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Cytochrome P450, flavin-containing monooxygenase, metabolic intermediate complex, mechanism-based inactivation, suicide substrate

**Abstract:**
The inactivation of cytochrome P450 enzymes by cyclopropylamines has been attributed to a mechanism involving initial one-electron oxidation at nitrogen followed by scission of the cyclopropane ring leading to covalent modification of the enzyme. Herein we report that in liver microsomes *N*-cyclopropylbenzylamine (**1**) and related compounds inactivate P450 to a large extent via formation of metabolic intermediate complexes (MICs) in which a nitroso metabolite coordinates tightly to the heme iron, thereby preventing turnover. MIC formation from **1** does not occur in reconstituted P450 systems with CYP2B1/2, 2C11 or 2E1, or in microsomes exposed to gentle heating to inactivate the flavin-containing monooxygenase (FMO). In contrast, *N*-hydroxy-*N*-cyclopropylbenzylamine (**3**) and *N*-benzylhydroxylamine (**4**) generate MICs much faster than **1** in both reconstituted and microsomal systems. MIC formation from nitrone **5** (PhCH=N(O)*c*Pr) is somewhat faster than from **1**, but very much faster than the hydrolysis of **5** to a primary hydroxylamine. Thus the major overall route from **1** to a P450 MIC complex would appear to involve FMO oxidation to **3**, further oxidation by P450 and/or FMO to nitrone **5'** (C2H4C=N(O)CH2Ph), hydrolysis to **4**, and P450 oxidation to α-nitrosotoluene as the precursor to oxime **2** and the major MIC from **1**.

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# Cyclopropylamine Inactivation of Cytochromes P450:

# Role of Metabolic Intermediate Complexes

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## Introduction

The time- and cofactor-dependent inactivation of cytochrome P450 enzymes by cyclopropylamines such as **1** has long been thought to occur via single electron transfer (SET) oxidation at nitrogen, leading to ring opening of the cyclopropane ring and covalent modification of the enzyme as shown in Figure 1 [1]. This mechanism is supported by direct observations of ring-opened products in chemical model systems [2-5], and in the oxidation of cyclopropylanilines by peroxidases [6-8]. While the formation of cinnamaldehyde as a metabolite of an *N*-(2-phenylcyclopropyl)amine has been reported, direct evidence for the formation of ring-opened metabolites of a simple *N*-cyclopropylamine has not been demonstrated for a P450 system. On the contrary, P450 systems have shown only formation of cyclopropanone hydrate from the *N*-dealklylation of cyclopropylanilines [7]. *N*-Cyclopropylanilines, however, do not inactivate P450, which leaves open the possibility that they are oxidized differently from aliphatic cyclopropylamines such as **1**.

To address this question we undertook an investigation of the metabolism of **1** by rat liver microsomes. The metabolites observed included benzylamine, benzaldehyde, benzyl alcohol, cyclopropylamine, cyclopropanone hydrate and benzaldoxime (structure **2** in Figure 2) [9]. The formation of oxime **2** as a metabolite of **1** was an unexpected but interesting observation. Oxime metabolites of amines arise via *N*-oxidation leading to a *C*-nitroso species that tautomerizes to the oxime [10]. *C*-Nitroso intermediates can also form metabolic intermediate (MI) complexes via coordinate-covalent bonding from the nitrogen lone pair to the ferrous heme iron of P450 enzymes [11-13]. This type of MI complex (MIC) is recognizable by a prominent absorption maximum at approximately 455 nm in the difference spectrum of incubation mixtures. Since MI

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complexes are generally quite robust, their formation constitutes a form of suicide inactivation of P450 activity.

In this report we provide evidence that MI complex formation is a significant contributor to the net loss of P450 activity observed upon incubation of rat liver microsomes with compound **1** and NADPH. We also characterize the mechanism of the process leading to MIC formation through studies with purified isozymes and through elucidation of structure-activity relationships.

## Materials and Methods

### Reagents and General Procedures

[7-$^{14}$C]-Benzoic acid and sodium [$^{14}$C]-formate were purchased from Moravek Biochemicals (Brea, CA) with specific activities of 53 and 56 Ci/mol, respectively. [7-$^{13}$C]-Benzoic acid and sodium [$^{13}$C]-formate were purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA). Compounds **2**, **4**, **6**–**9** and cyclopropylamine were obtained from Aldrich (Milwaukee, WI). NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO). Aminopyrine was from Mallinckrodt Inc. (St. Louis, MO). All other chemicals and reagents were of reagent grade or higher purity and were purchased from commercial suppliers.

Melting points were obtained using a Uni-melt capillary melting point apparatus and are uncorrected. The $^{1}$H and $^{13}$C NMR spectra of the synthesized compounds were recorded on Bruker DRX 400 or DRX 500 spectrometers. Analytical thin layer chromatography (TLC) was performed on Analtech Uniplate 250 μ silica gel plates with detection by UV and $I_2$. GC/EIMS

analyses were performed on an HP5890 Series II GC fitted with a DB-5 capillary column (0.26 mm x 30 m, J & W Scientific, Folsom, CA) coupled to an HP5971A Mass Selective Detector. Data were collected in full scan mode (40–350 AMU, 0.77 scans/sec) and analyzed using an HP5970 ChemStation. Samples were analyzed using the following oven temperature program: 60 ºC for 3 min; 10 ºC/min to 250 ºC; hold at 250 ºC for 5 min. UV-vis spectra were recorded using a Hitachi U-3010 spectrophotometer. Radiochemical purity of [1'-$^{14}$C]-**1**·HCl and [7-$^{14}$C]-**1**·HCl was assessed by HPLC using a Kromasil C4 column (5 μm, 4.6 x 150 mm) and an isocratic solvent system consisting of 15% acetonitrile in 100 mM $NaClO_4$ (pH 2.25) at a flow rate of 1 mL/min. Column effluent was passed through a UV detector (210 nm) in series with a Ramona radioactivity flow detector with a solid scintillant cell. Data were collected on an SRI chromatography data system and analyzed using Peak Simple software.

**Chemistry**

Compounds **1** [14, 15], **5** [16] and **13** [17] were prepared by literature procedures and gave $^1$H NMR and mp that agreed with those reported. Compounds **10** [18], **11** [19] and **14** [17] were available from previous work in our laboratory [14] and were indistinguishable by $^1$H NMR and MS characterization from the previously reported preparations of these compounds.

***N,N*-Dibenzyl-[$^{13}$C]-formamide.** To a 30 mL culture tube (2 x 10 cm) was added dibenzylamine·HCl (1.46 g, 6.25 mmol), sodium [$^{13}$C]-formate (0.35 g, 5.00 mmol) and 15 mL of acetonitrile. The tube was capped with a Teflon-lined screw cap and the contents stirred at 82 ºC for 48 h. The reaction was cooled to room temperature and diluted with ether (50 mL), and the resulting solution rinsed with 1 M HCl (3 x 25 mL). The ether solution was then dried over

magnesium sulfate, filtered and the solvent removed in vacuo. This yielded a pale yellow liquid that solidified upon standing to form a white solid (0.79 g, 70 % yield). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 4.26 (d, 2H, $J$ = 5.23 Hz), 4.41 (d, 2H, $J$ = 2.98 Hz), 7.17-7.21 (m, 4H), 7.26-7.40 (m, 6H), 8.52 (d, 1H, $J$ = 118.51 Hz). $^{13}$C NMR (100.6 MHz, $CDCl_3$) δ 44.81, 50.47, 127.86, 127.90, 128.34, 128.70, 128.89, 129.11, 135.76, 136.14, 136.06 ($^{13}$C enriched carbon). GC/MS: $t_R$ 19.8 min, m/z 226 [$M^+$].

