effectively assists the binding of chromophore with substrates or inhibitors.

Registry No. BSAO, 9059-11-4; Cu, 7440-50-8; $PhNHNH_2$, 100-63-0.

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1-Phenylcyclobutylamine, the First in a New Class of Monoamine Oxidase Inactivators. Further Evidence for a Radical Intermediate†

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ABSTRACT: 1-Phenylcyclobutylamine (PCBA) is shown to be both a substrate and a time-dependent irreversible inactivator of monoamine oxidase (MAO). Inactivation results in attachment to the flavin cofactor. For every molecule of PCBA leading to inactivation, 325 molecules are converted to product. The first metabolite formed is identified as 2-phenyl-1-pyrroline; then after a lag time, 3-benzoylpropanal and 3-benzoylpropionic acid are generated. The 3-benzoylpropanal is a product of MAO-catalyzed oxidation of 2-phenyl-1-pyrroline (presumably, of its hydrolysis product, γ-aminobutyrophenone). The aldehyde is nonenzymatically oxidized by nascent hydrogen peroxide to the carboxylic acid. These results are consistent with a one-electron oxidation of PCBA to the amine radical cation followed by homolytic cyclobutane ring cleavage. The resulting radical can partition between cyclization (an intramolecular radical trap) to the 2-phenylpyrrolinyl radical and attachment to the flavin. The cyclic radical can be further oxidized by one electron to 2-phenyl-1-pyrroline. PCBA represents the first in the cyclobutylamine class of MAO inactivators and strongly supports involvement of a radical mechanism for MAO-catalyzed amine oxidations.

Mitochondrial monoamine oxidase (MAO)[1] was discovered almost 60 years ago, yet its mechanism for oxidation of biogenic amines is unknown. On the basis of studies of the mechanism of inactivation of MAO by various cyclopropylamine analogues (Scheme I) (Silverman et al., 1980; Silverman & Hoffman, 1981; Silverman, 1983, 1984; Silverman & Ya-

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[1] Abbreviations: MAO, mitochondrial monoamine oxidase (EC 1.4.3.4); PCBA, 1-phenylcyclobutylamine.

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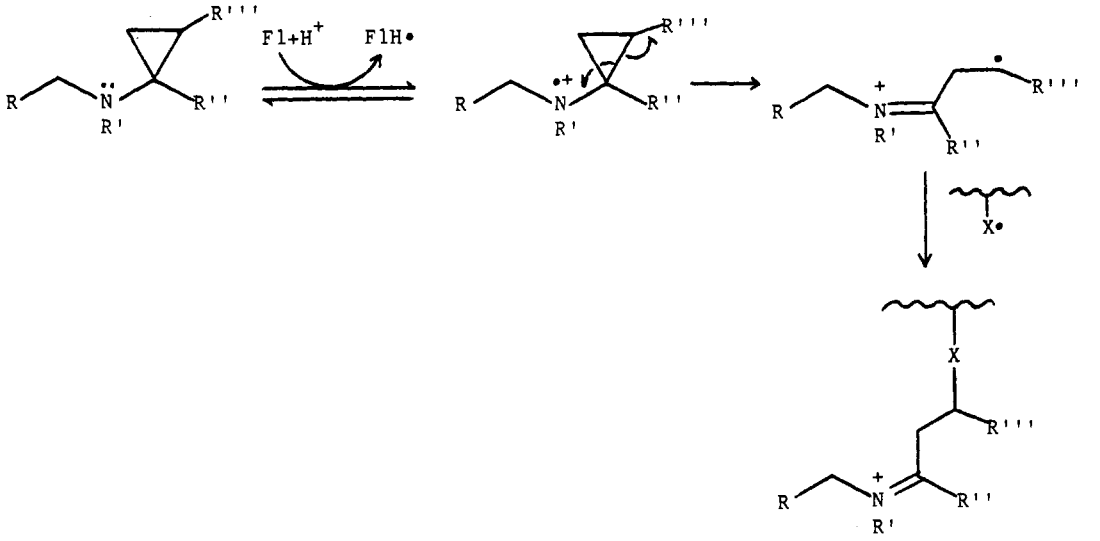
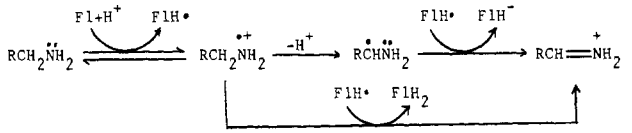
Scheme I: Proposed Mechanism of MAO Inactivation by Cyclopropylamines[a]



