were recorded on a Varian XL-100 spectrometer operating at 94.1 MHz. Chemical shifts are reported as ppm downfield from trichlorofluoromethane. Gas–liquid chromatography (GC) was carried out on a Hewlett-Packard 402 unit equipped with flame ionization detectors. The columns (6 ft × $^1/_4$ in.) were packed with 20% QF-1 on Gas Chrom Q. Trimethylsilyl ether/ester derivatives of organic acids were prepared by treating the compound with an excess of a reagent consisting of hexamethyldisilazane, trimethylchlorosilane, and pyridine (13:8:10 v/v) at room temperature for 10 min. Coupled gas–liquid chromatography–mass spectrometry (GC–MS) was performed on a Hewlett-Packard 5992 system. Low- and high-resolution mass spectra of solid samples were obtained on an AEI MS9 mass spectrometer. All mass spectra were recorded by using an electron beam energy of 70 eV. High performance liquid chromatography (HPLC) was carried out on μBondapak C_{18} columns (20 × 0.39 cm) using isocratic solvent systems on a

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[1] Abbreviations: HPP, (4-hydroxyphenyl)pyruvic acid; DCIP, 2,6-dichloroindophenol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; 2-fluoro-HPP, (2-fluoro-4-hydroxyphenyl)pyruvic acid; 2,6-difluoro-HPP, (2,6-difluoro-4-hydroxyphenyl)pyruvic acid; 2-chloro-HPP, (2-chloro-4-hydroxyphenyl)pyruvic acid; 2-methyl-HPP, (2-methyl-4-hydroxyphenyl)pyruvic acid; THF, tetrahydrofuran.

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Waters Associates chromatograph.

Enzymes and Assays. Catalase (bovine liver), D-amino acid oxidase (porcine kidney), and L-amino acid oxidase (*Crotalus adamanteus* venom) were purchased from Sigma Chemical Co. HPP dioxygenase was purified from *Pseudomonas* sp. P.J. 874 by the method of Lindstedt et al. (1977). Routine assay of the enzyme utilized the spectrophotometric enol borate method of Lindstedt & Rundgren (1982). The assay mixtures contained potassium phosphate (0.8 M), sodium borate (0.4 M), Tris-HCl (0.2 M), ascorbate (25 mM), catalase (0.1 mg/mL), HPP, and HPP dioxygenase at pH 7.5. The reaction was followed by the decrease in absorbance at 308 nm, the absorption maximum for the enol form of HPP (ϵ = 7100 M^{-1} cm^{-1}). Assays were conducted at either 30 or 37 °C. The specific activity of the HPP dioxygenase used in these studies was 2.8 μmol min^{-1} mg^{-1} at 37 °C. A somewhat different reaction mixture was used for the testing of various α-keto acids as substrates for HPP dioxygenase. A typical reaction contained Tris-HCl (0.1 M, pH 7.5), reduced glutathione (10 mM), 2,6-dichloroindophenol (DCIP; 0.1 mM), catalase (0.2 mg/mL), HPP dioxygenase (0.1 mg/mL), and the potential substrate (0.5 mM). Incubations were conducted for 1 h at 25 °C. Products were isolated by passage of the reaction mixture through a short column of Dowex-50 (H^+), saturation of the effluent with sodium chloride, and extraction with ethyl acetate. The organic extract was dried over sodium sulfate, the solvent was evaporated, and the residues were taken up in methanol for analysis or fractionation by HPLC (Waters μBondapak C_{18}; water–methanol–formic acid, 70:30:1, or a similar solvent system).

Synthetic Precursors and Chromatographic Standards. 2-Fluoro-DL-tyrosine (McCord et al., 1975), 2-chloro-DL-tyrosine (McCord et al., 1975), 2-Methyl-DL-tyrosine (Jen & Miu, 1965), DL-2-amino-4-(4-hydroxyphenyl)butanoic acid (Evans & Walker, 1947), [(4-hydroxyphenyl)thio]acetic acid (Cisney, 1973), and [(4-hydroxyphenyl)sulfinyl]acetic acid (Shibuya et al., 1979) were prepared by slight modifications of the literature procedures. The synthesis of 2,6-difluoro-DL-tyrosine has been described previously (Pascal & Chen, 1984).

(2-Fluoro-4-hydroxyphenyl)pyruvic Acid (**3**).[2] 2-Fluoro-DL-tyrosine (40 mg, 0.20 mmol) was dissolved in Tris-HCl buffer (pH 8, 10 mL). D-Amino acid oxidase (0.5 mg), L-amino acid oxidase (0.3 mg), and catalase (0.2 mg) were added, and the solution was stirred at 24 °C for 9 h. The reaction mixture was acidified, saturated with sodium chloride, and extracted 4 times with ether. The combined extracts were dried over sodium sulfate and concentrated to dryness under a stream of nitrogen. The residue was crystallized from benzene to give 2-fluoro-HPP (**3**; 20 mg, 50% yield): mp 181–183 °C dec; ^{1}H NMR (acetone-d_6) δ 4.16 (s, 2 H, CH_2), 6.60 (dd, 1 H, J = 11.5, 2.5 Hz, C-3′-H), 6.65 (dd, 1 H, J = 8, 2.5 Hz, C-5′-H), 7.12 (dd, 1 H, J = 8, 8 Hz, C-6′-H); ^{19}F NMR (acetone-d_6) δ −117.0 (dd, J = 11.5, 8 Hz, C-2′-F); mass spectrum, m/z 198 (M^+, 10), 125 (M − $COCO_2H$, 100). Exact mass: Calcd for $C_9H_7FO_4$: 198.0328. Found: 198.0326. GC analysis (20% QF-1, 200 °C) of the trimethylsilyl ether/ester derivative indicated a purity of 96%. This derivative showed the following prominent ions upon analysis by GC–MS: m/z 414 (M^+, 24), 399 (M − CH_3, 100).

