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Characterization of 3-Phenylpropenes as Mechanism-Based Inhibitors of Dopamine β-Hydroxylase[1]

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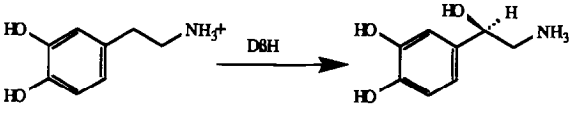
An extension of the characterization of a series of 3-phenylpropenes as mechanism-based inhibitors of dopamine β-hydroxylase was made to include 2-CH_3-3-(*p*-HO-phenyl)-1-propene **(4)**. Kinetic data with **4** establish that this compound has all of the inactivation characteristics of other 2-substituted 3-phenylpropenes previously studied in this laboratory [P. F. Fitzpatrick, D. R. Flory, Jr., and J. J. Villafranca (1985) *Biochemistry* **24,** 2108–2114]. Comparison of the inactivation rate constants and partition ratios of 2-H, 2-Cl, 2-CH_3, and 2-Br-3-(*p*-HO-phenyl)-1-propenes leads to the conclusion that there is a steric contribution to the magnitude of the inactivation rate parameters exerted by substitution at the 2-position. The 2-Br- and 2-CH_3-containing compounds have slower inactivation rates than the 2-H congener and the partition ratios are lower than that found with 3-(*p*-HO-phenyl)-1-propene. The radiolabeled compound 2-[^{3}H]-3-(*p*-HO-phenyl)-1-propene **(8c)** was synthesized and used to determine the stoichiometry of inhibitor bound to the enzyme. A stoichiometry of 1.0 mol of inhibitor/mol of enzyme monomer was found. The bound inhibitor is labile to conditions of protein denaturation and reaction with hydroxylamine preventing further isolation and identification of the modified amino acids.

INTRODUCTION

Dopamine β-hydroxylase (DβH)[3] (EC 1.14.17.1) is a copper-utilizing monooxygenase which catalyzes the benzylic hydroxylation of dopamine to yield norepinephrine (*1–3*). The enzyme shows a broad substrate specificity *in vitro,* which has allowed several classes of mechanism-based inhibitors to be developed in the study of the enzymatic reaction mechanism (for a review see Ref. (*4*)).



One class of mechanism-based inhibitors which we have used extensively is the substituted 3-phenylpropenes (*5–7*). In our continuing efforts at understanding

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[3] Abbreviations used: DβH, dopamine β-hydroxylase; Mes, 2[*N*-morphilino]ethanesulfonic acid; DMF, dimethylformamide; THF, tetrahydrofuran; DTT, dithiothreitol; TFA, trifluoroacetic acid; Con A, concanavalin A; TPCK, L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone; HPLC, high performance liquid chromatography.

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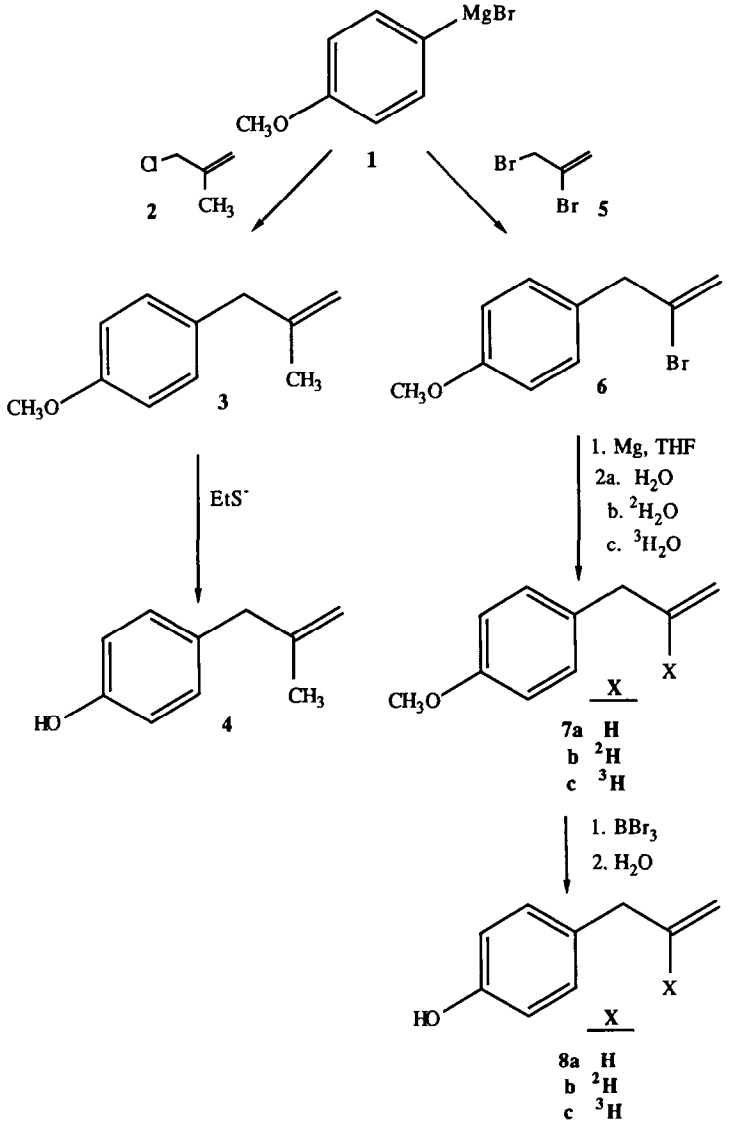
electronic and steric factors governing the inactivation mechanism by this class of DβH inhibitors we have synthesized and characterized as a mechanism-based inhibitor 2-CH_3-3-(*p*-HO-phenyl)-1-propene **(4)** (Scheme 1). Previously, we proposed that 3-phenylpropenes lead to inactivation of DβH through covalent modification at the active site by an enzymatically generated intermediate (*5–7*). Further, we have synthesized a tritium-labeled analog of 3-(*p*-HO-phenyl)-1-propene, namely, 2-[^{3}H]-3-(*p*-HO-phenyl)-1-propene **(8c)**, to determine the stoichiometry of bound inhibitor and to investigate the nature of the enzyme–inhibitor complex formed upon inactivation by this class of mechanism-based inhibitors (Scheme 1).



