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2-Ethynylnaphthalene as a Mechanism-Based Inactivator of the Cytochrome P-450 Catalyzed N-Oxidation of 2-Naphthylamine

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Since the N-oxidation of several carcinogenic arylamines has been shown to be catalyzed preferentially by cytochrome P-450IA2 in several species, homologous ethynyl-substituted aromatic hydrocarbons, 2-ethynylnaphthalene, 1-ethynylnaphthalene, and 2-ethynylfluorene, were synthesized and examined as potential mechanism-based inactivators of this monooxygenase. By use of 2-naphthylamine, whose N-oxidation was known to be selectively catalyzed by rat cytochrome P-450$_{ISF-G}$ (P-450IA2), and hepatic microsomes from isosafrole-treated rats, each of these ethynyl derivatives was found to be strongly inhibitory at concentrations of 1 and 10 μM. However, only inhibition by 2-ethynylnaphthalene was significantly enhanced by prior incubation with the microsomal system. The inactivation of 2-naphthylamine N-oxidation was found to be NADPH- and time-dependent and to follow pseudo-first-order kinetics, demonstrating that 2-ethynylnaphthalene is a potent mechanism-based inactivator of the enzymatic activity. The extrapolated $k_{inactivation}$ and K_I were 0.23 min^{-1} and 8 μM, respectively. By use of 2-aminofluorene, whose N-oxidation was known to be catalyzed by both cytochromes P-450$_{ISF-G}$ and P-450$_{\beta NF-B}$ (P-450IA1), and the purified enzymes in a reconstituted system, both 2-ethynylnaphthalene and 1-ethynylnaphthalene were found to be strongly inhibitory. However, 2-ethynylnaphthalene was a more potent inhibitor of the purified P-450$_{ISF-G}$ than of P-450$_{\beta NF-B}$; and it was also found to be a more potent inhibitor of P-450$_{ISF-G}$ than was 1-ethynylnaphthalene. With radiolabeled 2-ethynylnaphthalene and purified P-450$_{ISF-G}$, the metabolic formation of [^{3}H]-2-naphthylacetic acid was measured and used to calculate a partition ratio of 64 (molecules of 2-ethynylnaphthalene metabolized per molecule of P-450 inactivated). Covalent binding of 2-ethynylnaphthalene to the purified P-450s was also demonstrated, resulting in 0.62–0.77 mol of [^{3}H]-2-ethynylnaphthalene bound per mole of P-450$_{ISF-G}$ and 0.72 mol of [^{3}H]-2-ethynylnaphthalene bound per mole of P-450$_{\beta NF-B}$. These studies suggest that 2-ethynylnaphthalene may be useful as a inhibitor of arylamine N-oxidation and as an active site probe for cytochrome P-450IA2.

The hepatic cytochrome P-450IA2 (P-450$_{ISF-G}$),[1] which is preferentially inducible by isosafrole in rats, is a major enzyme catalyzing the N-oxidation of carcinogenic primary aromatic amines (*1*). Since this conversion is an obligatory step in the carcinogenesis of these arylamines, specific mechanism-based inactivators of the enzyme could prove to be useful as modulators of carcinogenesis. Additionally, such inactivators could be valuable as probes in learning more about the nature of the catalytic site of this monooxygenase.

Certain compounds containing the acetylenic functional group have been established as inhibitors of P-450s (*2*) and cause the suicidal destruction (mechanism-based inactivation) of these enzymes. By a strategy of incorporating the reactive acetylenic functional group into structural analogues of substrate molecules, selective inhibition of specific P-450s has been reported. For example, 1-ethynylpyrene and methyl-1-pyrenylacetylene were found to be mechanism-based inactivators of P-450-dependent benzo[*a*]pyrene hydroxylase activity (*3*, *4*). Similarly, Ortiz de Montellano and Reich (*5*) have shown that an acetylenic fatty acid, 11-dodecynoic acid, is a selective inactivator of the P-450s catalyzing ω- and (ω – 1)-hydroxylation of lauric acid.

In an effort to apply this strategy to the design of a potential inhibitor for P-450$_{ISF-G}$, 2-ethynylnaphthalene, 1-ethynylnaphthalene, and 2-ethynylfluorene were prepared (Figure 1) as structural homologues of 2-naphthylamine, 1-naphthylamine, and 2-aminofluorene. The oxidation of these arylamines by different rat P-450s has been previously characterized (*6*); while P-450$_{ISF-G}$ had the highest catalytic activity, the regioselectivity with respect to the substrate differed markedly. Specifically, the

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[1] Abbreviations: P-450, liver microsomal cytochrome P-450. P-450$_{ISF-G}$ and P-450$_{\beta NF-B}$ are two forms of rat liver P-450 whose properties have been described in detail elsewhere (*7*, *8*). These proteins correspond to P-450IA2 and P-450IA1, respectively, in the gene classification of Nebert et al. (*9*) and have been termed P-450d and P-450c and also P-448H and P-448L in other laboratories; for these and other comparisons in the literature, see refs 10 and 11.