***N,N*-Dibenzyl-[1'-$^{13}$C]-cyclopropylamine.** In a 3-neck round bottom flask *N,N*-dibenzyl-[$^{13}$C]-formamide (0.50 g, 2.21 mmol) was dissolved in 10 mL of dry tetrahydrofuran and stirred vigorously under nitrogen. Titanium isopropoxide (850 µL, 2.88 mmol) was added, followed by 2.20 mL of a 3.00 M solution of ethylmagnesium bromide in ether (6.60 mmol). Upon addition of the Grignard reagent, gas evolution could be seen and a yellow color formed. The mixture was heated for 15 min at 45 ºC and an additional 1.50 mL of 3.00 M ethylmagnesium bromide (4.50 mmol) was added, whereupon the reaction mixture became black. The mixture was heated to 45 ºC for 15 min and then allowed to cool to room temperature and stir for 12 h. The reaction was quenched by addition of 10 mL of saturated aqueous ammonium chloride and the mixture concentrated in vacuo, resulting in formation of a white precipitate. After adding 15 mL of 1 M NaOH the resulting mixture of precipitate and solution was divided into two 50 mL culture tubes. Each tube was then extracted with ether (2 x 25 mL each) with the aid of centrifugation. The extracts were pooled, dried over magnesium sulfate, filtered and concentrated in vacuo giving a yellow liquid (0.46 g). This material was then applied to a silica gel column wluted with 4:1 hexanes/ethyl acetate to obtain a mixture of *N,N*-dibenzylcyclopropylamine and *N,N*-dibenzyl-3-pentylamine (0.16 g). This material was then applied to a second silica gel column eluted with 2.5% ether in pentane to obtain 0.11 g of *N,N*-

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dibenzyl-[1'-$^{13}$C]-cyclopropylamine as a clear colorless liquid (32% yield). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 0.30-0.41 (m, 4H), 1.81 (doublet of septets, 1H, $J$ = 167.13 Hz), 3.67 (d, 4H, $J$ = 4.34 Hz), 7.23-7.50 (m, 10H). $^{13}$C NMR (100.6 MHz, CDCl3) δ 7.71, 7.87, 36.50 ($^{13}$C enriched carbon), 58.43, 126.95, 128.17, 129.65, 138.98. GC/MS: $t_R$ 17.8 min, m/z 238 [$M^+$].

**[1'-$^{13}$C]-1·HCl.** To a 16 x 100 mm culture tube containing *N*,*N*-dibenzyl-[1'-$^{13}$C]-cyclopropylamine (0.10 g, 0.42 mmol) in 2 mL of dry 1,2-dichloroethane was added 1-chloroethyl chloroformate (140 µL, 1.26 mmol). The tube was then purged with nitrogen, sealed with a Teflon-lined screw cap and heated to 90 ºC for 16 h with stirring. TLC showed the presence of starting material so additional 1-chloroethyl chloroformate (45.3 µL, 0.42 mmol) was added, and the reaction stirred an additional 5 h at 90 ºC prior to cooling and concentration in vacuo. Methanol (2 mL) was added to the resulting brown solution was heated at 70 ºC for 30 min. Removal of solvent in vacuo gave 0.11 g of a brown viscous liquid that solidified upon standing. This solid was then dissolved in a minimum volume of hot absolute ethanol, the solution cooled to room temperature and ether added to precipitate the desired product, [1'-$^{13}$C]-**1**·HCl, as a white crystalline solid (36 mg, 45% yield). mp 157-159 ºC. $^{1}$H NMR (400 MHz, $D_2O$) δ 0.81-0.93 (m, 4H), 2.74 (dm, 1H, $J$ = 186.28 Hz), 4.33 (s, 2H), 7.50 (bs, 5H). $^{13}$C NMR (100.6 MHz, $D_2O$) δ 3.31, 3.45, 29.99 ($^{13}$C enriched carbon), 52.07, 129.54, 129.92, 130.23. GC/MS (as free base): $t_R$ 9.6 min, m/z 148 [$M^+$].

**[1'-$^{14}$C]-1·HCl** was prepared in 24% yield (after purification) by the above method [1'-$^{14}$C]-**1**·HCl using sodium [$^{14}$C]-formate mixed with ordinary sodium formate to achieve a specific activity of 0.50 Ci/mol. HPLC indicated a chemical and radiochemical purity ≥ 98%.

***N*-Cyclopropyl-[7-$^{13}$C]-benzamide.** A solution of [7-$^{13}$C]-benzoic acid (0.31 g, 2.50 mmol) and 240 µL of oxalyl chloride (2.75 mmol) in 3 mL dry benzene was heated to 80 ºC for

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5 h in a 10 mL culture tube with a Teflon-lined screw cap. The resulting solution was cooled and added dropwise to a stirred solution of cyclopropylamine (0.52 mL, 7.50 mmol) in 3 mL of benzene with cooling to 0 ºC. A white precipitate formed and the mixture was stirred at room temperature overnight. The reaction mixture was then diluted with 10 mL of ether and washed with 1 M NaOH (2 x 5 mL) and 1 M HCl (2 x 5 mL). The aqueous rinsings were back-extracted with ether (1 x 10 mL each) and the organic portions pooled, dried over magnesium sulfate and concentrated in vacuo giving 0.31 g of a pale yellow solid (76% yield). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 0.62-0.66 (m, 2H), 0.88-0.94 (m, 2H), 2.91-2.96 (octet, 1H, $J$ = 3.51 Hz), 6.25 (s, 1H), 3.80 (s, 3H), 7.42-7.46 (m, 2H), 7.49-7.53 (m, 1H), 7.74-7.77 (m, 2H). $^{13}$C NMR (100.6 MHz, $CDCl_3$) δ 7.04, 23.32, 127.01, 128.75, 128.79, 131.70, 169.07 ($^{13}$C enriched carbon). GC/MS: $t_R$ 15.1 min, m/z 162 [$M^+$].

**[7-$^{13}$C]-1·HCl.** *N*-Cyclopropyl-[7-$^{13}$C]-benzamide (0.31 g, 1.89 mmol) was added to 3 mL of dry tetrahydrofuran in a three neck round bottom flask fitted with a nitrogen inlet and a condenser. Under an atmosphere of nitrogen and with cooling to 0 ºC a 1 M solution of $BH_3$·THF (11.7 mL, 11.71 mmol) was then added dropwise with stirring. The solution was then heated to reflux for 3 h, at which time TLC showed the reaction to be complete. The reaction was quenched by dropwise addition of 5 mL of 6 M HCl, and the tetrahydrofuran was removed in vacuo. The mixture was adjusted to pH > 13 by addition of 4 M NaOH and extracted with ether (3 x 15 mL). The extracts were pooled, dried over magnesium sulfate, filtered and concentrated *in vacuo* giving 0.16 g of a clear colorless liquid. The liquid was then dissolved in 10 mL of ether and bubbled with HCl gas. The white precipitate that formed was collected by suction filtration and dissolved in a minimum volume of hot absolute ethanol, cooled to room temperature, and the product precipitated by addition of ether giving 0.15 g of white crystalline

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solid (44% yield). mp 155-156 ºC. $^{1}$H NMR (400 MHz, $D_2O$) δ 0.80-0.93 (m, 4H), 2.69-2.75 (m, 1H), 4.31 (d, 2H, $J$ = 144.61 Hz) 7.47 (m, 5H). $^{13}$C NMR (100.6 MHz, $D_2O$) δ 3.33, 29.95 ($^{13}$C enriched carbon), 52.03, 129.50, 129.54, 129.93, 130.19, 130.23. GC/MS (as free base): $t_R$ 10.4 min, m/z 148 [$M^+$].

**[7-$^{14}$C]-1·HCl** was prepared by the above method in 44% yield from [7-$^{14}$C]-benzoic acid at a specific activity of 0.45 Ci/mol. HPLC indicated a chemical and radiochemical purity ≥ 98%.