(2,6-Difluoro-4-hydroxyphenyl)pyruvic Acid (**4**). 2,6-Difluoro-DL-tyrosine (40 mg, 0.18 mmol) was oxidized enzymatically as described above to give 2,6-difluoro-HPP (**4**; 22.5 mg, 56% yield): mp 137–139 °C; ^{1}H NMR (acetone-d_6) δ 4.19 (t, 2 H, J = 1 Hz, CH_2), 6.49 (m, 2 H, C-3′,5′-H_2); mass spectrum, m/z 216 (M^+, 35), 143 (M − $COCO_2H$, 100). Exact mass: Calcd for $C_9H_6F_2O_4$: 216.0234. Found: 216.0236. GC analysis (20% QF-1, 190 °C) of the trimethylsilyl ether/ester derivative indicated a purity of 94%. This derivative showed the following prominent ions upon analysis by GC–MS: m/z 432 (M^+, 14), 417 (M − CH_3, 100), 269 (M − Me_3Si − Me_3SiOH, 44).

(2-Chloro-4-hydroxyphenyl)pyruvic Acid (**5**). 2-Chloro-DL-tyrosine (43 mg, 0.20 mmol) was enzymatically oxidized as described above to give 2-chloro-HPP (**5**; 17 mg, 40% yield): mp 134–138 °C dec; ^{1}H NMR (acetone-d_6) δ 4.28 (s, 2 H, CH_2), 6.79 (dd, 1 H, J = 8, 2 Hz, C-5′-H), 6.92 (d, 1 H, J = 2 Hz, C-3′-H), 7.19 (d, 1 H, J = 8 Hz, C-6′-H); mass spectrum, m/z 214 (M^+, 14), 141 (M − $COCO_2H$, 100). Exact mass: Calcd for $C_9H_7ClO_4$: 214.0033. Found: 214.0028. GC analysis (200 °C) of the trimethylsilyl ether/ester derivative indicated a purity of 93%. This derivative showed the following prominent ions upon analysis by GC–MS: m/z 430 (M^+, 25), 415 (M − CH_3, 100).

(2-Methyl-4-hydroxyphenyl)pyruvic Acid (**6**). 2-Methyl-DL-tyrosine (49 mg, 0.25 mmol) was enzymatically oxidized as described above to give 2-methyl-HPP (**6**; 12 mg, 25% yield): mp 103–104 °C dec; ^{1}H NMR (acetone-d_6) δ 2.16 (s, 3 H, CH_3), 4.11 (s, 2 H, CH_2), 6.62 (dd, 1 H, J = 8, 2.5 Hz, C-5′-H), 6.69 (d, 1 H, J = Hz, 2.5 C-3′-H), 6.99 (d, 1 H, J = 8 Hz, C-6′-H); mass spectrum, m/z 194 (M^+, 21), 121 (M − $COCO_2H$, 100). Exact mass: Calcd for $C_{10}H_{10}O_4$: 194.0579. Found: 194.0574. GC analysis (190 °C) of the trimethylsilyl ether/ester derivative indicated a purity of 98%. This derivative showed the following prominent ions upon analysis by GC–MS: m/z 410 (M^+, 43), 395 (M − CH_3, 100).

2-Oxo-4-(4-hydroxyphenyl)butanoic Acid (**7**). DL-2-Amino-4-(4-hydroxyphenyl)butanoic acid (39 mg, 0.20 mmol) was enzymatically oxidized as described above to give compound **7** (13 mg, 33% yield): mp 142–144 °C; ^{1}H NMR (acetone-d_6) δ 2.83 (t, 2 H, J = 7 Hz, CH_2), 3.16 (t, 2 H, J = 7 Hz, CH_2), 6.74 and 7.07 (AA′BB′ system, 4 H, ring H_4); mass spectrum, m/z 194 (M^+, 14), 149 (M − CO_2H, 9), 107 (M − CH_2COCO_2H, 100). Exact mass: Calcd for $C_{10}H_{10}O_4$: 194.0579. Found: 194.0568. HPLC analysis (70:30:1 water–methanol–formic acid) indicated a purity of 98%.

[(4-Hydroxyphenyl)thio]pyruvic Acid (**8**). Bromopyruvic acid hydrate (334 mg, 2 mmol) was dissolved in 0.4 N NaOH (5 mL). A solution of *p*-hydroxythiophenol (126 mg, 0.1 mmol) in 0.2 N NaOH (5 mL) was added. The resulting solution was left at room temperature overnight under nitrogen. The solution was acidified with HCl, and it was extracted 4 times with ether. After being dried over sodium sulfate, the solvent was evaporated. The residue was crystallized from ether–benzene to give compound **8** (169 mg, 80% yield): mp 135–136 °C; ^{1}H NMR (acetone-d_6) δ 3.94 (s, 2 H, CH_2), 6.81 and 7.31 (AA′BB′ system, 4 H, ring H_4); mass spectrum, m/z 212 (M^+, 70), 139 (M − $COCO_2H$, 100), 126 (M − $CHCO_2H$, 80), 125 (M − CH_2COCO_2H, 52). Exact mass: Calcd for $C_9H_8O_4S$: 212.0143. Found: 212.0144. HPLC analysis (70:30:1 water–methanol–formic acid) indicated a purity of 99%.

4-Hydroxyoxanilic Acid (**9**). A slurry of 4-aminophenol (1.08 g, 10 mmol) in dichloromethane (10 mL) was cooled to 0 °C under a nitrogen atmosphere. Solutions of ethyl oxalyl chloride (2.68 mL, 24 mmol) and pyridine (2.00 mL, 25 mmol) in dichloromethane (5 mL each) were added. After being

[2] The structures of the analogues of HPP used in this study are illustrated in Figure 1.

stirred for 2 h, the reaction mixture was allowed to warm to room temperature, and it was washed 3 times with 5 mL of 1 N HCl. The organic phase was shaken with 25 mL of 1 N NaOH for 15 min. The aqueous phase was separated and acidified with 3 N HCl. Collection of the resulting precipitate yielded 4-hydroxyoxanilic acid (0.54 g, 30%): mp >350 °C; ^{1}H NMR (D_2O) δ 6.91 and 7.36 (AA'BB' system, 4 H, ring H_4); ^{13}C{^{1}H} NMR (D_2O) δ 115.7, 123.9, 128.9, 153.3, 163.2, 166.1; mass spectrum, m/z 182 (M^+, 15), 137 (M – CO_2, 10), 109 (M – $COCO_2$, 100); IR ν_{max} 1645, 1673 cm^{-1}. Anal. Calcd for $C_8H_7NO_4$: C, 53.04; H, 3.90; N, 7.73. Found: C, 53.12; H, 3.95; N, 7.73.

