Olefin Oxygenation and N-Dealkylation by Dopamine β-Monooxygenase: Catalysis and Mechanism-Based Inhibition†

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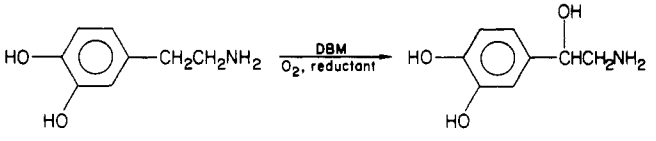
ABSTRACT: In an initial communication [May, S. W., Mueller, P. W., Padgette, S. R., Herman, H. H., & Phillips, R. S. (1983) *Biochem. Biophys. Res. Commun. 110*, 161–168], we reported that 1-phenyl-1-(aminomethyl)ethene hydrochloride (PAME) is an olefinic substrate for dopamine β-monooxygenase (DBM; EC 1.14.17.1) which inactivates the enzyme in an apparent mechanism-based manner. The present study further characterizes this reaction. The inactivation reaction yields k_{inact} = 0.23 min^{-1} at pH 5.0 and 37 °C and is strictly dependent on reductant (ascorbate) and oxygen. The DBM/PAME substrate reaction (apparent k_{cat} = 14 s^{-1}), shown to be stimulated by fumarate, gives the corresponding epoxide as product, identified by derivatization with 4-(*p*-nitrobenzyl)pyridine. However, the lack of DBM inhibition by α-methylstyrene oxide, and the observation of identical PAME/DBM inactivation rates in the absence and presence of preformed enzymatic PAME epoxide, indicates that free epoxide is not the inactivating species. A structure–activity study revealed that 4-hydroxylation of PAME (to give 4-HOPAME) increases both k_{inact} (0.81 min^{-1}) and apparent k_{cat} (56 s^{-1}) values, while 3-hydroxylation (to give 3-HOPAME) greatly diminishes inactivation activity while retaining substrate activity (apparent k_{cat} = 47 s^{-1}). 4-Hydroxy-α-methylstyrene was found to be a DBM inhibitor (k_{inact} = 0.53 min^{-1}) with weak substrate activity (apparent k_{cat} = 0.71 s^{-1}), while 3-hydroxy-α-methylstyrene and α-(cyanomethyl)styrene were found not to exhibit detectable DBM substrate activity and only weak inhibitory activity. 3-Phenylpropargylamine hydrochloride showed no detectable DBM substrate activity but rapidly inactivated the enzyme. A new substrate activity for DBM was discovered, N-dealkylation of *N*-phenylethylenediamine and *N*-methyl-*N*-phenylethylenediamine, and the lack of O-dealkylation activity with phenyl 2-aminoethyl ether and 4-hydroxyphenyl 2-aminoethyl ether indicates that DBM N-dealkylation proceeds via initial one-electron abstraction from the benzylic nitrogen heteroatom. With this new substrate and inhibitor reactivity information in hand, along with the other known substrate reactions, a DBM oxygenation mechanism analogous to that for cytochrome P-450 is proposed.

Dopamine β-monooxygenase (DBM; EC 1.14.17.1),[1] a copper-containing monooxygenase, catalyzes the in vivo hydroxylation of dopamine (DA) to norepinephrine (NE) and thus plays a key role in neurotransmitter interconversion (Levin & Kaufman, 1961; Freidman & Kaufman, 1965; van der Schoot & Creveling, 1965; Skotland & Ljones, 1979; Rosenberg & Lovenberg, 1980; Villafranca, 1981):



DA NE

In appropriately designed substrate and product analogues, DBM has been found to also catalyze sulfoxidation (May & Phillips, 1980; May et al., 1981a), oxygenative ketonization (May et al., 1981b, 1982; Klinman & Kreuger, 1982; Mangold & Klinman, 1984), selenoxidation (May et al., 1984; Padgette et al., 1985), and olefin oxygenation (May et al., 1984; Padgette et al., 1985) reactions, and alkyne reactivity has also been reported (Colmbo & Villafranca, 1984). In addition, the pharmacological and therapeutic potentials of alternate DBM substrates and inhibitors are being actively investigated in our laboratory (Herman et al., 1983; Padgette et al., 1984). We have also been interested in chemically probing the nature of DBM–copper catalysis for comparison with our mechanistic studies on non-heme iron monooxygenase catalysis (Katopodis et al., 1984; May et al., 1977) and to complement recent kinetic studies on DBM hydroxylation (Miller & Klinman, 1983; Ahn & Klinman, 1983) and on the nature of the enzyme-bound copper (Ash et al., 1984; Klinman et al., 1984).

In an initial communication (May et al., 1983), we reported that 1-phenyl-1-(aminomethyl)ethene hydrochloride (PAME) was the first known benzylic olefinic oxygenation substrate for DBM and that enzyme inactivation proceeded concomitant with turnover. The expected stoichiometry of the reaction was observed, with the product being identified as the corresponding diol, 1,2-dihydroxy-2-phenylpropylamine (DHPPA) after acid treatment, suggesting that the epoxide derivative of PAME was being formed by DBM and hydrolyzed to DHPPA under acidic conditions.

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[1] Abbreviations: BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Con A, concanavalin A; DBM, dopamine β-monooxygenase; DHPPA, 1,2-dihydroxy-2-phenylpropylamine; DMF, dimethylformamide; 3-HOPAME, 3-amino-2-(3-hydroxyphenyl)propene; 4-HOPAME, 3-amino-2-(4-hydroxyphenyl)propene; 4-HOPAEE, 4-hydroxyphenyl 2-aminoethyl ether; *N*-MePEDA, *N*-methyl-*N*-phenylethylenediamine; NBP, 4-(*p*-nitrobenzyl)pyridine; NBS, *N*-bromosuccinimide; PAEE, phenyl 2-aminoethyl ether; PAME, phenyl(aminomethyl)ethene hydrochloride; PEA, phenylethylamine; PEDA, *N*-phenylethylenediamine; SNPA, *N*-succinimidyl (*p*-nitrophenyl)acetate; *N*-TFA-PAME, *N*-(trifluoroacetyl)phenyl(aminomethyl)ethene; *N*-TFA-DHPPA, *N*-(trifluoroacetyl)-1,2-dihydroxy-2-phenylpropylamine; THF, tetrahydrofuran; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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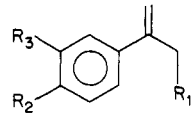
Chart I: Olefinic DBM Substrate Analogues



no.	compd	R_1	R_2	R_3
I	α-methylstyrene	H	H	H
II	4-hydroxy-α-methylstyrene	H	OH	H
III	3-hydroxy-α-methylstyrene	H	H	OH
IV	α-(cyanomethyl)styrene	CN	H	H
V	PAME	$NH_3^+Cl^-$	H	H
VI	4-HOPAME	$NH_3^+Cl^-$	OH	H
VII	3-HOPAME	$NH_3^+Cl^-$	H	OH

The DBM/PAME inactivation reaction showed saturable pseudo-first-order kinetics, and no reversal of inactivation was seen upon dialysis, which suggests that DBM was being inactivated either by an intermediate along the pathway to the epoxide product, as has been demonstrated for cytochrome P-450 olefin inactivation (Ortiz de Montellano et al., 1982), or by the epoxide product itself.

We have now extended our initial findings on the DBM/PAME reaction, including identification of the epoxide product itself, and also present additional DBM reactivity data on a number of new olefinic substrate analogues. All alternate olefinic substrate analogues studied can be considered derivatives of α-methylstyrene (Chart I), with this terminal double-bond configuration apparently favored over that of the straight-chain PAME isomer cinnamylamine. Cinnamylamine was found not to exhibit DBM substrate activity, and only minor inhibitory activity was observed (May et al., 1983).

A number of the new olefins tested show potent, ascorbate-dependent reactivity with DBM, and all analogues that inhibit the enzyme appear to be operating in a mechanism-based mode of inactivation. Because of the similarities between the emerging view of DBM–Cu^{2+} catalysis and the well-studied chemistry of the P-450 family of oxygenases, additional compounds have been designed and synthesized to see how far DBM proceeds in mimicking P-450 reactivity. Along these lines, we also report herein that DBM indeed does catalyze another classic P-450 reaction, N-dealkylation (Miwa et al., 1983), although time-dependent irreversible inactivation occurs at longer reaction times. N-Dealkylation obeys the usual DBM stoichiometry and proceeds with significant turnover activity. With the extended olefin data in hand, along with the newly discovered DBM N-dealkylation activity, a general DBM catalytic mechanism is proposed analogous to P-450 catalysis.

Experimental Procedures

Materials and Methods

Materials. Tyramine hydrochloride, ascorbic acid, and sodium fumarate were obtained from Sigma Chemical Co. PEDA (Aldrich) was crystallized as the monohydrochloride ($EtOH/Et_2O$), mp 210–211 °C. 4-(*p*-Nitrobenzyl)pyridine (NBP) was also from Aldrich. *N*-Succinimidyl (*p*-nitrophenyl)acetate (SNPA) was from Pierce. [^{3}H]PAME was prepared by nonspecific T_2 exchange by the method of Bush et al. (1981) and was kindly supplied by Dr. James Powers. Beef liver catalase (65 000 units/mg) was obtained from Boehringer Mannheim. Other chemicals and solvents were obtained from standard commerical sources. HPLC analyses were performed on an LDC Constametric III system with an LDC SpectroMonitor III Model 1204D variable wavelength detector.

Enzyme Isolation and Assay. Dopamine β-monooxygnenase was isolated and purified from bovine adrenals according to the procedure of Ljones et al. (1976), with minor modifications, and exhibited a specific activity of 20–32 units/mg (1 unit is defined as 1 μmol/min of oxygen with 10 mM tyramine as substrate in the standard oxygen monitoring assay). The concentration of purified DBM tetramer [M_r 290,000 (Friedman & Kaufman, 1965)] was estimated by absorbance ($\epsilon_{280}^{1\%} = 12.4$). Kinetic constants (apparent k_{cat} and K_m) of substrates with dopamine β-monooxygenase were determined by using the polarographic oxygen monitor assay and were calculated by computer fit of the data to the hyperbolic form of the Michaelis–Menten equation (Cleland, 1967). The standard DBM assay mixture, utilizing a YSI Model 53 polarographic oxygen monitor, contained 0.12 M sodium acetate buffer, pH 5.0, in the presence of 10 mM sodium fumarate, 0.3 mg/mL catalase, 5 μM $CuSO_4$, 10 mM ascorbic acid, and 4–7 μg of DBM, at atmospheric oxygen saturation in a total volume of 2.5 mL. In our assay system, atmospheric oxygen saturation was found to be 250 μM (May et al., 1981a,b). Enzymatic reactions were initiated with substrate unless otherwise noted, and the initial rate was measured as the rate of oxygen consumption minus the small background ascorbic acid autoxidation rate.

DBM Reactivity with Olefinic Substrates and Inhibitors

Time-Dependent Inactivation of DBM: Dilution Method 1. Inactivation reactions, performed at 30 °C, contained 0.12 M sodium acetate buffer (pH 5.0), 9–10 mM sodium fumarate, 0.5 μM $CuSO_4$, and 0.275 mg/ml catalase, at the indicated DBM concentration, at various inhibitor concentrations in a 0.400 mL total volume. The inactivation reactions were initiated by adding ascorbate to a final concentration of 10.0 mM. The activity of DBM vs. time was assayed by the dilution of 40-μL aliquots of the inactivation mixture into the standard DBM assay mixture (2.5 mL total volume). First-order inactivation constants were determined by linear regression analysis as the slopes of lines (k_{obsd}) resulting from plots of ln (percent activity remaining) vs. time. Plots of $1/k_{obsd}$ vs. 1/[inhibitor] were linear and were fitted by linear regression analysis, with k_{inact} calculated as $1/(y$ intercept) and K_I as $-1/x$ intercept). This protocol was used for inhibition assays with compounds requiring 15% DMF cosolvent.

Time-Dependent Inactivation of DBM: Dilution Method 2. An aliquot (30 μL withdrawn, 25 μL injected) of enzyme inactivation mixture (0.280 mL) was assayed for initial DBM activity before initiation with an inhibitor–ascorbate solution (32 μL). The final inactivation solution (0.282 mL) contained 10 mM sodium fumarate, 0.3 mg/mL catalase, 0.5 or 5 μM $CuSO_4$, and 10 mM ascorbate, at varying inhibitor concentrations and the indicated DBM concentration, in 0.12 M NaOAc buffer, pH 5.0, at either 25 or 37 °C. Aliquots (28 μL) were assayed under standard DBM assay conditions vs. time. Data (percent initial activity vs. time) were analyzed as given above for dilution method 1.

