Design and Kinetic Characterization of Multisubstrate Inhibitors of Dopamine β-Hydroxylase

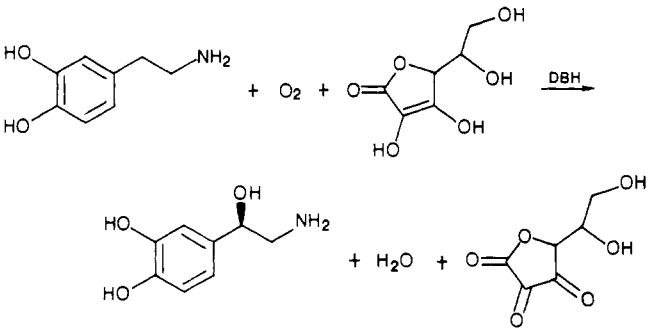
Lawrence I. Kruse,* Walter E. DeWolf, Jr., Pamela A. Chambers, and Paula J. Goodhart

Department of Medicinal Chemistry, Research and Development Division, Smith Kline & French Laboratories, Philadelphia, Pennsylvania 19101

Received May 28, 1986

ABSTRACT: The synthesis and kinetic characterization of a new class of dopamine β-hydroxylase (DBH; EC 1.14.17.1) inhibitor, 1-(4-hydroxybenzyl)imidazole-2-thiol, is reported. These inhibitors, which incorporate a phenethylamine substrate mimic and an oxygen mimic into a single molecule, exhibit both the kinetic properties and the potency ($K_{is} \sim 10^{-9}$ M) expected for a multisubstrate inhibitor and are therefore classified as such. Steady-state kinetic experiments with these multisubstrate inhibitors and their substructural analogues support the recently proposed pH-dependent changes in substrate binding order [Ahn, N., & Klinman, J. P. (1983) *Biochemistry 22*, 3106] and a mechanism whereby the inhibitor binds specifically to the reduced Cu^+ form of enzyme at both the phenethylamine substrate site and the active-site copper atom(s). A Yonetani–Theorell double-inhibition experiment indicates mutually exclusive binding of the inhibitor substructures *p*-cresol and 1-methylimidazole-2-thiol to suggest an extremely short intersite distance between the phenethylamine binding site and the active-site copper atom(s).

Dopamine β-hydroxylase (DBH; EC 1.14.17.1) is a tetrameric, copper-containing, mixed-function oxidase that catalyzes the conversion of dopamine to norepinephrine (Skotland & Ljones, 1979a; Rosenberg & Lovenberg, 1980; Villafranca, 1981; Ljones & Skotland, 1984):



The capacity of DBH to bind and activate diatomic oxygen in a copper atom mediated event (Miller & Klinman, 1985) demonstrates a functional relationship to other copper proteins such as hemocyanin or tyrosinase (Solomon, 1981; Preaux & Gielens, 1984), which, interestingly, possess a binuclear, magnetically coupled copper site. While a 2:1 copper:subunit stoichiometry has been shown to yield maximal catalytic activity for DBH with phenethylamine substrate (Klinman et al., 1984) and k_{cat} inactivators (Ash et al., 1984), the copper atoms appear magnetically noninteractive. Moreover, relative to tyrosinase or hemocyanin, DBH binds the prosthetic copper atoms rather weakly (Skotland & Ljones, 1979b; Klinman et al., 1984). Despite these ambiguities, an activated copper–oxygen complex seems the likely catalytic intermediate. The chemical reactivity of this species, which hydroxylates an unactivated C–H bond, would argue in support of a proximate binding of phenethylamine substrate to yield product rather than single turnover autoxidation of enzyme. A small intersite distance between phenethylamine substrate and the activated metal–oxygen complex, while still tentative for DBH, is now well established for the protein tyrosinase (Wilcox et al., 1985) or the more distantly related family of P-450 enzymes (Poulos et al., 1985). Despite a paucity of direct experimental evidence for a small intersite distance in DBH, we have used this concept to design inhibitors, which, as evidenced by steady-state kinetic analysis, bind DBH in a highly specific fashion and bind DBH approximately 10^6-fold more tightly than substrates. Here we report these inhibitors and the results of detailed steady-state kinetic experiments that suggest a multisubstrate mode of binding of the inhibitors to enzyme and lend support to a small intersite distance.

Experimental Procedures

Materials

Tyramine hydrochloride, L-ascorbic acid, and DL-octopamine were purchased from Sigma. Crystalline catalase (specific activity 65 000 U/mg as claimed by supplier) was from Boehringer. AG 50W-X8 ion exchange resin was obtained from Bio-Rad. *p*-Cresol (**4**), 1-methylimidazole-2-thiol (**3**), 4-methoxybenzaldehyde, and 2,6-difluorophenol were all products of Aldrich. All other chemicals were commercial products of the highest purity available. Homogeneous bovine dopamine β-hydroxylase was isolated from either whole adrenal medulla or partially purified chromaffin vesicles according to our improved procedure.[1] Enzyme from whole medulla (specific activity 22–31 U/mg, octopamine assay, at 37 °C and pH 5.0 with 10 mM ascorbate, 10 mM tyramine, 10 mM fumarate, and 10 μM $CuCl_2$) or DBH from partially purified vesicles (specific activity 42 U/mg, octopamine assay) each gave identical kinetic results.

Chemical Syntheses

3,5-Difluoro-4-hydroxybromobenzene. A solution of 2,6-difluorophenol (143.5 g, 1.10 mol) in DMF (600 mL) was stirred during the slow addition of *N*-bromosuccinimide (196.5 g, 1.10 mol) over 30 min according to the general procedure of Mitchell et al. (1979). The resulting red solution was stirred at room temperature for 12 h and then poured into H_2O (1.5

[1] W. E. DeWolf, Jr., and L. I. Kruse, unpublished results.

0006-2960/86/0425-7271$01.50/0

L). The resulting oil was separated, and the aqueous phase was extracted three times with ethyl acetate. The ethyl acetate extracts were combined with the oil, and the resulting solution was washed three times with water and once with saturated aqueous NaCl and dried over anhydrous Na_2SO_4. The solution was concentrated to yield 228 g (99%) of crude product, which was used without purification: TLC R_f 0.63 with 7:3 hexane–ethyl acetate as eluant; NMR ($CDCl_3$) δ 7.15 (apparent d, J = 8 Hz).