SCHEME 1. Synthesis of **4**, **8b**, and **8c**.

RESULTS

Inactivation by 2-CH_3-3-(p-HO-phenyl)-1-propene **(4).** 2-CH_3-3-(*p*-HO-Phenyl)-1-propene **(4)** was tested as a mechanism-based inhibitor of DβH and the results compared with those from inactivation with several 2-X-3-(*p*-HO-phenyl)-1-propenes (*5, 6*). Inactivation of dopamine β-hydroxylase with 2-CH_3-3-(*p*-HO-phenyl)-1-propene **(4)** leads to a first order loss of enzyme activity which is dependent on inhibitor concentration (Fig. 1). Inactivation is also dependent on catalytically functional enzyme since no inactivation takes place if ascorbate is absent from the incubation mixture (data not shown). Protection against inactivation is afforded when 36.0 mM tyramine (a substrate of DβH) is included in the incubation mixture with 1.2 mM **4** indicating inactivation at the active site. The rate of inactivation is also dependent on the oxygen concentration as expected. A plot of $1/k_{obs}$ versus 1/[I] at 0.25 mM and 1.2 mM O_2 shows that the rate of inactivation increases with increased oxygen concentration (Fig. 1). The apparent k_{inact} is determined by extrapolation to infinite inhibitor concentration at a given oxygen concentration (*y*-intercept). The apparent K_i is determined by extrapolation to the *x*-intercept. Thus all the substituted 3-phenylpropenes meet the kinetic criteria required for mechanism-based inhibition (*8, 9*).

The kinetics of inactivation and partition ratios for several 2-X-3-(*p*-HO-phenyl)-1-propenes are given in Table 1. The partition ratios shown were determined using the HPLC method described in Rajashekhar *et al.* (*6*) by incubation under 1.2 mM O_2. The results shown in Table 1 indicate that all of the 3-phenylpropenes show oxygen dependent inactivation. The K_i values range from approximately 1.0 to 5.0 mM and are effected little by [O_2]. As one replaces 2-X (X = H) of the propene side chain with any of the other substituents (X = Cl, Br, CH_3) the k_{inact} decreases while the K_i values remain essentially unchanged. Further, the

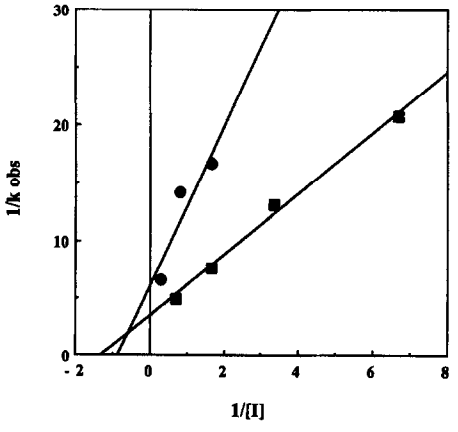


FIG. 1. Double reciprocal plot of $1/k_{obs}$ vs 1/[I] at 0.25 (●) and 1.2 mM (■) oxygen for the inactivation of dopamine β-hydroxylase by 2-CH_3-3-(*p*-HO-phenyl)-1-propene **(4)** at various inhibitor concentrations. Conditions: 14% DMF, 12 mM ascorbate, 0.5 mg/ml catalase, 0.2 mg/ml dopamine β-hydroxylase, and 100 mM Mes, pH 5.5, at room temperature.