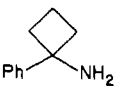
[a] Fl is the oxidized flavin cofactor; X can be the flavin or an amino acid residue.

Scheme II: Proposed Mechanism for MAO-Catalyzed Amine Oxidation[a]



[a] Fl is the oxidized flavin cofactor.

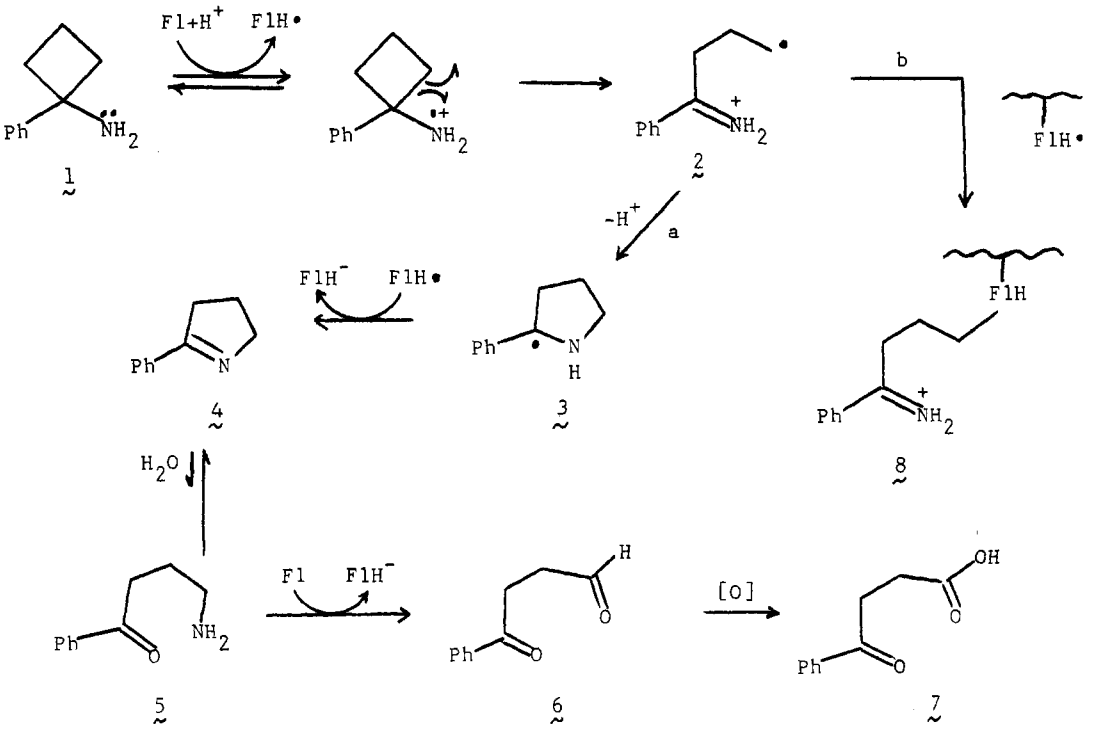
masaki, 1984; Silverman & Zieske, 1985; Vazquez & Silverman, 1985) and by allenic amines (Krantz et al., 1979; Simpson et al., 1982), a one-electron mechanism for MAO-catalyzed amine oxidation was proposed (Scheme II). To the best of our knowledge, no cyclobutylamines have been reported as inactivators of MAO; however, if the mechanisms shown in Schemes I and II are relevant to MAO, certain cyclobutylamines, e.g., PCBA (**1**), should inactivate the enzyme.