Time-Dependent Inactivation of DBM: Progress Curve Method. Aliquots (2.5 mL) of the standard DBM assay mixture (without DBM), containing 0.12 M sodium acetate buffer, pH 5.0, in the presence of 10 mM sodium fumarate, 0.3 mg/mL catalase, 0.5 μM $CuSO_4$, 10 mM ascorbic acid, and 1–10 mM 4-HOPAME·HCl at atmospheric oxygenation, were incubated at 37 °C in the polarographic oxygen-monitoring apparatus. Base lines stabilized without any oxygen consumption in 1–2 min. The reaction was initiated by the addition of 9 μg of DBM (specific activity 33.4 units/mg). A progress curve was recorded for the first 3 min, a time period during which substrate concentration is essentially constant. The method of Main (1973) was used to calculate k_{inact} and K_I from these data.

[^3H]PAME Radioactive Labeling of DBM. An inactivation solution was prepared containing 0.120 M NaOAc (pH 5.64), 10 mM sodium fumarate, 5.6 μM $CuSO_4$, 0.30 mg/mL catalase, 10 mM ascorbate, 0.60 mg of DBM, and 39.1 mM (0.60 mg) [^{3}H]PAME (specific activity 3.26–5.64 μCi/μmol), in a total volume of 0.80 mL. The inactivation was allowed to proceed with intermittent stirring at room temperature for 3 h. No activity was detectable in an identical parallel inactivation reaction after 2 h. The inactivation solution was loaded onto a concanavalin A– (Con A–) Sepharose (Sigma) affinity column at 10 mL/h and then washed with 50 mM potassium phosphate buffer (KP_i) containing 0.20 M NaCl (pH 6.5) at 40 mL/h until the eluent fractions contained 0.2% of the maximal radioactivity contained by a wash fraction (unretained [^{3}H]PAME). Separate control experiments verified that neither catalase (determined by enzymatic assay) nor PAME (determined by HPLC) is retained by Con A–Sepharose. The Con A resin was eluted by pumping 10 mL of 0.50 M methyl α-D-mannoside (in 50 mM KP_i, pH 6.5, 0.20 M NaCl) up through the column. The collected resin solution was stirred for 1 h at room temperature and centrifuged, and the supernatant was transferred to an Amicon cell with XM100 membrane, concentrated, and ultrafiltered against 10 volumes of 10 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) (pH 7.0). The retentate was removed, and DBM concentration (A_{280}) and radioactivity were determined. The control reaction was treated identically except ascorbate was omitted from the inactivation solution.

Oxygen to Product Stoichiometry of the DBM/PAME Reaction. For each experiment, the O_2-monitor assay mixture contained 0.127 M NaOAc (pH 5.0), 10 mM sodium fumarate, 0.29 mg/mL catalase, 5.7 μM $CuSO_4$, 20 mM PAME·HCl, and 10 mM ascorbate. Enzymatic reactions were initiated by adding 25 μL (0.13 mg) of DBM, and the O_2 uptake was followed until inactivation had decreased the rate to equal the previously recorded ascorbate background. Enzymatic reactions were run paired with an identical control reaction, minus DBM, and reactions and controls were worked up identically. The reaction was quenched with 170 μL of 9 M H_2SO_4 (by adjusting pH to 0.3–0.5) and shaken at 40 °C for 2 h (unless otherwise noted) followed by the addition of 7.4 μL of 67.9 mM phenethylamine hydrochloride (PEA) (0.50 μmol) as internal standard. The hydrolysate was centrifugated at ~3000 rpm for 10 min, and the supernatant was basified to pH 12.5–13.0 with 50% NaOH and extracted with EtOAc. The combined EtOAc extracts were dried and dissolved in 2.0 mL of THF plus 2.0 mL of H_2O, and 25–27 mg of *N*-succinimidyl (*p*-nitrophenyl)acetate (SNPA) was added with swirling. After the mixtures were allowed to stand at room temperature for 30 min, 1 drop of saturated Na_2CO_3 was added, and the resulting solution was analyzed by HPLC.

The HPLC analysis (20-μL filled injection loop) were performed on a Laboratory Data Control C-18 reverse phase column (10 cm, 5-Å pore, low load) with 50% MeOH and 50% 10 mM NaOAc (v/v), pH 5.0, as mobile phase, at 1.50 mL/min, with UV detection at 280 nm at 0.032 absorbance unit full scale. Samples for standard curves were prepared by mixing DHPPA with PEA (0.50 μmol) in 2.5 mL of 0.127 M NaOAc (pH 5.0) followed by basification, EtOAc extraction, and SNPA derivatization exactly as described for the enzymatic reactions and controls. A standard based on derivatized DHPPA peak heights was linear from 0.10 to 0.90 μmol of DHPPA.

NBP Derivatization of Epoxides. The following procedures are based on the general method of Nells et al. (1982), with modifications. Prior to analyzing the DBM/PAME reaction product, detection of α-methylstyrene oxide, a model compound for PAME epoxide, was optimized. Solutions of 6% 4-(*p*-nitrobenzyl)pyridine (NBP) in ethylene glycol (w/v) (1.0 mL), 100 mM Tris-HCl, pH 7.4/27% absolute EtOH (v/v) (0.70 mL), and 0.24 μmol α-methylstyrene oxide (0.10 mL in absolute EtOH) were incubated at 60 °C for varying time periods with shaking. Sample tubes were removed and immersed in ice, and 50% Et_3N in acetone (v/v) (2.0 mL) was added, and the tubes were vortexed. Absorbance at 560 nm was measured exactly 1 min after Et_3N addition.

For the detection of the DBM/PAME epoxide reaction product, the following procedure was followed. Standard DBM assay conditions (pH 5.0) were employed, with 23 mM PAME and 14.8 μg of DBM in a total volume of 1.04 mL. Controls (–enzyme; –ascorbate; –PAME; reaction blank without enzyme, DBM, or ascorbate) were run in parallel, substituting buffer for the omitted components. The reaction and controls were incubated at 37 °C with shaking for 64 min, when 0.40-mL aliquots were removed. To each solution was added 6% NBP in ethylene glycol (w/v) (1.0 mL) and 200 mM Tris-HCl, pH 7.63/27% absolute EtOH (v/v) (0.40 mL), and the resulting solutions were incubated at 60 °C with shaking for 1 h. These solutions were cooled in ice, and 50% Et_3N in acetone (v/v) (2.0 mL) was added followed by vortexing and recording the spectrum vs. distilled water exactly 1 min after Et_3N addition on a Hewlett-Packard 8451A photodiode array spectrophotometer.

HPLC Detection of N-Dealkylation Products. Aniline, one product of the N-dealkylation of PEDA, was identified and quantified by reverse-phase HPLC, with 65% 0.10 M NaOAc, pH 5.83, 5 mM sodium octylsulfonate (SOS), and 35% MeOH (v/v) as mobile phase at 1.0 mL/min. Dealkylated product were monitored by UV detection at 250 nm. Enzymatically derived aniline was quantitated by using a standard curve based on peak height. Identical HPLC procedures and conditions were used for the identification and quantification of the N-dealkylated product of *N*-MePEDA, *N*-methylaniline.

Syntheses

3-Bromo-2-phenylpropene. This material was prepared by the method of Pines et al. (1957), subsequently modified by Reed (1965). Distillation gave 3-bromo-2-phenylpropene (allylic isomer) plus 1-bromo-2-phenylpropene (vinylic isomer) codistilling side product (bp 99.5–100.5 °C, 5 mm). ^{1}H NMR showed 56% allylic isomer and 44% vinylic isomer: ^{1}H NMR (allylic isomer) (neat) δ 7.47–6.96 (m), 5.30 (s, 1 H), 5.20 (s, 1 H), 4.08 (s, 2 H).

1-Phenyl-1-(aminomethyl)ethene Hydrochloride (PAME) (3-Amino-2-phenylpropene). The distilled 3-bromo-2-phenylpropene mixture (154.88 g, 0.440 mol of allylic isomer) in THF (50 mL) was added dropwise to a stirring solution of liquid NH_3 (300 mL). After the solution was stirred for 1 h, the NH_3 was allowed to evaporate, the resulting solid was filtered and washed with THF, and the filtrate was concentrated. To the residual liquid was added 2 N HCl (250 mL), and the resultant mixture was extracted with ether (2 × 100 mL). The aqueous phase was basified to pH 11, and the solution again was extracted with ether. The ether extract was dried, filtered, and evaporated, giving light orange, crude 3-amino-2-phenylpropene (58.0 g, 99.1%), which was distilled to give clear, colorless pure material (bp 86.5–105.5 °C, 2 mm) (36.99 g, 63.2%). The free amine was dissolved in EtOH (30 mL) and cooled to 0 °C, concentrated HCl (25.5 mL, 1.1 equiv) was added, and the resulting solution was triturated with ether to give white, crystalline PAME·HCl (45.25 g,

60.60%): mp 178–179 °C; ^{1}H NMR (trifluoroacetic acid) δ 7.46 (s, 5 H), 5.76 (s, 1 H), 5.62 (s, 1 H), 4.40 (q, 2 H); mass spectrum (EI), *m/e* 133 (M^+). Anal. Calcd for $C_9H_{11}N$·HCl: C, 63.71; H, 7.13; N, 8.26. Found: C, 63.78; H, 7.18; N, 8.25. Similar syntheses have been published (Panzik & Mulvaney, 1972; Patanova et al., 1975).

N-(Trifluoroacetyl)-PAME (N-TFA-PAME). The following procedure is based on that of Weygand & Csendes (1952). PAME free amine (4.304 g, 32.4 mmol) and Et_3N (5.0 mL, 35.6 mmol) were dissolved in benzene (30 mL) with stirring, and trifluoroacetic anhydride (6.86 mL, 48.6 mmol) was added. The reaction mixture was heated at 50 °C for 12 h, cooled to room temperature, and washed with saturated $NaHCO_3$, 1 N HCl, and saturated NaCl. The benzene phase was dried, filtered, and evaporated, giving crude *N*-TFA-PAME, which was recrystallized to give white *N*-TFA-PAME (4.154 g, 49%): mp 101.5–103 °C; ^{1}H NMR δ 7.37 (s, 5 H), 5.53 (s, 1 H), 5.30 (s, 1 H), 4.42 (d, 2 H); mass spectrum (EI), *m/e* 229 (M^+).

N-(Trifluoroacetyl)-1,2-dihydroxy-2-phenylpropylamine (N-TFA-DHPPA). The general method of Baran (1960) was followed. A mixture of pyridine (60 mL), *N*-TFA-PAME (2.677 g, 11.7 mmol), and OsO_4 (3.0 g, 11.8 mmol) was stirred at room temperature for 2 h, and a mixture of $NaHSO_3$ (5.4 g), H_2O (81 mL), and pyridine was added. After being stirred an additional 15 h, the clear, dark orange reaction mixture was extracted with $CHCl_3$, washed with 0.2 N HCl–saturated NaCl, dried, filtered, and evaporated, to give *N*-TFA-1,2-dihydroxy-2-phenylpropylamine as a yellow oil: mass spectrum (CI), *m/e* 264 (M + 1).

1,2-Dihydroxy-2-phenylpropylamine Hydrochloride (DHPPA). The crude *N*-TFA-DHPPA was stirred in absolute EtOH and 1 N NaOH overnight. The resulting pale yellow solution was extracted with $CHCl_3$, dried, filtered, and evaporated, giving DHPPA free amine as a yellow oil, which was crystallized as the HCl salt by triturating with ether after treatment with HCl in EtOH. White, crystalline DHPPA·HCl (1.17 g, 49% yield based on *N*-TFA-PAME), mp 152–153 °C, was obtained after ether washing and vacuum drying: ^{1}H NMR (D_2O) δ 7.53 (s, 5 H), 3.90 (s, 2 H), 3.50 (s, 2 H); mass spectrum (CI), *m/e* 168 (M + 1). Anal. Calcd for $C_9H_{13}NO_2$·HCl: C, 53.08; H, 6.93; N, 6.88. Found: C, 53.22; H, 6.93; N, 6.87.