TABLE 1

Inactivation Kinetics and Partition Ratios for Several 2-X-3-(*p*-HO-Phenyl)-1-propenes

	[Oxygen]						
	1.2 mM		0.54 mM		0.25 mM		
X	k_{inact} (min^{-1})	K_i (mM)	k_{inact} (min^{-1})	K_i (mM)	k_{inact} (min^{-1})	K_i (mM)	Partition ratio[a]
H[b]	1.8	0.8	1.3	1.5	0.51	1.8	88 ± 9
Cl[b]	0.67	4.9	—	—	0.20	3.6	41 ± 4
Br[b,c]	0.26	4.9	0.15	2.0	0.08	2.3	63 ± 6
CH_3	0.33	1.0	—	—	0.14	1.2	68 ± 7

[a] Partition ratios were determined by incubation under 1.2 mM O_2 as described in Ref. (6).

[b] Ref. (5).

[c] Ref. (6).

partition ratio also varies. Replacement of the 2-X (X = H) with X = Cl shows a twofold decrease in the partition ratio.

Inactivation by 2-[^2H]-3-(p-HO-phenyl)-1-propene **(8b)** *and 2-[^3H]-3-(p-HO-phenyl)-1-propene* **(8c).** The deuterated analog of 3-(*p*-HO-phenyl)-1-propene **(8a)** was synthesized for two purposes: first, to test the synthetic method for incorporation of tritium at the 2-position of **8a,** and second, to determine if there was an isotope effect at the 2-position on the rate of inactivation. Dopamine β-hydroxylase was incubated with various concentrations of **8b** following the general methods described later. Aliquots were removed and assayed for activity. The experiment was also done with the 2-[^{1}H] analog **(8a)** and the rates of inactivation compared. The rate of enzyme inactivation by the 2-[^{1}H] and 2-[^{2}H] analogs, **8a** and **8b,** respectively, was determined at several inhibitor concentrations. A replot of $1/k_{obs}$ vs 1/[I] shows that there is no apparent isotope effect (Fig. 2).

Dopamine β-hydroxylase was incubated with **8c** having a specific radioactivity of from 0.5 to 1.6 cpm/pmol depending on the batch used. Aliquots were removed and assayed for activity as described later. A plot of ln specific activity (μmol/min/mg of enzyme) versus time for the inactivation of DβH by **8c** is shown in Fig. 3. The rate of inactivation is equal to that with **8a** as expected (data not shown). Also shown is a control experiment in which ascorbate has been left out of the incubation mixture. Without ascorbate the enzyme does not turn over and no inactivation occurs. Inactivation leads to incorporation of approximately 1 mol of inhibitor bound/mol of enzyme subunit (Table 2). The stoichiometry of bound inhibitor was also determined under conditions of enzyme denaturation. Inactivated enzyme was dialyzed versus 4 M urea and 6 M guanidine–HCl at pH 6.0 and 8.0 (four changes over 2 h) The amount of bound radioactivity (inhibitor) was then determined. It was found that 34–48% of the initially bound radioactivity re-

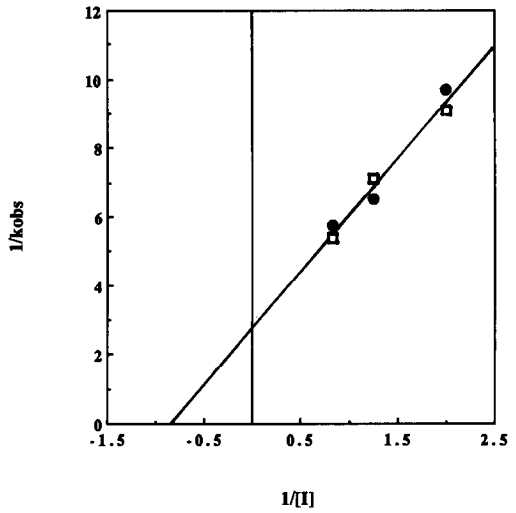


FIG. 2. Double reciprocal plot of $1/k_{obs}$ vs 1/[I] at various concentrations of 3-(*p*-HO-phenyl)-1-propene **(8a)** (●) and 2-[^{2}H]-3-(*p*-HO-phenyl)-1-propene **(8b)** (□). Conditions: 14% DMF, 1.2 mM oxygen, 12 mM ascorbate, 0.5 mg/ml catalase, 0.2 mg/ml dopamine β-hydroxylase, and 100 mM Mes, pH 5.5, at room temperature.