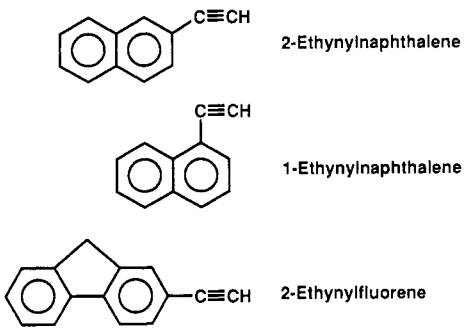


Figure 1. Structures of the ethynyl-substituted compounds prepared for this study.

enzyme catalyzed both N-oxidation and ring oxidation of 2-naphthylamine, but only ring oxidation of 1-naphthylamine. Moreover, P-450$_{ISF-G}$ was the only P-450 of the eight surveyed to catalyze the N-oxidation of 2-naphthylamine. The N-oxidation of 2-aminofluorene, on the other hand, was catalyzed effectively by P-450$_{ISF-G}$ and P-450$_{\beta NF-B}$, and to a lesser extent by four other P-450s. Given these differences in regioselectivity, initial experiments were carried out in which each of the ethynyl-substituted analogues was examined for its ability to inhibit the 2-naphthylamine N-oxidation activity of hepatic microsomes prepared from isosafrole-treated rats. The results of these initial experiments led to the selection of 2-ethynylnaphthalene for further characterization of its inhibitory effect. 2-Ethynylnaphthalene was thus found to be a potent mechanism-based inactivator of 2-naphthylamine and 2-aminofluorene N-oxidation and to result in covalent binding to the purified P-450s.

Experimental Procedures

Chemicals. [5,6,7,8-^{3}H]-2-Naphthylamine (47 mCi/mmol) was obtained from ChemSyn Science Laboratories, Lenexa, KS. The following materials were purchased: glucose 6-dehydrogenase (from *Leuconostoc mesenteroides*), L-α-dilauroyl-*sn*-glycero-3-phosphocholine, NADPH, and 2′,5′-ADP agarose gel from Sigma Chemical Company Co., St. Louis, MO; glucose 6-phosphate, $NADP^+$, and NAD^+ from P-L Biochemicals, Milwaukee, WI; desferrioxamine mesylate from CIBA Pharmaceuticals, Summit, NJ; isosafrole from Eastman Kodak Chemical Co., Rochester, NY; 1-acetonaphthone, 2-acetonaphthone, *n*-butyllithium, PCl_5, triphenylphosphine, dibromomethane, 2-fluorenecarboxaldehyde, and 2-naphthylacetic acid from Aldrich Chemical Co., Milwaukee, WI; and potassium *tert*-butoxide from Pfaltz and Bauer, Waterbury, CT. *N*-Hydroxy-2-naphthylamine (*12*) and 2-nitrosonaphthalene (*13*) were synthesized according to published procedures. Pyridine was dried by treatment with solid KOH, distilled, and then stored over KOH pellets. Tetrahydrofuran was distilled from purple sodium benzophenone dianion under an N_2 atmosphere. Toluene was distilled from Na metal under an N_2 atmosphere. Thin-layer chromatography was done on silica gel 60 plates (F-254, 0.25-mm thickness, EM Laboratories, Inc., Cherry Hill, NJ). Silica gel 60 (230–500 mesh) from Merck & Co., Darmstadt, was used for flash chromatography.

Instrumentation. ^{1}H-NMR spectra were obtained on a Bruker AC200 NR/200 FT NMR spectrometer. Mass spectral data were obtained with a Hewlett-Packard Model 5995 gas chromatograph/quadrapole mass spectrometer equipped with a capillary column operating in the electron impact mode at 70 eV. UV spectra were obtained with a Hewlett-Packard 8451A diode array spectrophotometer. Analyses were performed by Galbraith Laboratories, Knoxville, TN, or by Spang Microanalytical Laboratory, Eagle Harbor, MI.