3,5-Difluoro-4-methoxybromobenzene. A 1-L flask was charged with a 50% NaH dispersion in oil (53 g, 1.1 mol), and this dispersion was washed three times with hexane. The hexane-washed NaH was suspended under argon in DMF (1 L), and iodomethane (103 mL, 1.65 mol) was added with stirring. The mixture was stirred in an ice–water bath as the crude 3,5-difluoro-4-hydroxybromobenzene (228 g, 1.1 mol) was added dropwise. After the addition was completed, additional iodomethane (52 mL, 0.825 mol) was added cautiously and the thick mixture was stirred at room temperature overnight. The reaction mixture was poured into H_2O (1.5 L), the oily layer was separated, and the aqueous phase was washed three times with hexane. The combined oily layer and hexane washes were washed sequentially with 3 N NaOH and twice with H_2O and saturated aqueous NaCl and then dried over anhydrous Na_2SO_4. The solution was concentrated, and the residual oil was purified by distillation to yield the product (178 g, 72%) as a colorless oil: bp 80–100 °C (15 torr); TLC R_f 0.4 with hexane as eluant; NMR ($CDCl_3$) δ 3.95 (s, 3 H), 7.15 (apparent d, J = 8 Hz, 2 H).

3,5-Difluoro-4-methoxybenzonitrile. A mixture of 3,5-difluoro-4-methoxybromobenzene (170 g, 0.76 mol) and Cu^ICN (138 g, 1.54 mol) in *N,N*-dimethylacetamide (600 mL) was heated at reflux under argon for 3.5 h and then cautiously poured onto a mixture of ice (1 kg) and ethyl acetate (1 L). The resulting mixture was filtered through diatomaceous earth, the organic layer of the filtrate was separated, and the aqueous layer was extracted twice with ethyl acetate. The combined ethyl acetate layers were washed three times with water and once with saturated aqueous NaCl and then dried with anhydrous Na_2SO_4. The solution was concentrated and the product was recrystallized from heptane to yield the product (109 g, 84%): mp 82–85 °C; IR (Nujol mull) 2240, 1570, 1520 cm^{-1}; NMR ($CDCl_3$) δ 4.06 (t, J = 2 Hz, 3 H), 7.2 (m, 2 H total).

3,5-Difluoro-4-methoxybenzaldehyde. A solution of 3,5-difluoro-4-methoxybenzonitrile (14.1 g, 0.083 mol) and Raney alloy (14.1 g) in 88% formic acid (140 mL) and H_2O (30 mL) was stirred and heated at reflux for 2 h according to the method of van Es and Staskun (1965). The resulting mixture was filtered, the filtrate was extracted twice with hexane, and the combined hexane extracts were washed twice with water and once with saturated aqueous NaCl and then dried over anhydrous Na_2SO_4. The solution was concentrated to yield the product (11.3 g, 78%) as an unstable solid: mp 40–41 °C; TLC R_f 0.4 with 5:1 hexane–ethyl acetate as eluant; IR (Nujol mull) 1710, 1580, 1540 cm^{-1}; NMR ($CDCl_3$) δ 4.2 (t, J = 3 Hz, 3 H), 7.2–7.5 (m, 2 H total), 9.8 (t, J = 2 Hz, 1 H).

1-(3,5-Difluoro-4-methoxybenzyl)imidazole-2-thiol. A mixture of 3,5-difluoro-4-methoxybenzaldehyde (6.8 g, 40 mmol) and aminoacetaldehyde dimethyl acetal (4.2 g, 40 mmol) was heated at 100 °C for 30 min, the mixture was dissolved in ethanol (75 mL), and the resulting solution was stirred at room temperature during the slow (10 min) addition of $NaBH_4$ (1.5 g, 40 mmol). After being stirred overnight at room temperature, the solution was concentrated and the residue was partitioned between water and ethyl acetate. The ethyl acetate layer was washed with water and saturated aqueous NaCl and dried over anhydrous Na_2SO_4. The solution was concentrated to yield 10.65 g (93%) of the intermediate (*N*-(3,5-difluoro-4-methoxybenzyl)amino)acetaldehyde dimethyl acetal as an unstable oil. The crude oil was heated at reflux with KSCN (4.0 g, 37 mmol) in a mixture of ethanol (55 mL), water (92 mL), and 12 N HCl (9.3 mL) for 1 h. The reaction mixture was diluted to a volume of 500 mL with water and cooled, and the white crystalline product was filtered and dried: mp 156–158 °C; TLC R_f 0.2 with 1:1 hexane–ethyl acetate as eluant; IR (Nujol mull) 3300, 1450, 1030 cm^{-1}; NMR (Me_2SO-d_6–$CDCl_3$) δ 3.9 (s, 3 H), 5.2 (s, 2 H), 6.9–7.3 (m, 4 H total). Anal. Calcd for $C_{11}H_{10}N_2F_2SO$: C, 51.56; H, 3.93; N, 10.93. Found: C, 51.61; H, 4.01; N, 10.81.

1-(3,5-Difluoro-4-hydroxybenzyl)imidazole-2-thiol. A mixture of 1-(3,5-difluoro-4-methoxybenzyl)imidazole-2-thiol (2.0 g, 7.8 mmol) in dichloromethane (10 mL) was stirred under argon at room temperature during the dropwise addition of a 1.0 M solution of BBr_3 in dichloromethane (24 mL, 24 mmol). After the resulting solution had been stirred at room temperature for 1.5 h, anhydrous methanol (10 mL) was added cautiously, the mixture was concentrated, and the residue was boiled in anhydrous methanol (25 mL) and concentrated. The residue was purified by flash chromatography on silica gel (Still et al., 1978) with 2:1 ethyl acetate–hexane as eluant to yield 1.23 g (65%) of product: mp 213–215 °C; TLC R_f 0.2 with 1:1 hexane–ethyl acetate as eluant; IR (Nujol mull) 3250, 1560, 1505, 1220, 1050 cm^{-1}; NMR (Me_2SO-d_6-$CDCl_3$) δ 5.2 (s, 2 H), 6.8–7.2 (m, 4 H total). Anal. Calcd for $C_{10}H_8F_2N_2SO$: C, 49.58; H, 3.33; N, 11.56. Found: C, 49.89; H, 3.35; N, 11.52.

1-(4-Hydroxybenzyl)imidazole-2-thiol. The reaction of 4-methoxybenzaldehyde, as for 3,5-difluoro-4-methoxybenzaldehyde, followed by demethylation of the intermediate methyl ether with BBr_3 yielded the product (42%) after recrystallization from ethanol: mp 188 °C; IR (Nujol mull) 1610, 1590 cm^{-1}; NMR (Me_2SO-d_6–$CDCl_3$) δ 5.1 (s, 2 H), 6.6 (s, 2 H), 6.75 (d, J = 9 Hz, 2 H), 7.2 (d, J = 9 Hz, 2 H), 9.0 (s, 1 H). Anal. Calcd for $C_{10}H_{10}N_2SO$: C, 58.23; H, 4.89; N, 13.58. Found: C, 58.48; H, 5.04; N, 13.98.

3,5-Difluoro-4-methoxy-1-(2-nitroethenyl)benzene. A solution of 3,5-difluoro-4-methoxybenzaldehyde (5 g, 29 mmol) and nitromethane (4.5 mL) in acetic acid (20 mL) was heated at 100 °C for 3 h, additional nitromethane (10 mL) was added, and the solution was heated for 1 h and poured into water (100 mL). The oily product was extracted twice with ethyl acetate, and the combined extracts were washed with water, dried over anhydrous $MgSO_4$, and concentrated. The residue was recrystallized from ethanol to yield 2.17 g (35%) of product: IR (Nujol mull) 1580, 1380 cm^{-1}; NMR ($CDCl_3$) δ 4.0 (t, J = 2 Hz, 3 H), 6.8–7.3 (m, 2 H total), 7.6 (d, J = 14 Hz, 1 H), 7.8 (d, J = 14 Hz, 1 H).