mained after dialysis (Table 3A). Inactivated enzyme was also treated with hydroxylamine under nondenaturing conditions. In this case 48% of the initially bound radioactivity remained after dialysis (Table 3B). Inactivated enzyme was reduced, *S*-carboxymethylated, and digested with trypsin as described under Experimental Procedures. The resulting peptides were mapped by HPLC and analyzed for radioactivity. The analysis indicated a low level of tritium incorporation

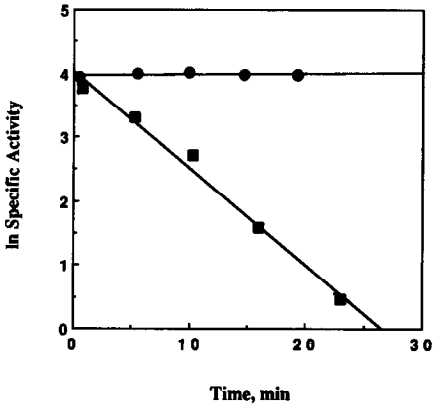


FIG. 3. Time course for the inactivation of dopamine β-hydroxylase by 2-[^{3}H]-3-(*p*-HO-phenyl)-1-propene **(8c)** at 1.0 mM plus (■) and minus (●) ascorbate. Conditions: 5% DMF, 0.25 mM oxygen, 12 mM ascorbate, 0.5 mg/ml catalase, 0.67 mg/ml dopamine β-hydroxylase, and 100 mM Mes, pH 5.5, at room temperature.

TABLE 2

Determination of the Stoichiometry of Bound **8c**

Experiment	mol inhibitor **8c**/ mol monomer of DβH
All components	1.0 ± 0.1
−ascorbate	0.01

in several peptides (results not shown), indicating the possibility that more than one active site amino acid was modified. Further characterization of the peptides was precluded by this observation.

DISCUSSION

Several years ago Colombo *et al.* (*5*) demonstrated mechanism-based inhibition of DβH by 2-Br-3-(*p*-HO-phenyl)-1-propene. In order to further investigate the nature of the inactivating species, Fitzpatrick *et al.* (*7*) used a number of ring-substituted 3-phenylpropenes in a structure–reactivity study. The results of this study indicated that the species leading to enzyme inactivation was the radical formed by abstraction of the benzylic hydrogen atom. This radical then partitions between product formation and enzyme inactivation (Fig. 4). Miller and Kinman (*10, 11*) published results of studies in which they also proposed the formation of a radical during catalysis. A benzylic radical was trapped and characterized using benzyl hydrazine and phenyl hydrazine as inactivators of DβH by Fitzpatrick *et al.* (*12*).

It had become apparent that substitution of halides at the 2-position of several 3-(*p*-HO-phenyl)-1-propenes has an effect on both k_{inact} and the partition ratio (*6*). In

TABLE 3

Determination of Bound Radioactivity under Various Conditions

Dialysis conditions	Percentage of initially bound radioactivity
A	
4 M urea, 50 mM Mes, pH 6.0	34
4 M urea, 0.25 M Tris, pH 8.0	35
6 M guanidine–HCl, 50 mM Mes, pH 6.0	48
6 M guanidine–HCl, 0.25 M Tris, pH 8.0	32
B	
100 mM KPi, pH 7.0	97
1 M hydroxylamine–HCl, 100 mM KPi, pH 7.0	48

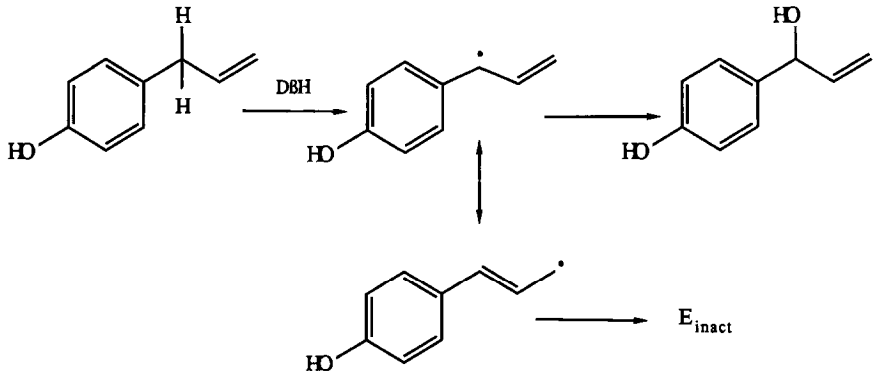


FIG. 4. Mechanism of inactivation of dopamine β-hydroxylase by 3-(HO-phenyl)-1-propene.