(3-Hydroxyphenyl)dimethylcarbinol. This compound was synthesized by the method of Gilman et al. (1954) from methyl *m*-hydroxybenzoate and 3.3 equiv of methylmagnesium iodide: mp 97–98 °C (lit. mp 97–101 °C); ^{1}H NMR (D_2O) δ 6.29–7.18 (m, 4 H), 1.48 (s, 6 H); mass spectrum (EI), *m/e* 152 (M^+).

(4-Hydroxyphenyl)dimethylcarbinol. This compound was synthesized by the general procedure of Gilman et al. used above for the meta isomer, except starting with methyl *p*-hydroxybenzoate and omitting the base hydrolysis workup. The crude solid was recrystallized (benzene/ethyl acetate) to give a white crystalline solid: mp 107–110 °C (49%); ^{1}H NMR (acetone-d_6) δ 8.18 (s, 1 H), 7.11 (d, d, 4 H), 3.90 (s, 1 H), 1.49 (s, 6 H); mass spectrum (EI), *m/e* 152 (M^+).

3-Acetoxy-α-methylstyrene (m-Isopropenylphenyl Acetate). This compound was synthesized by the method of Gilman et al. (1954), by refluxing (3-hydroxyphenyl)dimethylcarbinol with an excess of acetic anhydride followed by solvent evaporation and distillation (bp 138–139 °C, 11 mmHg): ^{1}H NMR (acetone-d_6) δ 6.90–7.47 (m, 4 H), 5.45 (s, 1 H), 5.15 (m, 1 H), 2.22 (s, 3 H), 2.12 (m, 3 H); mass spectrum (EI), *m/e* 176 (M^+).

4-Acetoxy-α-methylstyrene (p-Isopropenylphenyl Acetate). (4-hydroxyphenyl)dimethylcarbinol (50.0 g, 0.329 mol) was refluxed under nitrogen with acetic anhydride (140 mL, 1.27 mol, 3.85 equiv) overnight. The reaction mixture was concentrated and then distilled to give 4-acetoxy-α-methylstyrene (41.4 g, 76%) (bp 83–85 °C, 0.3 mmHg) as a clear, colorless liquid: ^{1}H NMR ($CDCl_3$) δ 7.23 (d, d, 4 H), 5.31 (m, 1 H), 5.05 (m, 1 H), 2.23 (s, 3 H), 2.12 (m, 3 H); mass spectrum (EI), *m/e* 176 (M^+).

4-Hydroxy-α-methylstyrene (p-Isopropenylphenol). A mixture of *p*-isopropenylphenyl acetate (0.734 g, 4.16 mmol), 10% NaOH (3.0 mL, 7.5 mmol, 1.8 equiv), and MeOH (2.0 mL) was stirred under nitrogen for 2.5 h at room temperature in the dark. The MeOH was evaporated, and the solution was acidified to pH 6.8 with HCl (4 M), which caused the precipitation of a white solid. The solid was collected, washed with cold water, and vacuum dried, giving *p*-isopropenylphenol as a pale yellow solid (0.488 g, 88%). After recrystallization (cyclohexane), the material was a white solid: mp 80–82 °C [lit. mp 83–84 °C (Corson et al., 1958)]; ^{1}H NMR (acetone-d_6) δ 7.10 (d, d, 5 H), 5.29 (m, 1 H), 4.95 (m, 1 H), 2.10 (m, 3 H).

3-Hydroxy-α-methylstyrene (m-Isopropenylphenol). This compound was prepared as above and was isolated as a clear, colorless liquid and distilled: bp 78–81 °C (1.0 mmHg); ^{1}H NMR (acetone-d_6) δ 7.43–6.67 (m, 4 H), 5.39 (m, 1 H), 5.09 (m, 1 H), 2.90 (s, 1 H), 2.13 (m, 3 H). The liquid crystallized upon refrigeration.

3-Acetoxy-α-(bromomethyl)styrene. A mixture of *m*-isopropenylphenyl acetate (8.8 g, 50 mmol), *N*-bromosuccinimide (NBS) (recrystallized) (8.9 g, 50 mmol), and CCl_4 (3 mL) was heated to reflux with stirring until all of the NBS went into solution. The reaction mixture was cooled to room temperature, and the white solid formed was filtered and washed with CCl_4. Upon evaporation, ^{1}H NMR showed complete conversion to the allylic/vinylic bromide mixture (55% allylic isomer by ^{1}H NMR). This material could be used without purification; simple distillation yielded the bromide mixture: bp 96–122 °C (1.6 mmHg); ^{1}H NMR ($CDCl_3$) (allylic isomer) δ 6.92–7.45 (m, 4 H), 5.56 (s, 1 H), 5.51 (s, 1 H), 4.33 (s, 2 H), 2.28 (s, 3 H).

4-Acetoxy-α-(bromomethyl)styrene. A mixture of 4-acetoxy-α-methylstyrene (21.0 g, 0.120 mol), *N*-bromosuccinimide (21.4 g, 0.120 mol), and CCl_4 (5.0 mL) was heated to reflux, and 5 mg of benzoyl peroxide was added. The reaction mixture refluxed rapidly giving a yellowish brown clear solution. After CCl_4 evaporation, the crude product (20.0 g, 63% allylic isomer by ^{1}H NMR) showed complete conversion to the bromide isomers and was used without further purification: ^{1}H NMR (allylic isomer) ($CDCl_3$) δ 6.94–7.74 (m), 5.54 (s, 1 H), 5.50 (s, 1 H), 4.32 (s, 2 H), 2.24 (s, 3 H).

N-[2-(3-Acetoxyphenyl)-2-propenyl]phthalimide. The following procedures are based on the general method of Panzik & Mulvaney (1972). A solution of potassium phthalimide (0.650 g, 3.53 mmol) in DMF (6.2 mL), under N_2 atmosphere, was heated to 70 °C and 3-acetoxy-α-(bromomethyl)styrene (0.853 g of allylic isomer, 3.34 mmol) was added dropwise, causing the reaction temperature to rise to 78 °C. The reaction was stirred for 2 h at 70 °C, the DMF was removed by evaporation and $CHCl_3$ (15 mL) added, and the resulting solution was extracted with H_2O, 0.1 N NaOH, and H_2O. After drying, filtration, and evaporation, the resulting oil was taken up in MeOH and triturated with H_2O, causing the precipitation of a white solid. Collection yielded the product phthalimide derivative (0.556 g, 52%): mp 86–87

°C; ^{1}H NMR ($CDCl_3$) δ 7.60–8.07 (m, 4 H), 6.92–7.54 (m, 4 H), 5.49 (m, 1 H), 5.25 (m, 1 H), 4.72 (s, 2 H), 2.30 (s, 3 H).

N-[2-(4-Acetoxyphenyl)-2-propenyl]phthalimide. A mixture of potassium phthalimide (1.0 g, 5.4 mmol), DMF, K_2CO_3, and 4-acetoxy-α-(bromomethyl)styrene (1.38 g of allylic isomer by NMR, 5.4 mmol) was heated to 60 °C under N_2. After the reaction temperature rose to 90 °C, the mixture was stirred for an additional 30 min, cooled, and taken up in $CHCl_3$. After the mixture was washed with H_2O, the $CHCl_3$ layer was dried, filtered, and evaporated, giving a pale yellow crystalline solid which was recrystallized ($CHCl_3$/hexane): ^{1}H NMR ($CDCl_3$) δ 7.70–8.10 (m, 4 H), 7.33 (d, d, 4 H), 4.48 (s, 1 H), 5.23 (s, 1 H), 4.73 (s, 2 H), 2.30 (s, 3 H).

3-HOPAME [3-Amino-2-(3-hydroxyphenyl)propene]. A mixture of *N*-[2-(3-acetoxyphenyl)-2-propenyl]phthalimide (3.5 g, 10.4 mol), anhydrous hydrazine (1.0 mL, 32.3 mmol), and 95% EtOH (55 mL) was refluxed under N_2 for 1 h. After the mixture was cooled, the EtOH was removed by evaporation, and the resulting solid was dissolved in 1 N NaOH and adjusted to pH 9.3 with HCl. After extraction of the resulting solution with warm EtOAc, the EtOAc extract was dried, filtered, and evaporated to give 3-HOPAME free amine (1.67 g). The free amine was dissolved in MeOH, cooled on ice, and concentrated HCl (0.93 mL) was added followed by trituration with Et_2O to give a light brown solid, 3-HOPAME·HCl (0.606 g, 31%). This material was recrystallized (EtOH/Et_2O) to give a light tan crystalline solid: mp 143–145 °C; ^{1}H NMR (D_2O) δ 6.86–7.59 (m, 4 H), 5.73 (s, 1 H), 5.54 (m, 1 H), 4.13 (s, 2 H); mass spectrum (EI), *m/e* 149 (M^+), (CI) *m/e* 150 (M + 1).

4-HOPAME [3-Amino-2-(4-hydroxyphenyl)propene]. A mixture of *N*-[2-(4-acetoxyphenyl)-2-propenyl]phthalimide (4.0 g, 11.9 mmol), anhydrous hydrazine (0.80 mL, 25.8 mmol), and 95% EtOH (25.0 mL) was refluxed under N_2 for 1 h. After EtOH removal, the resulting solid was dissolved in 1 N NaOH and extracted with $CHCl_3$, and the aqueous layer was carefully adjusted to pH 9.5 with concentrated HCl, causing the precipitation of a white solid which was separated by filtration and air dried, giving 4-HOPAME free amine (0.60 g, 34%). The free amine was converted to the HCl salt as described above to give 4-HOPAME·HCl: mp 175 °C (dec); ^{1}H NMR (free amine) (Me_2SO-d_6/acetone-d_6) δ 7.06 (d, d, 4 H), 5.33 (s, 1 H), 5.12 (m, 1 H), 4.25 (s, 2 H); mass spectrum (EI), *m/e* 149 (M^+), (CI) *m/e* 150 (M + 1). Anal. Calcd for $C_9H_{12}NOCl$: C, 58.22; H, 6.47, N, 7.55; Cl, 19.14. Found: C, 58.17; H, 6.49; N, 7.51; Cl, 19.07.

α-(Cyanomethyl)styrene. A mixture of α-(bromomethyl)styrene (23.4 g of allylic isomer, 0.119 mol), acetonitrile (190 mL), and CuCN (28.7 g, 0.32 mol) was stirred under nitrogen for 9 h at 60 °C, and after cooling, the reaction mixture was filtered and washed with several portions of acetonitrile [see Reed (1965, 1967) and Supniewski et al. (1932)]. The combined acetonitrile solution was evaporated, H_2O and Et_2O were added, and the resulting mixture was stirred vigorously and separated. The combined Et_2O extracts were washed with saturated NaCl, dried, filtered, and evaporated to give crude α-(cyanomethyl)styrene (41.32 g, 39% allylic cyanide by HPLC). A portion of the crude product (21.2 g) was distilled, giving 97% pure α-(cyanomethyl)styrene by HPLC (bp 77–78 °C, 0.4 mm) (5.85 g, 67% based on starting allylic bromide): ^{1}H NMR (acetone-d_6) δ 7.71–7.21 (m, 5 H), 5.65 (s, 1 H), 5.49 (m, 1 H), 3.75 (s, 1 H).

α-Methylstyrene Oxide. The procedure of Guss & Rosenthal (1955) was followed, giving a clear, colorless liquid; bp 67–68 °C (5 mmHg); ^{1}H NMR ($CDCl_3$) δ 7.30 (s, 5 H), 2.89 (d, d, 2 H, J = 12, 6 Hz), 1.67 (s, 3 H); TLC, one spot, R_f 0.35 ($CHCl_3$).

Phenylpropargylamine Hydrochloride. This compound was prepared by the method of Klemm et al. (1971) and gave an identical NMR spectrum, mp 223 °C dec (lit. mp 217 °C).

N-Methyl-N-phenylethylenediamine (N-MePEDA). A solution of *N*-methyl-*N*-phenylethanolamine (7.0 g, 46 mmol) and tosyl chloride (10.0 g, 52.5 mmol) in Et_3N (50 mL) was stirred for 2 h at room temperature, refrigerated for 24 h, diluted with Et_2O, filtered, and evaporated. The product tosylate derivative (12 g, 86%, 40 mmol) was dissolved in MeOH, and the resulting solution was saturated with NH_3(g) and stirred for 2 h at 0 °C followed by 2 h at room temperature. The reaction mixture was evaporated to dryness, dissolved in H_2O, basified to pH 13, and extracted with Et_2O. The extracts were dried, filtered, and evaporated to yield *N*-MePEDA free amine (4.5 g, 75%), which was crystallized from EtOH/Et_2O to give a white solid: mp 199–200 °C; ^{1}H NMR (D_2O) δ 7.63 (m, 5 H), 4.07 (m, 2 H), 3.37 (s, 3 H), 3.27 (m, 2 H).