2-(Bromomethyl)-2-hydroxy-3-(4-hydroxyphenyl)propionic Acid (**10**). A solution of (4-methoxybenzyl)magnesium chloride in THF (30 mL) was prepared from 4-methoxybenzyl chloride (4.69 g, 30 mmol) and magnesium (1.46 g, 60 mmol). The Grignard reagent was added to a solution of ethyl bromopyruvate (5.89 g, 30 mmol) in THF (20 mL) at −78 °C. After 30 min the reaction was warmed to room temperature and stirred for 5 h. At this time 1 N HCl was added, and the mixture was extracted twice with ether. The combined extracts were washed successively with aqueous sodium bicarbonate and brine, and they were dried over magnesium sulfate. Evaporation of the solvent gave 8.15 g of ethyl 2-(bromomethyl)-2-hydroxy-3-(4-methoxyphenyl)propionate as a yellow oil. A portion of this material (3.0 g) was heated at reflux in a 1:1 (v/v) solution of 48% aqueous HBr and glacial acetic acid for 5 h. The solution was concentrated under vacuum, water was added, and the product was extracted with ether. The extracts were concentrated to dryness, and the resulting residue was triturated with chloroform to give compound **10** as a light brown powder (1.52 g): mp 185–186.5 °C; ^{1}H NMR (acetone-d_6) δ 2.95 and 3.06 (AB quartet, 2 H, J = 14 Hz, $ArCH_2$), 3.56 and 3.86 (AB quartet, 2 H, J = 10 Hz, CH_2Br), 6.88 and 6.99 (AA'BB' system, 4 H, ring H_4); ^{13}C NMR (acetone-d_6) δ 40.0 (t), 43.2 (t), 78.1 (s), 115.6 (d), 126.0 (s), 132.1 (d), 157.0 (s), 174.2 (s); mass spectrum, m/z 276 [M^+ (^{81}Br), 2], 274 [M^+ (^{79}Br), 2), 150 (M – Br – COOH, 10), 107 (M – $CH_2BrCOHCOOH$, 100). Exact mass: Calcd for $C_{10}H_{11}BrO_4$: 275.9821. Found: 275.9806.

2-Hydroxy-2-(hydroxymethyl)-3-(4-hydroxyphenyl)-propionic Acid (**11**). A solution of compound **10** (0.500 g, 0.91 mmol) in 1 N NaOH (50 mL) was heated at reflux for 1.5 h. After the solution was cooled, the aqueous phase was acidified with concentrated HCl and washed 5 times with ether. The aqueous phase was concentrated to dryness, and the product was extracted from the salts with ethanol. The ethanol was evaporated, and the residue was triturated with chloroform to yield compound **11** as a tan powder (148 mg): mp 165–167 °C; ^{1}H NMR (D_2O) δ 2.81 and 3.00 (AB quartet, 2 H, J = 14 Hz, $ArCH_2$), 3.65 and 3.95 (AB quartet, 2 H, J = 12 Hz, CH_2O), 6.84 and 7.14 (AA'BB' system, 4 H, ring H_4); ^{13}C NMR (D_2O) δ 39.7 (t), 66.7 (t), 79.5 (s), 115.5 (d), 127.0 (s), 131.4 (d), 154.3 (s), 176.7 (s); mass spectrum, m/z 212 (M^+, 6), 107 (M – $CH_2OHCOHCOOH$, 100). Exact mass: Calcd for $C_{10}H_{12}O_5$: 212.0685. Found: 212.0681.

2,3-Epoxy-2-[(4-hydroxyphenyl)methyl]propanoic Acid (**12**). An aqueous solution of compound **10** (100 mg, 0.36 mmol) and sodium carbonate (220 mg, 1.8 mmol) was stirred at room temperature for 8 h. The reaction mixture was acidified and extracted 4 times with ether. The combined extracts were washed with brine and dried over magnesium sulfate. Evaporation of the solvent gave compound **12** as a white powder (60 mg): mp 102–104 °C dec; ^{1}H NMR (acetone-d_6) δ 2.76 and 3.01 (AB quartet, 2 H, J = 6 Hz, epoxide CH_2), 2.95 and 3.28 (AB quartet, 2 H, J = 15 Hz $ArCH_2$), 6.75 and 7.12 (AA'BB' system, 4 H, ring H_4); mass spectrum, m/z 194 (M^+, 6), 107 (M – $CH_2OCCOOH$, 100). Exact mass: Calcd for $C_{10}H_{10}O_4$: 194.0579. Found: 194.0566.

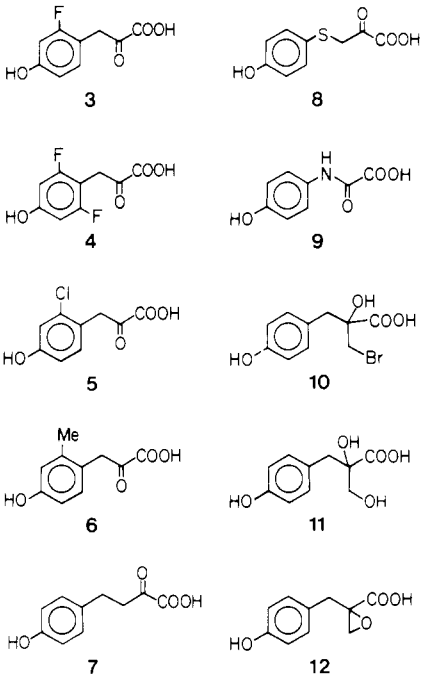


FIGURE 1: Analogues of (4-hydroxyphenyl)pyruvic acid.

RESULTS

Enzymatic Oxidation of 2-Fluoro-HPP (**3**) *to (3-Fluoro-2,5-dihydroxyphenyl)acetic Acid* (**13**). We began our mechanistic studies by examining the effects of rather small perturbations of substrate structure. 2-Fluoro-HPP (Figure 1) was the first analogue examined, and it proved to be a substrate for HPP dioxygenase. Its enzymatic processing was easily observed by a slight modification of the standard enol borate spectrophotometric assay. The absorption spectrum of 2-fluoro-HPP in the borate assay buffer (after 30-min equilibration at 25 °C) shows a maximum at 313 nm with an extinction coefficient of 6900 M^{-1} cm^{-1} (for HPP, λ_{max} = 308 nm, ϵ = 7100 M^{-1} cm^{-1}), so the decrease in absorbance at 313 nm was used to monitor the progress of the enzymatic reaction. At a concentration of 0.1 mM, **3** was consumed at one-tenth the rate of HPP oxidation under the same conditions. V_{max} was estimated to be 0.36 μmol min^{-1} mg^{-1} at 37 °C, and the K_M was 24 μM (see Table I). Substrate inhibition was observed at concentrations greater than 0.1 mM, but no time-dependent inactivation of HPP dioxygenase by **3** was detected.