order to investigate if part of this effect was steric in nature, we synthesized 2-CH_3-3-(*p*-HO-phenyl)-1-propene **(4)**. Methyl and chloro substituents are nearly identical sterically yet differ considerably electronically, chlorine being more electron withdrawing than a methyl group. Any differences in the behavior of 2-Cl versus 2-CH_3-3-(*p*-HO-phenyl)-1-propene toward the inactivation of DβH would be attributed to electronic effects. As shown in Table 1, the following trends are found for inactivation by 2-X-3-(*p*-HO-phenyl)-1-propenes: k_{inact}, H > Cl > CH_3 ≈ Br, partition ratio, Cl < Br ≈ CH_3 < H. A consistent explanation of these trends is that there is a contribution from steric factors on both k_{inact} and the partition ratio; substitution of the 2-H with any substituents decreases the rate of inactivation and lowers the partition ratio. These results suggest that the electronic effect on the rate of inactivation is minimal.

We previously proposed that the intermediate formed in the enzymatic mechanism is a radical and model studies have shown that radicals are stabilized by both electron withdrawing and donating groups (*13, 14*). Further, the stability of a radical center is enhanced by the combined action of an electron withdrawing (captor) and an electron releasing (donor) substituent (*15*). This combined action has been called the captodative effect. The results seen with the 2-substituted 3-phenylpropenes appear to exhibit behavior indicating a captodative effect adding further support to the proposal that the intermediate formed during inactivation by this class of mechanism-based inhibitor is indeed a radical species.

The type of chemistry that a radical would be expected to undergo with active site residues is difficult to predict a priori. Radicals classically undergo a limited number of reactions which include recombination with other radicals, hydrogen atom abstraction, and, less often, insertion reactions. These possibilities make it hard to define the nature of the covalent enzyme–inhibitor adduct which forms during mechanism-based inactivation by 3-phenylpropenes. The radical could also undergo a further one electron oxidation to generate a carbocation. The chemistry of carbocations is much more predictable and one can envision trapping of an active site nucleophile to form a covalent adduct. For example, reaction with an active site carboxylate anion would lead to the formation of a bound ester. The

results described in this paper on the stability of the adduct formed during mechanism-based inactivation with 2-[^{3}H]-3-(*p*-HO-phenyl)-1-propene **(8c)** are indicative of esters, viz., susceptibility to hydrolysis and nucleophilic attack. Tentatively, we propose formation of an ester by reaction of a carbocation species and an active site carboxylate anion. Hydrolysis of the bound species would be expected to release an unsaturated alcohol. The species released during hydrolysis and the nature of the peptide adduct are under investigation.

EXPERIMENTAL PROCEDURES

Spectroscopy and physical methods. NMR analyses were on a Varian EM-360 60 MHz continuous wave spectrometer and/or a Bruker 360 MHz Fourier transform spectrometer. Mass spectral analyses were from the Penn State Mass Spectrometry Facility on a Kratos MS 9150 spectrometer. All solvents were dried before use. Prior to use, **2** and **5** were distilled. TLC plates and silica gel for flash chromatography were from Merck. Products were purified as required by flash chromatography, distillation, HPLC, and/or preparative TLC as described under Syntheses.

Materials. Ascorbic acid, fumaric acid, potassium phosphate (monobasic), iodoacetate, DTT, methyl α-mannoside, and Mes were from Sigma. *p*-Anisyl magnesium bromide **(1)**, 3-chloro-2-methyl-propene **(2)**, 2,3-dibromo-propene **(5)**, acetic acid, DMF, and tyramine hydrochloride were from Aldrich. HPLC grade acetonitrile and THF were from Baker. Oxygen was from Burdette. Catalase was from Boehringer-Mannheim. ScintiVerse II scintillation fluid and TFA were from Fisher. TPCK–trypsin was from Cooper Biomedical and β-mercaptoethanol was from Bio-Rad. Ultrapure urea and guanidine–HCl were from Schwartz-Mann. Tritiated water was from NEN and deuterated water (98%) was from MSD Isotopes.

Enzyme isolation and purification. Dopamine β-hydroxylase was isolated from whole frozen bovine adrenal medulla by chromatography on Con A Sepharose and DE-53 cellulose following the procedure of Colombo *et al.* (*16*). Purified enzyme was mixed with an equal volume of 4.2 M ammonium sulfate in 50 mM potassium phosphate, pH 6.5, and the resulting precipitate stored at 4°C. Ammonium sulfate-precipitated enzyme was prepared for use by removing 1–2 ml of the ammonium sulfate suspension and centrifuging for 15 min at 4°C in an Eppendorf tabletop high speed centrifuge. The resulting pellet was resuspended and dialyzed in four changes of 50 mM Mes, pH 6.0, at 4°C. The first buffer change contained 10 mM $CuCl_2$ and the second change contained 1 mM $CuCl_2$. Under these conditions DβH containing 8 Cu per tetramer was routinely obtained.