Synthesis of Ethynyl Compounds. 1-Ethynylnaphthalene. 1-Ethynylnaphthalene was synthesized by a modification of the method of Wade et al. (*14*). PCl_5 (22 g, 0.105 mol) was dissolved in 75 mL of dry pyridine and warmed to 50 °C in a round-bottom flask fitted with a reflux condenser and a $CaCl_2$ drying tube. 1-Acetonaphthone (3.4 g, 0.02 mol), dissolved in 75 mL of dry pyridine, was added over a 15-min period, and the mixture was stirred at 55 °C for an additional 6 h. The reaction mixture was then cooled to room temperature and poured into 150 mL of cold aqueous 2 N HCl containing 25 g of ice. The product was extracted with petroleum ether, and the extract was sequentially washed with 2 N HCl, water, and a saturated NaCl solution. The petroleum ether was removed in vacuo, and the resulting brown liquid was purified by flash chromatography as described by Still et al. (*15*) using 5% toluene in cyclohexane as the eluting solvent. The yellow liquid 1-ethynylnaphthalene (0.26 g, 9% yield) gave a single spot on thin-layer chromatography (R_f = 0.73, hexane/toluene, 19:1): ^{1}H NMR (in $CDCl_3$) δ 3.46 (s, 1 H, ≡CH), 7.37–7.62 (m, 3 H, ArH), 7.71–7.75 (d, 1 H, ArH), 7.81–7.85 (d, 2 H, ArH), 8.37–8.38 (d, 1 H, ArH); mass spectrum, *m/z* 152.1 (M^+); UV (CH_3CN) λ_{max} 238, 286, and 296 nm, shoulder at 308 nm. Anal. Calcd for $C_{12}H_8$: C, 94.70, H, 5.30. Found: C, 95.02, H, 5.23.

2-Ethynylnaphthalene. 2-Ethynylnaphthalene was prepared from 2-acetonaphthone by the procedure described above. NMR analysis of the initial 2-ethynyl product indicated the presence of about 25% of the chlorovinyl byproduct. Thus, the crude material was dissolved in 15 mL of hexane and cooled to −78 °C under an N_2 atmosphere; 1.0 mL of 2.5 M *n*-butyllithium was added, and the mixture was stirred for 3 h at −78 °C under N_2. Five milliliters of cold H_2O was added, and the reaction mixture was allowed to warm to room temperature overnight. The hexane was separated, and the water layer was washed with additional hexane. The combined hexane extracts were washed sequentially with 1 N HCl and water and then evaporated to dryness in vacuo. The product was purified by flash chromatography, by using 5% toluene in hexane, to give 0.375 g (12%) of white crystalline 2-ethynylnaphthalene, which give a single spot on thin-layer chromatography (R_f = 0.71, hexane/toluene, 19:1): mp 40–42 °C; ^{1}H NMR (in $CDCl_3$) δ 3.14 (s, 1 H, ≡CH), 7.47–7.55 (m, 3 H, ArH), 7.77–7.83 (m, 3 H, ArH), 8.03 (s, 1 H, ArH); mass spectrum, *m/z* 152.1 (M^+); UV (CH_3CN) λ_{max} 276, 286, and 296 nm, shoulder at 244 nm. Anal. Calcd for $C_{12}H_8$: C, 94.70, H, 5.30. Found: C, 94.36, H, 4.99.

The characteristics of 2-ethynylnaphthalene prepared above are consistent with data reported by Takahashi et al. (*16*), who prepared this derivative by a bis(triphenylphosphine)palladium dichloride/CuI catalyzed coupling of (trimethylsilyl)acetylene and 2-iodonaphthalene. The chemical shift for the acetylenic proton of 2-ethynylnaphthalene reported above (δ 3.14) is also consistent with the shift previously observed by Okamoto et al. (*17*), as is the chemical shift of the acetylenic proton of 1-ethynylnaphthalene. However, the properties for 2-ethynylnaphthalene differ from those reported by Wilcox and Weber (*18*), who prepared this compound from the 2-ethenyl derivative by bromination and dehydrogenation.

2-Ethynyl[*ring*-G-^{3}H]naphthalene was prepared from 2-acetyl[*ring*-G-^{3}H]naphthalene (ChemSyn Science Laboratories, Lenexa, KS) and purified as described above. The radiolabeled 2-acetylnaphthalene (231 mg, 1.36 mmol) yielded 21 mg of purified [^{3}H]-2-ethynylnaphthalene (0.137 mmol, 10%). Analysis of the purified product by thin-layer chromatography showed a single spot whose R_f was identical with that of a reference sample of the unlabeled compound. The specific activity, based upon that of the starting 2-acetylnaphthalene, was 512 mCi/mmol.