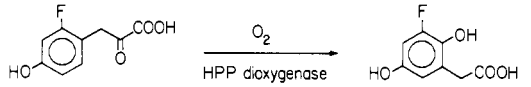
The following "large-scale" reaction was carried out to obtain sufficient product for spectroscopic characterization. 2-Fluoro-HPP (1.0 mg), DCIP (0.2 mg), reduced glutathione (18 mg), catalase (1 mg), and HPP dioxygenase (1.1 mg) were incubated in 10 mL of 200 mM Tris-HCl buffer (pH 7.5) at 24 °C for 60 min. The reaction mixture was passed through a 10-mL column of Dowex-50 (H^+), and it was saturated with sodium chloride. The solution was extracted 5 times with ethyl acetate, the combined extracts were dried over sodium sulfate, and the solvent was evaporated under a stream of nitrogen. A control incubation containing all components except HPP dioxygenase was treated identically. The concentrated organic extracts were first examined by proton NMR. The extract

Table I: Kinetic Constants for Reactions of Substrate Analogues with HPP Dioxygenase

alternate substrates	V_{max} (units/mg)	K_M (μM)
2-fluoro-HPP (**3**)	0.36[a]	24
[(4-hydroxyphenyl)thio]pyruvic acid (**8**)	0.082[b]	140
inhibitors	**type[c]**	**K_{is} (μM)**	**K_{ii} (μM)**
2,6-difluoro-HPP (**4**)	C	1.3	
2-chloro-HPP (**5**)	NC	4	10
2-methyl-HPP (**6**)	NC	9	9
2-oxo-4-(4-hydroxyphenyl)butanoic acid (**7**)	NC	300	500
4-hydroxyoxanilic acid (**9**)	C	6	
2,3-epoxy-2-[(4-hydroxyphenyl)methyl]-propanoic acid (**12**)	NC	540	2500

[a] Assays conducted at 37 °C. [b] Assays conducted at 24 °C. [c] C = competitive inhibitor; NC = noncompetitive inhibitor.

of the control incubation contained unreacted **3**, but the spectrum of the "live" incubation extract indicated that approximately 80% of the substrate had been converted to an as yet unidentified product. The product was purified by HPLC (μBondapak-C_{18}; 70:30:1 water–methanol–formic acid), and it was subjected to analysis by mass spectrometry and ^1H and ^{19}F NMR spectroscopy. The mass spectrum of the trimethylsilyl derivative of the product showed a molecular ion at m/z 402 and a fragmentation pattern consistent with that expected for a tris(trimethylsilyl) ether/ester derivative of a (dihydroxyfluorophenyl)acetic acid. The ^1H and ^{19}F NMR spectra of the pure underivatized product are illustrated and analyzed in Figure 2. The ABX spin system containing two protons and a fluorine clearly defines the substitution pattern of the aromatic ring. The singlet at δ 3.56 is due to the methylene group of the side chain. Thus, we conclude that the product is (3-fluoro-2,5-dihydroxyphenyl)acetic acid (**13**).



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We were unable to detect any other products formed by enzymatic oxidation of compound **3**, but both the control and live incubations contained a small amount of (2-fluoro-4-hydroxyphenyl)acetate produced by nonenzymatic oxidation and decarboxylation of **3**. In summary, compound **3** appears to be a slow but normal substrate for HPP dioxygenase; in this reaction the side-chain migration occurs only to the side of the ring opposite the fluorine substituent, and the product is a fluorinated derivative of homogentisate.

Inhibition of HPP Dioxygenase by Ortho-Substituted Analogues of HPP. 2,6-Difluoro-HPP (**4**), 2-chloro-HPP (**5**), and 2-methyl-HPP (**6**) were prepared as possible alternate substrates for HPP dioxygenase. No products were detected after incubation of these compounds with HPP dioxygenase as described above for 2-fluoro-HPP. However, all of these compounds proved to be potent reversible inhibitors of the enzyme. The most effective was 2,6-difluoro-HPP, a competitive inhibitor with a K_i of 1.3 μM. The results of these inhibition studies are summarized in Table I.

Inhibition of HPP Dioxygenase by 4-Hydroxyoxanilic Acid (**9**). Compound **9** is isosteric with HPP, but it contains a relatively unreactive oxamide group in the side chain rather than an α-keto acid moiety. No oxidation of **9** was observed upon incubation with HPP dioxygenase; however, it was a powerful competitive inhibitor with a K_i of 6 μM.

Enzymatic Oxidation of [(4-Hydroxyphenyl)thio]pyruvate (**8**) *to [(4-Hydroxyphenyl)sulfinyl]acetate* (**14**). Compound **8** (0.5 mg), DCIP (0.2 mg), reduced glutathione (18 mg),

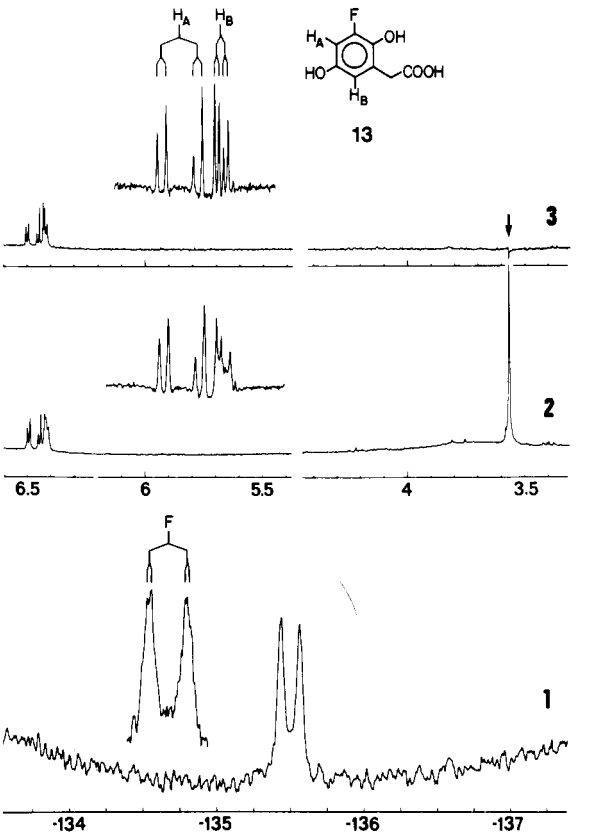
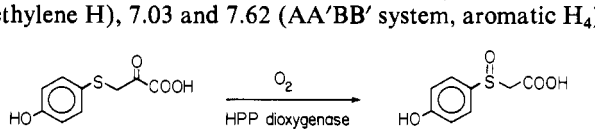