Enzyme activity. Activity was measured using the standard assay which measures the rate of oxygen disappearance using a YSI Model 53 oxygen monitor. The assay conditions were 200 mM acetate, pH 5.0, 15 mM tyramine, 10 mM fumarate, 42 μg/ml catalase, 20 mM ascorbate, and 0.22 mM O_2 in a total volume of 3 ml at 37°C. The assay cells were equilibrated for 10 min after which ascorbate was added and the nonenzymatic rate of oxygen uptake determined. This background

rate arises from the autoxidation of ascorbate and was subtracted from the enzyme-derived rate for each assay. The assay was initiated by the addition of DβH. Catalase was included in the assay solution in order to prevent inactivation of DβH by hydrogen peroxide which is generated during the autoxidation of ascorbate.

Enzyme inactivation. The conditions for a typical incubation experiment were 100 mM Mes, pH 5.5, 100–250 μg/ml DβH, 250–500 μg/ml catalase, 12 mM ascorbate, varied concentrations of inhibitor, and 5–14% DMF (v/v) in 0.2 ml at room temperature. The incubation mixture was stirred under the desired oxygen concentration and aliquots (5–25 μl) removed through a septum cap in order to assay for activity as a function of time. The incubation experiment was initiated by the addition of either DβH or ascorbate. The rate of inactivation was obtained by determining the slope of the first order plot of ln specific activity (μmol O_2/min/mg enzyme) versus time.

Partition ratio determination. The partition ratio was determined using a similar reaction mixture. At 5- to 10-min intervals 15-μl aliquots were withdrawn and mixed with 30 μl of THF/methanol (2/1) on ice. The amount of product formed was determined by reverse phase HPLC analysis at 280 nm on a Waters system using a μ-Bondapak C-18 column. The column was eluted with 50 mM acetic acid/acetonitrile (70/30). The time course of product formation was followed and the amount of product at complete inactivation was determined as a measure of the partition ratio. Product was generated enzymatically as previously described (*6*).

Enzyme inactivation with 2-[^3H]-3-(p-HO-phenyl)-1-propene (**8c**) *and removal of catalase.* The specific radioactivity (cpm/mol) of **8c** was determined by counting a known quantity of the compound. Dopamine β-hydroxylase was incubated with **8c** at room temperature and 0.25 mM oxygen. Aliquots were removed and assayed for enzyme activity using the standard assay. After >95% loss of activity the incubation mixture was dialyzed against four changes of 200 mM NaCl, 50 mM potassium phosphate, pH 6.5, buffer at room temperature. Typically two control experiments were also run: (minus) ascorbate and (minus) DβH. These were treated in the same manner. In a typical stoichiometry experiment, 0.5–1.0 mg of DβH was labeled and an aliquot of the incubation mixture was counted to determine the amount of radioactive inhibitor bound to the enzyme. In experiments where peptides were to be generated, 4.0–6.0 mg of enzyme was labeled.

For determination of the stoichiometry of inhibitor bound to enzyme, the mixture was then loaded on a 3 mm × 0.5 cm Con A column preequilibrated in the above buffer to remove catalase. The column was washed until the effluent showed no absorbance above that due to the buffer. Routinely the first milliliter of effluent contained the catalase from the incubation mixture as evidenced by an absorbance at 410 nm due to the heme in catalase. Dopamine β-hydroxylase was eluted from the Con A column as follows. One column volume of a solution of 10% methyl α-mannoside in the above buffer was loaded on the column and the column clamped for 8 h. Upon reopening, the first milliliter of effluent contained greater than 95% of the DβH originally loaded. An aliquot was counted for the amount of radioactivity bound to the enzyme. The same procedure was used to generate peptides for analysis with the following changes. The fractions contain-

ing labeled DβH were combined and dialyzed versus 6 M guanidine–HCl, 0.25 M Tris, at pH 8.0 and room temperature in preparation for reduction and *S*-carboxymethylation.