(3,5-Difluoro-4-methoxyphenethyl)amine. A mixture of $LiAlH_4$ (1.5 g, 39.5 mmol) in dry diethyl ether (100 mL) was stirred under argon during the dropwise addition of a solution of 3,5-difluoro-4-methoxy-1-(2-nitroethenyl)benzene (2.32 g, 10.8 mmol) in dry diethyl ether (25 mL) and then heated at reflux for 3 h, and allowed to stand overnight at room temperature. The reaction mixture was cooled to 0 °C, quenched by the sequential addition of water (1.5 mL), 10% NaOH solution (1.5 mL), and water (3 mL), and allowed to stir at room temperature until a granular white precipitate formed. The mixture was filtered, the precipitate was washed well with diethyl ether, and the combined filtrates were dried over an-

hydrous $MgSO_4$ and concentrated. The residue was purified by flash chromatography on silica gel first using 91:8.5:0.5 dichloromethane–methanol–15 N NH_4OH as eluant and then eluting with 4:1 dichloromethane–methanol to yield 1.27 g (63%) of product: IR (neat) 3400, 3300, 1580, 1520, 1445 cm^{-1}; NMR ($CDCl_3$) δ 1.2 (br s, 2 H), 2.6 (t, J = 6 Hz, 2 H), 2.9 (t, J = 6 Hz, 2 H), 3.9 (s, 3 H), 6.7 (m, 2 H total).

(3,5-Difluoro-4-hydroxyphenethyl)amine Hydrochloride. A solution of (3,5-difluoro-4-methoxyphenethyl)amine (1.27 g, 6.8 mmol) in dry dichloromethane (50 mL) was cooled to −15 °C under argon and stirred while a solution of BBr_3 in dry dichloromethane (13.8 mmol total BBr_3) was added dropwise. The solution was allowed to warm to room temperature and stir for 1 h and was then cooled to 0 °C and quenched by the cautious addition of methanol (10 mL). The resulting reaction mixture was concentrated, the residue was dissolved in methanol and concentrated, and this process was repeated three times. The residual solid was triturated with acetonitrile to yield the product (1.25 g, 73%) as the hydrobromide salt: mp 286–288 °C. The hydrochloride was prepared as follows. A solution of the hydrobromide salt (0.72 g, 2.87 mmol) in methanol (10 mL) was stirred and treated with silver acetate (0.49 g, 2.95 mmol), and the resulting AgBr was filtered off. The filtrate was concentrated, the residue was dissolved in methanol (5 mL), treated with activated carbon, and then treated with a saturated solution of ethereal hydrogen chloride. The resulting solution was concentrated and the residue was recrystallized from methanol/acetonitrile to yield 0.441 g (74%) of the hydrochloride: mp 270–272 °C; IR (Nujol mull) 1625, 1540 cm^{-1}; NMR (Me_2SO-d_6–$CDCl_3$) δ 3.0 (s, 4 H), 6.8–7.0 (m, 2 H total), 8.0–9.0 (br s, 4 H total). Anal. Calcd for $C_8H_{10}ClF_2NO$: C, 45.84; H, 4.81; N, 6.68. Found: C, 45.95; H, 4.85; N, 6.60.

Enzyme Assays

General Methodology. The activity of DBH was determined at 37 °C either by measuring production of octopamine from tyramine substrate with a periodate cleavage procedure similar to that reported already (Nagatsu & Udenfriend, 1972) or by following the uptake of oxygen substrate with a Yellow Springs Instrument Model 53 oxygen electrode. All reaction mixtures contained 50 mM buffer (acetate, pH 4.5; phosphate, pH 6.6), 10 mM ascorbic acid when this was not the varied substrate, 200 μg/mL crystalline bovine catalase, and sufficient $CuCl_2$ (10 μM, pH 4.5; 5 μM, pH 6.6) to give maximal stimulation of enzyme activity. Ionic strength was adjusted to a constant value of 0.2 by the addition of appropriate amounts of NaCl. When fumarate was present, its concentration was 10 mM. Inhibitors, when present, were included at concentrations approximating 0, 1, 2, and 3 times the K_i. Control experiments were always performed to insure linearity of product formation under all experimental conditions.

Oxygen Electrode Assay. This assay was used when oxygen was the variable substrate. An incubation mixture of approximately 4.0-mL final volume was employed at 37 °C with typical incubation times of 2–3 min or 30 s in the case of *p*-cresol. Tyramine, a nonvaried substrate, was held at a constant 0.5 to 2.0 mM ($\sim K_m$), and ascorbic acid was included at a concentration of 10 mM.

The following experiment is typical. Stock buffer solution (4 mL) containing tyramine, $CuCl_2$, and inhibitor was placed into the oxygen electrode assembly at 37 °C and gassed with the appropriate O_2/N_2 mixture for 3 min. The oxygen electrode plunger was lowered and crystalline catalase suspension (40 μL of 20 mg/mL suspension) was added followed by sodium ascorbate stock solution (20 μL of 2.0 M solution). The oxygen uptake due to ascorbate autoxidation was monitored for 3–5 min, and 2–10 μL of DBH stock solution was added to initiate the reaction. Enzyme velocities were corrected for oxygen consumption due to autoxidation of ascorbate. When *p*-cresol was used as inhibitor, the reaction was stopped by the addition of 3.0 M trichloroacetic acid (0.8 mL) and velocity was determined from the octopamine produced. This procedure allowed for a facile correction for oxygen consumption due to *p*-cresol turnover.

Other Kinetic Assays. With the exception of experiments where oxygen was the varied substrate, all assays were performed at 37 °C in a New Brunswick R-76 shaker/bath oscillating at 120 Hz in air. Assay mixtures were identical with those used for oxygen electrode experiments.

Kinetic Analyses. Inhibitors were typically present in each experiment at concentrations approximating 0, 1, 2, and 3 times the K_i, and a minimum of four and normally five or six substrate concentrations were employed in each experiment. The standard regression analyses of Cleland (1979) were employed to fit the initial velocity data to eq 1–3 for competitive,

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A} \quad (1)$$

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (2)$$

$$v = \frac{VA}{K_a + A(1 + I/K_{ii})} \quad (3)$$

noncompetitive, and uncompetitive patterns, respectively, and the suggested statistical criteria were applied to decide between patterns. For the calculation of apparent substrate dissociation constants from binary and ternary enzyme complexes, eq 4

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (4)$$

was used. In the experiment where induced substrate inhibition was observed, the initial velocity data were fit to eq 5 and the

$$v = \frac{VA}{K_a + A + A^2/K_i} \quad (5)$$

resulting lines were replotted manually. For the double-inhibition experiment, initial velocity data were fit to eq 6 where

$$v = \frac{VA}{K_a[1 + I/K_i + J/K_j + IJ/(\alpha K_iK_j)] + A} \quad (6)$$

I and J represent the concentrations of the two inhibitors which are competitive with tyramine substrate A. As expressed in eq 6, α is the term that defines the degree of interaction in binding of I and J. When it was necessary to manipulate inhibitor or substrate dissociation constants, standard errors were propagated through the appropriate calculations.