**Compound 3** was prepared by lithium aluminum hydride reduction of nitrone **5** as described by Morgan and Beckett [20]. Briefly, *N*-cyclopropylbenzylidene nitrone (0.79 g) was dissolved in dry tetrahydrofuran and cooled under nitrogen to 0 ºC on ice. Lithium aluminum hydride (0.75 g, 19.70 mmol in 3 mL dry tetrahydrofuran) was added dropwise with continuous cooling. The mixture was stirred for 1 h at which time both TLC and GC/MS showed hydroxylamine **3** to be the major product ($R_f$ 0.66, 1:1 ether/pentane. $t_R$ 11.3 min). The reaction was quenched by addition of 10 mL $H_2O$ resulting in formation of a white precipitate that was removed by suction filtration. The filtrate was extracted with ether (4 x 50 mL) and the extracts pooled, dried over magnesium sulfate and concentrated under vacuum giving a viscous yellow-orange liquid (0.43 g). After silica gel chromatography using 1:1 ether/pentane as solvent 0.18 g of the desired product was obtained as a white solid (22% yield from **1** as a precursor to **5**). mp 63-66 °C. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 0.60-0.64 (m, 4H), 2.36-2.41 (m, 1H), 4.02 (s, 2H), 5.05 (broad s, 1H), 7.25-7.39 (m, 5H). $^{13}$C NMR (100.6 MHz, $CDCl_3$) δ 6.71, 40.55, 64.57, 127.51, 128.48, 129.72. MS (CI, $NH_3$): m/z 164 [$MH^+$].

**12·HCl** was prepared from *N,N*-dibenzylacetamide using titanium isopropoxide and ethylmagnesium bromide as described by Chaplinski and de Meijere [21]. Debenzylation of the

resulting *N*,*N*-dibenzyl(1'-methyl)cyclopropylamine was achieved at room temperature utilizing atmospheric pressure hydrogenation over palladium on carbon catalyst in acidic methanol [22]. The crude reaction product was dissolved in hot absolute ethanol and precipitated with ether resulting in 60% yield of **12**·HCl from the *N*,*N*-dibenzyl(1'-methyl)cyclopropylamine. The $^{1}$H NMR, $^{13}$C NMR, EIMS (as the free base) and mp of the product were in agreement with those previously reported [19].

**15·HCl**. Using the procedure of Chaplinski and de Meijere [21] **15**•HCl was prepared from *N*-benzyl-*N*-methylformamide (derived from the reaction of *N*-benzyl-*N*-methylamine with ammonium formate) [23], titanium isopropoxide and ethylmagnesium bromide in 15% yield. The $^{1}$H NMR, $^{13}$C NMR and EIMS (as the free base) matched those reported for this compound prepared by another method [15].

**Biochemical studies**

**Enzyme preparations.** Liver microsomes were prepared from untreated and phenobarbital-treated male Sprague-Dawley rats (UT- and PB-microsomes, respectively) following literature protocols [7, 24]. Isolation of CYP2B1/2 from PB-microsomes was performed as previously reported [25]. Plasmid EL2, constructed [26] to express the cDNA of CYP2C11 [27] in *E. coli* was kindly provided by Dr. M. A. Correia (with permission from Dr. T. Omura). This plasmid was expressed, and the CYP2C11 isolated and purified as described [26]. A culture of *E. coli* engineered to express CYP2E1 was kindly provided by Prof. F.P. Guengerich; the desired protein was isolated and purified as described [28]. CYP2C11 and CYP2B1/2 (0.5-1.0 nmol) were reconstituted with reductase (1.0-2.0 nmol) and DLPC (30 μg)

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for 40 min at 0 ºC [7]. CYP2E1 (1.0 nmol) was reconstituted with reductase (3.0 nmol), cytochrome $b_5$ (5.0 nmol) and DLPC (30 μg) for 40 min at 0 ºC [29]. A culture of C-1A *E. coli* cells engineered [30] to express rat NADPH-cytochromme P450 oxidoreductase was generously provided by Dr. C. B. Kasper; the protein was expressed and purified as described.

**Determination of mechanism-based inactivation of cytochrome P450 by 1 and 2.** Mechanism-based inactivation by was determined using a two-stage assay procedure. P450 inactivation in a primary incubation consisting of concentrated enzyme and inhibitor was subsequently assessed after dilution of an aliquot of the primary incubation into a secondary assay to measure remaining enzyme activity. For the primary incubation PB-microsomes were diluted with 0.1 M potassium phosphate buffer, pH 7.6, (buffer A) to a protein concentration of 2 mg/mL and kept on ice until use. Stock solutions of **1** at concentrations of 2.5, 5.0, 12.5, 25.0 and 50.0 mM were prepared by dissolving **1**·HCl in buffer A. Stock solutions of **2** at concentrations of 5.0, 12.5, 25.0 or 50.0 mM were prepared by dissolving **2** in acetonitrile. Incubations were conducted in 1.7 mL Eppendorf tubes to which were added 384 μL of microsome suspension and 8 μL of either buffer A, acetonitrile or a stock solution of **1** or **2**. The resulting mixtures were incubated in an oscillating water bath at 33 ºC for 3 min prior to inititiating the reaction by adding 8 μL of NADPH solution (50 mM in buffer A), bringing the final incubation volume to 400 μL.

For the second stage assays, tubes were prepared containing 75 μL of aminopyrine solution (20 mM in buffer A), 10 μL of NADPH solution (50 mM in buffer A), 3 μL of an NADPH regenerating system (0.3 units/μL glucose-6-phosphate dehydrogenase in 1 M glucose-6-phosphate) and 362 μL of buffer A.

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At various times 50 μL aliquots of the primary incubation were withdrawn and added to a secondary assay tube (prewarmed to 33 ˚C) thereby achieving a final volume of 500 μL and a protein concentration of 0.2 mg/mL. Secondary incubations were then incubated for an additional 10 min in an oscillating water bath at 33 ºC and quenched by addition of 250 μL each of 15% zinc sulfate and saturated aqueous barium hydroxide solution. After centrifugation (20,800 x g for 10 min), 800 μL of the supernatant was removed and combined with 400 μL of Nash reagent [31] followed by heating at 60 ºC for 10 min. The absorbance of each incubation sample was then measured at 415 nm versus a similarly treated buffer (blank) solution. Formaldehyde formation was quantified using a calibration curve prepared in a similar manner from standard solutions.

**Measurement of absorption spectra of P450 metabolic intermediate (MI) complexes.** Aliquots of a suspension of PB-microsomes, or a solution of a purified resonstituted P450 (final P450 concentration 0.5-1.0 μM unless otherwise noted) were transferred to matched sample and reference cuvettes (480 μL each). Each cuvette then received 10 μL of a 50 mM solution of NADPH in buffer A followed by zeroing of the baseline between 400-500 nm. Reaction was started by addition of 10 μL of 5 mM test compound (dissolved in buffer A or methanol) to the sample cuvette and 10 μL of solvent to the reference cuvette. For determination of absorbtion maxima and the extent of MIC formation, spectral scans (400-500 nm) were acquired once every 60 sec from 400-500 nm until the amplitude of the difference spectrum plateaued at a maximum value. Kinetic scans were performed by monitoring absorbance at 455 nm for up to 30 min.

**Heat-treatment of microsomes.** Individual 1 mL aliquots of diluted PB-microsomes (1 mg protein/mL) in thin-walled glass test tubes were partly immersed in a water bath at 50 ºC, shaken for 90 sec, and then immediately cooled by immersion in ice water [32]. Inactivation of

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FMO was assessed by monitoring the oxidation of thiobenzamide to thiobenzamide *S*-oxide using the method of Cashman and Hanzlik [33]. The effect of heat treatment on P450 activity was assessed using the APD assay described above.

**Results**

Incubation of varying concentrations of **1** with rat liver microsomes and NADPH leads to a time-, concentration-, and cofactor-dependent loss of cytochrome P450 activity as assessed by the aminopyrine *N*-demethylase (APD) assay. A replot of the reciprocals of the slopes of Figure 3 vs. the reciprocals of inhibitor concentration [34] leads to the kinetic constants reported in Table 1. As described in earlier literature, the loss of APD activity in microsomes incubated with **1** does not proceed to completion but instead plateaus with ca. 25-30% activity remaining [35-37]. This behavior can also be seen in the apparent curvature and leveling off of the bottom line in Figure 3.