2-Ethynylfluorene. 2-Ethynylfluorene was synthesized by the general method of Matsumoto and Kuroda (*19*) from 2-fluorenecarboxaldehyde. (Bromoethyl)triphenylphosphonium bromide was first prepared by heating 60 g (0.23 mol) of triphenylphosphine and 36 mL (0.5 mol) of dibromomethane under reflux in 500 mL of dry toluene for 24 h. The mixture was cooled to 0 °C, and the solid product was removed by suction filtration and washed with cold toluene. The filtrate was then heated an additional 24 h under reflux and cooled to 0 °C to obtain a second crop of crystals. The combined solids were dried in vacuo to yield 69.5 g (70%) of (bromomethyl)triphenylphosphonium bromide. A portion of this product (6.73 g, 0.015 mol) was mixed with potassium *tert*-butoxide (5.04 g, 0.045 mol) in 25 mL of tetrahydrofuran at −78 °C under an N_2 atmosphere for 1 h. 2-Fluorenecarboxaldehyde (3.0 g, 0.015 mol) was added to the solution, which was then stirred for 2 h at −78 °C and allowed to warm to room temperature over several h. The reaction mixture

was dried in vacuo, dissolved in water, and extracted with benzene. The benzene extract was washed with 30% sodium bisulfite solution, concentrated to dryness in vacuo, and purified by flash chromatography (*15*) with petroleum ether as the eluting solvent. The yield of the white 2-ethynylfluorene product, which gave a single spot on thin-layer chromatography (R_f = 0.3, petroleum ether), was 1.14 g (40%): mp 83 °C; ^{1}H NMR (in $CDCl_3$) δ 3.1 (s, 1 H, ≡CH), 3.85 (s, 2 H, $ArCH_2$), 7.25–7.85 (m, 7 H, ArH); mass spectrum, m/z 190 (M^+); UV (CH_3CN) λ_{max} 228, 300, and 312 nm, shoulder at 284 nm. Anal. Calcd for $C_{15}H_{10}$: C, 94.70, H, 5.30. Found: C, 94.52, H, 5.08.

Tissue Preparations. Male Sprague-Dawley rats (100–175 g) were purchased from the Charles River Breeding Laboratories, Inc., Wilmington, MA. Induction of cytochrome P-450 by isosafrole was carried out according to the procedure of Dickens et al. (*20*), by giving ip injections of isosafrole on three successive days (150 mg of isosafrole in 2.5 mL of trioctanoin/kg body weight). The rats were then sacrificed on the fourth day; liver microsomes were prepared (*8*) and stored at −70 °C. No difference in metabolic activity could be detected between fresh microsomes and those stored by this method. Protein concentrations were measured by the biuret reaction (*21*).

NADPH–cytochrome *c* (P-450) reductase was prepared from rat liver as described by Yasukochi and Masters (*22*) and modified elsewhere (*8*). Rat hepatic P-450 enzymes P-450$_{\beta NF\text{-}B}$ and P-450$_{ISF\text{-}G}$ were purified as described previously (*8*). P-450 concentrations were estimated by the method of Omura and Sato (*23*).

Enzyme Assays and Inactivation. Incubation mixtures (1 mL) used to measure the microsomal N-oxidation of 2-naphthylamine contained 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM EDTA, 5 mM glucose 6-phosphate, 0.4 mM $NADP^+$, 0.3 mM NAD^+, glucose-6-phosphate dehydrogenase (1 unit/mL), [^{3}H]-2-naphthylamine (either 0.1 or 0.5 mM added in 10 μL of ethanol), and 1 mg of liver microsomal protein. The assays were conducted in open 10-mL Erlenmeyer flasks, and reaction mixtures were preincubated without substrate at 37 °C for 3 min. The reactions were then started by the addition of [^{3}H]-2-naphthylamine. Each of the purified P-450s (0.25 nmol) was reconstituted immediately prior to enzyme assay by mixing with 0.5 nmol of purified rat liver NADPH–P-450 reductase (2-fold molar excess over P-450) and 30 μg of L-α-dilauroyl-*sn*-diglycero-3-phosphocholine in 10 mM sodium phosphate buffer (pH 7.4) for 3 min at 37 °C. For reactions with purified P-450s, 0.1 mM 2-amino[^{3}H]fluorene was added (in 10 μL of ethanol) as substrate, and the reactions were then initiated by the addition of 1 mM NADPH. After incubation at 37 °C for 5 min, the reactions were terminated by extraction with 0.5 mL of water-saturated ethyl acetate. Aliquots (50 μL) of the organic extracts were immediately analyzed by HPLC as described previously (*6*).

In studies involving the inactivation of enzyme activity, various concentrations of 2-ethynylnaphthalene, 1-ethynylnaphthalene, or 2-ethynylfluorene [in 2 μL of $(CH_3)_2SO$] were added to the incubation mixture after the 3-min preincubation. At specific time intervals (0–10 min), substrate was added and the assay continued for 5 min as described above. Control experiments were carried out in which either $(CH_3)_2SO$ was added alone (without inhibitor) or incubation with inhibitor was done in the absence of the NADPH-generating system (or NADPH).