Phenyl 2-Aminoethyl Ether (PAEE). A mixture of phenol (14.0 g, 0.20 mol), KOH (14.0 g, 0.25 mol), chloroacetonitrile (14 mL, 0.21 mol), and MeOH (200 mL) was stirred under N_2 for 24 h at room temperature. The reaction mixture was poured into ice-cold water and extracted with Et_2O, and the Et_2O extracts were washed with 0.1 N NaOH and brine, then dried, filtered, and evaporated to give phenoxyacetonitrile (15.2 g, 59%): ^{1}H NMR ($CDCl_3$) δ 7.50–6.80 (m, 5 H), 4.4 (s, 2 H).

A mixture of phenoxyacetonitrile (13.3 g, 0.10 mol), $LiAlH_4/AlCl_3$ (0.10 mol each), and anhydrous Et_2O (300 mL) was refluxed for 3 h under nitrogen. The excess $LiAlH_4$ was destroyed by careful addition of H_2O, and the product was extracted with Et_2O, dried, filtered, and evaporated to give phenyl 2-aminoethyl ether (PAEE) free amine, which was crystallized as the HCl salt from EtOH/Et_2O: mp 221–222 °C; ^{1}H NMR (D_2O) δ 7.00–7.65 (m, 5 H), 3.50 (t, 2 H), 4.40 (t, 2 H). Anal. Calcd for $C_8H_{11}NO$·HCl: C, 55.34; H, 6.97; N, 8.06. Found: C, 55.50; H, 7.01; N, 8.03.

4-Hydroxy-PAEE. *p*-hydroxyphenoxyacetic acid was esterified with methanolic HCl in quantitative yield. The methyl ester was recrystallized from $CHCl_3$/hexane: ^{1}H NMR (acetone-d_6) δ 6.80 (s, 4 H), 4.30 (s, 2 H), 3.70 (s, 3 H). The ester (3.0 g, 16.5 mmol) was dissolved in concentrated NH_4OH, stirred at room temperature overnight, and then evaporated under reduced pressure to give the amide derivative (2.3 g, 83%), which was used without further purification. The amide (2.3 g, 13.8 mmol) was suspended in dry THF (100 mL) and added to a suspension of $LiAlH_4$ (2.0 g, 52.6 mmol) in 50 mL of dry THF dropwise, under N_2. The reaction mixture was refluxed 12 h and cooled to room temperature, and water was cautiously added followed by acidification with 6 N H_2SO_4. The aqueous layer was washed with Et_2O, adjusted to pH 10 with KOH pellets, and extracted with Et_2O. The Et_2O extracts were dried, filtered, and evaporated, and the resultant product, 4-hydroxy-PAEE free amine, was crystallized as the HCl salt from EtOH/Et_2O: mp 172–174 °C dec (0.81 g, 31%); ^{1}H NMR (D_2O) δ 6.93 (s, 4 H), 4.25 (t, 2 H), 3.42 (t, 2 H).

Results

DBM/PAME Reaction

The olefinic β-phenethylamine derivative, PAME, was shown to be a substrate for DBM, and the kinetic parameters are given in Table I. The DBM apparent k_{cat} value with

Table I: DBM Substrate Reaction Kinetic Parameters[a]

	apparent k_{cat} (s^{-1})	K_M (mM)	K_I (mM)
PAME	14[b]	6.7	
4-HOPAME	56	1.3	
3-HOPAME	47	57	
α-methylstyrene[c]	0[d]		
4-hydroxy-α-methylstyrene[c]	0.7	3.7	
3-hydroxy-α-methylstyrene[c]	0[d]		
α-(cyanomethyl)styrene[c]	0[d]		
PEDA[e]	44	14	
PEDA	21	9.1	
N-MePEDA[e]	11	5.4	
tyramine[f]	121	2.0	
tyramine[c]	66	9.2	
PAEE			8.2

[a] Kinetic values were obtained under standard assay conditions using DBM of 21 units/mg specific activity; DBM tetramer molecular weight of 290 000 was used in apparent k_{cat} calculation. [b] Determined as 17 s^{-1} with 26 units/mg DBM, and normalized to 21 units/mg activity by multiplying by 21/26. [c] 15% DMF present in assay mixtures. [d] No detectable O_2 consumption in standard assay. [e] NaOAc buffer, 0.12 M, pH 5.6 [f] Apparent k_{cat} values higher than previously reported due to increased specific activity of DBM.

Table II: Controls for DBM/PAME Reaction

expt	condition	rate of O_2 uptake (μmol/min)
1[a]	+10 mM PAME	3.03
	+10 mM ascorbate	4.58
	+0.5 μM $CuSO_4$, 10 mM fumarate	5.14
	+DBM (0.70 unit)	27.8
2[b]	+DBM (0.26 unit)	1.81
	+10 mM ascorabte	1.81
	+20 mM PAME	23.6
3[b]	+DBM (0.26 unit)	1.81
	+20 mM PAME	1.81
	+10 mM ascorbate	24.3

[a] Assay mixture contained 0.29 mg/mL catalase in 0.127 M NaOAc buffer, pH 5.0; final volume was 2.57 mL in the standard O_2 monitor assay. [b] Assay mixture contained 10 mM fumarate, 0.5 μM $CuSO_4$, and 0.30 mg/mL catalase in 0.127 M NaOAc, pH 5.0; final volume was 2.50 mL in the standard O_2 monitor assay.

PAME as substrate was found to be 14 s^{-1}, which, although lower than many DBM substrates, is still about 12% of the apparent k_{cat} of the best known DBM substrate, tyramine. As shown in Table II, no reaction was seen between PAME and ascorbate or $CuSO_4$, and DBM/PAME reaction is dependent on reductant (ascorbate). The DBM/PAME reaction was also supported by ferrocyanide as reductant, albeit with a lower apparent k_{cat} (1 s^{-1}) and K_m (4.6 mM) (May et al., 1983). It should be noted that the K_m for PAME (ascorbate reductant) determined in this study, 6.7 mM, differs slightly from the K_m of 8.3 mM reported earlier (May et al., 1983).

Previously, we have determined that reaction of PAME with DBM exhibits the expected stoichiometry of electron:O_2:product of 2:1:1 (May et al., 1983), characteristic of monooxygenase reactions. As has been reported repeatedly in the literature for other substrates, sodium fumarate activates PAME oxygenation by DBM, with maximal activation occurring at about 5 mM fumarate. Fumarate activation parallels that found for DBM oxygenative ketonization (May et al., 1981b) and sulfoxidation reactions (Phillips and May, unpublished results).

The V/K pH dependence of the PAME–DBM substrate reaction was determined between pH 4.4 and pH 6.8 and is shown in Figure 1. The maximal V/K is seen at about pH 5.5, with values dropping off below pH 5.0 and above pH 6.0. Nonlinear least-squares fitting of the data to the equation $V/K = C/(1 + H/K_a + K_b/H)$ yielded values of 5.0 ± 0.2 and 6.0 ± 0.2 for pK_a and pK_b, respectively. For this calculation of V_{max}, the DBM tetramer molecular weight of 290 000 was used. This curve and the corresponding pK values agree well with those published for the DBM hydroxylation reaction of tyramine, pK_a = 5.2 and pK_b = 5.8 (Colombo et al., 1984a).

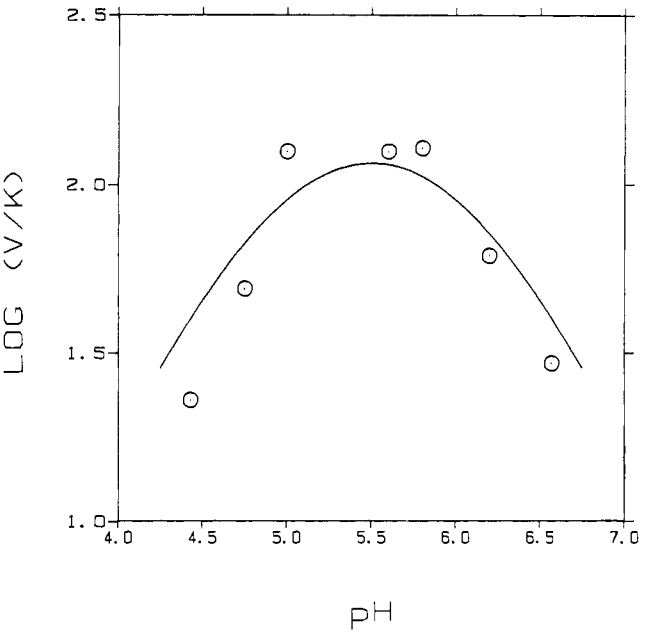


FIGURE 1: DBM/PAME reaction: pH dependence of substrate reaction. Substrate kinetic constants were determined at various pH values at 37 °C by using the oxygen monitor apparatus, with 0.1 M NaOAc/0.1 M MES as buffer, with 20.5 nM DBM tetramer (18.0 units/mg), 0.29 mg/mL catalase, 10 mM sodium fumarate, 5.5 μM $CuSO_4$, and 10 mM ascorbate. V/K values were determined by using the program of Cleland to fit the data to the hyperbolic form of the Michaelis–Menten equation.

Inactivation by Olefinic Compounds

Inactivation Kinetics. DBM undergoes a time-dependent inactivation during the course of the PAME oxygenation reaction, exhibiting, as with all olefinic inhibitors presented herein, pseudo-first-order kinetics (May et al., 1983). The values for k_{inact} and K_I were determined previously to be 0.04 min^{-1} and 13 mM, respectively (May et al., 1983). In the determination of these early PAME inactivation kinetic values by dilution assay, the initial DBM activity was determined as the activity of an enzyme aliquot as soon as possible after the inhibitor (PAME) was added. Subsequently, the initial DBM activity has been determined before inhibitor/ascorbate addition, followed by addition of an inhibitor/ascorbate solution to initiate the inactivation reaction. In addition, catalase of higher specific activity was used. By use of this method, strictly first-order concentration-dependent inactivation kinetics were obtained (Figure 2A), and the double-reciprocal plot (Figure 2B) yields the values of k_{inact} = 0.23 min^{-1} and K_I = 4.7 mM (Table III) assuming the simplest kinetic scheme for suicide inactivation (Walsh, 1977). Thus, the kinetic partition ratio for PAME, apparent k_{cat}/k_{inact}, is 900 turnovers per inactivation. For this calculation, and for all partition ratios calculated herein, apparent k_{cat} is based on a monomer molecular weight of 73 000.

Inclusion of the substrate tyramine at 10 mM in the inactivation mixture protects against inactivation, and the inactivation process is strictly dependent on the presence of ascorbate as reductant in the reaction mixture (data not shown).

Inactivation of DBM by PAME goes completely to 100% inactivation, and no reappearance of enzyme activity occurs

Table III: DBM Inactivation Kinetic Parameters

	k_{inact}[a] (min^{-1})	K_I (mM)	apparent k_{cat}/k_{inact}[b]
PAME[c]	0.23	4.7	900
4-HOPAME[d]	0.81	0.52	1000
3-HOPAME[c]	*f*		
4-hydroxy-α-methylstyrene[e]	0.53	2.5	20
3-hydroxy-α-methylstyrene[e]	*f*		

[a] Determined by linear regression analysis, as described under Experimental Procedures, at three or more inhibitor concentrations. All k_{inact} and K_I values are apparent values obtained at a single oxygen concentration (atmospheric equilibration). [b] For partition ratio calculation, the apparent k_{cat} constants were calculated on the basis of a DBM active site molecular weight of 73 000. [c] Dilution assay method 2 under Experimental Procedures, at 37 °C, pH 5.0. [d] Progress curve method under Experimental Procedures at 37 °C, pH 5.0 [e] Dilution assay method 1 under Experimental Procedures, at 30 °C. [f] Very weak ascorbate-dependent DBM inactivation was observed, but the low activity made k_{inact} determinations nonreproducible.