When **1** is incubated with microsomes and NADPH a characteristic difference spectrum with a maximum at 455 nm appears and increases in amplitute with time (Figure 4A). This spectrum is consistent with the formation of a P450 metabolic intermediate (MI) complex in which the ferrous heme iron is ligated by the nitrogen lone pair of a *C*-nitroso metabolite of an amine [12]. Such complexes are generally quite robust, but characteristically, oxidation of the iron to Fe(III) with ferricyanide leads to destruction of the complex and loss of the 455 nm absorption associated with the MIC chromophore, as shown in Figure 4B. MI complexes have extinction coefficients in the difference spectrum ($A_{490}$ - $A_{455}$) of approximately 65 $mM^{-1}cm^{-1}$ [10, 38]. Using this value one can estimate the fractional conversion of P450 to MI complex and compare that to the fractional loss of catalytic activity. For example, incubation of PB-

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microsomes with 1 mM **1** for 10 min at 33 ˚C results in conversion of 19% of total P450 to MI complex, but at the same time the loss of APD activity is ca. 60%. On the other hand incubation of PB-microsomes with 0.1 mM **1** for 10 min at 33 ˚C results in conversion of 26% of total P450 to MI complex but only 34% of the APD activity is lost. Others have also noted that low concentrations of amine substrates are optimum for generating MI complexes, while higher concentrations actually generate less MIC [11, 12, 39]. The reasons for the unusual biphasic dependence of MIC formation on amine concentration are not clear, but this pattern is decidedly different from the concentration dependence of P450 inactivation by **1** (Figure 3). The lack of direct correlation between MIC formation and loss of enzyme activity suggests that the P450 isozyme(s) participating in MI complex formation are not identical to those contributing to APD activity (as discussed further below).

To investigate which isozymes of P450 give rise to MI complexes during the metabolism of **1** we turned to reconstituted systems with CYP2B1/2, the major isoforms in PB-microsomes, and with 2C11 and 2E1 which are constitutive isoforms in male rat liver [40]. To our surprise we observed no MIC formation from **1** with any of these reconstituted systems (Table 2). This observation caused us to consider a potential role for the flavin-containing monooxygenase (FMO) in MI complex formation. Since FMO enzymes are known to form secondary hydroxylamine metabolites from secondary amines like **1**, we tested hydroxylamines **3** and **4** for MIC formation. Both compounds gave rise to substantial MIC formation in both reconstituted systems and in microsomes, and compared to **1**, the rates were much greater with **3** and **4** (Table 2 and Figure 5A). Interestingly, nitrone **5**, which is a potential FMO metabolite of **3**, also gives rise to an MI complex in microsomes, although it hardly does so in reconstituted systems (Table 2). Some nitrones are easily hydrolyzed to hydroxylamines, but repetitive UV scans (200-450

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nm) of solutions of **5** in the phosphate buffer used for microsomal incubations, with or without added microsomal protein, showed that **5** is unchanged for at least 2 hr at room temperature. Thus, MIC formation from **5** must involve a mechanism not requiring hydrolysis.

Further support for an obligatory role for FMO activity in MIC formation from **1** comes from experiments with heat treatment of microsomes. FMO enzymes are very heat-labile in the absence of NADP(H) [41]. Thus microsomes heated briefly without NADPH added (50 ˚C for 90 sec, then rapidly cooled to ≤ 37 ˚C) are unable to form a MI complex from **1** plus NADPH, yet they retain ≥ 90% of their original P450 activity, and they give a strong MI response (≥ 95% of control) when treated with NADPH and **4** (data not shown). These observations suggest that the *N*-oxidation of **1** by FMO is an obligatory step toward the generation of the MIC derived from **1**.

Additional insight into MIC formation from cyclopropylamines comes from results with analogs of **1**, as shown in Table 2 and Figure 5B. As noted in earlier SAR studies of microsomal P450 inactivation by **1**, benzylamine and *N*-alkylbenzylamines do not inactivate P450 [35, 42], while *N*-cyclobutylbenzylamine (**11**) does so only weakly [19]. The current work shows that neither do any of these compounds give rise to MI complexes in PB-microsomes (Table 2). Adding a methyl group on C-1' of the cyclopropyl moiety (**12**) actually increases MIC formation slightly compared to **1** (Table 2 and Figure 5B). On the other hand, results with compounds **13** and **15** indicate that adding one methyl to the benzylic carbon of **1** significantly decreases the rate and extent of MIC formation, while adding two methyl groups blocks MIC formation entirely. Finally, studies with **15** show that adding a methyl to the nitrogen of **1** results in a prominent lag period, after which MIC formation proceeds at the same rate as with **1** itself,

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suggesting that **15** must first be *N*-demethylated to **1** by P450 and then oxidized to **3** by the FMO prior to forming an MI complex.

Although incubation of oxime **2** (100 μM) with PB-microsomes (1 mM NADPH, 25 ˚C, 30 min) does not result in MIC formation, we tested the ability of **2** to inactivate P450 as a suicide substrate. Figure 6 shows that under conditions capable of supporting P450 activity, oxime **2** does cause time-, concentration- and cofactor-dependent loss of APD activity in PB-microsomes; the relevant kinetic parameters are given in Table 1. Although the kinetic parameters for **2** are very similar to those for **1**, oxime **2** is unlikely to contribute significantly to the inactivation of microsomal P450 observed with **1** because at a concentration of 1 mM, only 20% of **1** is metabolized during a 60 minute incubation, and oxime **2** accounts for only 19% of the total metabolites formed. Thus during a normal (10 min) assay for P450 inactivation or MIC formation, the concentration of **2** remains far too low for it to be an effective P450 inactivator, especially in the presence of a much higher concentration of **1**.

## Discussion

The discovery of **1** as a suicide substrate for cytochrome P450 enzymes was based on the observation of time- and cofactor-dependent loss of the aminopyrine *N*-demethylase and *p*-nitroanisole *O*-demethylase activites of PB rat liver microsomes [35, 42]. Results presented in Figure 3 confirm the time-dependence and also indicate the concentration dependence of the inactivation process. They also confirm that as originally described, the inactivation of APD activity does not go to completion but rather levels off with ca. 25-30% of the original activity remaining. In retrospect, we now know that as substrates, aminopyrine and *p*-nitroanisole are relatively non-selective among common P450 isoforms [43]. Thus there could be several

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possible explanations for the observed incompleteness of the inactivation process. One is that **1** is moderately selective in inactivating some but not all of the P450 isoforms which metabolize aminopyrine and *p*-nitroanisole. Another is that the catalytic activity of P450 enzymes covalently modified by reactive metabolites of **1** is decreased but not eliminated. A third is that at longer incubation times, the FMO enzyme loses its activity due to thermal or other natural degradative processes, thus halting the *N*-oxidation of **1** that is apparently required, at least for MI complex formation, and possibly for P450 inactivation by other mechanisms as well. Since any or all of the above scenarios could be operating in rat liver microsomes, the remainder of this discussion will be focussed primarily on the issue of MIC formation from **1**.

The inactivation of P450 by **1** was first attributed to a putative electrophilic cyclopropylidene Schiff base metabolite formed by the classical hydrogen atom transfer (HAT) mechanism that was thought to be involved in numerous *C*-hydroxylation and *N*- and *O*-dealkylation reactions of P450 [35]. However, the observation that **12**, the C-1' methyl derivative of **1,** which can not form a cyclopropylidene Schiff base, was as effective a P450 inactivator as **1,** led to the proposal of the single electron transfer (SET) mechanism as a specific mechanism for the inactivation of P450 by cyclopropylamines (see Figure 1) [36, 37], and as a potentially *general* mechanism for *N*-dealkylation of amines. While these mechanisms have been much studied and much debated for more than 20 years [44-48], the new observation of extensive P450 MIC formation from **1** provides an important alternative to the SET mechanism for at least a portion, and possibly a significant portion, of the observed inactivation of P450 by **1** and related cyclopropylamines.