Results

Chemical Synthesis and Physical Properties of Inhibitors and 3,5-Difluorotyramine Substrate. Inhibitors **1** and **2** (Figure 1) were synthesized from the corresponding benzaldehyde by standard procedures (Figure 2). These compounds proved to be stable, well-characterized, highly crystalline compounds, which showed no tendency to undergo oxidation of the imidazole-2-thiol to the disulfide. Although inhibitors **1–3** are expected to exist in solution as an equilibrium mixture of thiol and thione tautomeric forms, the thione tautomer appears to be the exclusive form (>95%) in Me_2SO-d_6–$CDCl_3$ solution as evidenced by NMR chemical shift values for the imidazole 4-H and 5-H protons relative

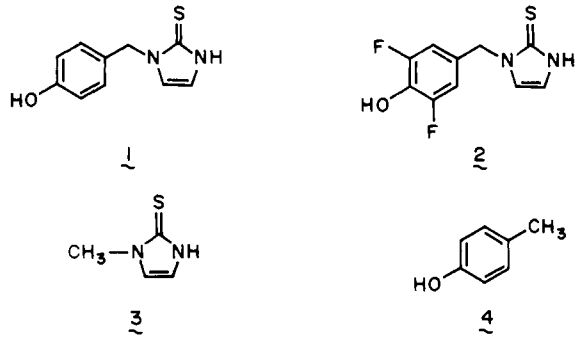


FIGURE 1: Structures of DBH inhibitors **1**–**4**.

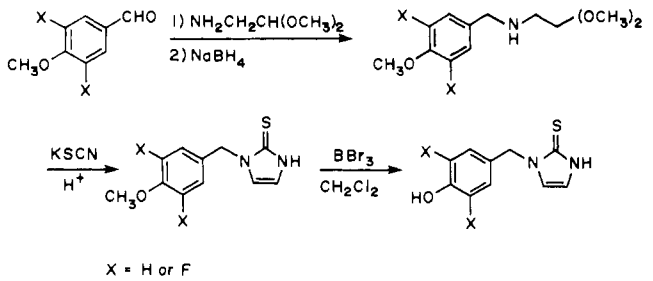


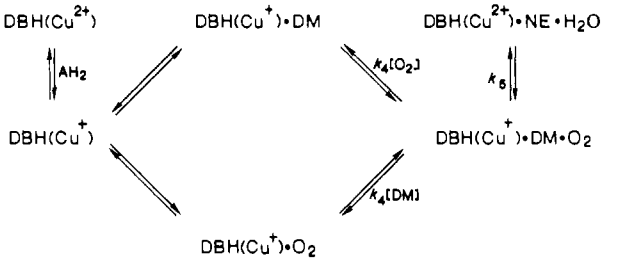
FIGURE 2: Synthetic route to **1** (X = H) and **2** (X = F).

to the corresponding *S*-methyl ethers. Under the conditions of our kinetic assays (pH 4.5–6.6) the inhibitors will exist overwhelmingly in the neutral, un-ionized form since the functional groups present are very weakly acidic (-SH pK_a 12.5; **1** -OH pK_a 10.65; **2** -OH pK_a 8.0). The 3,5-difluorotyramine substrate was used in enzymatic assays as the hydrochloride salt to avoid any possible complications arising from the inclusion of millimolar amounts of bromide ion.

Steady-State Kinetic Assays. DBH, as prepared by our isolation procedures, was found to contain highly variable amounts of copper.[1] Preliminary experiments were performed to establish the concentrations of Cu^{2+} that afforded the maximal stimulation of V_{max} under our assay conditions at pH 4.5 and 6.6, and these were found to be 10 and 5 μM, respectively. In separate control experiments where exogenous copper was absent or where copper was present in excess of the optimal concentration, except for changes in V_{max}, identical results were obtained for compounds **1**–**4**.[1] Ionic strength was found to affect V_{max} at both pH 4.5 and 6.6 with optimal stimulation of enzymatic activity occurring at an ionic strength, μ, of 0.2. This ionic strength was maintained in all assays by the addition of an appropriate amount of NaCl solution. Stimulation of DBH activity by mono- and particularly dicarboxylic acids (such as fumarate) appears to be highly dependent upon pH (Kaufman, 1974). Acetate was chosen as the buffer for experiments conducted at pH 4.5 only after thorough experimentation documented the inability of this carboxylic acid to stimulate DBH activity at this concentration and pH.[1] It was found that nonenzymatic autoxidation of ascorbic acid was rapid in some experiments at pH 6.6 when this was the varied substrate. Assay times in these cases were sufficiently short to insure substrate depletion did not cause product formation to deviate from linearity.

Predicted and Observed Kinetic Patterns. Catalysis by DBH requires the binding of ascorbate, phenethylamine, and oxygen. The kinetic mechanism of DBH remains to be completely elucidated, although previous studies have provided sufficient information to be of use in defining, by steady-state kinetics, the interaction of our multisubstrate inhibitors with enzyme. Ascorbate substrate appears to bind enzyme prior to oxygen or phenethylamine substrates but an unambiguous

Scheme I[a]



pH (conditions)	microscopic rate constants[b] k_5/k_4[DM]	$k_5/k_4[O_2]$	$k_4[O_2]/k_4$[DM]
4.5 (– fumarate)	14.9	1.62	9.2
6.6 (– fumarate)	0.140	1.49	0.094
6.6 (+ fumarate)	5.89	0.832	7.1

[a] Abbreviations: AH_2, ascorbate; DM, dopamine; NE, norepinephrine. [b] Data reproduced from Ahn and Klinman (1983).

distinction between a ping-pong mechanism (Goldstein et al., 1968) and an ordered sequential mechanism (Ljones & Skotland, 1984) has not been made. Despite this ambiguity, if the minimal kinetic mechanism suggested by Ahn and Klinman (1983) is considered (Scheme I), so long as ascorbic acid binding precedes the binding of tyramine or oxygen, for the purpose of predicting the inhibition patterns in Table I, it is immaterial which kinetic mechanism obtains. Furthermore, ascorbic acid was maintained at high, nearly saturating concentrations (unless this was the varied substrate) to minimize ambiguities that could result from ascorbate binding enzyme "downstream" from either oxygen or tyramine.