Table IV: [^{3}H]PAME DBM Radiolabeling[a]

	reaction[b]	control[a,c]
[DBM] (nmol/mL)	0.538	0.150
[[^{3}H]PAME] (nmol/mL)	2.90	0.051
[[^{3}H]PAME]/[DBM]	5.4	0.3

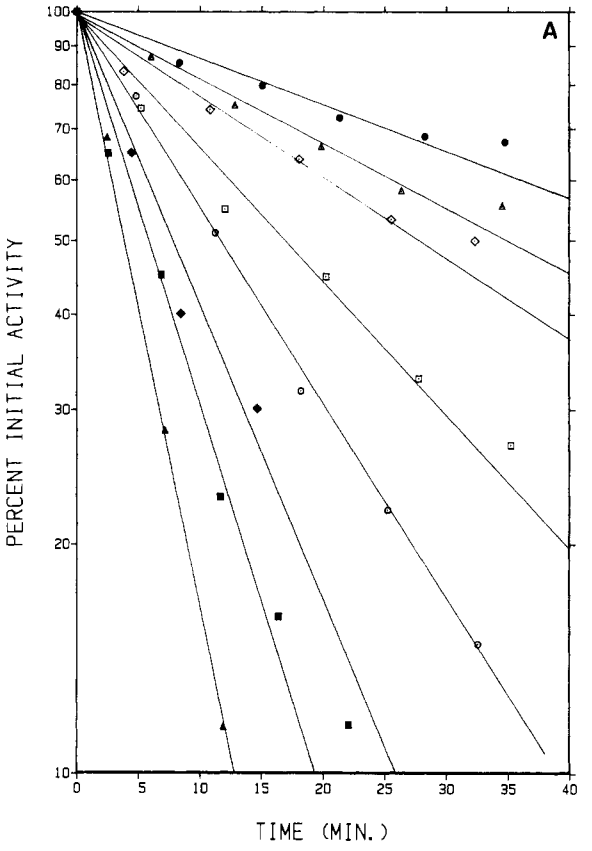
[a] Procedure given under Experimental Procedures. [b] Standard assay conditions. [c] Minus ascorbate.

even after 9 h of incubation in the presence of acetate (data not shown). Similarly, no reversal of inactivation was observed after prolonged dialysis. When standard assay conditions were used, the control solution (0 mM PAME and 10 mM ascorbate) retained 73.4% of initial activity after dialysis, while the inhibited enzyme (40 mM PAME and 10 mM ascorbate) had zero activity before and after dialysis (Mueller, 1981).

Anaerobic Inactivation. As shown in Figure 3, the inactivation of DBM by PAME is clearly dependent on the presence of oxygen in the reaction mixture. The anaerobic reaction mixture retains about 90% of the initial enzyme activity at 25 min after mixing, whereas the plus O_2 control showed no remaining activity. Upon introduction of O_2 to the reaction, inactivation occurred rapidly.

[^{3}H]PAME Labeling Experiment. A major concern in obtaining valid radiolabeling stoichiometry data is the determination of the DBM concentration. Absorption at 280 nm by catalase present in the reaction mixture complicates a simple absorbance measurement, but inclusion of catalase is necessary to prevent inactivation of the enzyme by H_2O_2 formed by ascorbate autoxidation. Since the molecular weight of catalase is very close to that of DBM tetramer, size exclusion separation was not possible. Catalase was found not to bind to Con A–Sepharose (data not shown), and thus, this affinity resin was used to reisolate the radiolabeled DBM. Table IV summarizes DBM radiolabeling by [^{3}H]PAME: reisolated DBM (plus ascorbate) showed definite label incorporation, 5.4 nmol/nmol of DBM tetramer, while the minus ascorbate control gave only 0.34 nmol/nmol of DBM tetramer. Thus, after subtraction of the noncatalytic incorporation, one obtains 5.1 mmol of [^{3}H]PAME/nmol of DBM tetramer, confirming that PAME forms a nondialyzable adduct with the enzyme. We emphasize that in the absence of an active site titrant, no quantitative conclusions should be drawn from the value obtained for the tritium incorporation per tetramer.

Product Identification and Oxygen:Product Stoichiometry. In analogy to the olefin epoxidation reaction catalyzed by some other "hydroxylases" [e.g., cytochrome P-450 (Ortiz de Montellano et al., 1982) and *Pseudomonas oleovorans* ω-hydroxylase (May et al., 1977; Katopodis et al., 1984)], it



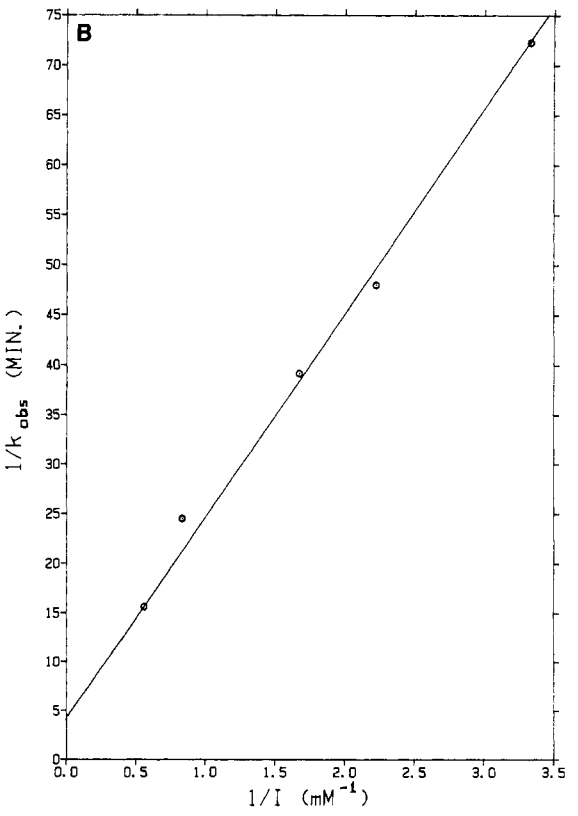


FIGURE 2: Kinetics of DBM/olefin inactivation reactions. (Panel A) (●) 0.30 mM PAME; (▲) 0.45 mM PAME; (◇) 0.60 mM PAME; (□) 1.20 mM PAME; (○) 1.80 mM PAME; (◆) 0.35 mM 4-HOPAME; (■) 0.50 mM 4-HOPAME; (▲) 0.75 mM 4-HOPAME. (Panel B) A plot of the PAME data from panel A, as $1/k_{obsd}$ vs. $1/I$, using the program of Cleland (1967) to fit the data to the hyperbolic form of the inactivation expression $k_{obsd} = [I]k_{inact}/([I] + K_I)$.

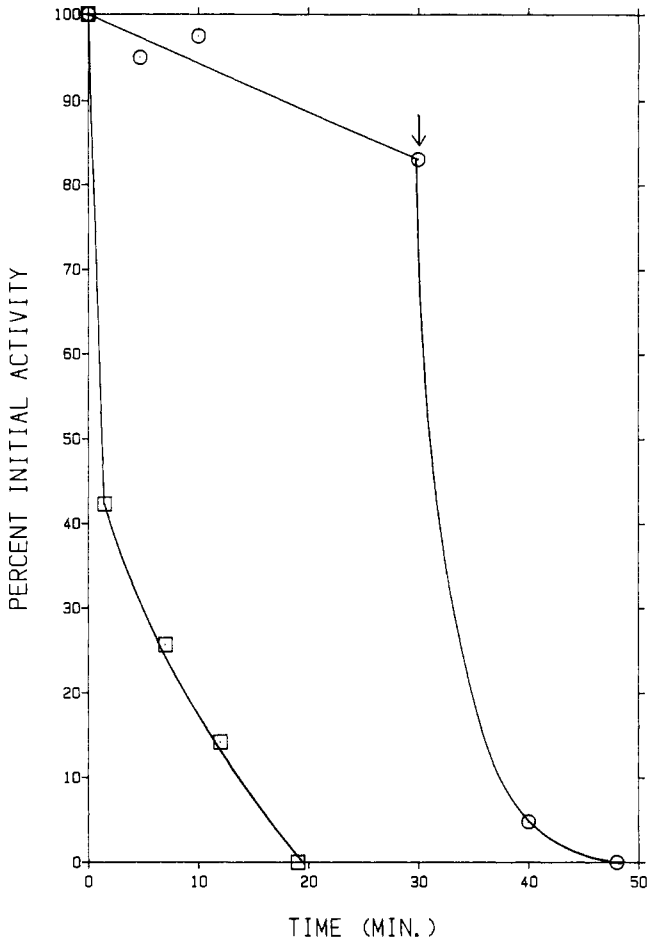


FIGURE 3: DBM/PAME inactivation reaction: dependence on oxygen. Protocol was according to inactivation dilution method 2, with the following alterations. A vial with serum cap containing 0.125 M NaOAc, pH 5.0, 10 mM sodium fumarate, 5 μM $CuSO_4$, and 40 μg of DBM (total volume of 0.280 mL) was connected to a vacuum manifold via a vacuum line with needle inserted through the septum and evacuated (P = 11 mmHg) and flushed with Ar 10 times. Aliquots for activity determination (standard assay) were withdrawn with a Hamilton syringe. To the anaerobic inactivation mixture was added 32 μL of an anaerobic PAME/ascorbate solution, prepared in the same manner, giving a final concentrations of 40 mM for PAME and 5 mM for ascorbate. Activity was followed vs. time, and after 31 min the septum cap was removed (indicated by arrow), the inactivation mixture swirled to introduce air to the system, and the remaining DBM activity determined. The control reaction of identical composition was left open to air at all times. (○) Anaerobic; (□) aerobic.

seemed likely that the product of PAME oxygenation by DBM was the corresponding epoxide. However, undoubtably owing to the presence in the molecule of a primary amine salt and a β-epoxide, numerous attempts at the synthesis of the putative product were unsuccessful. Acid trapping of the epoxide was therefore chosen to convert any epoxide formed to the corresponding diol, 1,2-dihydroxy-2-phenylpropylamine (DHPPA).

Initial attempts to identify DHPPA from hydrolyzed reaction mixes by using HPLC with fixed-wavelength UV detection (254 or 280 nm) were unsuccessful due to the low extinction coefficients at these wavelengths. However, EtOAc extraction of the basified acid hydrolysate followed by derivatization of the DHPPA amine group with the chromophoric activated ester reagent N-succinimidyl (p-nitrophenyl)acetate (SNPA), when analyzed by reverse-phase HPLC, gave a peak corresponding to synthetic derivatized DHPPA. The presence of a small amount of nonenzymatic DHPPA seen in the control can be attributed to the autoxidation of ascorbate, where the resultant H_2O_2 epoxidizes

Table V: Stoichiometry of DBM-Catalyzed Oxygenation Reactions

oxygenated substrate	$[Fe(CN)_6^{4-}]/[O_2]$	$[O_2]$/[product]
PEDA	1.8[a]	0.98[b]
N-MePEDA		0.96[b]
PAME[c]	1.9	1.1

[a] The reaction system contained 5 μM $CuSO_4$, 10 mM sodium fumarate, and 2.0 mM $K_4Fe(CN)_6$ in 0.1 M MES buffer, pH 6.0, with 5 mM substrate (PEDA). The reaction was initiated by adding DBM and was followed spectrophotometrically at 420 nm (ϵ = 1100 M^{-1} cm^{-1}) at 37 °C. [b] Product aniline was determined by RP-HPLC with 65% 0.1 M NaOAc, pH 5.8, 5 mM SOS, and 35% MeOH as mobile phase, monitored at 250 nm. Identical HPLC procedures and conditions were used for the identification of the N-dealkylation product of N-MePEDA, N-methylaniline. [c] Reported previously (May et al., 1983).

the olefin which would hydrolyze to the diol in situ. Control experiments showed that omission of ascorbate or PAME abolishes the formation of DHPPA, and shorter acid hydrolysis times gave lower percentages of nonenzymatic diol in the control as compared to the enzymatic reaction: in a typical procedure, a 15-min acid treatment yielded 21% nonenzymatic DHPPA, while a 2-h acid treatment gave 46% nonenzymatic diol. Quantitation of DHPPA was accomplished by HPLC standard curves made with synthetic DHPPA by using the identical SNPA derivatization protocol as for the enzymatic reaction mixtures and controls. Subtracting the nonenzymatic DHPPA formed in identical parallel reactions (minus DBM) from the total DHPPA formed in the enzymatic reactions gave an average of 1.1 μmol of DHPPA/μmol of O_2 (May et al., 1983) (Table V).