1

The N-substituted cyclobutylamino radical is known to undergo homolytic ring cleavage similar to that for the cyclopropylaminyl radical, albeit at a much slower rate (Maeda & Ingold, 1980); consequently, a mechanism similar to that for cyclopropylamine inactivation of MAO may become important for certain cyclobutylamines (Scheme III, pathway b). Furthermore, if **2** (Scheme III) is generated, it has a built-in

Scheme III: Proposed Mechanism for PCBA Oxidation and MAO Inactivation[a]



[a] Fl is the oxidized flavin cofactor.

Table I: TLC R_f Values and HPLC[a] Retention Times of Relevant Compounds

compound	R_f value A[b]	B[c]	C[d]	D[e]	T_R (min)
PCBA			0.09	0.21	4.03
2-phenyl-1-pyrroline			0.19	0.29	2.64
3-benzoylpropanal			0.72	0.79	5.32
3-benzoylpropanoic acid					7.95
3-benzoylpropene			0.77	0.81	2.75
butyrophenone			0.74	0.83	2.52
β-aminopropiophenone					2.88
3-benzoylpropanal, DNP[f]	0.46	0.79	0.75		
3-benzoylpropene, DNP	0.65	0.90	0.73		
butyrophenone, DNP	0.68	0.91	0.76		
2,4-dinitrophenylhydrazine	0.08	0.10			

[a] Alltech analytical 5-μm silica gel column; 3:1 *n*-hexane–ethyl acetate; 2 mL/min. [b] In 3:1 *n*-hexane–ethyl acetate. [c] In 1:1 *n*-hexane–ethyl acetate. [d] In 1:1 ether–ethyl acetate. [e] In 12:3:5 1-butanol–acetic acid–water. [f] 2,4-Dinitrophenylhydrazone.

intramolecular trap for the radical (Scheme III, pathway a); identification of products of this trap should be evidence for a radical intermediate. PCBA (**1**) was selected as the prototype for this potential new class of MAO inactivators because it is an analogue of the substrate benzylamine and is a homologue of 1-phenylcyclopropylamine, an inactivator of MAO (Silverman & Zieske 1985). PCBA was found to be both an inactivator and a substrate for MAO. The results described here provide strong support for involvement of a radical mechanism.

Materials and Methods

Analytical Methods. MAO activity assays and protein concentration determinations were carried out on a Perkin-Elmer Lambda 1 UV/vis spectrophotometer. Optical spectra were recorded in semi-microcuvettes in a Perkin-Elmer 330 spectrophotometer equipped with a Perkin-Elmer data station. Infrared spectra were obtained on a Perkin-Elmer 283 spectrophotometer. NMR spectra were recorded on a Varian EM-390 90-MHz spectrometer. Thin-layer chromatography (TLC) was performed on silica gel 60 coated plastic plates (Merck). Table I lists the R_f values for the relevant compounds with the various solvent systems. An Orion Research Model 601A pH meter with either a general combination electrode or a microcombination probe (Microelectrodes, Inc., Model MI-410) was used to measure pH values. High-performance liquid chromatography (HPLC) was carried out on a Beckman Model 330 liquid chromatograph equipped with a UV detector (254 nm) and a Hewlett-Packard 3390A recording integrator; Table I lists the HPLC retention times for the relevant compounds. Mass spectrometry was carried out on a Hewlett-Packard Model 5985A instrument.

Reagents. Benzylamine hydrochloride was prepared by bubbling HCl gas through an ethereal solution of benzylamine and then recrystallizing the salt from ethanol. 1-Phenylcyclobutanecarbonitrile, butyrophenone, and leuco crystal violet were purchased from Aldrich. 3-Benzoylpropionic acid was bought from Sigma. 1-Phenylcyclobutylamine was prepared by the method of Kalir & Pelah (1967) starting from 1-phenylcyclobutanecarbonitrile. 2-Phenyl-1-pyrroline was prepared by the procedure of Kemppainen et al. (1976). β-Aminopropiophenone was prepared as previously described (Silverman & Zieske, 1985). 3-Benzoylpropene was prepared by the pyridinium chlorochromate oxidation of the corresponding alcohol (Minasian et al., 1982). 2,4-Dinitrophenylhydrazone derivatives of the carbonyl compounds were prepared by the method of Shriner et al. (1980).