FIGURE 2: NMR spectra (4:1 $CDCl_3$–acetone-d_6) of the purified product of the reaction of 2-fluoro-HPP with HPP dioxygenase. Spectrum 1: ^{19}F NMR (94.1 MHz). Spectrum 2: ^1H NMR (250 MHz). Spectrum 3: ^1H NMR spectrum recorded while irradiating the methylene proton resonance at 3.56 ppm. The magnitudes of the observed coupling constants are J_{AB} = 2.8 Hz, J_{AF} = 11.5 Hz, and J_{BF} = 1.5 Hz.

catalase (1 mg), and HPP dioxygenase (1.1 mg) were incubated in 10 mL of 200 mM Tris–HCl buffer (pH 7.5) at 24 °C for 60 min. The reaction mixture was passed through a 10-mL column of Dowex-50 (H^+), and it was saturated with sodium chloride. The solution was extracted 5 times with ethyl acetate, the combined extracts were dried over sodium sulfate, and the solvent was evaporated under a stream of nitrogen. A control incubation containing all components except HPP dioxygenase was treated identically. The concentrated organic extracts were taken up in 0.5 mL of methanol and analyzed by HPLC. The chromatographic results are shown in Figure 3. The extract of the control incubation (chromatogram 1) contains only unreacted **8** and a trace of [(4-hydroxyphenyl)thio]acetic acid formed by nonenzymatic decarboxylation of **8**. The extract of the incubation which included HPP dioxygenase (chromatogram 2) contains an additional major peak corresponding in mobility to an authentic standard of [(4-hydroxyphenyl)sulfinyl]acetic acid (**14**; see chromatogram 3). Coinjection of the enzymic product with authentic **14** in several solvent systems yielded single peaks in each case. Furthermore, the ^1H NMR spectrum (in acetone-d_6) of the organic extract displayed the following signals (in addition to those due to unreacted **8**) which correspond exactly to those in the spectrum of the synthetic sample of compound **14**: δ 3.78 (d, J = 14 Hz, methylene H), 3.84 (*d*, J = 14 Hz, methylene H), 7.03 and 7.62 (AA'BB' system, aromatic H_4).



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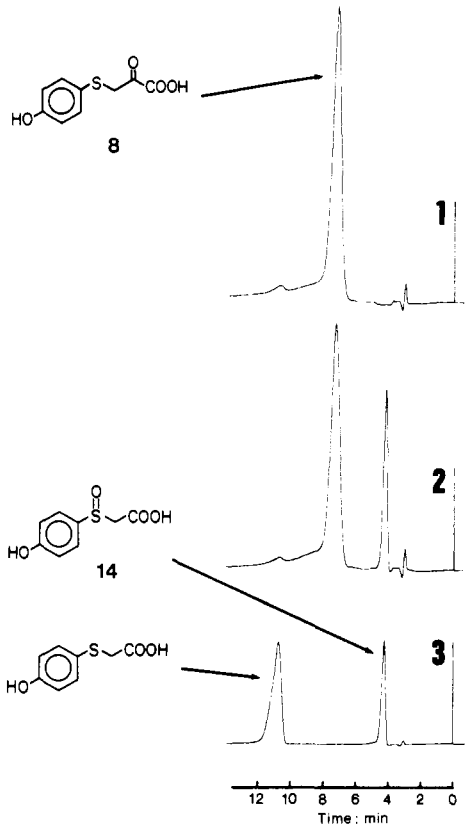


FIGURE 3: HPLC analysis of the reaction of [(4-hydroxyphenyl)-thio]pyruvate (**8**) with HPP dioxygenase. Chromatogram 1: Extract of an incubation of **8** with all reaction components except HPP dioxygenase. Chromatogram 2: Extract of an incubation of **8** with a complete reaction mixture. Chromatogram 3: Synthetic samples of [(4-hydroxyphenyl)thio]acetic acid and [(4-hydroxyphenyl)-sulfinyl]acetic acid (**11**). Reaction conditions and isolation procedures are described under Results. The analyses were carried out on a Waters μBondapak C_{18} column using water–methanol–formic acid (70:30:1) as the eluting solvent at 1.0 mL/min; detection was at 254 nm.

Compound **8** does not give a satisfactory chromophore in the borate-containing buffer to permit a continuous assay of its enzymic oxidation, so K_M and V_{max} were estimated by using a discontinuous assay method. Compound **8** (at various concentrations) was incubated with HPP dioxygenase as described above for periods of 5–30 min. The product, [(4-hydroxyphenyl)sulfinyl]acetic acid, was isolated as before, and it was quantified by HPLC using standard curves generated with the synthetic material. The assay was linear for 15–20 min at 24 °C. V_{max} was found to be 0.082 μmol min^{-1} mg^{-1} at 24 °C, and the K_M was approximately 140 μM.

Attempted Active Site Labeling of HPP Dioxygenase. Substrate analogues **10** and **12**, which contain a bromomethyl group and an epoxide, respectively, in place of the ketone of HPP, were prepared as possible active site labeling agents. Displacement of the bromine or opening of the epoxide by an active site nucleophile would lead to covalent modification and presumably inactivation of HPP deoxygenase. However, in tests for time-dependent irreversible inactivation of HPP dioxygenase by compounds **10** and **12**, incubations of the analogues at concentrations of up to 10 mM with the enzyme for up to 30 min failed to result in any inactivation of the enzyme relative to control experiments. Compound **12** proved to be a noncompetitive inhibitor of HPP dioxygenase (see Table I), and so it probably binds in the enzyme active site; compound **10** has no effect on enzyme activity whatsoever. Trihydroxy analogue **11** was prepared as a possible active site directed iron-chelating agent, but it had no effect on enzyme activity at concentrations as high as 10 mM.