Epoxide Trapping with NBP. The time course of α-methylstyrene oxide derivatization with NBP at 60 °C was determined in a series of kinetic experiments, with the reaction monitored by measuring the increase in A_{560} after quenching in ice and addition of Et_3N/acetone. It was apparent that complete reaction is reached by about 50 min. The visible spectrum of the NBP–DBM/PAME reaction product adduct is shown in Figure 4 (frame A) and shows an absorbance maximum at 560–570 nm characteristic of N-alkylated NBP in basic solution (Barbin et al., 1975). Control reactions showed no such absorbance maximum. In frame B, the spectral contribution of ascorbate was subtracted to more clearly show the DBM dependence of adduct formation.

Inactivation in the Presence of Preformed Product. To test whether the epoxide product of the DBM/PAME reaction can return to the active site and inactivate the enzyme independent of turnover, the experiment shown in Figure 5 (frame A) was performed. As seen in Figure 5, the time course of O_2 uptake and concomitant enzyme inactivation were identical between a reaction mixture and the same reaction mixture when a fresh identical aliquot of DBM was added after 100% enzyme inactivation and reequilibration with air. If any moderately stable reactive species were released upon initial PAME turnover, one would expect to see a more rapid inactivation rate for the second DBM aliquot. However, examination of the time courses of the two reactions shows that the inactivation decay of O_2 consumption is identical with and without preformed reaction product present. In frame B, the identical experiment with 4-HOPAME (vide infra) also showed no significant change in inactivation time course with preformed product present.

As a further demonstration that free epoxide is not responsible for inactivation by olefinic substrates, α-methylstyrene oxide was examined for DBM inhibitory activity and compared with α-methylstyrene inactivation of DBM. Under conditions where α-methylstyrene inhibits DBM to a signif-

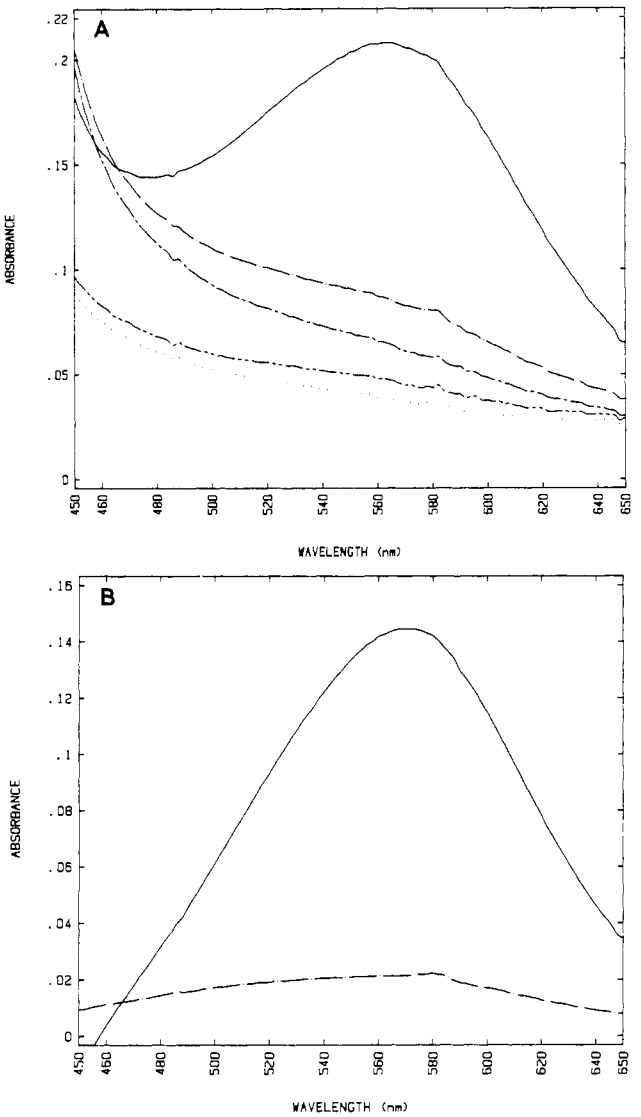


FIGURE 4: Identification of DBM/PAME epoxidation product by NBP derivatization. Procedure is as given under Experimental Procedures. Enzymatic reactions and controls were performed under standard assay condition at 37 °C for 64 min, with the indicated changes, followed by NBP derivatization at 60 °C for a minimum of 1 h. Spectra were recorded exactly 1 min after ice quenching and Et_3N/acetone addition. (Frame A) (1) (—) +DBM + 10 mM ascorbate + 23 mM PAME; (2)(–·–) –DBM + 10 mM ascorbate + 23 mM PAME; (3) (– – –) +DBM + ascorbate – PAME; (4) (– · · –) +DBM – ascorbate + PAME; (5) (···) standard assay mix – DBM – ascorbate – PAME. (Frame B) Corrected for ascorbate contribution: (—) curves 1–3 from frame A; (–·–) curves 2–3 from frame A.

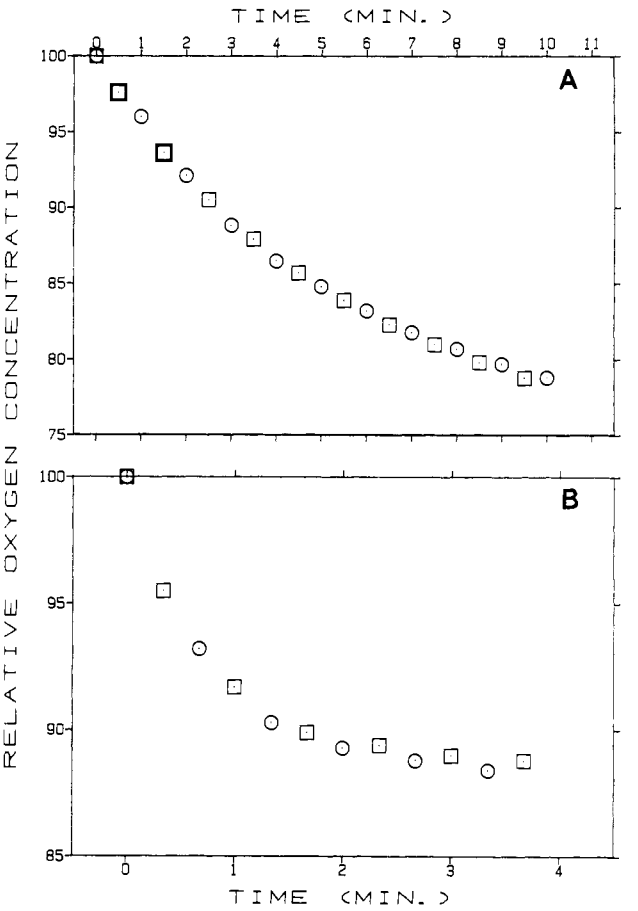


FIGURE 5: Time course of oxygen uptake and inactivation rate in the absence and presence of preformed product. The reaction mixture contained 10 mM fumarate, 0.36 mg/mL catalase, 5.6 μM copper sulfate, 10 mM ascorbate, and 40 mM PAME·HCl in 0.12 M NaOAc, pH 5.64 (2.575 mL total volume). The reaction, run at 37 °C, was initiated by adding 0.012 mg of DBM (10 μL) and was monitored by the standard dissolved oxygen electrode assay. After total inactivation of the enzyme, the system was resaturated with air, and an identical aliquot of DBM was added and the reaction followed. The initial portions of each of the two reactions were plotted vs. time to allow comparison of the inactivation rates. (Frame A) 40 mM PAME. (Frame B) Identical procedure with 13.7 mM 4-HOPAME and 0.0059 mg of DBM per injection. (○) Initial injection; (□) second injection in the presence of preformed product.

icant extent, giving $k_{obsd} = 0.02\ min^{-1}$, α-methylstyrene oxide at the same concentration showed no DBM inhibitory activity whatsoever. The fact that no inhibition by the epoxide is observed, even at concentrations several orders of magnitude higher than could be formed by olefin turnover, indicates that free epoxide is not responsible for the ascorbate-dependent DBM inactivation by α-methylstyrene.

4-Hydroxy-PAME, as shown in Table I, is an excellent DBM substrate that gives an apparent k_{cat} of 56 s^{-1}, a full 46% of tyramine apparent k_{cat} activity, and 4 times that of PAME itself. The dilution assay showed an ascorbate-dependent pseudo-first-order inactivation of DBM, which was much more rapid than that observed with similar concentrations of the parent compound, PAME (Figure 2A). Because of this very rapid inactivation activity and high apparent k_{cat}, the dilution method does not give accurate values for k_{inact} and K_I, because inactivation is so rapid that inhibitor concentrations at the levels required for analysis change significantly during the observation interval. Therefore, kinetic parameters for inactivation by 4-HOPAME were determined by using a modification of the methods of Main (1973) that involves analysis of the progress curve over an interval such that substrate concentration is not significantly depleted (see Experimental Procedures). This type of kinetic analysis is commonly used for suicide inactivators whose potency is such that inactivation is too rapid for application of the standard dilution method [see, for example, Daniels et al. (1983)]. The kinetic parameters obtained for inactivation by 4-HOPAME were $k_{inact} = 0.81$ and $K_I = 0.52$ (Table III), making this compound the most potent olefinic DBM inhibitor discovered in these studies. However, because the compound has such a large initial rate substrate activity, the partition ratio for 4-HOPAME is 1000, a value which is close to that of PAME itself. 3-Hydroxy-PAME, as shown in Table I, is also a DBM substrate, with an apparent k_{cat} value, 47 s^{-1}, similar to that of 4-HOPAME. The K_m value for this compound was found to be 57 mM, very much higher than the K_m's determined for PAME and especially 4-HOPAME. 3-HOPAME also inactivated DBM in an ascorbate-dependent manner but to such a small extent that

reproducible inactivation constants could not be obtained.

In addition to these compounds, a number of other unsaturated DBM substrate analogues were tested for DBM substrate and inhibitory activity (Table I and III). Turnover of α-methylstyrene and α-(cyanomethyl)styrene was so slow that significant O_2 uptake could not be determined over the time course of the experiment. Because of the limited aqueous solubility of the non-amine-containing compounds, assay solutions for these compounds contained 15% DMF. It can be seen from Table I that the K_m of tyramine is raised from 2.0 to 9.2 mM upon inclusion of 15% DMF in the assay solution and the k_{cat} value is lowered from 121 to 66 s^{-1}, about 55% of the rate with no DMF. For comparison, the tyramine K_m with 6% DMF has been reported to be 4.2 mM (Colombo et al., 1984a).

4-Hydroxy-α-methylstyrene was found to exhibit weak but measurable DBM substrate activity (Table I) and gave K_m = 3.7 mM and apparent k_{cat} = 0.7 s^{-1}. 4-Hydroxy-α-methylstyrene was found to be a potent DBM inhibitor (Table III). This inhibition is strictly dependent on ascorbate, and dilution assay inactivation kinetics yielded k_{inact} = 0.53 min^{-1} and K_I = 2.5 mM, giving a partition ratio of 20 turnovers per inactivation. This value is about 45 times lower than that found for PAME and represents a large increase in inhibition efficiency. 3-Hydroxy-α-methylstyrene showed no DBM activity as measured by the standard oxygen monitor assay but was found to possess weak ascorbate-dependent DBM inhibitory activity.

3-Phenylpropargylamine was found to be a very potent DBM inhibitor. Inactivation analysis using the dilution method yielded an approximate value of k_{inact}/K_I of 1700 min^{-1} M^{-1}. With this compound the potency of inactivation precluded separate determinations of k_{inact} and K_I by the dilution method, and kinetic analysis by the method of Main (1973) gave an anomalous plot of I/ρ vs. I. Furthermore, a small but significant degree of ascorbate-independent inactivation was observed. Clearly, inactivation by this acetylenic compound is a complex process which will require further detailed study.

Interaction of DBM with Benzylic Nitrogen Compounds

Our results to this point do not bear on the steps of "Cu–O" attack on the olefinic substrates. Because reaction of olefins with P-450 is thought to proceed via an initial one-electron abstraction from the olefin to the active site Fe(V)=O species (Ortiz de Montellano et al., 1982), the feasibility of such a one-electron abstraction mechanism in DBM catalysis was explored by determining DBM reactivity with benzylic nitrogen heteroatom substrates. Because N-dealkylation by P-450 proceeds analogously via initial one-electron transfer from heteroatom to Fe(V)=O, it was reasoned that N-dealkylation activity by DBM would provide strong support for such an initial one-electron abstraction capability.