3-Benzoylpropanal. 3-Benzoylpropionic acid was reduced to the diol as described by Cousseau & Lamant (1967): NMR ($CDCl_3$) δ 1.2–1.6 (m, 4 H), 3.1 (t, 1 H), 3.4 (br s, 2 H), 3.7 (m, 5 H), and 7.2–7.9 (m, 5 H). A portion of the diol (310 mg, 1.9 mmol) was dissolved in methylene chloride (10 mL), and pyridinium chlorochromate (750 mg, 3.4 mmol) was added. After 2 h at room temperature, ether (10 mL) and isopropyl alcohol (0.5 mL) were added, and the mixture was shaken vigorously. The solution was then passed through Florisil, dried ($MgSO_4$), and filtered, and the solvent was removed by rotary evaporation, giving the aldehyde as a colorless oil (0.16 g, 51%): NMR ($CDCl_3$) δ 2.9 (m, 2 H), 3.3 (t, 2 H), 7.3–8.0 (m, 5 H), and 9.9 (br s, 1 H).

Enzymes and Assays. These were reported previously (Silverman & Zieske, 1985). Protein concentration determinations were done with the Pierce BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). Horseradish peroxidase (160 purpurogallin units/mg) was bought from Sigma. When PCBA was the substrate, the amount of enzymatic oxidation, as measured by the production of hydrogen peroxide, was determined by the leuco crystal violet method of Mottola et al. (1970). The incubation period was such that the production of hydrogen peroxide was linear with time.

Identification of PCBA Oxidation Products. Method 1. MAO (14 μM) containing PCBA (13 mM) in 50 mM potassium phosphate, pH 7.2, buffer (800-μL total volume) was incubated at 25 °C in the dark for 4 days. The solution was then made strongly basic with 2 N sodium hydroxide, extracted with ether (4 × 1 mL), acidified with 6 N hydrochloric acid, and extracted with ether (4 × 1 mL). All extracts were combined, and the solvent was evaporated. The samples were analyzed by TLC with PCBA, 2-phenyl-1-pyrroline, β-aminopropiophenone, 3-benzoylpropanal, 3-benzoylpropionic acid, butyrophenone, and 3-benzoylpropene as standards (Table I).

Studies also were carried out in which the amines and non-amines were initially separated by ion-exchange chromatography [Dowex-50 (H^+)] before TLC and in which the extracts were treated with 2,4-dinitrophenylhydrazine reagent (Shriner et al., 1980) and chromatographed with the 2,4-dinitrophenylhydrazone derivatives of 3-benzoylpropanal, 3-benzoylpropionic acid, butyrophenone, and 3-benzoylpropene as standards (Table I).

Method 2. MAO was incubated with PCBA, and the oxidation products were extracted as described under Method 1. The samples then were analyzed by GC/MS with PCBA, 2-phenyl-1-pyrroline, β-aminopropiophenone, 3-benzoylpropanal, 3-benzoylpropionic acid, butyrophenone, and 3-benzoylpropene as standards.

Method 3. MAO (30 μM) containing PCBA (12 mM) in 50 mM potassium phosphate, pH 7.2, buffer (2-mL total volume) was incubated at 25 °C in the dark. At indicated times, 250-μL aliquots were withdrawn and extracted as described under Method 1. The samples were then dissolved in 50 μL of 3:1 *n*-hexane–ethyl acetate and analyzed by HPLC with PCBA, 2-phenyl-1-pyrroline, β-aminopropiophenone, 3-benzoylpropanal, 3-benzoylpropionic acid, butyrophenone, and 3-benzoylpropene as standards (Table I).