Elegant isotope effect studies have been employed in the study of DBH catalysis (Klinman et al., 1980; Miller & Klinman, 1983; Ahn & Klinman, 1983; Miller & Klinman, 1985). An analysis of the microscopic rate constants that describe flux through the ternary DBH–oxygen–dopamine complex has demonstrated a striking dependence upon pH and activation by fumarate anion. Results of these studies, which are critical for interpreting our steady-state kinetics, are reproduced in Scheme I. A random kinetic mechanism has been demonstrated for the addition of oxygen and phenethylamine substrates at high pH, and this converts to a predominantly ordered mechanism at low pH or in the presence of fumarate with phenethylamine binding preceding oxygen binding.

A consideration of the proposed pH- and fumarate-dependent changes in kinetic mechanism in light of the rules of Cleland (1977) (Segel, 1975) for predicting kinetic patterns leads to the kinetic patterns in Table I. Implicit in these predictions are the following assumptions: (1) pH 6.6, random oxygen and phenethylamine binding; (2) pH 4.5 or added fumarate, predominantly ordered binding of phenethylamine prior to oxygen; (3) compound **3** binding the active-site copper atom(s) as an oxygen mimic but, unlike oxygen, possibly binding either free or tyramine-bound enzyme irrespective of order of substrate addition; (4) compounds **1** and **2**, as multisubstrate inhibitors, competing with both phenethylamine and oxygen at the active site. Tyramine was used in the present study since this substrate has been shown to be kinetically indistinguishable from dopamine (Miller & Klinman, 1985).

The experimental results in Table I demonstrate an excellent correspondence between predicted and experimentally observed kinetic patterns. All inhibitors are uncompetitive with ascorbic acid, indicating they bind exclusively to reduced Cu^+ enzyme downstream from this substrate. At pH 6.6 under apparent random conditions, the multisubstrate inhibitor **1** binds com-

Table I: Predicted and Observed Kinetic Patterns and Apparent Kinetic Constants as a Function of pH and Fumarate Activation[a]

compd	1	2	3	4
		pH 6.6		
ascorbate	U (U)		U (U)	U (U)
K_m = 0.42 ± 0.4 mM	0.533 ± 0.035		639 ± 53	3470 ± 340
oxygen	C (C)		C (C)	N (N)
K_m = 0.61 ± 0.23 mM	0.965 ± 0.148		266 ± 34	K_{is} = 11 300 ± 1900
				K_{ii} = 5840 ± 460
tyramine	C (C)	C (C)	N (C)	C (C)
K_m = 4.02 ± 1.77 mM	0.344 ± 0.016	0.039 ± 0.008	716 ± 126	4870 ± 390
oxygen (+10 mM fumarate)	N (N)		N (N)	
K_m = 0.084 ± 0.005 mM	K_{is} = 1.95 ± 0.69		K_{is} = 284 ± 29	
	K_{ii} = 1.26 ± 0.13		K_{ii} = 711 ± 31	
		pH 4.5		
ascorbate	U (U)		U (U)	U (U)
K_m = 0.18 ± 0.02 mM	0.093 ± 0.008		73.4 ± 3.0	249 ± 17
oxygen	N (N)		N (N)	N (I)[b]
K_m = 0.39 ± 0.22 mM	K_{is} = 0.114 ± 0.037		K_{is} = 65.3 ± 11.2	
	K_{ii} = 0.0805 ± 0.0085		K_{ii} = 76.9 ± 8.8	
tyramine	C (C)	C (C)	N (C)	C (C)
K_m = 2.04 ± 0.68 mM	0.0549 ± 0.0016	0.0057 ± 0.0006	45.5 ± 1.9	168 ± 13

[a] Kinetic patterns: C, competitive; N, noncompetitive; U, uncompetitive; I, indeterminate. Experimentally observed patterns are given in parentheses. Binding constants are reported in μM units with standard errors. Inhibition constants are K_{is} values for C patterns and K_{ii} values for U patterns. Experimental conditions and data analysis are reported in Materials and Methods. [b] Apparent induced substrate inhibition precludes a simple analysis of data. However, fitting the data for each inhibitor concentration to the equation for substrate inhibition and replotting the theoretical lines yields a noncompetitive pattern.

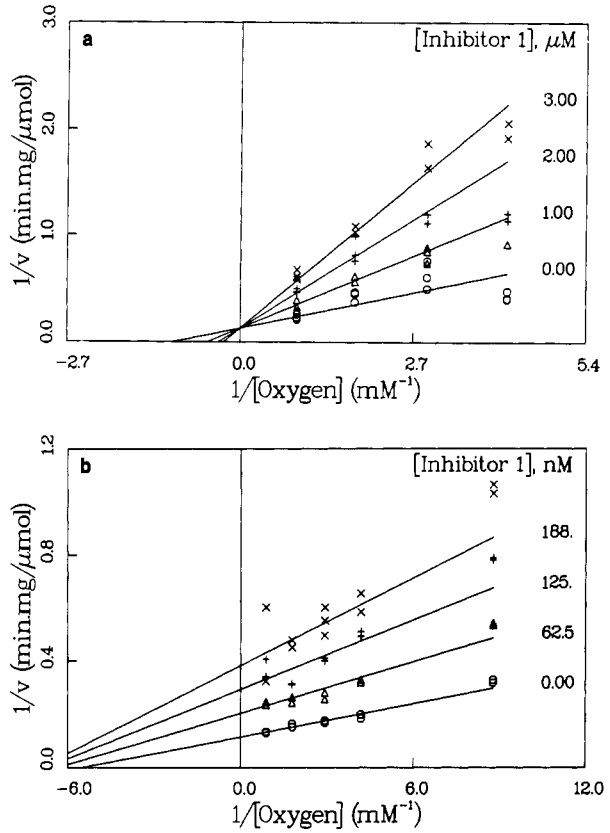


FIGURE 3: Lineweaver–Burk plot of **1** vs. oxygen: (a) pH 6.6; (b) pH 4.5.

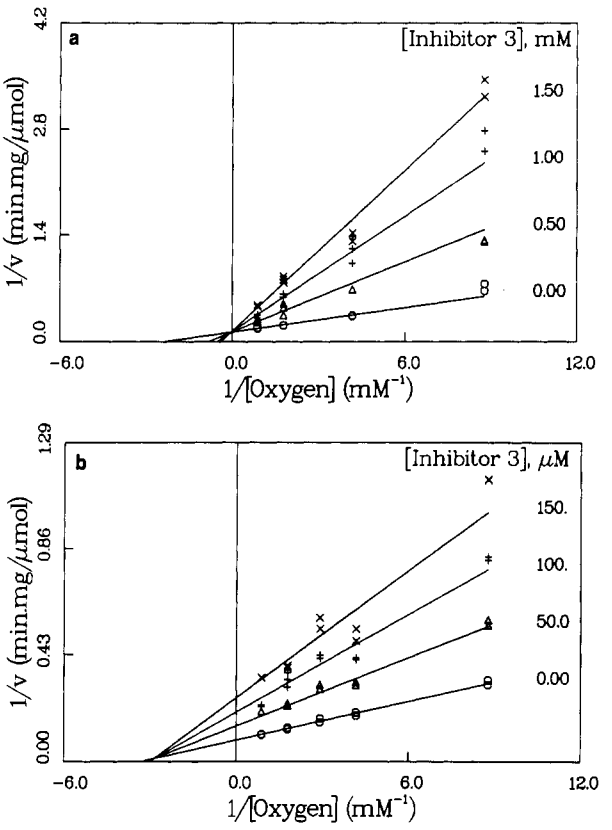


FIGURE 4: Lineweaver–Burk plot of **3** vs. oxygen: (a) pH 6.6; (b) pH 4.5.