Discussion

At the outset of this investigation, three compounds were known to be alternate substrates of HPP dioxygenase: phenylpyruvate (Tanaguchi et al., 1964), (4-fluorophenyl)pyruvate (Tanaguchi et al., 1964), and 3,4-dihydroxyphenyl)pyruvate (Fellman et al., 1972). However, these molecules contained structural modifications only in places remote from the α-keto acid moiety and the site of ring hydroxylation. We hoped to obtain information concerning the mechanism of HPP dioxygenase by examining the reactions of the enzyme with analogues of HPP containing additional functionality near the sites of enzymatic attack.

The substitution of hydrogen by fluorine in organic molecules introduces few steric demands, but the high electronegativity of fluorine and the strength of the C–F bond may produce large changes in reactivity. 2-Fluoro-HPP (**3**) was an interesting choice for an initial investigation. The presence of the electron-withdrawing group on the aromatic ring should reduce its susceptibility to oxygenation, and the placement of this group ortho to the keto acid side chain might interfere with the side-chain migration, perhaps leading to the accumulation of reaction intermediates. In fact 2-fluoro-HPP is smoothly converted to fluorinated homogentisate (**13**) by HPP dioxygenase at about 10% of the rate observed for the natural substrate HPP. The product was characterized by ^1H and ^{19}F NMR spectroscopy and by mass spectrometry of its tris(trimethylsilyl) ether/ester derivative. Inasmuch as compound **13** appears to be the only product of this reaction, oxygenation and side-chain migration are directed away from the fluorine atom. It is not clear whether this regiospecificity results from the preferred binding of **3** in a particular orientation or from an electronic perturbation by the fluorine.

2,6-Difluoro-HPP (**4**) was prepared in order to leave no choice concerning the direction of side-chain migration—fluorine would be encountered in either direction. Unfortunately, no product was detected upon incubation of **4** with HPP dioxygenase. Compound **4** is, however, an extremely potent competitive inhibitor of bacterial HPP dioxygenase; the K_i of 1.3 μM is more than 2 orders of magnitude lower than the inhibition constants for any previously observed competitive inhibitors of this enzyme [see Lindstedt & Rundgren (1982) for a tabulation of many of these inhibitors]. The inhibition by **4** is reversible; there is no time-dependent loss of activity of the enzyme upon preincubation with this compound. We cannot, of course, completely rule out the possibility that this or any other of our inhibitors is a very slow substrate, but rates of turnover as low as 0.1–0.5% of that of HPP should have been detectable, depending on the method of analysis.

Two other ortho-substituted HPP analogues were prepared in connection with this study: 2-chloro-HPP (**5**) and 2-methyl-HPP (**6**). Neither of these compounds is a substrate for the dioxygenase, but both are potent reversible inhibitors. The aromatic ring of 2-methyl-HPP is at least as electron rich as that of HPP itself, so the failure of **6** as a substrate must be ascribable to a steric effect of the methyl group. Indeed, the Taft steric parameters E_s (Unger & Hansch, 1976) for the substituents H, F, Cl, and CH_3 are 0, −0.46, −0.97, and −1.24, respectively. We speculate that the failures of 2-chloro- and 2-methyl-HPP as substrates are due to the higher steric demands of their ring substituents. Perhaps the interaction of these relatively bulky substituents with some active site group results in a rotation of the aromatic ring away from the enzymatic oxygenating agent or, alternatively, results in some

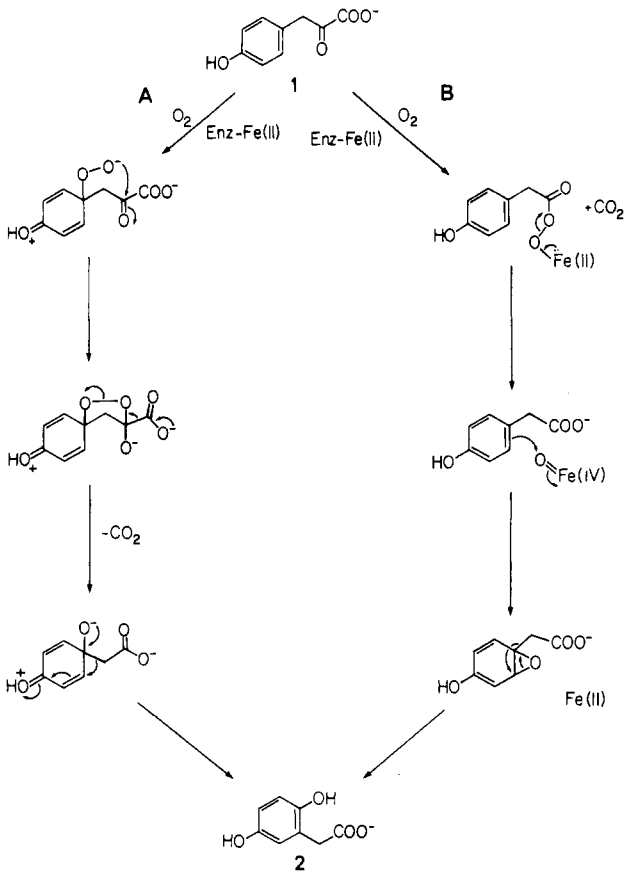


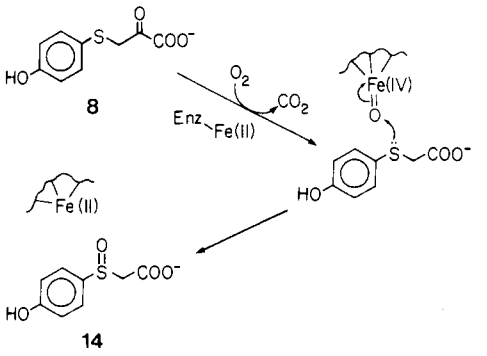
FIGURE 4: Some possible mechanisms for the HPP dioxygenase reaction.

conformational change in the enzyme that prevents catalysis.