Formation of MICs from **1** can not account for *all* the loss of P450 activity, however. For example, analog **14** inactivates P450 but does not form an MI complex. In addition, the

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percentage loss of P450 activity observed with **1** also greatly exceeds the percentage of P450 converted to MI complex(es) in microsomes. Since the available data can not rule out the possibility that this reflects extensive MI complex formation with a small subset of P450s that happen to make a major contribution to the oxidation of aminopyrine by PB-microsomes, further studies will be required to elucidate the contribution of individual P450 isoforms to both the loss of activity and the MIC formation observed in microsomes.

The conversion of a secondary amine to a C-nitroso compound, the presumed precursor of oxime **2** and the presumed ligand responsible for heme-based MI chromophore formation, requires a formal six-electron oxidation. This process almost certainly occurs in three two-electron steps as outlined in Figure 7 . At the outset, either *N*-oxidation of **1** (step a) or alpha carbon oxidation of **1** (step g) are logical possibilities. Although several studies indicate that P450 can *N*-hydroxylate some primary and secondary amines [49, 50], the observation that mild heating of microsomes abolishes MIC formation from **1** with little effect on P450 activity as assessed by APD activity argues strongly for the importance of FMO enzyme(s) in the formation of MI complexes from **1**. FMO involvement could occur at steps a, b, or e in Figure 7, but since FMO enzymes in rat liver generally do not *N*-oxidize primary amines [11, 41, 51, 52], and since benzylamine (**6**) and cyclopropylamine do not give rise to MIC formation, step i in Figure 7 is probably not significant. On the other hand step g, which is likely to be catalyzed by P450 enzymes, seems highly probable because benzaldehyde, cyclopropylamine, benzylamine and cyclopropanone hydrate are all formed during the microsomal metabolism of **1**; none of these compounds, however, give rise to MICs in microsomes.

In agreement with earlier studies MIC formation from **3** or **4** occurs much more rapidly than from **1** (or from **12**, **13** or **15**), and MIC formation is generally faster and more extensive in

PB-microsomes than in UT-microsomes [11, 12, 39, 53]. Deuteration of the alpha carbon of amphetamine (**18**) results in a 1.8-fold *increase* in the rate of MIC formation, which probably reflects a primary kinetic deuterium isotope effect on the partitioning of the nitroso compound between oxime formation vs. MIC formation (Figure 7) [10]. Methyl substitution around the nitrogen also strongly affects MIC formation. For example, *N*-hydroxyamphetamine (**16**) and *N*-hydroxy-2-phenylethylamine (**17**) form MI complexes much faster than amphetamine, but 2-phenylethylamine (**19**) does not form an MIC [12]. On the other hand neither phentermine (**20**) nor its *N*-hydroxy derivative, nor even the corresponding nitroso compound, form P450 MI complexes [10], probably for reasons of steric hindrance in the P450 active sites. Methylation also influences MIC formation from congeners of **1** (Figure 5B). For example **14**, like phentermine (**20**), does not form an MI complex in PB-microsomes, but whereas adding a methyl group to **19** to make **18** confers MIC-forming ability, adding one or two methyl groups to the benzylic carbon of **1** decreases or abolishes MIC formation, respectively. Finally, *N*-methylation of **1** delays the start of MIC formation, suggesting, as noted by others [11, 12], that tertiary amines must first be *N*-dealkylated to secondary amines en route to MIC formation.

The role of nitrone metabolites in MIC formation has never been fully elucidated. Nitrone metabolites of various amines have occasionally been reported in vivo and in vitro with microsomes and even reconstituted P450 systems [54, 55], but rarely are they observed as major metabolites, possibly because many nitrones are hydrolytically labile (steps c and d in Figure 7). Nitrone **5** is one of two conceivable nitrone metabolites of **1** (see Figure 8), but **5** is observed to be very stable in solution at pH 7.5, possibly because it is conjugated [55, 56]. The isomeric nitrone **5'**, however, would be quite electrophilic and reactive, like cyclopropanone itself, because of the $sp^2$ carbon in the strained three-membered ring. For this reason, and because **5'** is

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not stabilized by conjugation like **5**, hydrolysis of **5'** should occur quite readily. This would release hydroxylamine **4**, which is very efficient at forming MI complexes in microsomes and in reconstituted systems (Table 2). That oxime **2** is a significant metabolite of **1** (19% of total) further emphasizes the probable importance of the route through **5'** to MIC formation.

Two major questions remain about the role of nitrone metabolites in MIC formation from **1** (or **3**). First, why is exogenously introduced nitrone **5** such a sluggish precursor to MIC formation compared to exogenously introduced **3** or **4**? Second, given that hydrolysis of **15** is too slow to be involved in MIC formation, how does nitrone C-N bond cleavage occur? It is possible, although not necessarily predictable, that the major route to MIC formation from **1** or **3** is actually via **5'** and not **5**, and that *exogenously introduced* **5** undergoes a *direct* oxidative conversion to a nitroso ligand for MIC formation, thereby obviating the need for hydrolysis. A potential mechanism for the direct oxidation of nitrone to nitroso is depicted in Figure 9. There is now considerable evidence that the hydroperoxy-iron derivative of P450 can be a nucleophilic as well as electrophilic oxidant and oxygen atom transfer agent (for leading references see [57]). Nitrones are electrophilic; thus addition of an EnzFe(III)-O-O-H group to the nitrone $sp^2$ carbon could lead to the bond rearrangements suggested in Figure 9. This would provide a non-hydrolytic mechanism for C-N bond cleavage, and would generate the putative nitroso MIC ligand directly in front of the heme iron. One-electron reduction could then form the stable (ferrous) MIC complex shown in Figure 7. Precedent for this suggestion can be found in a review by Lindeke [58] which suggested that a structure analogous to **21** (i.e., R = $PhCH_2$-, R' = Ph, but with H replacing EnzFe) could undergo decomposition to form benzaldehyde, water and α-nitrosotoluene ($PhCH_2NO$) as a precursor to benzaldoxime (**2**). Further precedent comes from work of Sang et al. who showed that alkylhydroperoxy radicals (ROO•) add to the spin-trap

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reagent *C*-phenyl-*N*-*tert*-butyl nitrone (PBN) to form unstable adducts resembling structure **21** that decompose with C-N and O-O bond scission [59].

In summary, the inactivation of cytochrome P450 enzymes by cyclopropylamines such as **1** (and **14**) *may* involve SET oxidation followed by ring opening and covalent modification of enzyme as suggested in Figure 1, but evidence presented above makes it clear that MIC formation occurs and can potentially account for a significant fraction of the P450 inactivation by **1** and analogs **3**, **12** and **13** (but not **14**). Thus secondary amine **1** first undergoes an obligatory (and possibly rate-limiting) oxidation by the flavin containing momooxygenase to the secondary hydroxylamine **3**. The latter is further oxidized by either P450 or the FMO to form nitrones **5** and/or **5'**. Nitrones that can hydrolyze rapidly (possibly **5'**, for example) can release a primary hydroxylamine to be oxidized rapidly by P450 to the MIC-forming nitroso species. Although nitrone **5** is hydrolytically-stable, when added exogenously it is converted to an MIC faster much than it can hydrolyze to a primary hydroxylamine, suggesting that a non-hydrolytic mechanism like that of Figure 9 is required for MIC formation. Since MI complexes are fairly robust their formation can also account, at least in part, for several other characteristics of P450 inactivation by **1**, such as the time-dependent loss of heme spectrum ($A_{417}$) and the apparent covalent binding of radioactivity to microsomal protein [36, 37, 42]. The formation of ring-opened metabolites of cyclopropylamines by P450 enzymes, and their role in inactivation of P450 activity, remains an open question and is the subject of ongoing work in our laboratory.