Similar studies also were carried out by using all three methods substituting 2-phenyl-1-pyrroline for PCBA. Samples containing 3-benzoylpropanal, MAO, and up to 5 mM hydrogen peroxide were incubated in the same manner, and extracts were analyzed by HPLC. Control samples containing no enzyme also were carried out in all cases.

Determination of the Turnover Number for PCBA. Method 1. MAO (7.5 μM) containing various concentrations of PCBA

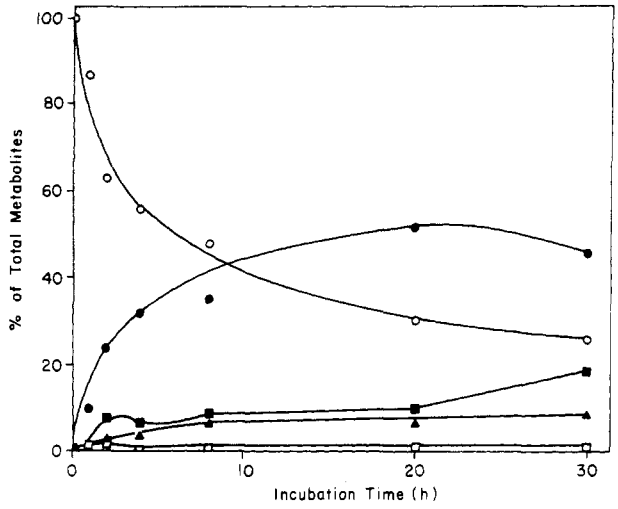


FIGURE 1: MAO-catalyzed metabolism of PCBA: PCBA (○); 2-phenyl-1-pyrroline (●); 3-benzoylpropanal (■); 3-benzoylpropanoic acid (▲); γ-aminobutyrophenone (□).

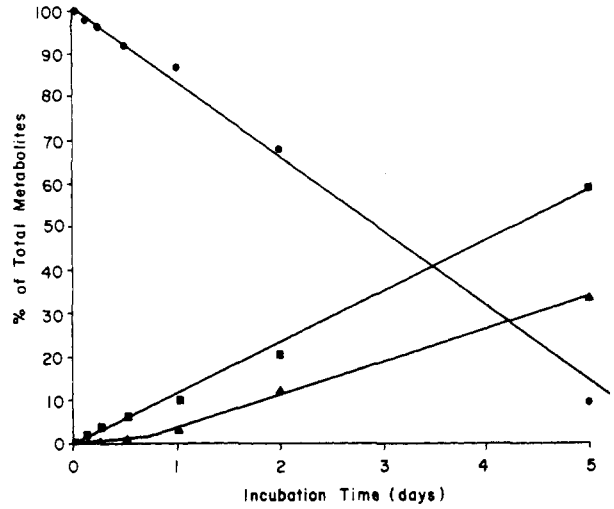


FIGURE 2: MAO-catalyzed metabolism of 2-phenyl-1-pyrroline: 2-phenyl-1-pyrroline (●); 3-benzoylpropanol (■); 3-benzoylpropanoic acid (▲).

(0–4 mM) in 50 mM potassium phosphate, pH 7.2, buffer (250-μL total volume) was incubated at 25 °C in the dark. At indicated times, 20-μL aliquots were removed and assayed for enzyme activity. The final enzyme activities were replotted vs. the PCBA/MAO ratios to determine the turnover number.