Reduction and S-carboxymethylation. The disulfide bonds of the enzyme were reduced and *S*-carboxymethylated using established procedures (*17*). A known amount of labeled DβH was dialyzed in the guanidine containing buffer described above followed by reaction with a 10- to 20-fold excess of DTT over the number of sulfhydryl groups. DβH has been reported to have 14 sulfhydryl groups per monomer (*2*). The final concentration of DTT was adjusted to approximately 1.5 mM. The labeled enzyme and the DTT were mixed in the following fashion. The labeled enzyme was put into the bottom of a reaction vessel, which has a side arm. An aliquot of a stock DTT solution was placed into the side arm of the vessel. By means of an attached stopcock the vessel was alternately evacuated and refilled with nitrogen several times. After the last cycle, the vessel was tipped so that the two solutions were mixed together. The vessel was placed in a water bath at 37°C for 4 h. Next, 1.15 equivalents of iodoacetate over the total sulfhydryl content were added to the vessel. To do this the vessel was momentarily opened to add iodoacetate, reclosed, and immediately evacuated. The reaction was allowed to proceed at room temperature for 1 h. At this time the excess iodoacetate was quenched by the addition of one drop of β-mercaptoethanol. The reaction mixture was then dialyzed versus 50 mM potassium phosphate, pH 6.5, at room temperature in preparation for proteolysis.

Trypsin proteolysis. Reduced and *S*-carboxymethylated enzyme in 50 mM potassium phosphate, pH 6.5, was brought to 4 M in urea by the addition of solid urea. TPCK–trypsin was added to 2% (w/w) and allowed to react for 4 h at room temperature (*18*). This was followed by a second addition of trypsin, 2% (w/w), which was allowed to react overnight. The trypsin was added as an aliquot of a stock trypsin solution in 0.1 N hydrochloric acid. The stock solution was made fresh for each addition.

Peptide mapping. Proteolytically generated peptides were separated or mapped by reverse phase HPLC using a Vydac C-18 column (5 mm × 30 cm) on a Waters system. The peptides were eluted with 0.1% TFA and acetonitrile containing 0.1% TFA using a gradient from 0 to 70% acetonitrile over 100 min. The peptides were monitored with a uv detector at 214 nm. Fractions were collected every minute and analyzed for radioactivity by mixing with 10–15 ml of ScintiVerse II scintillation cocktail and counted on a Beckman LS 7500 liquid scintillation counter.

SYNTHESES

2-CH_3-3-(p-CH_3O-Phenyl)-1-propene **(3).** 3-CH_3-2-Cl-1-Propene **(2)** (9.0 g, 0.1 mol) was dissolved in 50 ml of THF and cooled to 0°C. To this was slowly added 77 ml (0.1 mol) of a 1.3 M THF solution of *p*-anisyl bromide **(1)** under nitrogen. The reaction was brought to room temperature and stirred for 6 h. The reaction mixture was poured into 100 ml of crushed ice containing 10 ml of concentrated HCl and stirred for 15 min. This was extracted 3× with ether and the ether layers

combined and washed with water. The ether layer was dried through magnesium sulfate and the solvent removed under vacuum leaving a yellow liquid which contained crystals. The crystals were filtered off and washed with hexane. The removal of the hexane left crude product which was purified by silica gel chromatography in hexane/benzene (3/1) yielding **3** (11.8 g, 73%): ^{1}H NMR δ 1.7 (3H, s), 3.2 (2H, s), 3.7 (3H, s), 4.6–4.9 (2H, m), 6.8–7.2 (4H, dd, J = 2, 9 Hz); mass spectrum, m/z 162 (M^+, 100), 147 (88), 131 (20), 121 (59).

2-CH_3-3-(p-HO-Phenyl)-1-propene **(4).** A NaH–oil dispersion was weighed into a flask under nitrogen (1.0 g, 0.025 mol). The dispersion was washed several times with dry hexane, in order to remove the oil, leaving white crystals of NaH. The NaH was covered with 3 ml of dry DMF. There was a small evolution of gas as the NaH reacted with trace water in the DMF. To this was added ethane thiol (1.13 ml, 0.015 mol) in 1 ml of DMF (*19*). The reaction mixture bubbled vigorously during the addition and was stirred under nitrogen for 10 min. To the generated ethane thiol anion was added **3** (1.0 g, 0.006 mol) dropwise. After complete addition, the reaction was gently refluxed for 2 h and then stirred at room temperature for 12 h. The reaction mixture was then poured into 100 ml of 10% HCl and extracted with ether. The ether layers were combined, washed with water, dried through magnesium sulfate, and then removed under vacuum. Purification by silica gel chromatography in $CHCl_3$ and vacuum distillation gave **4** (0.59 g, 66%): ^{1}H NMR δ 1.6 (3H, s), 3.2 (2H, s), 4.6–4.8 (2H, m), 5.1 (1H, s, exchangeable), 6.6–7.1 (4H, dd, J = 2, 10); mass spectrum, m/z 148 (M^+, 18), 133 (18), 122 (9), 107 (27), 28 (100).