Metabolism of 2-Ethynylnaphthalene by P-450$_{ISF\text{-}G}$. [^{3}H]-2-Ethynylnaphthalene (100 μM) was incubated at 37 °C with purified P-450$_{ISF\text{-}G}$, reconstituted as described above, in the presence of 1 mM NADPH for 6 min. Analyses of the ethyl acetate extracts of the resulting solutions were done by HPLC on a 4.6 × 25 cm Alltech Nucleosil C_{18} column eluted with H_2O–CH_3OH (1:9 v/v) at a flow rate of 1 min. A single metabolite was detected and identified as 2-naphthylacetic acid on the basis of coelution of the radioactive material with an authentic standard.

Stoichiometry of Binding of [^{3}H]-2-Ethynylnaphthalene Products to P-450s. Twenty nanomoles of purified P-450$_{ISF\text{-}G}$ or P-450$_{\beta NF\text{-}B}$ was reconstituted with 24 nmol of purified rabbit NADPH–P-450 reductase (*10*) and 240 μg of L-α-dilauroyl-*sn*-glycero-3-phosphocholine in 8.0 mL of 8 mM sodium phosphate buffer (pH 7.4) and incubated with 100 nmol of [^{3}H]-2-ethynylnaphthalene and 1.6 μmol of NADPH. After a 30-min incubation at 37 °C, the reaction mixture was cooled in an ice bath and activated charcoal (1 mg/mL of reaction mixture) was added. The charcoal was removed by centrifugation, and the sample was concentrated to <2-mL volume on a Centricon 10 microconcentrator (Amicon, Lexington, MA). The concentrated protein was loaded on a column of 2′,5′-ADP agarose and washed with 0.1 M potassium phosphate buffer (pH 7.25) containing 0.1% cholate (w/v), 1 mM EDTA, and 20% glycerol. The radiolabeled P-450 eluted in the wash fractions. Unbound [^{3}H]-2-ethynylnaphthalene eluted later after increasing the amount of cholate in the column buffer [approximately 0.5% (w/v) cholate]. The fraction containing radiolabeled enzyme was concentrated and dialyzed extensively against 0.05 M potassium phosphate buffer (pH 7.8). The amount of P-450 was determined, and the ^{3}H content was measured by liquid scintillation spectrometry. The stoichiometry of binding of enzyme with [^{3}H]-2-ethynylnaphthalene was calculated from measurements of incorporated ^{3}H radioactivity and of recovered amounts of P-450.

Table I. Effect of Ethynyl-Substituted Compounds on 2-Naphthylamine (2-NA) N-Oxidation Activity of Liver Microsomes from Isosafrole-Treated Rats[a]

concn of substrate 2-NA, mM	inhibitor	percentage loss of activity		
		0 min[b]	6 min	10 min
0.1	1 μM 2-EF	41	51	
0.1	10 μM 2-EF	77	84	
0.1	1 μM 2-EN	32	84	
0.1	10 μM 2-EN	82	100	
0.5	1 μM 1-EN	19		30
0.5	10 μM 1-EN	34		41
0.5	1 μM 2-EN	8		31
0.5	10 μM 2-EN	29		79

[a] Incubations were carried out as described under Experimental Procedures. The values presented are the means of results of duplicate determinations. 2-NA, 2-naphthylamine; 2-EF; 2-ethynylfluorene; 2-EN, 2-ethynylnaphthalene; 1-EN, 1-ethynylnaphthalene. [b] Time of incubation of microsomes with inhibitor before assaying enzyme activity.

Results

The ethynyl-substituted compounds 2-ethynylnaphthalene, 1-ethynylnaphthalene, and 2-ethynylfluorene were synthesized, and each was examined for its inhibitory effect on rat hepatic microsomal 2-naphthylamine N-oxidation activity, tested at concentrations of 1 and 10 μM. Enzyme activity, determined after incubating microsomes with each compound, was compared with activity without prior incubation. The results of these initial screening experiments are shown in Table I. Each of these compounds was found to be inhibitory even at low concentrations. Some inhibition of activity was also observed without prior incubation of microsomes with the ethynyl derivatives, and this decrease in activity is most probably due to competitive inhibition. This view is further suggested, at least with 1-ethynylnaphthalene and 2-ethynylfluorene, by the observation that preincubating microsomes with these compounds under the conditions of these experiments resulted in little additional loss of enzyme activity. However, with 2-ethynylnaphthalene, prior incubation did cause appreciable loss of activity and suggested that mechanism-based inactivation may be important in the inhibitory effect of 2-ethynylnaphthalene.