petitively with both tyramine and oxygen. Under ordered conditions (pH 4.5 or added fumarate) binding of **1** (or **3**) vs. oxygen changes dramatically to a noncompetitive pattern (Figures 3a,b and 4a,b). The simple phenethylamine mimic **4** shows a noncompetitive pattern with oxygen at pH 6.6, which becomes indeterminate under ordered conditions, a result of induced substrate inhibition with oxygen. A replot of the theoretical lines resulting from data analysis by the SUBIN program (eq 5) does yield a family of intersecting lines, which appears noncompetitive (not shown). The simple oxygen mimic **3** has consistently and unexpectedly exhibited competitive inhibition vs. tyramine substrate. This provides the only apparent exception to the predicted kinetic patterns.

Yonetani–Theorell Double-Inhibition Study. The unexpected mutually exclusive binding of tyramine substrate and oxygen mimic **3** suggested a very small distance in the enzyme active site between oxygen and phenethylamine binding sites. It therefore became of interest to determine whether binding of the two substructural inhibitors **3** and **4** occurred with complete mutual exclusion. The Yonetani–Theorell double-

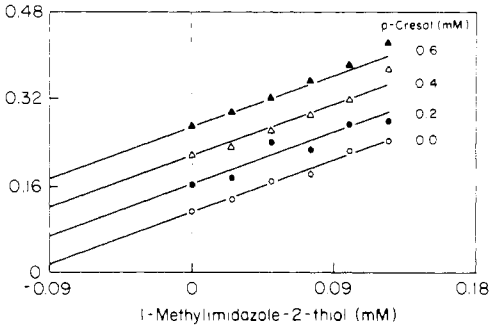


FIGURE 5: Yonetani–Theorell double-inhibition experiment with inhibitors **3** and **4**.

Table II: Apparent Dissociation Constants for Substrates and Inhibitors at pH 6.6

compd	dissociation const from binary complex[a]	dissociation const from ternary complex[b]
tyramine	9.6 ± 4.3 mM	1.0 ± 0.3 mM (0.5 ± 0.1 mM)[c]
oxygen	0.8 ± 0.3 mM	0.08 ± 0.03 mM (0.12 ± 0.03 mM)[c]
3,5-difluorotyramine	0.9 ± 0.3 mM	0.4 ± 0.1 mM
oxygen	0.4 ± 0.1 mM	0.21 ± 0.07 mM
1	344 ± 16 nM[d]	
2	39 ± 8 nM[d]	

[a] The data were fit to the kinetic equation $1/v = (K_aB + K_bA + AB + K_{ia}K_b)/VAB$. Dissociation constants reported here are K_{ia} and K_{ib} values. [b] These are K_A and K_B values. [c] This value is the true dissociation constant calculated by Miller and Klinman (1985) at pH 6.07. [d] This value is the slope inhibition constant (K_{is}) for the inhibitor vs. tyramine substrate. Under conditions of random binding of tyramine and oxygen substrates to enzyme a fraction of total enzyme will be present as the binary enzyme–oxygen complex and this will make the K_{is} vs. tyramine an overestimate of the true K_i value for inhibitor. However, the ratio of dissociation constants will not depend upon the concentation of nonvaried substrate so long as this is held constant (oxygen, here 0.24 mM).

inhibition method (Yonetani, 1982) was employed. This method, in which two inhibitors are varied in the presence of fixed, subsaturating substrates, yields the factor α (eq 6), which defines the degree of interaction between inhibitors at the enzyme active site. The family of nearly parallel lines produced (Figure 5) and extremely large α values, which result from this analysis (21.5 ± 16.8, pH 4.5; 10.6 ± 3.9, pH 6.6), demonstrate a high degree of interference in binding between oxygen mimic **3** and the phenethylamine mimic **4**.

Analysis of Substrate Binding. Isotope effect studies suggest a steady-state random kinetic mechanism for DBH at high pH. Velocity data for the binding of tyramine and oxygen substrate to DBH at pH 6.6 were fit to eq 4 to give the results shown in Table II. Data analysis by eq 4 presupposes a rapid equilibrium random kinetic mechanism where k_5 (Scheme I) becomes small relative to the interconversion of enzyme–substrate complexes. At pH 6.6 a considerable flux of ternary complex back to the binary DBH-oxygen complex (k_4[DM], Scheme I) occurs, whereas the $k_5/k_4[O_2]$ ratio is still greater than 1, suggesting a substantial commitment to catalysis relative to oxygen dissociation even at high pH. Thus the simple treatment of the velocity data by eq 4 is an approximation, but leads nevertheless to apparent dissociation constants of substrate from ternary complex which are in good agreement with true values calculated from isotope effect studies at a similar pH (Table II). A comparison of apparent dissociation constants derived for tyramine and 3,5-difluorotyramine substrates demonstrates approximately 10-fold tighter binding of the fluorinated substrate in the binary DBH–tyramine complex relative to the parent tyramine.

Discussion

The present study describes inhibitors of DBH that bind enzyme in the nanomolar range, fully 10^5–10^6-fold more tightly than tyramine substrate. Despite the very low K_i for **2** (Table I) we have not observed any evidence for "tight-binding" behavior (Williams & Morrison, 1979). Kinetic data for these inhibitors and substructural analogues **3** and **4** are supportive of a multisubstrate mode of inhibition and, we believe, suggest a very small intersite distance between the phenethylamine binding site and the active-site copper atom(s).

Multisubstrate inhibitors combine structural features of two or more substrates into a single molecule, which, if appropriately constructed, binds enzyme with a dissociation constant substantially lower than the product of the dissociation constants for the individual substrates, a result of favorable entropic effects (Byers, 1978). An application of this approach to the inhibition of DBH requires the utilization of an appropriate mimic for oxygen since the valency of this molecule obviously precludes its incorporation into an inhibitor. We have found the imidazole-2-thiol **3**, a known, comparatively weak inhibitor of DBH (Stolk & Hanlon, 1973; Fuller et al., 1977), to serve this purpose well. Indeed, the selectivity of this soft sulfur-containing ligand is such that it appears to bind exclusively to the reduced, Cu^+ form of enzyme as evidenced by uncompetitive inhibition vs. ascorbic acid and, under random conditions, competitive inhibition vs. oxygen and tyramine. This is remarkable for the simple ligand **3** in light of the known affinity of this compound for Cu^{2+} ions in solution (Hanlon & Shuman, 1975). Nevertheless, our inability to detect any meaningful slope inhibition constant for **3** vs. ascorbate together with the quality of our data for these experiments allows us to state with confidence that binding to the Cu^+ form of enzyme is 10- to 100-fold tighter than to the Cu^{2+} form. The inhibitors **1** and **2** resulted from a systematic study of 1-(arylalkyl)imidazole-2-thiols with methylene bridging chains of various length between the phenethylamine mimic and oxygen mimic groups. The simple 1-benzyl-substituted imidazole-2-thiol is an inhibitor of optimal chain length.