2-Br-3-(p-CH_3O-Phenyl)-propene **(6).** To a solution of 2,3-dibromopropene **(5)** (14.5 g, 0.073 mol) in 50 ml of THF was slowly added 60.8 ml (0.073 mol) of a 1.2 M solution of *p*-anisyl magnesium bromide **(1).** The addition was performed under nitrogen and stirred for 18 h. This solution was then added to 100 ml of crushed ice containing 25 ml of concentrated HCl and stirred for 20 min. This was extracted with ether, the ether layers washed with water and dried by filtration through magnesium sulfate, and the solvent removed under vacuum. Silica gel chromatography in hexane/benzene (3/1) and vacuum distillation (110°C, 2 mm Hg) gave **6** (5.0 g, 30%): ^{1}H NMR δ 3.7 (2H, s), 3.8 (3H, s), 5.4–5.6 (2H, m), 6.7–7.2 (4H, dd, J = 2, 9); mass spectrum, m/z 226, 228 (M^+, 40, 41), 147 (53), 121 (100).

2-[^2H]-3-(p-CH_3O-Phenyl)-1-propene **(7b).** To a 50-ml pear-shaped flask with a septum-sealed side arm was added freshly cut magnesium ribbon, 0.15 g (0.0062 mol). The magnesium was covered with 0.5 ml of dry THF and stirred under nitrogen. To this was added one-half of 1.0 g (0.0044 mol) of **6** neat. After 5 min, three to four drops of dibromoethane was added. The reaction began to evolve heat after several minutes of stirring; the temperature was controlled with an ice-water bath. The remainder of the **6** was slowly added over 15 min. When the reaction had returned to room temperature, 0.1 ml of deuterated water (98%) was added via a syringe. The reaction mixture bubbled upon addition. This was stirred for an additional 15 min and then extracted with ether. The ether layers were washed with water and dried over magnesium sulfate. The solvent was removed under vacuum and the crude product purified by silica chromatography in hexane/benzene (3/1) and vacuum distillation to give **7b:** ^{1}H NMR δ 3.3 (2H, s), 3.8 (3H,

s), 4.9–5.2 (2H, m), 6.7–7.2 (4H, dd, J = 2, 8); mass spectrum, m/z 149 (M^+, 100), 147 (21), 134 (21), 121 (45).

2-[^2H]-3-(p-HO-Phenyl)-1-propene **(8b).** To 25 ml of dry CH_2Cl_2 was added **7b** (0.15 g, 0.001 mol). The solution was cooled to 0°C and stirred under nitrogen. To this was added 2 ml (0.002 mol) of a 1.0 M solution of BBr_3 in CH_2Cl_2 (*20*). Upon addition of the BBr_3 the reaction mixture turned purple. This was stirred for 1 h after reaching room temperature. The reaction mixture was poured into 50 ml of ice water and stirred for 15 min followed by extraction with CH_2Cl_2. The organic layer was washed and then dried over magnesium sulfate. The solvent was removed under vacuum. The product was purified by silica chromatography and vacuum distillation to give **8b** (0.05 g, 37%): ^1H NMR δ 3.3 (2H, s), 4.8–5.1 (2H, m), 5.3 (1H, s), 6.6–7.1 (4H, dd, J = 2, 8); mass spectrum, m/z 135 (M^+, 100), 134 (97), 133 (28), 107 (43).

2-[^3H]-3-(p-CH_3O-Phenyl)-1-propene **(7c).** This compound was synthesized in the same manner as **7b.** Magnesium and **6** were reacted together to generate the Grignard which was then quenched with tritiated water. The reaction was performed twice with water of different specific radioactivity. The first synthesis used tritiated water having a specific radioactivity of 100 mCi/mmol, the second having a specific radioactivity of 1 Ci/mmol. The compound was extracted from the reaction mixture as with **7b** followed by purification by silica chromatography in hexane/benzene (3/1). The recovered product behaved on silica TLC and HPLC identically as the nonradioactively labeled analog **(7a).**

2-[^3H]-3-(p-HO-Phenyl)-1-propene **(8c).** Demethylation of **7c** was accomplished as in the case of **7b** by treatment with BBr_3 in dry CH_2Cl_2. From the first synthesis of **7c,** 0.31 g (0.0014 mol) was demethylated yielding **8c** (0.085 g, 42%) (after silica chromatography purification) having a specific radioactivity of 0.49 cpm/pmol. From the second synthesis of **7c,** 0.46 g (0.002) was demethylated to give **8c** (0.003 g, 1%) (after purification by HPLC) having a specific radioactivity of 1.68 cpm/pmol. The conditions of HPLC purification were 50 mM acetic acid/acetonitrile (70/30) on a Waters C-18 semi-prep reverse phase column. The peak having a retention time of 16 min, which corresponds to the nonradioactive analog **8a,** was collected and extracted with ether. The ether was washed several times with water and then removed under vacuum. The specific radioactivity of **8c** was determined by counting the radioactivity of a known amount of compound.

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