The loss of activity in the presence of 2-ethynylnaphthalene was also time-dependent. The straight lines obtained for the plots of logarithm of remaining activity versus incubation time obtained with varying 2-ethynylnaphthalene concentrations (1–50 μM) demonstrate that the process follows pseudo-first-order kinetics (Figure 2). First-order inactivation constants were determined by linear regression analysis as the slopes of these lines. Following the method of Kitz and Wilson (*24*), the dou-

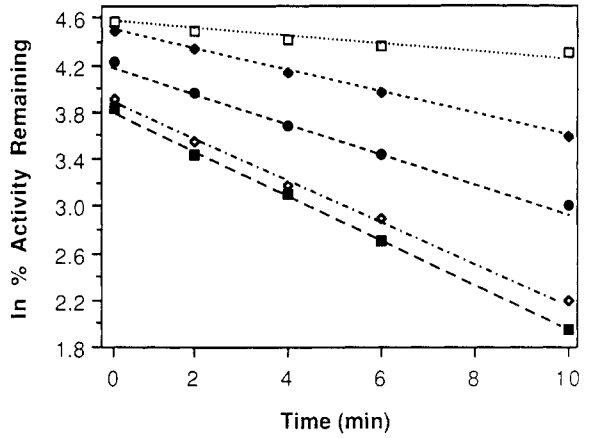


Figure 2. Time course of the inactivation of 2-naphthylamine N-oxidation activity by 2-ethynylnaphthalene. Liver microsomes from isosafrole-treated rats were incubated with five different concentrations of 2-ethynylnaphthalene at 37 °C, as described under Experimental Procedures. The concentrations of 2-ethynylnaphthalene were (□) 1 μM, (♦) 5 μM, (●) 10 μM, (◇) 20 μM, and (■) 50 μM. At the time points indicated, 2-naphthylamine was added to a concentration 0.5 mM and N-oxidation activity was assayed as described under Experimental Procedures.

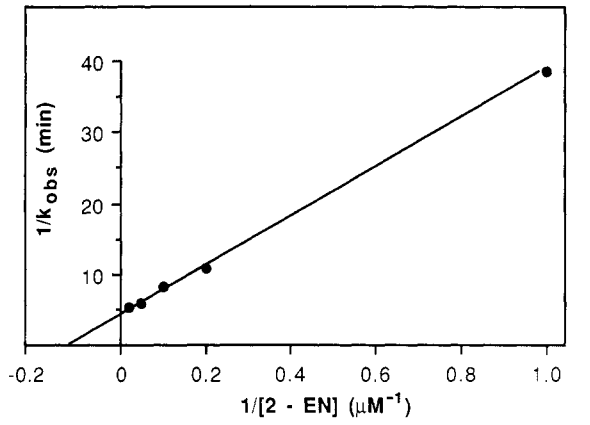


Figure 3. Double-reciprocal plot of the rate of inactivation of 2-naphthylamine N-oxidation activity as a function of inhibitor concentration. K_I is determined from the intercept on the abscissa and $k_{inactivation}$ from the intercept on the ordinate. 2-EN, 2-ethynylnaphthalene.

ble-reciprocal plot of the observed inactivation rate constants as a function of 2-ethynylnaphthalene concentration (Figure 3) was linear. The line, fitted by linear regression analysis, did not pass through the origin but intercepted the positive ordinate, thus showing that the changes in the rate of enzyme inactivation as a function of inactivator concentration obey saturation kinetics. From the ordinate intercept, a rate constant for inactivation under conditions of saturating inactivator ($k_{inactivation}$) was found to be 0.23 min^{-1}, and extrapolating the line to the abscissa intercept ($-1/K_I$) yields a K_I (the inactivator concentration required for half-maximal inactivation) of 7.7 μM. A limiting $t_{1/2}$ of 3.0 min was calculated from the $k_{inactivation}$. The time-dependent inactivation of enzyme activity is superimposed upon a competitive (reversible) inhibition (Figure 2) due to the presence of unmetabolized inactivator during the subsequent assay of 2-naphthylamine N-oxidation activity (*25*, *26*). The extent of the reversible inhibition is evident at the intercept on the ordinate and increases with increasing 2-ethynylnaphthalene concentration. In control experiments, incubations with NADPH alone resulted in loss of activity of approximately 10% in 10 min, and in the absence of NADPH, enzyme activity was essentially unchanged after incubation for 10 min with 50 μM 2-ethynylnaphthalene. To determine the loss of P-450 content by 2-ethynylnaphthalene, microsomes that had been incubated with the inhibitor (100 μM) for 10 min were reisolated and assayed for total P-450 content by CO difference spectra (*23*). Only a 15% loss in total P-450 was observed (data not shown).