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Table 1. Kinetic constants for inactivation of aminopyrine *N*-demethylase activity in PB-microsomes by **1** and **2**.

| Rate constant, units | **1** | **2** |
|---|---|---|
| $k_{inact}$ , $min^{-1}$ | 0.049 | 0.027 |
| $K_i$ , μM | 165 | 116 |
| $k_{inact}/K_i$ , $M^{-1}sec^{-1}$ | 4.92 | 3.88 |
| n, $(r^2)^a$ | 5, (0.9569) | 4, (0.9718) |

[a] Number of data points (observed rates) used in replot, and square of the regression coefficient of the replot from which the reported kinetic constants were derived.

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Table 2. Formation of MI complexes in microsomes and reconstituted P450 systems in vitro.

| | Percent of total P450 converted to MI complex | | | | |
|---|---|---|---|---|---|
| | Microsomes | | Reconstituted P450 systems | | |
| Compound | PB | UT | CYP2B1 | CYP2C11 | CYP2E1 |
| **1** | 26 | 20 | 0 | 0 | 0 |
| **2** | 0 | 0 | | | |
| **3** | 38 | 36 | 27 | 30 | 3 |
| **4** | 54 | 39 | 38 | 60 | 0 |
| **5** | 28 | 33 | 6 | 0 | 0 |
| **6** | 0 | | | | |
| **7** | 0 | | | | |
| **8** | 0 | | | | |
| **9** | 0 | | | | |
| **10** | 0 | | | | |
| **11** | 0 | | | | |
| **12** | 29 | | | | |
| **13** | 8 | | | | |
| **14** | 0 | | | | |
| **15** | 14 | | | | |

Blanks means experiment not done; zero means no MIC observed. Incubation conditions were [P450] = 0.5 or 1.0 μM, temperature =25 ˚C, [test compound] = 100 μM. Generally the maximum amount of MIC formation occurred within 15 min and remained stable for at least 30 additional min.

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Figure 1. Single electron transfer (SET) mechanism for inactivation of P450 enzymes by cyclopropylamine **1**.

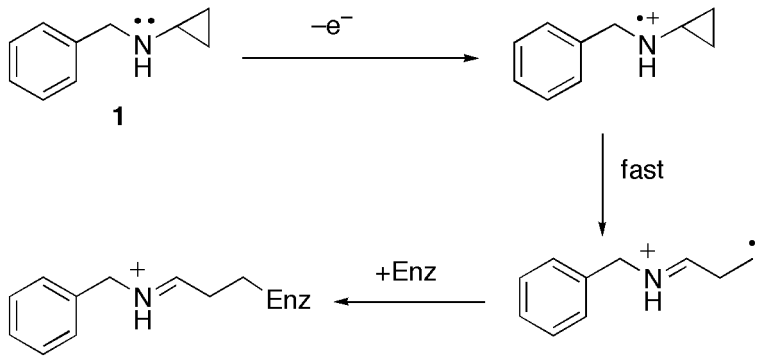


Figure 2. Structures and numbering of compounds studied or mentioned in the text.

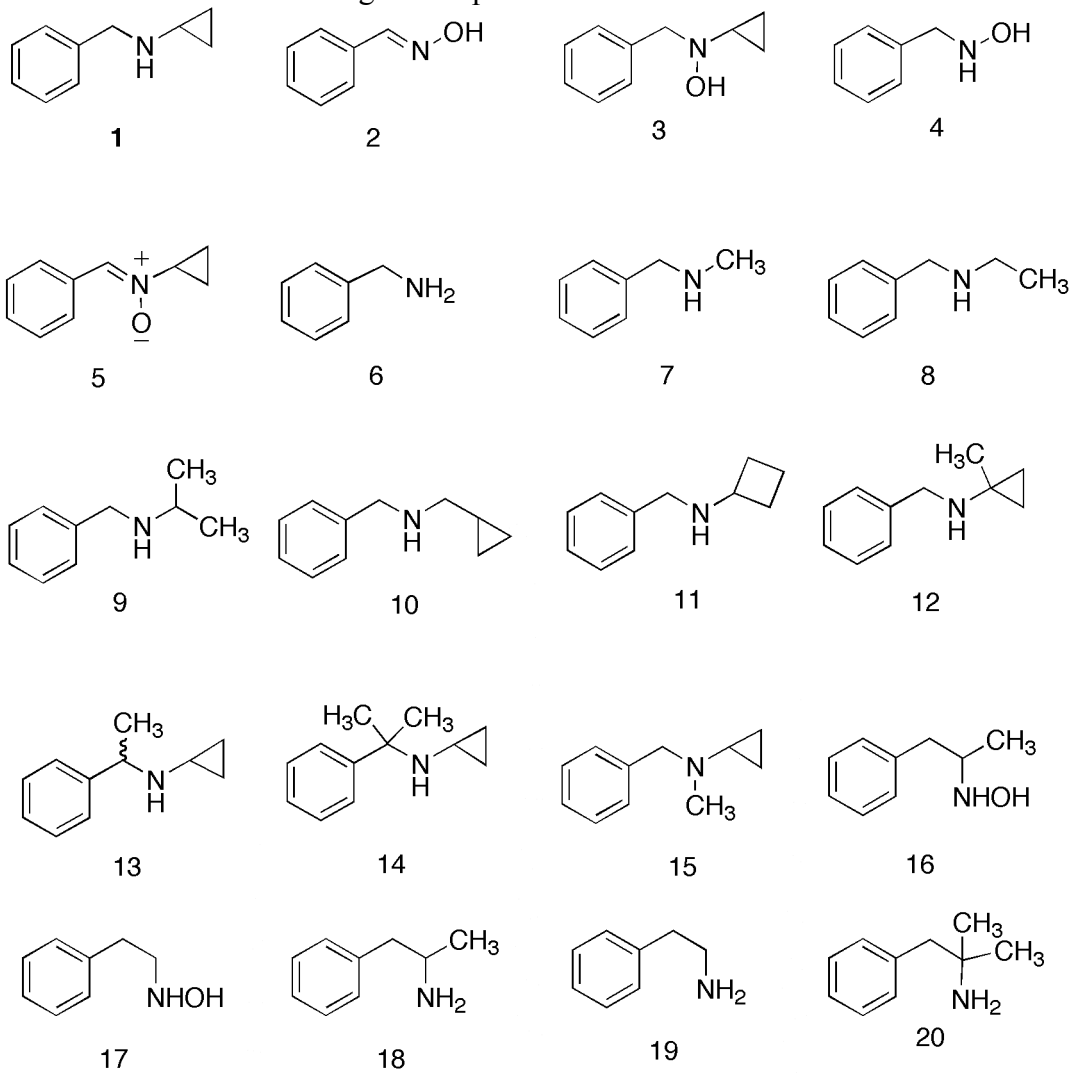

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Figure 3. Kinetics of inactivation of microsomal aminopyrine *N*-demethylase by **1** at 33 ˚C.