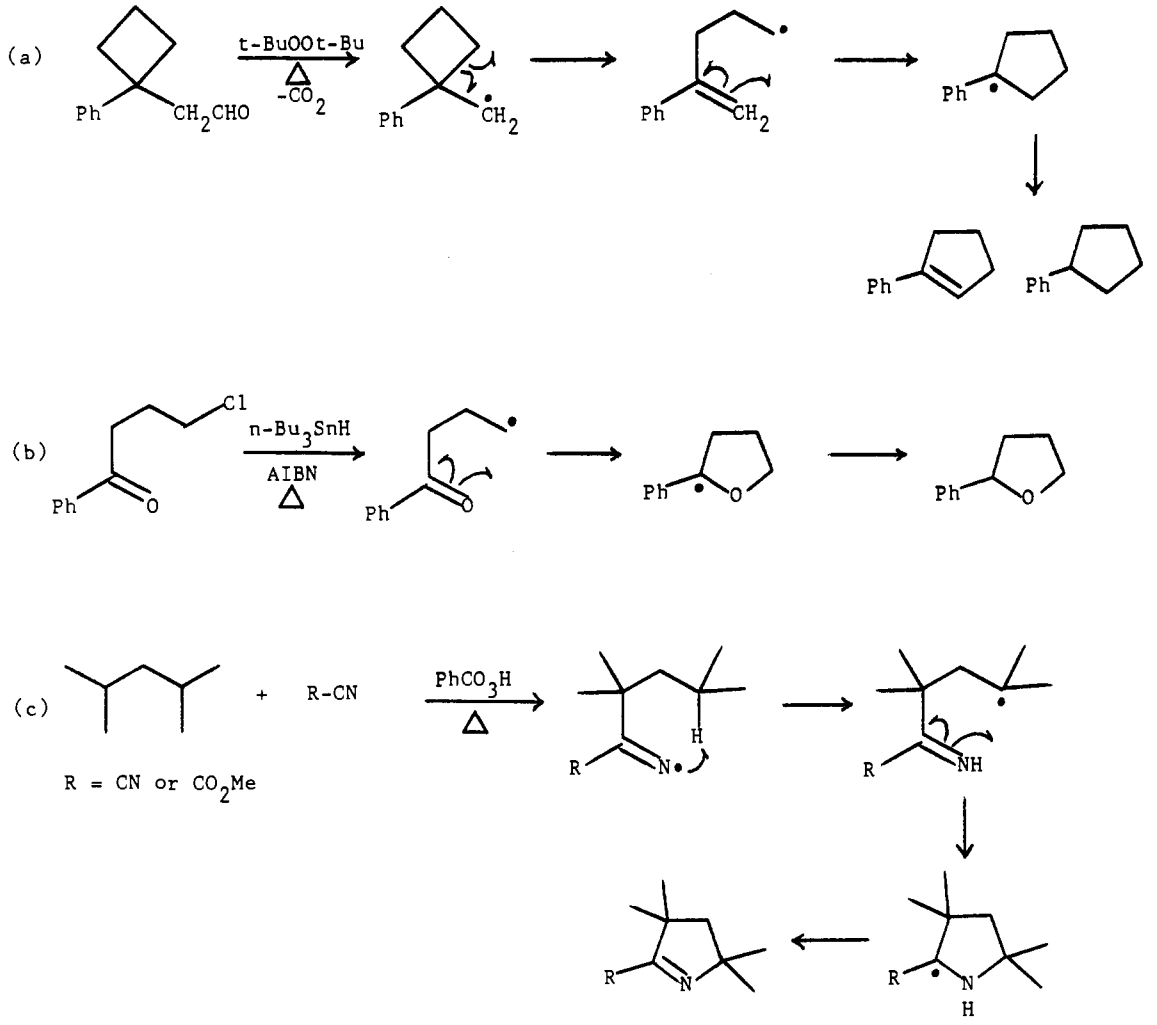
Method 2. MAO (22 μM) containing PCBA (8 mM) in 50 mM potassium phosphate, pH 7.2, buffer (2-mL total volume) was incubated at 25 °C in the dark. After 0, 2, 4, 6, 8, 10, and 24 h, 270-μL samples were withdrawn of which 70-μL aliquots were immediately placed onto Sephadex G-25 (0.7 × 10 cm) equilibrated with and eluted with 20 mM potassium phosphate, pH 7.2, buffer. The fraction containing the majority of the protein was collected. Each of these fractions for the various time points was assayed for enzyme activity and protein concentration. The remaining 200-μL portions from above were extracted with ether and analyzed by HPLC as described above. The turnover number was determined by comparing the amount of PCBA metabolized to the amount of MAO inactivated.