Of major importance with regard to the elucidation of the catalytic mechanism of HPP dioxygenase is the determination of the order in which the various functional groups of HPP are attacked. Previously proposed mechanisms for HPP dioxygenase fall into two classes: those which favor initial oxygenation of the aromatic ring and those in which oxidative decarboxylation of the α-keto acid is first. The former class is exemplified by the mechanism due to Goodwin & Witkop (1957) which is illustrated by path A of Figure 4. The crucial first step here is the hydroperoxidation of the aromatic ring. This process is certainly possible for a phenol, but Hamilton (1971) has argued that it would be very difficult for the simple phenyl group of phenylpyruvate to exhibit analogous reactivity. However, the identity of the initially formed oxygenating agent is anything but clear, and it may be sufficiently powerful to carry out such a step. The second class of mechanisms is illustrated in path B of Figure 4. In these schemes, first proposed by Hamilton (1971) and later in modified forms by others (Jefford & Cadby, 1981a,b), the α-keto acid is oxidatively decarboxylated to a peroxy acid which is the ultimate ring-oxygenating agent itself or a precursor of an oxoiron(IV) species (Groves & Van der Puy, 1976) which then attacks the ring. Unfortunately, several groups have incubated (4-hydroxyphenyl)peroxyacetic acid with HPP dioxygenases from various sources, but all have failed to observe conversion of this putative intermediate to normal products [Denum et al. (1982) and Jefford & Cadby (1981b) briefly discuss these unpublished experiments].

It might be possible to distinguish between the two main classes of mechanisms (path A vs. path B) by means of heteroatom-containing substrate analogues which should only react by one of the two pathways. 4-Hydroxyoxanilic acid (**9**) is virtually isosteric with HPP, but the substitution of a nitrogen for the methylene of HPP has two major effects on the chemical reactivity of this analogue. The electron-donating properties the nitrogen atom render the aromatic ring even more electron rich than that of HPP and thus increase its susceptibility to electrophilic attack. At the same time, however, this nitrogen substitution converts the normally electron-deficient α-keto acid moiety into a relatively unreactive oxamide. If the Witkop mechanism (path A) is correct, for which ring oxygenation is the initial step, then one would expect the aromatic ring of compound **9** to be readily oxidized by HPP dioxygenase. On the other hand, if an α-keto acid is required for the generation of the enzyme-bound oxygenating agent, then compound **9** should do no more than bind at the active site of the dioxygenase. In fact the 4-hydroxoxanilic acid is a potent competitive inhibitor of HPP dioxygenase, but we have been unable to detect any oxidation of this compound by the enzyme, a result that is consistent with mechanisms of type B.

Scheme I

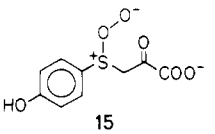


We wished to buttress this argument by finding a good alternate substrate that would be unlikely to react via the Witkop pathway. In the Witkop mechanism the aromatic ring has a very specific function, but in mechanism B the ring has no role in the generation of the oxygenating agent. Indeed, according to the latter mechanism any electron-rich group in the proper orientation should be oxygenated. We wished to add a functional group to HPP that might compete successfully with the aromatic ring for oxygenation. Insertion of a sulfur atom between the phenol and α-keto acid side chain was an obvious initial modification.

[(4-Hydroxyphenyl)thio]pyruvate (**8**) proved to be a good substrate for HPP dioxygenase, undergoing oxidative decarboxylation and sulfoxidation to yield [(4-hydroxyphenyl)-sulfinyl]acetate (*14*) as the only detectable product. Product **14** was characterized by chromatographic and NMR spectroscopic comparison with an authentic standard prepared as previously described (Shibuya et al., 1979). Unfortunately, we have been unable to prepare a satisfactory derivative of **14** for mass spectrometric analysis, so a rigorous demonstration (using oxygen-18 labeling) that the process is a dioxygenation has not yet been achieved. The results are consistent with the reaction path illustrated in Scheme I. Oxidative decarboxylation of the α-keto acid yields a potent oxygenating agent that attacks the nearest electron-rich site, in this case, the thioether. The scheme shows the oxygenating agent as a high-valent oxoiron species, but it might as easily be peracid, since both sorts of reagents can effect sulfoxidation.

Since the aromatic ring of **8** is not oxidized by HPP dioxygenase, we consider it unlikely that the Witkop mechanism is operating for this substrate. However, a referee has pointed out that if the enzyme can catalyze the conversion of **8** to persulfoxide **15**, then this intermediate might decompose to

give product **14** in a process not unlike the Witkop mechanism. Persulfoxides have not been directly observed, but strong evidence of their existence as intermediates in the photooxidation of sulfides has been presented (Foote & Peters, 1971; Gu et al., 1981, and references cited therein), and significantly, the photooxidation of diethyl sulfide in the presence of α-keto acids resulted in their oxidative decarboxylation (Ando et al., 1982). To our knowledge, however, persulfoxides have been implicated as intermediates only in processes involving singlet oxygen, but inasmuch as the catalytic, thermal generation of singlet oxygen by metalloenzymes is itself problematic (Jefford & Cadby, 1981b), the mechanism for the possible enzymatic formation of **15** is left unclear.



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With regard to other α-keto acid dioxygenases, to which HPP dioxygenase is often considered to be closely related, it is noteworthy that mechanisms requiring initial oxidative attack on the α-keto acid generally seem to have received greater experimental support. Most dioxygenases of this class, unlike HPP dioxygenase, utilize α-ketoglutarate as the source of the α-keto acid moiety in reactions where the methylene group of a cosubstrate is hydroxylated. The uncoupling of α-ketoglutarate oxidation from methylene hydroxylation has been observed for prolyl 4-hydroxylase (Tuderman et al., 1977; Counts et al., 1978; Rao & Adams, 1978), lysyl hydroxylase (Puistola et al., 1980), thymine 7-hydroxylase (Holme et al., 1979), and γ-butyrobetaine hydroxylase (Holme et al., 1982) which would seem to favor the path B mechanisms. However, incubations of peroxysuccinic acid with prolyl 4-hydroxylase and thymidine 2′-hydroxylase (Abbott & Udenfriend, 1974) have failed to result in the hydroxylation of the cosubstrate. More compelling are the studies of hydrogen kinetic isotope effects in the γ-butrobetaine dioxygenase reaction (Blanchard & Englard, 1983). These data indicate that a change in hybridization accompanies the methylene C–H bond cleavage, a result consistent with the presence of a radical intermediate such as that which might be formed by the abstraction of a hydrogen atom by an oxoiron active site reagent as discussed by Siegel (1979).