Incubation of the prototype diamine substrate *N*-phenylethylenediamine (PEDA) or its *N*-methyl derivative *N*-methyl-*N*-phenylethylenediamine (*N*-MePEDA), with purified DBM in the presence of fumarate, Cu^{2+}, and ascorbate or $K_4Fe(CN)_6$ as electron donor, results in an enzyme-dependent consumption of both electrons and oxygen in the stoichiometry diagnostic of monooygenase-catalyzed oxygenations. Kinetic constants obtained for ascorbate-supported oxygenation of PEDA and *N*-MePEDA are presented in Table I, and it is evident from the data that the reaction of DBM with both of these substrates is kinetically comparable to the DBM-catalyzed hydroxylation, sulfoxidation, ketonization, and epoxidation reactions.

Initial identification of the enzymatic dealkylation products was accomplished by extracting the basified enzymatic reaction mixtures with ether. The concentrated product from the reaction of DBM with PEDA was analyzed by TLC and shown to contain a major product with an R_f value identical with authentic aniline in two different solvent systems (5% MeOH/95% $CHCl_3$; 5% MeOH/95% C_6H_6). Similarly, the product from the *N*-MePEDA substrate was identified as *N*-methylaniline by TLC comparisons in the same solvent systems. Quantitative HPLC procedures for isolation and identification of aniline were developed in order to allow comparison with authentic aniline and determination of the stoichiometry of product formation. Control experiments using authentic aniline established that the amount of aniline present in the enzymatic reaction mixture could be quantitatively determined by using this procedure. The same HPLC conditions and procedures were also successfully used for the identification and quantitation of *N*-methylaniline, the product from the reaction of DBM with *N*-MePEDA. Table V summarizes the stoichiometry for DBM catalyzed *N*-dealkylation of PEDA and those for other known DBM-catalyzed reactions.

Appropriate control experiments revealed that formation of products from either benzylic nitrogen substrate is strictly dependent on the presence of DBM and a reducing agent, either ascorbate or ferrocyanide, in the reaction mixture. Furthurmore, if the N-dealkylation reaction is a direct result of DBM catalysis, the time courses of product formation and O_2 consumption should correlate stoichiometrically throughout the course of the reaction. We find that product formation and oxygen consumption are indeed stoichiometrically consistent throughout the time period and no lag period in product formation is observed. Furthermore, it is also clear from the data presented in Figure 6 that the rate of *N*-methylaniline formation is retarded by tyramine in a competitive manner, as would be expected for reactions occurring at the same active site. Formation of aniline from PEDA is also competitively inhibited by tyramine (data not shown). It should be noted that a rapid fall off in turnover rate occurs at longer reaction times with both diamine substrates. Control experiments have shown that this is due to a time- and concentration-dependent inhibition phenomenon that is not freely reversible, the mechanism of which has been investigated (K. Wimalasena, unpublished results). However, the data presented here clearly establish that, under initial rate conditions, both PEDA and *N*-MePEDA are well-behaved substrates for DBM, with the proper oxygen, electron, and product stoichiometries.

In contrast to these results, incubation of DBM with phenyl 2-aminoethyl ether (PAEE), the oxygen analogue of PEDA, under the usual reaction conditions did not result in significant consumption of oxygen or electrons. Kinetic studies established that PAEE is in fact a potent competitive inhibitor of DBM (K_I = 8.2 mM). Similarly, competitive inhibition (K_I = 60 mM) was observed with *p*-hydroxyphenyl 2-aminoethyl ether. Thus, the results establish that DBM is unable to carry out oxidative O-dealkylations under the usual reaction conditions.

Discussion

Identification of an olefinic DBM substrate was attempted for several reasons. First, when we began these studies, no DBM substrate or inhibitor containing a benzylic olefinic carbon had ever been reported in the literature, and oxygenation of such compounds by DBM would further extend the known reactivity of the enzyme. Second, study of such an olefinic reaction might provide insight into the chemical mechanism of DBM catalysis. Finally, the possibility existed that enzyme inactivation might occur concomitant with olefin oxygenation, as is the case for the P-450 enzymes (Ortiz de

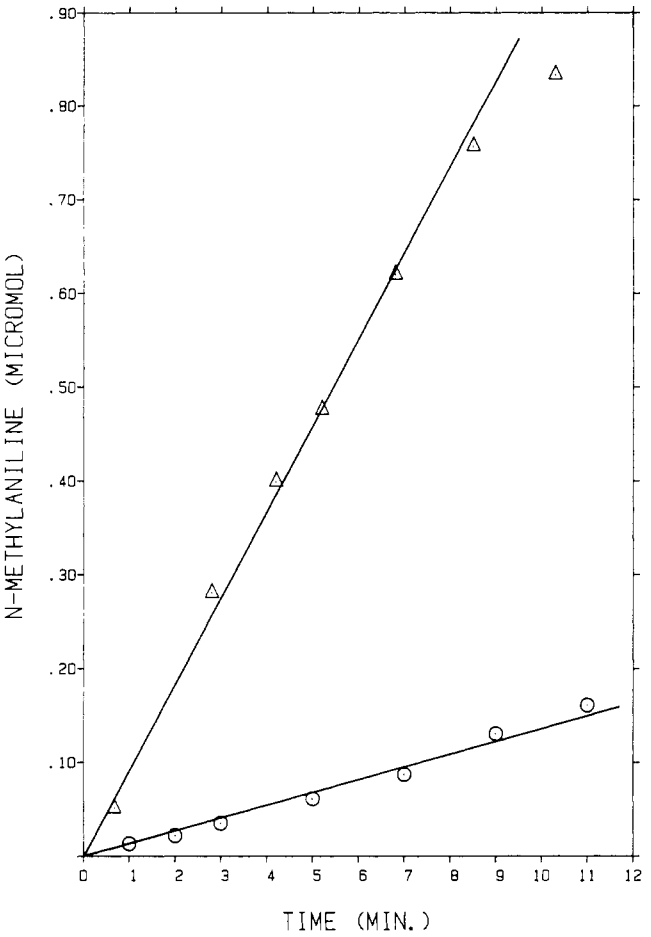


FIGURE 6: Time course of *N*-methylaniline formation in the absence and presence of tyramine during the N-dealkylation of *N*-MePEDA by DBM. The reaction mixture contained 10 mM sodium fumarate, 5.5 μM $CuSO_4$, 0.4 mg/mL catalase, 21 mM *N*-MePEDA, and 60 μg of DBM, with and without tyramine, in 0.125 M NaOAc buffer, pH 5.64, in 2.525 mL total volume. At each time interval 150-μL samples were withdrawn, added into 20 μL of concentrated HCl, and filtered, and 15 μL was analyzed by HPLC. The amount of *N*-methylaniline was determined from a HPLC standard curve with authentic material, as described under Experimental Procedures. (Δ) *N*-Methylaniline production with no tyramine present; (O) *N*-methylaniline production in the presence of 0.8 mM tyramine.

Montellano et al., 1982). If a class of specific DBM inhibitors could be designed that mimics catecholamine structure closely enough to be substitutable for dopamine in in vivo transport, uptake, and DBM catalysis, then such a class of compounds would perhaps be useful as pharmaceutical agents to modulate DA–NE interconversion.

Our results with the prototypic olefin substrate, PAME, establish that olefin oxygenation exhibits all of the characteristics of a reaction proceeding via the usual DBM catalytic pathway. The O_2:electrons:product stoichiometry is 1:2:1, as is diagnostic for all monooxygenase activities of DBM. PAME oxygenation exhibits reaction activation by the well-known DBM activator fumarate. The pH dependence of V/K and the resultant pK values correspond to those exhibited for the normal DBM hydroxylation reaction. Olefin oxygenation is a facile process; the kinetic parameters are comparable to those for the other known DBM substrate reactions of hydroxylation, sulfoxidation, oxygenative ketonization, and selenoxidation. Finally, the product of PAME oxygenation has been identified directly as the epoxide via NBP derivatization, and acid trapping of the epoxide product gives the corresponding diol in stoichiometric yield. By analogy with the *P. oleovorans* nonheme iron hydroxylase/epoxidase (May et al., 1977; Katopodis et al., 1984) and with P-450, the epoxide was, of course, the expected product of DBM olefin oxygenation. Thus, olefin epoxidation satisfies the criteria for a standard DBM reaction. The nonreactivity of PAME with $CuSO_4$ and ascorbate under assay conditions confirms epoxidation is DBM catalyzed [cf. olefin epoxidation by Cu^{2+} and iodosylbenzene (Franklin et al., 1984)].

All of our results support the conclusion that PAME and its derivatives inactivate DBM in a mechanism-based manner. Inactivation kinetics were found to be pseudo first order and saturable, and inactivation is strictly dependent on reductant and oxygen. No reappearance of enzyme activity occurs after extensive dialysis or prolonged incubation in the presence of acetate. Reaction of [^{3}H]PAME with DBM resulted in an ascorbate-dependent incorporation of radiolabel into enzyme.

Kinetic parameters reported here were obtained at a single oxygen concentration and thus obviously do not represent true limiting values. However, they do reflect the kinetic situation under atmospheric conditions and thus are relevant to an assessment of the possible pharmacological utility of these inhibitors. It is evident that the aromatic substituents affect both turnover and inactivation. 3-Hydroxylation and 4-hydroxylation of PAME increase the apparent k_{cat} by a factor of about 4, and 4-HOPAME has a k_{inact} 4 times larger than that of PAME. 3-HOPAME, although exhibiting an apparent k_{cat} comparable to that of 4-HOPAME, exhibits a K_m of 57 mM which is far greater than the value of 1.3 mM for 4-HOPAME. Similarly, the K_m's for *p*- and *m*-hydroxybenzyl cyanides have been reported to be 2.9 mM and 27.7 mM, respectively (Colombo et al., 1984a). Coupled with the long-known diminished reactivity of *m*-tyramine as compared with that of *p*-tyramine (van der Shoot et al., 1965), these results suggest that an increase in K_m going from 4- to 3-hydroxylation is a general phenomenon among DBM substrates.

It should be noted that 4-HOPAME is one of the most kinetically potent inhibitors discovered to date for DBM, with a k_{inact} of 0.8 min^{-1} at 37 °C, pH 5.0, and atmospheric O_2 (250 μM). By comparison, 2-bromo-3-(*p*-hydroxyphenyl)-1-propene, recently reported as a mechanism-based inhibitor for DBM by Villafranca and co-workers (Ash et al., 1984), has a k_{inact} of 0.09 min^{-1}, which increases severalfold when the [O_2] is raised above atmospheric saturation, and k_{inact} values as high as 0.5 min^{-1} have been reported for the benzyl cyanide class of inhibitors (Colombo et al., 1984a,c). Although k_{inact} values were not measured for our olefins at greater than atmospheric [O_2], the k_{inact} values would be predicted to be even greater at elevated [O_2]. In contrast to the potent DBM inactivation seen with 4-HOPAME, 3-HOPAME inactivates DBM only to a very minor extent. This result was quite surprising, considering that the apparent k_{cat} values for the 3- and 4-HO isomers were similar.

Removal of the amino group of 4-HOPAME to give 4-hydroxy-α-methylstyrene dramatically decreases substrate activity, going from an apparent k_{cat} of 56 s^{-1} down to only 0.7 s^{-1}. This reaffirms the pronounced effect of the amino group on turnover. In contrast, the k_{inact} with 4-hydroxy-α-methylstyrene is 0.5 min^{-1}, a full 62% of k_{inact} for 4-HOPAME. Thus, the kinetic partition ratio, apparent k_{cat}/k_{inact}, is 20 for 4-hydroxy-α-methylstyrene, with the greater partition ratio of 4-HOPAME reflecting its far greater substrate activity. From a pharmacological viewpoint, it might be argued that the presence of the amino group in this α-methylstyrene-derived class of inhibitors has increased substrate activity to such an extent that the partition ratios are higher than the optimal

range for in vivo efficacy. On the other hand, the amine-containing olefins are highly water soluble and, most significantly, maintain the critical structural requirements at adrenergic nerve terminals. Furthermore, the very low K_m and K_I values for 4-HOPAME ($\sim$1 mM) could allow for low dosing. The design of inhibitors with substituents which decrease substrate activity while maintaining both facile inactivation characteristics and similarities to catecholamine structure is currently an area of investigation in this group.