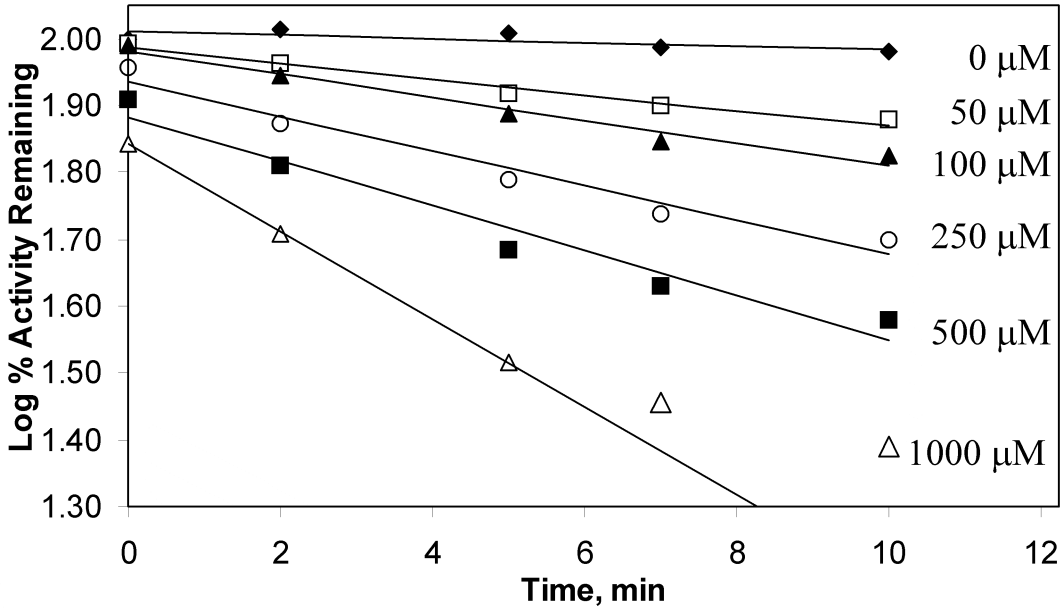

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Figure 4. Time course of spectral changes during oxidation of **1** at 25 ˚C by PB-microsomes (panel A) and their reversal by ferrricyanide (panel B). In panel A the spectrum was scanned every 2 min for 30 min. After the last spectrum of panel A was recorded sodium ferricyanide was added to a concentration of 50 μM and the scan shown in panel B was taken.

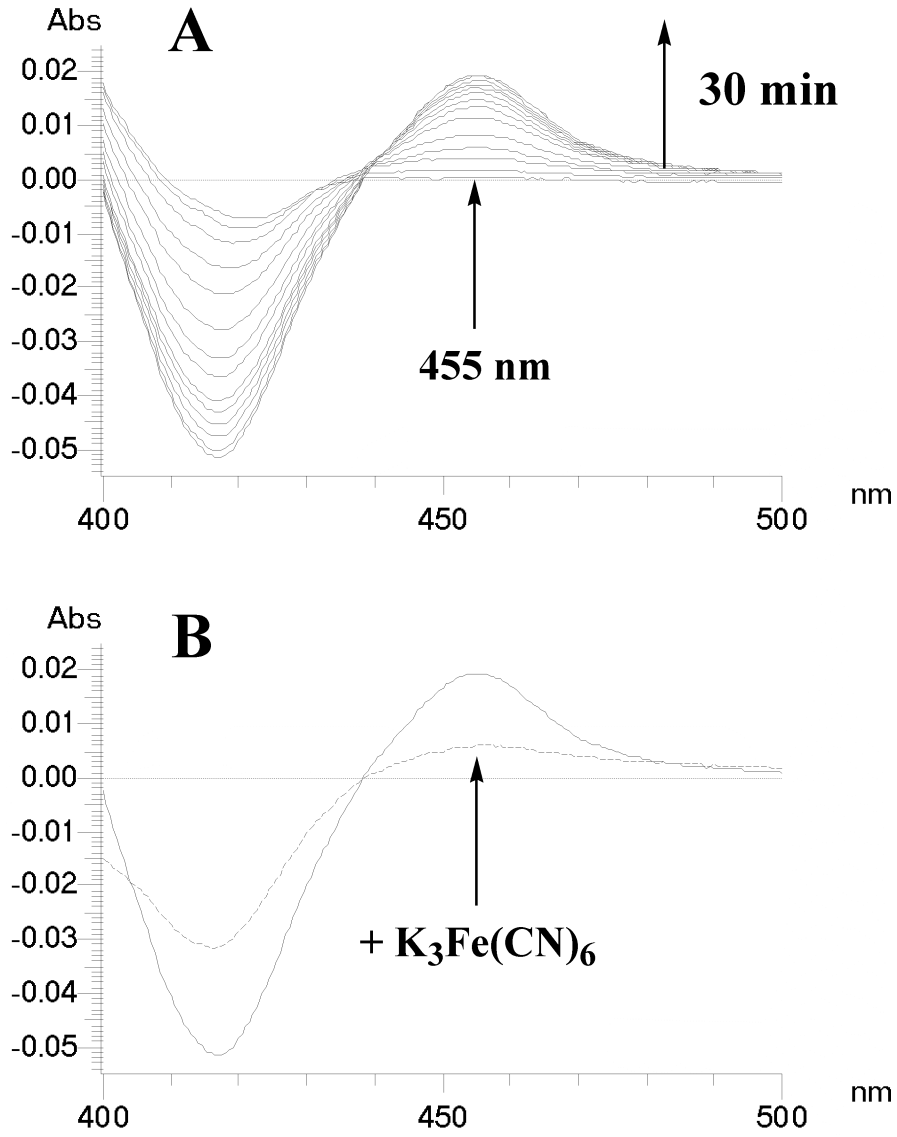

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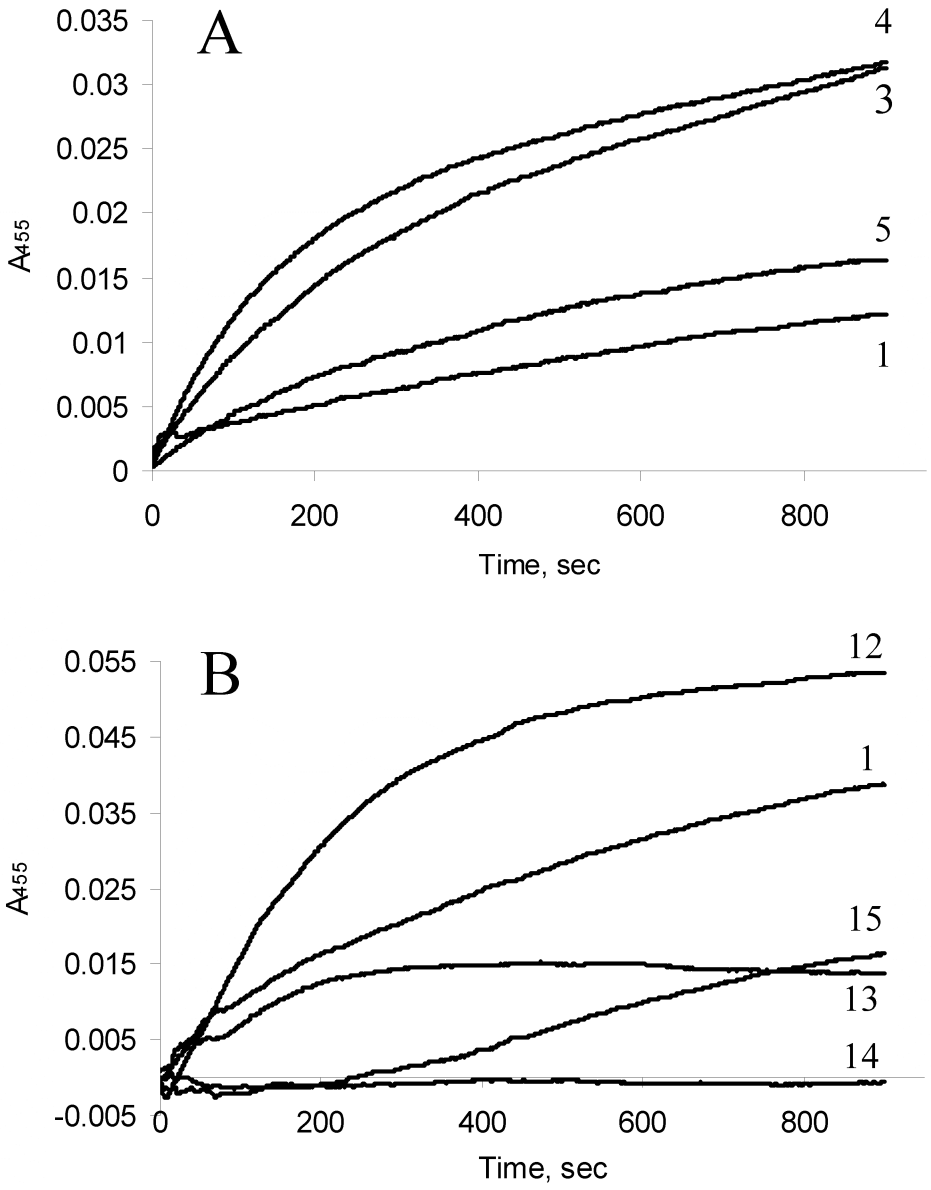
Figure 5. (A) Rate of formation of MI complexes by **1** and related N-oxidized compounds. PB-microsomes, [P450] = 1 μM, were incubated at 25 ˚C with test compounds (100 μM) and NADPH (1 mM) as described in Materials and Methods. Progress curves are numbered to correspond to individual test compounds (see Figure 2). (B) Rate of formation of MI complexes by **1** and methylated congeners. PB-microsomes, [P450] = 3 μM, were incubated at 25 ˚C with test compounds (100 μM) and NADPH (1 mM) as described in Materials and Methods. Progress curves are numbered to correspond to individual test compounds (see Figure 2).