The kinetic data of Table I are fully supportive of a multisubstrate mode of inhibition by **1** and **2** and are consistent with the recently proposed changes in kinetic mechanism for DBH (Ahn & Klinman, 1983). Like compound **3**, compounds **1**, **2**, and **4** also appear to interact exclusively with the reduced Cu^+ form of enzyme, as evidenced by their uncompetitive inhibition vs. ascorbate and competitive inhibition vs. tyramine. At high pH the multisubstrate inhibitor **1** shows the expected competitive binding with both substrates mimicked, oxygen and tyramine. Under conditions (low pH or in the presence of fumarate) where a preferential order of substrate addition is induced, inhibition of DBH by **1** and the oxygen mimic **3** changes rather dramatically to a noncompetitive pattern (Figures 3a,b and 4a,b). Under ordered conditions the noncompetitive pattern predicted for **4** vs. oxygen was not observed. Instead, induced substrate inhibition occurred with oxygen, a result that is characteristic of either an ordered mechanism with oxygen binding following *p*-cresol binding, or a much tighter binding of inhibitor to ternary complex than to binary complex (Cleland, 1977). Unfortunately, the concentrations of oxygen that can be achieved ($\sim 1.14 \times 10^{-3}$ M) under ambient pressure preclude a more detailed investigation of this phenomenon. Interestingly, as evidenced by oxygen uptake experiments, **4** is also a good alternate substrate for DBH ($V_{max} \approx 6.88$ μmol min^{-1} mg^{-1}, $K_m \approx 2.45$ mM at 0.24 mM O_2). Even though complex and nonlinear inhibition

patterns are frequently observed with alternate substrates (Radika & Northrop, 1984; Segel, 1975), under the conditions of our assays **4** exhibits "normal" linear inhibition.

Oxygen mimic **3** has consistently and unexpectedly (Rosenberg, 1983) exhibited competitive inhibition with tyramine substrate. The mutually exclusive binding of this oxygen mimic and tyramine substrate suggests a much smaller intersite distance than originally suspected. When **3** binds DBH, the increased steric bulk of this inhibitor relative to diatomic oxygen (13 atoms vs. 2) apparently occludes the adjacent phenethylamine binding site to prevent tyramine binding. Additional support for this can be drawn from the double-inhibition study with inhibitors **3** and **4**. Previous double-inhibition studies have been utilized to study the degree of interaction of inhibitors that bind to the same enzymatic site (Stone et al., 1984). However the competitive binding of both **3** and **4** with tyramine allow the utilization of eq 6 for the study of two enzymatic sites. The results of a double-inhibition study with **3** and **4** at pH 4.5 appear as a family of parallel lines (Figure 5), but an analysis of α with the appropriate error propagation provides large, but statistically significant values for this factor (21.5 ± 16.8, pH 4.5; 10.6 ± 3.9, pH 6.6). The relatively large standard errors in α arise from errors propagated from K_i and K_j, the inhibition constants for **3** and **4**, respectively. According to the original Yonetani–Theorell treatment (Yonetani, 1982) if $\alpha > 1$, the simultaneous binding of two inhibitors results in unfavorable steric interactions. The very large α values calculated in our experiments demonstrate that the binding of oxygen mimic **3** and the small phenethylamine substrate mimic **4** occurs with nearly complete mutual exclusion.

The multisubstrate inhibitor **1** was designed to bind simultaneously to both the phenethylamine site and the active-site copper atom(s). Insofar as steady-state kinetic experiments will support a postulated mode of inhibition, these appear to do so: compound **1** binds the reduced Cu^+ form of enzyme competitively with both oxygen and tyramine substrates. However, competitive inhibition with a substrate can conceivably occur as a result of binding to an enzymatic site which is distinct from but allosterically linked to the substrate binding site. Unequivocal spectroscopic demonstration of a direct sulfur ligand–copper interaction between **1** and DBH by EPR has been frustrated by the selective binding of **1** to the Cu^+ diamagnetic form of enzyme. Furthermore, an unambiguous demonstration of a direct sulfur–copper interaction between inhibitor **2** and Cu^+ DBH by EXAFS has been thwarted by the presence of a protein sulfur ligand at 2.30 Å from copper in the control, Cu^+ form of enzyme.[2] While further EXAFS experiments should ultimately allow a resolution of the nature of binding to the copper atom(s) in DBH by **1** and **2**, it is worth noting that the direct inner-sphere oxygen coordination to copper, which has been proposed as a catalytic mechanism (Miller & Klinman, 1985), does support our proposed multisubstrate interaction between **1** and DBH. This proposal, together with the direct dioxygen–copper interactions observed for other copper proteins (Solomon, 1981; Spiro et al., 1983; Robb, 1984) and model complexes (Karlin et al., 1981, 1984) as well as the affinity of **3** for copper ions in solution (Hanlon & Shuman, 1975) makes it difficult to understand competitive binding of inhibitor vs. oxygen arising from anything other than a direct binding to the active-site copper atom(s).

Evidence for a direct binding of **1** and **2** to the enzymatic site that binds phenethylamine substrates can be found in Table II. Under conditions (high pH) of diminishing catalysis the steady-state random kinetic mechanism of DBH is expected to approximate to a rapid equilibrium random mechanism. If this occurs, dissociation of substrates from enzyme will be approximated by eq 4, where K_{ia} and K_{ib} represent the dissociation of tyramine and oxygen substrates from the *binary* DBH–substrate complex and K_a and K_b represent the dissociation of substrates from the ternary complex. While this treatment is clearly only an approximation, it does reproduce the true dissociation constants calculated from isotope effects (Table II). This data analysis shows a ca. 10-fold greater affinity of 3,5-difluorotyramine for the enzyme than that found for tyramine. A similar increase in affinity is seen for the comparably substituted inhibitors **1** and **2**. The nearly identical increase in binding affinity of either substrate or inhibitor upon fluorine substitution makes binding at separate enzyme sites unlikely.

The substantial affinity of **1** and **2** for DBH is interesting in light of the mutually exclusive binding of substructures **3** and **4**. However, a comparison of the dissociation constant for **1** to the product of dissociation constants of **3** and **4** (Table I) suggests the "fit" of the multisubstrate inhibitor **1** to the DBH active site is not ideal. Thus, the entropic advantage (ca. 10^2–10^3-fold lowering of K_i) typically observed for other multisubstrate inhibitors (Byers, 1978) is lacking for **1**; the observed dissociation constant for this inhibitor is more closely approximated by the simple product of the dissociation constants for **3** and **4**.