No detectable DBM substrate activity was seen with α-methylstyrene or α-(cyanomethyl)styrene, although these compounds do inactivate DBM. For instance, k_{obsd} for α-methylstyrene at 20 mM inhibitor was ca. 0.02 min^{-1} at 25 °C and pH 5.0, with 15% DMF present. *m*-Hydroxy-α-methylstyrene also gave no detectable substrate activity and only trace inactivation activity. The acetylinic substrate 3-phenylpropagylamine exhibited rapid inactivation activity, but no measurable oxygen consumption in a substrate assay was observed with this compound. As noted under Results, complexities were observed in the kinetics and ascorbate dependency of inactivation, and further work is in progress to clarify the operative mechanism. However, the apparent k_{inact}/K_I value of 1700 min^{-1} M^{-1} determined for 3-phenylpropargylamine is comparable to that for 4-HOPAME (1600 min^{-1} M^{-1}), and thus inactivation by the alkyne does appear to be quite an efficient process. Similarly, Colombo et al. (1984d) have stated that 1-phenyl-1-propyne inactivation of DBM may proceed with one turnover per inactivation.

Whereas PAME epoxide was previously identified and quantitated by acid hydrolysis to the corresponding diol (May et al., 1983), we have now directly identified the epoxide by derivatization with NBP, a standard epoxide derivatizing reagent used successfully in identifying P-450 epoxidation products (Miller & Guengerich, 1982). The 560-nm spectral band is characteristic of the alkylated NBP species in basic media, and its formation is clearly dependent on DBM, ascorbate, and substrate. It is important to note that the amount of derivatized PAME epoxide formed corresponds to the amount expected from the enzymatic reaction stoichiometry on the basis of calibration of the trapping reaction with α-methylstyrene oxide. Thus, we conclude that products other than the epoxide are not formed in significant amounts during enzymatic oxygenation. NBP derivatization of DBM/3-HO-PAME reaction product also gave this characteristic absorbance at about 560 nm (data not shown).

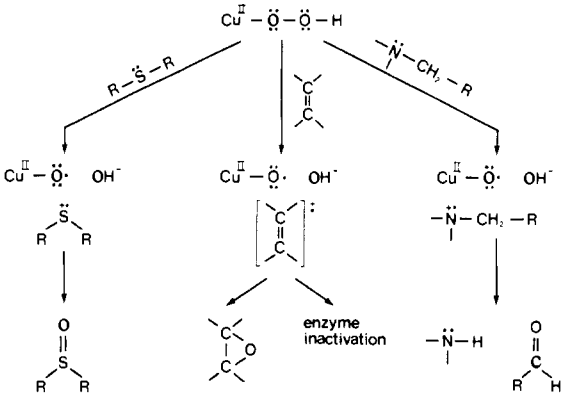
In our original report on PAME inhibition (May et al., 1983) we stated that the species responsible for inactivation may be either the epoxide or an intermediate along the pathway of olefin oxygenation, as has been proposed for P-450 inactivation by olefins. On the basis of two lines of evidence we favor the view that the respective epoxides are not the species that inactivate DBM: First, incubation of α-methylstyrene oxide with DBM resulted in no loss of enzymatic activity, whereas α-methylstyrene inactivates the enzyme to a significant extent at identical concentrations. Second, the double-addition experiments establish that the time course of enzyme inactivation by either PAME or 4-HOPAME is unchanged if preformed enzymatic product is present in solution. Importantly, NBP trapping of PAME epoxide under identical reaction conditions showed that the epoxide is stable throughout the double-addition experiment. Thus, free epoxide cannot be responsible for inactivation. While it might be argued that an enzyme-bound epoxide that does not freely equilibrate with solution is the actual inactivating species, we favor the analogy with olefinic inactivation of P-450 where the regiospecificity of inactivation represents strong evidence that intermediates along the inactivation pathway are the actural inactivating species (Ortiz de Montellano et al., 1982). Unfortunately, in the absence of a heme moiety or structural information regarding the DBM active site region, such arguments cannot yet be made for olefinic inactivation by DBM.

Our findings regarding DBM oxygenations of olefin, sulfide, and amine substrates reveal a remarkable similarity between DBM catalysis and P-450 catalysis. For DBM, the "Cu–oxygen" species responsible for catalysis has not been directly identified. Clearly, the electron-rich porphyrin environment of the P-450 iron could not be paralleled by DBM copper, where the ligands most likely could not stabilize a $[Cu(IV)=O]^{2+}$ species analogous to the P-450 $[Fe(V)=O]^{3+}$ species thought to be involved in P-450 reactions (Guengerich & MacDonald, 1984; Groves et al., 1978). However, the similarities in the chemistry catalyzed by the two monooxygenases cannot be ignored. Thus, even if a formal analogy to the ferryl P-450 intermediate is not chemically satisfactory for DBM, this does not preclude both enzymes operating via generation of analogous *substrate-derived* species along the reaction coordinate. In particular, if O–O bond cleavage in a DBM Cu(II)–O–O–H species [proposed by Ahn and Klinman (1983)] is not heterolytic but homolytic, O–O cleavage accompanied by one-electron substrate oxidation would yield OH^- and $[Cu(III)=O]^+$, formally analogous to the P-450 $[Fe(IV)=O]^{2+}$ species that results after initial one-electron abstraction from olefins and heteroatoms (Guengerich & MacDonald, 1984). Subsequent to such an initial one-electron abstraction from substrate, the resulting $[Cu(III)=O]^+$ species would be expected to carry out reactions similar to those observed for P-450.

Our demonstration of the heretofore unknown DBM-catalyzed N-dealkylation of PEDA and *N*-MePEDA provides support for such an initial one-electron oxidation of substrate by DBM. In the case of P-450, N-dealkylation is thought to proceed via initial one-electron abstraction, followed by H· or H^+ abstraction and then oxygen recombination (or imine hydrolysis) to give the α-hydroxylated carbinolamine that readily dealkylates (Guengerich & MacDonald, 1984; Augusto et al., 1982). A possible alternative pathway is initial H· abstraction from the carbon adjacent to the nitrogen atom followed by oxygenation of this α-carbon to generate directly the carbinolamine. This alternative pathway is apparently operative for O-dealkylations by P-450 (Watanabe et al., 1982; Miwa et al., 1984), and thus, if it were operative with DBM, the ether substrate, PAEE, should also undergo α-hydroxylation to yield the respective hemiacetal, which would O-dealkylate. However, no trace of DBM substrate activity was detected with either PAEE or its *p*-hydroxylated analogue, 4-HOPAEE, despite the fact that these compounds are good DBM competitive inhibitors and obviously do bind to the active site. Thus, we conclude that the α-hydroxylation mechanism operative for P-450 O-dealkylations cannot be initiated, and the specificity of DBM catalysis requires initial electron abstraction from only the benzylic position. While this occurs readily for N, the effective redox potential of the activated copper–oxygen species is insufficient to remove an electron from O, and dealkylation of ethers does not occur.

It is also important to note that N-dealkylation of *N*-MePEDA proceeds regiospecifically, producing only *N*-methylaniline as the product, in spite of the alternate possibility of N-demethylation generating PEDA as a product. This specificity may be ascribed to the specificity of the enzyme, and it also eliminates the possibility that N-dealkylation is a

Scheme I: Pathways for DBM Oxygenations[a]



[a] Note that two copper atoms may actually participate in reductive oxygen activation. Multiple chemical steps subsequent to initial cation radical formation are not illustrated (see text).

result of nonenzymatic decomposition in solution of such enzymatically generated intermediates as the *N*-oxide or a similar species.

Sulfur oxygenation by DBM, represented by the sulfoxidation of phenyl 2-aminoethyl sulfide and its structural analogues (May & Phillips, 1980), gives sulfoxide stoichiometrically as product, with no S-dealkylation occurring. This situation is also observed with P-450, where the strong tendency of the sulfur cation radical toward sulfoxidation also produces solely the sulfoxide and not the S-dealkylated product (Guengerich & MacDonald, 1984).

With the above results in hand, a general mechanism for DBM catalysis is proposed and shown in Scheme I. The mechanism is analogous to that currently favored for P-450 catalysis (Guengerich & MacDonald, 1984) and resembles that which we have recently proposed for non-heme iron monooxygenase catalysis (Katopodis et al., 1984). For either benzylic S- or N-containing substrates, reaction with active site Cu(II)–O–O–H generates the heteroatom radical cation, $[Cu(III)=O]^+$, and OH^-. In the case of benzylic N substrates (PEDA and *N*-MePEDA) this step may be followed by H^+ or H· abstraction from the adjacent carbon to give the respective radical or cationic intermediate, which recombines with the resulting copper–oxygen species at the α-carbon to generate the respective carbinolamine, leading to dealkylation. Alternatively, after loss of H^+, the second electron transfer may occur from nitrogen to give an imine species which hydrolyzes to the carbinolamine. In the case of benzylic S substrates, oxygen rebound to give sulfoxide is favored over H^+ or H· abstraction. DBM hydroxylation is readily accommodated by this mechanism, via initial generation of a benzylic radical followed by recombination and protonation to give the benzylic hydroxylated product.

Olefin oxygenation by DBM would start with initial one-electron oxidation to give the olefin radical cation followed by recombination with the resultant $[Cu(III)=O]^+$ to give a reactive carbocation species, which can partition between closure to the epoxide or alkylation of an active site residue leading to inactivation of the enzyme. At present, it is obviously not known which of the olefinic carbons represents the locus of initial oxygen attack. It is apparent that initial oxygen attack at the terminal methylene would produce a benzylic carbocation and the 4-hydroxy group of 4-HOPAME would be predicted to stabilize such a species.

Current research efforts are directed toward the goals of trapping the proposed intermediates with appropriately designed inhibitors and substrates. We note that in view of the central role of DBM in adrenergic neuronal function, those substrate analogues and inhibitors that retain the structural features essential for presynaptic uptake and storage, and are thus effectively targetable for DBM, would be expected to show the most promise for eventual utilization as clinical agents.

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Mechanism of "Uncoupled" Tetrahydropterin Oxidation by Phenylalanine Hydroxylase†

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ABSTRACT: Phenylalanine hydroxylase can catalyze the oxidation of its tetrahydropterin cofactor without concomitant substrate hydroxylation. We now report that this "uncoupled" tetrahydropterin oxidation is mechanistically distinct from normal enzyme turnover. Tetrahydropterins are oxygenated to 4a-carbinolamines only during catalytic events involving substrate hydroxylation. In the absence of hydroxylation tetrahydropterins are oxidized directly to quinonoid dihydropterins. Stoichiometry studies define a ratio of two tetrahydropterins oxidized per O_2 consumed in uncoupled enzyme turnover, thus indicating the complete reduction of O_2 to H_2O. Complementary results establish the lack of H_2O_2 production by the enzyme when uncoupled and define a tetrahydropterin oxidase activity for the enzyme. Thus, the hydroxylating intermediate of phenylalanine hydroxylase may be discharged in two ways, by substrate hydroxylation or by electron abstraction. A mechanism is proposed for the uncoupled oxidation of tetrahydropterins by phenylalanine hydroxylase, and the significance of these findings is discussed.

Oxygen activation by the mammalian mixed function oxidase phenylalanine hydroxylase (PAH)[1] requires a tetrahydropterin and appears to involve O_2 interaction with the ferrous center of the non-heme enzyme (Wallick et al., 1984; Marota & Shiman, 1984). However, the structure of the hydroxylating intermediate formed by PAH and the mechanism for its formation are, at present, unknown. Concurrent

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[1] Abbreviations: PAH, phenylalanine hydroxylase; DHPR, dihydropteridine reductase; 6-MPH_4, 6-methyltetrahydropterin; PH_4, tetrahydropterin; GC–MS, gas chromatography–mass spectrometry; *p*-Cl-Phe, DL-*p*-chlorophenylalanine; Phe, L-phenylalanine; DTT, dithiothreitol; HRP, horseradish peroxidase; PAR, 4-(2-pyridylazo)resorcinol; BHT, 2,6-di-*tert*-butyl-4-methylphenol; Tris, tris(hydroxymethyl)aminomethane; Dopa, L-3,4-dihydroxyphenylalanine.

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