Table II. Effect of Ethynyl-Substituted Compounds on 2-Aminofluorene N-Oxidation by Purified Cytochromes P-450[a]

P-450	inhibitor	percentage loss of activity: 0 min[b]	percentage loss of activity: 6 min
$P\text{-}450_{\beta NF\text{-}B}$	1 μM 1-EN	0	5
	10 μM 1-EN	5	64
	1 μM 2-EN	17	40
	10 μM 2-EN	31	87
$P\text{-}450_{ISF\text{-}G}$	1 μM 1-EN	26	36
	10 μM 1-EN	39	48
	1 μM 2-EN	31	92
	10 μM 2-EN	43	95

[a] Incubations were carried out as described under Experimental Procedures. The values presented are means of results of duplicate determinations. 1-EN, 1-ethynylnaphthalene; 2-EN, 2-ethynylnaphthalene. [b] Time of incubation of enzyme with inhibitor before assaying activity.

Because of the specific involvement of $P\text{-}450_{ISF\text{-}G}$ in the N-oxidation of 2-naphthylamine (*6*), studies were conducted with the purified P-450 in a reconstituted system to examine the inhibitory effect of 2-ethynylnaphthalene. These studies also included an assessment of the selectivity of the 2-ethynyl derivative as an inhibitor of $P\text{-}450_{ISF\text{-}G}$ by comparing its effect with that on $P\text{-}450_{\beta NF\text{-}B}$. The effect of 1-ethynylnaphthalene on each of these P-450s was also examined. As shown in Table II, 2-ethynylnaphthalene at concentrations of 1 and 10 μM inhibited the 2-aminofluorene N-oxidation activity of both P-450 enzymes, and this effect increased with prior incubation of this inhibitor with the P-450. However, at the lower concentration of 1 μM, the inhibition by 2-ethynylnaphthalene was much greater with $P\text{-}450_{ISF\text{-}G}$ than with $P\text{-}450_{\beta NF\text{-}B}$. 1-Ethynylnaphthalene also inhibited the 2-aminofluorene N-oxidation activity of both cytochrome P-450 enzymes; however, its inhibitory effect on $P\text{-}450_{ISF\text{-}G}$ was less dependent upon its prior incubation with enzyme than in the case of $P\text{-}450_{\beta NF\text{-}B}$, and the inhibition of $P\text{-}450_{\beta NF\text{-}B}$ was only observed at the higher concentration (10 μM).

A partition ratio, defined as the number of metabolite molecules produced per inactivation event, for the inactivation of $P\text{-}450_{ISF\text{-}G}$ by 2-ethynylnaphthalene was estimated by determining (via quantitative liquid chromatography) the amount of product formed from the incubation of [^{3}H]-2-ethynylnaphthalene with reconstituted $P\text{-}450_{ISF\text{-}G}$. From determination of the amount of 2-naphthylacetic acid formed, a partition ratio of 64 was obtained which indicates that inactivation of the enzyme by 2-ethynylnaphthalene occurs with reasonable efficiency. For example, values of ~40 and ~60 have been obtained for cyclopropylbenzylamine and (1-methylcyclopropyl)-benzylamine, respectively, in the inactivation of a reconstituted, purified cytochrome P-450 prepared from phenobarbital-treated rats (*27*). Additionally, in the inactivation of cytochrome P-450b by (*p*-methylphenyl)-acetylene, a partition ratio of 38 was determined (*28*). However, an exceptionally low ratio of ~2 was reported for 10-undecynoic acid in the inactivation of cytochrome $P\text{-}450_{LA\omega}$ (*29*).

Experiments were also carried out to determine whether or not covalent binding of 2-ethynylnaphthalene to purified

Table III. Rates of Mechanism-Based Inactivation of Microsomal and Purified P-450