In summary, in a novel approach to the inhibition of a mixed-function oxidase, we have prepared inhibitors which bind enzyme 10^5–10^6-fold more tightly than substrate. The steady-state kinetics described here in detail support a multisubstrate mode of interaction between these inhibitors and enzyme and the unique pH- and fumarate-dependent changes in kinetic order for DBH. Lastly, while a discussion of the in vivo effects of **1** and **2** is tangential to the present study, it is worth noting that these are not mere kinetic curiosities, but as evidenced in a recent pharmacological study (Kruse et al., 1986), these inhibitors and structural relatives produce substantial antihypertensive effects in vivo upon oral dosing in animals. One structural analogue of these inhibitors is presently undergoing preclinial evaluation as a novel antihypertensive agent.

ACKNOWLEDGMENTS

We thank Professor John F. Morrison (The Australian National University) for helpful discussions and a critical review of this study.

Registry No. **1**, 95333-64-5; **2**, 95333-60-1; **3**, 60-56-0; **4**, 106-44-5; DBH, 9013-38-1; $NH_2CH_2CH(OMe)_2$, 22483-09-6; *p*-$MeOC_6H_4CHO$, 123-11-5; O_2, 7782-44-7; 3,5-difluoro-4-hydroxybromobenzene, 104197-13-9; 2,6-difluorophenol, 28177-48-2; 3,5-difluoro-4-methoxybromobenzene, 104197-14-0; 3,5-difluoro-4-methoxybenzonitrile, 104197-15-1; 3,5-difluoro-4-methoxybenzaldehyde, 654-11-5; 1-(3,5-difluoro-4-methoxybenzyl)imidazole-2-thiol, 104197-16-2; [*N*-(3,5-difluoro-4-methoxybenzyl)amino]acetaldehyde dimethyl acetal, 101471-18-5; 1-(4-methoxybenzyl)imidazole-2-thiol, 95460-09-6; 3,5-difluoro-4-methoxy-1-(2-nitroethenyl)benzene, 104197-17-3; (3,5-difluoro-4-methoxyphenethyl)amine, 104197-18-4; (3,5-difluoro-4-hydroxyphenethyl)amine hydrobromide, 104197-19-5; (3,5-difluoro-4-hydroxyphenethyl)amine hydrochloride, 59043-65-1; tyramine hydrochloride, 60-19-5; L-ascorbic acid, 50-81-7; 3,5-difluorotyramine hydrochloride, 104197-20-8.

REFERENCES

Ahn, N., & Klinman, J. P. (1983) *Biochemistry 22*, 3096.

[2] R. A. Scott and L. I. Kruse, unpublished results.

Ash, D. E., Papadopoulos, N. J., Colombo, G., & Villafranca, J. J. (1984) *J. Biol. Chem. 259*, 3395.
Byers, L. D. (1978) *J. Theor. Biol. 74*, 501.
Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.
Fuller, R. W., Ho, P. P. K., Matsumoto, C., & Clemens, J. A. (1977) *Adv. Enzyme Regul. 15*, 267.
Goldstein, M., Joh, T. H., & Garvey, T. Q., III (1968) *Biochemistry 7*, 2724.
Hanlon, D. P., & Shuman, S. (1975) *Experienta 31*, 1005.
Karlin, K. D., Dahlstrom, P. L., Cozzette, S. N., Scensny, P. M., & Zubieta, J. (1981) *J. Chem. Soc., Chem. Commun.*, 881.
Karlin, K. D., Hayes, J. C., Gultneh, Y., Cruse, R. W., McKown, J. W., Hutchinson, J. P., & Zubieta, J. (1984) *J. Am. Chem. Soc. 106*, 2121.
Kaufman, S. (1974) *J. Psychiatr. Res. 11*, 303.
Klinman, J. P., Humphries, H., & Voet, J. G. (1980) *J. Biol. Chem. 255*, 11648.
Klinman, J. P., Krueger, M., Brenner, M., & Edmondson, D. E. (1984) *J. Biol. Chem. 259*, 3399.
Kruse, L. I., Kaiser, C., DeWolf, W. E., Jr., Frazee, J. S., Erickson, R. E., Ezekiel, M., Ohlstein, E. H., Ruffolo, R. R., Jr., & Berkowitz, B. B. (1986) *J. Med. Chem. 29*, 887.
Ljones, T., & Skotland, T. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 2, p 131, CRC, Boca Raton, FL.
Miller, S. M., & Klinman, J. P. (1983) *Biochemistry 22*, 3091.
Miller, S. M., & Klinman, J. P. (1985) *Biochemistry 24*, 2114.
Mitchell, R. H., Lai, Y.-H., & Williams, R. V. (1979) *J. Org. Chem. 44*, 4733.
Nagatsu, T., & Udenfriend, S. (1972) *Clin. Chem. (Winston-Salem, N.C.) 18*, 980.
Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., & Kraut, J. (1985) *J. Biol. Chem. 260*, 16122.
Preaux, G., & Gielens, C. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 2, p 159, CRC, Boca Raton, FL.
Radika, K., & Northrop, D. (1984) *Anal. Biochem. 141*, 413.
Robb, D. A. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 2, p 207, CRC, Boca Raton, FL.
Rosenberg, R. C. (1983) *Biochim. Biophys. Acta 749*, 276.
Rosenberg, R. C., & Lovenberg, W. (1980) *Essays Neurochem. Neuropharmacol. 4*, 163.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 342–343, 749–757, Wiley, New York.
Skotland, T., & Ljones, T. (1979a) *Inorg. Perspect. Biol. Med. 2*, 151.
Skotland, T., & Ljones, T. (1979b) *Eur. J. Biochem. 94*, 145.
Solomon, E. I. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) p 43, Wiley, New York.
Spiro, T. G., Woolery, G. L., Brown, J. M., Powers, L., Winkler, M. E., & Solomon, E. I. (1983) in *Copper Coordination Chemistry: Biochemical and Inorganic Perspectives* (Karlin, K. D., & Zubieta, J., Eds.) p 23, Adenine, New York.
Still, W. C., Kahn, M., & Mitra, A. (1978) *J. Org. Chem. 43*, 2923.
Stolk, J. M., & Hanlon, D. P. (1973) *Life Sci. 12*, 417.
Stone, S. R., Mark, A., & Morrison, J. F. (1984) *Biochemistry 23*, 4340.
van Es, T., & Staskun, B. (1965) *J. Chem. Soc.*, 5775.
Villafranca, J. J. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) p 263, Wiley, New York.
Wilcox, D. E., Porras, A. G., Hwang, Y. T., Lerch, K., Winkler, M. E., & Solomon, E. I. (1985) *J. Am. Chem. Soc. 107*, 4015.
Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437.
Yonetani, T. (1982) *Methods Enzymol. 87*, 500.