In conclusion, we have described two new alternate substrates for bacterial HPP dioxygenase: 2-fluoro-HPP (**3**) and [(4-hydroxyphenyl)thio]pyruvate (**8**). A normal catalytic cycle—including oxidative decarboxylation, ring oxygenation, and side-chain migration—is observed with the former substrate, which yields a fluorinated homogentisate. With compound **8**, however, oxidative decarboxylation and oxidation of the thioether to a sulfoxide occur instead. This is the first alternate substrate described for HPP dioxygenase which does not undergo ring hydroxylation, a result that tends to disfavor mechanisms for HPP dioxygenase which require ring oxygenation as the initial step in catalysis. We have also prepared a number of potent reversible inhibitors of HPP dioxygenase with K_i's in the low micromolar range, the most powerful being 2,6-difluoro-HPP (**4**). In addition to their possible utility in mechanistic studies of HPP dioxygenases from a variety of species, these inhibitors may have a wider potential practical application as herbicides, since HPP dioxygenase appears to be required for the biosynthesis of plastoquinones in higher plants (Weiss & Edwards, 1980).

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Inhibition of Aspartic Proteases by Pepstatin and 3-Methylstatine Derivatives of Pepstatin. Evidence for Collected-Substrate Enzyme Inhibition†

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ABSTRACT: The synthesis of 10 analogues of pepstatin modified so that statine is replaced by 4-amino-3-hydroxy-3,6-dimethylheptanoic acid (Me^3Sta) or 4-amino-3-hydroxy-3-methyl-5-phenylpentanoic acid (Me^3AHPPA) residues is reported. Both the 3*S*,4*S* and 3*R*,4*S* diastereomers of each analogue were tested as inhibitors of the aspartic proteases, porcine pepsin, cathepsin D, and penicillopepsin. In all cases the 3*R*,4*S* diastereomer (rather than the 3*S*,4*S* diastereomer) of the Me^3Sta and Me^3AHPPA derivatives was found to be the more potent inhibitor of the aspartic protease (K_i = 1.5–10 nM for the best inhibitors), in contrast to the results obtained with statine (Sta) or AHPPA derivatives, where the 3*S*,4*S* diastereomer is the more potent inhibitor for each diastereomeric pair of analogues. The Me^3Sta- and Me^3AHPPA-containing analogues are only about 10-fold less potent than the corresponding statine and AHPPA analogues and 100–1000-fold more potent than the corresponding inhibitors lacking the C-3 hydroxyl group. Difference NMR spectroscopy indicates that the (3*R*,4*S*)-Me^3Sta derivative induces conformational changes in porcine pepsin comparable to those induced by the binding of pepstatin and that the (3*S*,4*S*)-Me^3Sta derivatives do not induce the difference NMR spectrum. These results require that the C-3 methylated analogues of statine-containing peptides must inhibit enzymes by a different mechanism than the corresponding statine peptides. It is proposed that pepstatin and (3*S*)-statine-containing peptides inhibit aspartic proteases by a collected-substrate inhibition mechanism. The enzyme–inhibitor complex is stabilized, relative to pepstatin analogues lacking the C-3 hydroxyl groups, by the favorable entropy derived when enzyme-bound water is returned to bulk solvent. The water is displaced from the enzyme active site by the (3*S*)-statine hydroxyl group. The relative potencies of (3*R*)-statine, (3*S*)-Me^3Sta, and (3*R*)-Me^3Sta are rationalized in terms of the mechanism proposed for pepstatin. The relationship of this type of inhibition mechanism to transition-state-analogue inhibitor mechanisms is discussed.

Enzyme inhibitors can be used effectively to elucidate the nature of enzyme active sites when the geometry of the inhibitor closely approximates the pathway geometry of a reaction intermediate for a particular enzymic reaction. Pepstatin [isovalerylvalylvalyl-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoylalanyl-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (Iva-Val-Val-Sta-Ala-Sta) (**1**) (Figure 1)], a tight-binding inhibitor of aspartic proteases discovered by Umezawa et al. (1970), has been shown to bind to the active sites of most aspartic proteases with unusually small dissociation constants (4.57×10^{-11} M in the case of pepsin) (Workman & Burkitt, 1979). The extraordinarily tight binding to this class of proteases led to the hypothesis that pepstatin inhibits by approximating some reaction pathway intermediate, possibly the tetrahedral intermediate for amide bond hydrolysis shown in Figure 2 (Marciniszyn et al., 1976; Marshall, 1976). The relationship between the tetrahedral intermediate and the structure of pepstatin is most clearly evident at the statine residue in the third position of the peptide chain. Pepstatin is postulated to mimic the tetrahedral intermediate by placing the *pro-S* hydroxyl group[1] of statine in a position normally occupied by one of the oxygens of the tetrahedral intermediate produced during amide bond hydrolysis. Experiments with *O*-acetylpepstatin (**2**) and dideoxypepstatin (**3**), both of which are very much weaker inhibitors of pepsin, confirmed the importance of this *pro-S* hydroxyl group, and the latter compound, which is an over 4000-fold weaker inhibitor than pepstatin, established that the

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[1] Abbreviations: Sta, 4-amino-3-hydroxy-6-methylheptanoic acid; Sto, 4-amino-6-methyl-3-oxoheptanoic acid; AHPPA, 4-amino-3-hydroxy-5-phenylpentanoic acid; dSta, 4(*S*)-amino-6-methylheptanoic acid; Iva, isovaleric acid; dideoxypepstatin, Iva-Val-Val-dSta-Ala-dSta; Iaa, isoamylamide; O4MP, 4-(hydroxymethyl)pyridine; Boc, *tert*-butoxycarbonyl; NOE, nuclear Overhauser effect; TLC, thin-layer chromatography. Sta^3 refers to Sta in the third position of the peptide chain in pepstatin; C-3 refers to the third carbon in the backbone of statine or AHPPA. *pro-S* designates a substituent (hydroxyl or methyl) replacing the hydroxyl group in (3*S*,4*S*)-statine; *pro-R* designates a substituent replacing the C-3 proton in (3*S*,4*S*)-statine.

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