Results and Discussion

PCBA was found to be both a substrate and an inactivator of MAO. Its K_m (110 μM) is lower than the K_m for benzylamine (340 μM) (Yamasaki & Silverman, 1985), but its k_{cat} (0.41 min^{-1}) is well below that for benzylamine (270 min^{-1}). Preincubation of MAO with PCBA resulted in a time-dependent loss of enzyme activity, which was protected by substrate; the addition of β-mercaptoethanol did not protect the enzyme from inactivation. Dialysis or gel filtration did not restore enzyme activity. At all concentrations of PCBA, inactivation appeared to be progressively inhibited with time. This appears to be the result of product inhibition (vide infra). Because of this interference, no reliable kinetic data were obtained that could be used to determine the K_{inact} and k_{inact}. Inactivation of the enzyme by PCBA was accompanied by a conversion of the flavin spectrum to that of reduced flavin. Denaturation of the inactivated enzyme did not lead to reoxidation of the flavin, indicating that attachment was to the flavin, as is the case with 1-phenylcyclopropylamine (Silverman & Zieske, 1985). Consequently, these results suggest that PCBA is a mechanism-based (enzyme-activated) inactivator (Silverman & Hoffman, 1984; Walsh, 1984; Abeles, 1983; Rando, 1984) of MAO.

In order to determine the mechanism of inactivation of MAO by PCBA, we investigated the MAO-catalyzed metabolism of this compound. It was hoped that an understanding of the reaction leading to product formation may provide a basis for proposing an intermediate that could lead to inactivation as well as to product. The reaction was monitored with time by HPLC (Figure 1). The consumption of PCBA was accompanied by an increase in a metabolite identified by TLC, HPLC, and mass spectrometry as 2-phenyl-1-pyrroline (**4**, Scheme III). After a lag period, a second metabolite began to form, which was identified by TLC, HPLC, and mass spectrometry as 3-benzoylpropanal (**6**, Scheme III). This compound continued to increase in concentration with time while 2-phenyl-1-pyrroline reached a maximum concentration and then began to diminish. A third metabolite appeared to increase slowly and linearly after a lag period and was identified as 3-benzoylpropanoic acid (**7**, Scheme III). In the absence of MAO, PCBA was completely stable under the reaction conditions. Because of the initial increase, and then decrease, in the concentration of 2-phenyl-1-pyrroline, it was suspected as the intermediate in the formation of 3-benzoylpropanal and 3-benzoylpropanoic acid. Incubation of MAO with 2-phenyl-1-pyrroline resulted in the consumption of this compound and formation of 3-benzoylpropanal *with no lag period* (Figure 2). A control run in the absence of MAO proved 2-phenyl-1-pyrroline to be stable under these conditions. 2-Phenyl-1-pyrroline (5 mM) does not inactivate MAO even after 72 h. The oxidation of 3-benzoylpropanal to 3-benzoylpropanoic acid was shown to be the result of a nonenzymatic oxidation by the hydrogen peroxide that is formed during oxidation of the substrates. There was no difference in the rate of oxidation of 3-benzoylpropanal in the presence or absence of MAO, but the addition of a low concentration of hydrogen peroxide accelerated the oxidation rate. When the metabolism was observed over an extended period of time at a ratio of 460:1 (inactivator:enzyme), only about 60% of the PCBA was consumed and the production of the two metabolites ceased. This is consistent with concomitant enzyme inactivation. The partition ratio (ratio of number of turnovers to product per turnover to give inactivation) was determined by quantifying the amount of PCBA consumed and the loss of enzyme activity at each time point; an average value of 325 for all of the time points was obtained. The partition ratio also was determined by adding various concentrations of PCBA to MAO and monitoring the loss of enzyme activity until it reached a constant minimum. A plot of the PCBA/MAO ratio vs. percent of enzyme activity re-

Scheme IV: Examples of Intramolecular Radical Traps



maining (Figure 3) was linear to about 60% enzyme activity; then, an upward deviation from linearity was observed. If the linear portion is extrapolated to zero enzyme activity, a turnover number of 325 is obtained. This upward deviation probably results from competitive inhibition of PCBA by 2-phenyl-1-pyrroline or 2-benzoylpropanal; the K_i values against benzylamine for these compounds are 360 and 930 μM, respectively.