M. A. Cerny and R. P. Hanzlik, "Cyclopropylamine inactivation of cytochromes P450. Role of metabolic intermediate complexes." Arch. Biochem. Biophys. 436, 265-275 (2005). Publisher's official version:
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Figure 6. Kinetics of inactivation of microsomal aminopyrine *N*-demethylase by different concentrations of oxime **2** at 33 ˚C.

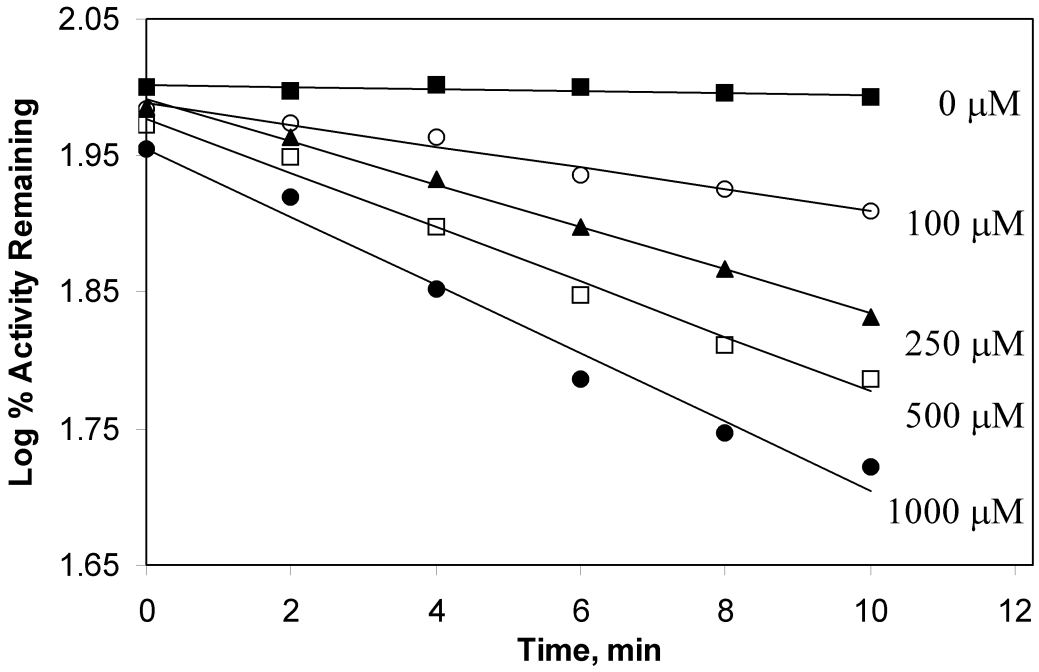
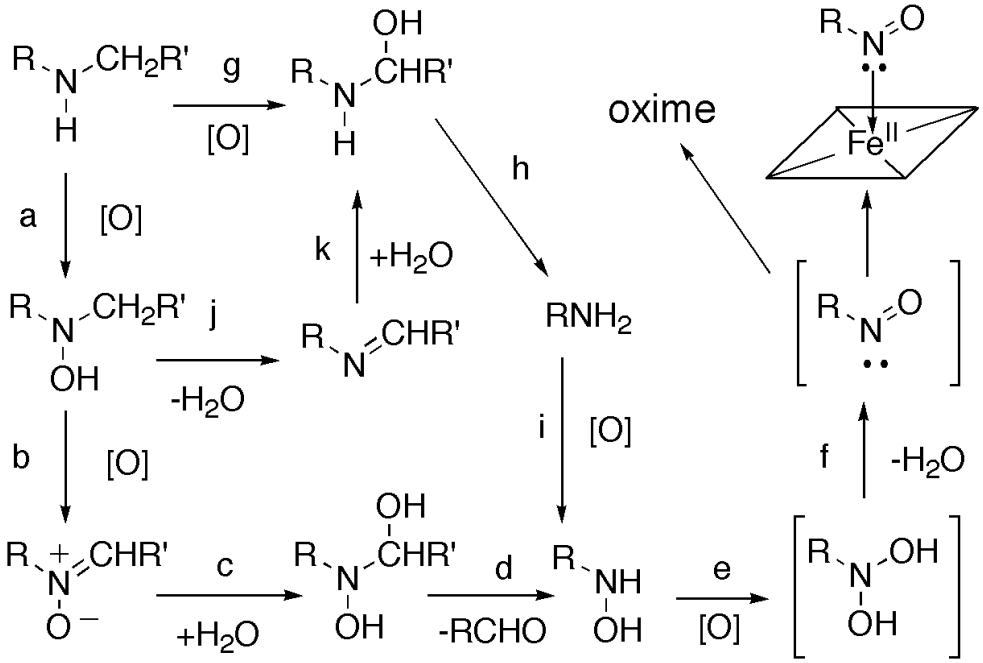



Figure 7. Potential pathways and intermediates in the six-electron oxidation of secondary amines to oximes and MI complexes.



M. A. Cerny and R. P. Hanzlik, "Cyclopropylamine inactivation of cytochromes P450. Role of metabolic intermediate complexes." Arch. Biochem. Biophys. 436, 265-275 (2005). Publisher's official version:
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Figure 8. Overall scheme for microsomal metabolism of *N*-benzyl-*N*-cyclopropylamine (**1**).

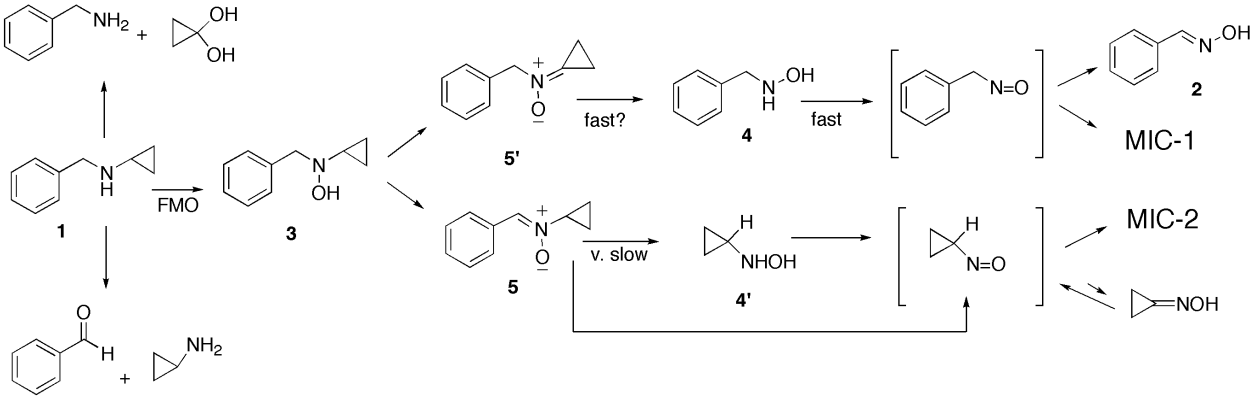


Figure 9. Proposed mechanism for oxidation of nitrone **5** to a nitroso intermediate for oxime formation and MIC formation.