The results of these metabolic and inactivation studies can be rationalized by the mechanism shown in Scheme III. A one-electron oxidation of PCBA would give the amine radical cation. Maeda & Ingold (1980) showed that *N*-*n*-propyl-*N*-cyclobutylamino radical generation results in homolytic cleavage of the cyclobutyl ring with an extrapolated rate constant at 25 °C of 1.2×10^5 s^{-1}, which is smaller than that for the cyclopropyl analogue (too fast to measure). Homolytic ring cleavage of the PCBA radical cation gives **2**, which is the proposed intermediate responsible for the partitioning between product formation (Scheme III, pathway a) and inactivation (Scheme III, pathway b). There is ample precedent in closely related systems for the cyclization of **2** to **3**. Wilt et al. (1966) generated the (1-phenylcyclobutyl)methyl radical, which yielded a mixture of 1-phenylcyclopentane and 1-phenylcyclopentene, the disproportion products of the 1-phenylcyclopentanyl radical (Scheme IVa). Menapace & Kuivila (1964) obtained 2-phenyltetrahydrofuran as the product of cyclization of 3-benzoylpropanyl radical (Scheme IVb). Tanner & Rahimi (1979) observed a similar ring closure for a substituted γ-iminopropyl radical (Scheme IVc). Cyclization, apparently, is quite efficient because the partition ratio in our case is 325. A second electron oxidation of **3** would give the initial metabolite **4**. 2-Phenyl-1-pyrroline is relatively stable to hydrolysis (Kemppainen et al., 1976), presumably because the back reaction (**5** → **4**) is favored. However, **5** should be a good substrate for MAO since 4-phenylbutylamine (Yamasaki & Silverman, 1985) and 3-keto-3-phenylpropylamine (Silverman & Zieske, 1985) are excellent substrates. This would explain why **5** was not observed as a metabolite;

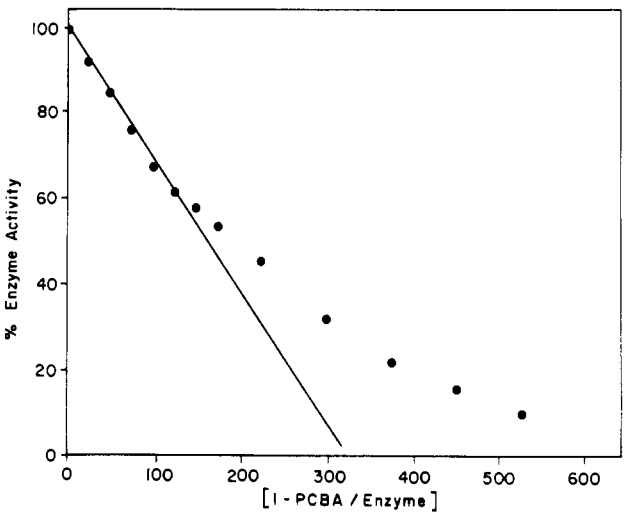


FIGURE 3: Effect on MAO activity of increasing concentrations of PCBA.

the low concentration of **5** generated from hydrolysis of **4** would be rapidly oxidized by MAO to **6**.

PCBA is the first example of a new class of mechanism-based inactivators of MAO containing a cyclobutylamine framework. This compound was designed as a substrate and inactivator on the basis of a radical mechanism for MAO-catalyzed amine oxidation. It is difficult to imagine a non-radical pathway for PCBA that produces the observed metabolites and also inactivates the enzyme. These results, therefore, provide excellent support for a radical mechanism of MAO-catalyzed amine oxidations.

Registry No. MAO, 9001-66-5; PCBA, 17380-77-7; **4**, 700-91-4; **5**, 7643-94-9; **6**, 56139-59-4; **7**, 2051-95-8; $PhCO(CH_2)_2CH(OH)_2$, 99397-75-8.

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