substrate	apparent $k_{inactivation}$, min^{-1}		reference
	microsomes	purified P-450	
2-ethynylnaphthalene	0.23	>0.46 ($P\text{-}450_{ISF\text{-}G}$)	Figures 2 and 3, Table II[a]
1-ethynylnaphthalene	~0.014		Table I[a]
2-ethynylfluorene	~0.015		Table I[a]
17α-ethynylnaphthalene	~0.01		39
20-(1,5-hexadiynyl)-5-pregnene-3β,20α-diol		0.063 ($P\text{-}450_{SCC}$)	40
11-dodecynoic acid	0.28		5
1-ethynylpyrene	0.10–0.20		3
fluroxene		0.28 ($P\text{-}450_{PB\text{-}B}$)	41
		0.14 ($P\text{-}450_{\beta NF\text{-}B}$)	
vinyl chloride	~0.083		42
allylisopropylacetamide	0.1		43
N-cyclopropylbenzylamine	0.063	0.48 ($P\text{-}450_{PB\text{-}B}$)	27, 32
N-(1-methylcyclopropyl)benzylamine		0.42 ($P\text{-}450_{PB\text{-}B}$)	32
N-(1-phenylcyclopropyl)benzylamine	0.36	0.47 ($P\text{-}450_{PB\text{-}B}$)	32
N-(2-phenylcyclopropyl)benzylamine	0.023	0.27 ($P\text{-}450_{PB\text{-}B}$)	32
N-(cyclobutyl)benzylamine	0.01		32
N-(1-phenylcyclobutyl)benzylamine	0.022	0.07 ($P\text{-}450_{PB\text{-}B}$)	32
chloramphenicol		0.40 ($P\text{-}450_{PB\text{-}B}$)	26
N-[2-(*p*-nitrophenethyl)]dichloroacetamide		0.59 ($P\text{-}450_{PB\text{-}B}$)	26
		0.19 ($P\text{-}450_{\beta NF\text{-}B}$)	26
N-[2-(*p*-nitrophenethyl)]dibromoacetamide		0.70 ($P\text{-}450_{PB\text{-}B}$)	26
N-(2-phenethyl)dichloroacetamide		0.53 ($P\text{-}450_{PB\text{-}B}$)	26
		0.14 ($P\text{-}450_{\beta NF\text{-}B}$)	26
N-(3-phenpropyl)dichloroacetamide		0.10 ($P\text{-}450_{PB\text{-}B}$)	38
N-(4-phenbutyl)dichloroacetamide		0.08 ($P\text{-}450_{PB\text{-}B}$)	38
N-(*p*-nitrobenzyl)dichloroacetamide		0.50 ($P\text{-}450_{PB\text{-}B}$)	38
N-(*p*-nitrophenyl)dichloroacetamide		0.54 ($P\text{-}450_{PB\text{-}B}$)	38
		0.69 ($P\text{-}450_{\beta NF\text{-}B}$)	38
N-(*p*-chlorophenyl)dichloroacetamide		0.42 ($P\text{-}450_{PB\text{-}B}$)	38
N-phenyldichloroacetamide		0.21 ($P\text{-}450_{PB\text{-}B}$)	38
(dichloromethyl)acetophenone		0.75 ($P\text{-}450_{PB\text{-}B}$)	38
		0.45 ($P\text{-}450_{\beta NF\text{-}B}$)	38
α,α-dichlorotoluene		0.35 ($P\text{-}450_{PB\text{-}B}$)	38
		0.06 ($P\text{-}450_{\beta NF\text{-}B}$)	38
N-octyldichloroacetamide		0.48 ($P\text{-}450_{PB\text{-}B}$)	38
		0.44 ($P\text{-}450_{\beta NF\text{-}B}$)	38
N-hexyldichloroacetamide		0.42 ($P\text{-}450_{PB\text{-}B}$)	38
N-(*n*-butyl)dichloroacetamide		0.19 ($P\text{-}450_{PB\text{-}B}$)	38
N-(*tert*-butyl)dichloroacetamide		0.13 ($P\text{-}450_{PB\text{-}B}$)	38
N-methyldichloroacetamide		0.16 ($P\text{-}450_{PB\text{-}B}$)	38
methyl-2,2-dichloroacetate		0.21 ($P\text{-}450_{PB\text{-}B}$)	38
1,1-dichloroacetone		0.18 ($P\text{-}450_{PB\text{-}B}$)	38
dichloroacetamide		0.03 ($P\text{-}450_{PB\text{-}B}$)	38
1,1,2,2-tetrachloroethane		0.35 ($P\text{-}450_{PB\text{-}B}$)	38

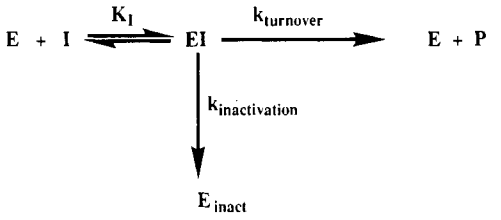
[a] This report.

$P\text{-}450_{ISF\text{-}G}$ or purified $P\text{-}450_{\beta NF\text{-}B}$ occurs under the conditions of inhibition. Each P-450 was incubated with radiolabeled 2-ethynylnaphthalene in reconstituted systems by using the general conditions employed in the inhibition experiments. After extensive removal of unbound ^3H, the amount of radioactivity associated with the enzyme was determined. Two different preparations of $P\text{-}450_{ISF\text{-}G}$ produced calculated binding ratios of 0.62 and 0.77 mol of [^3H]-2-ethynylnaphthalene/mol of $P\text{-}450_{ISF\text{-}G}$, and a single preparation of $P\text{-}450_{\beta NF\text{-}B}$ yielded a binding ratio of 0.72 mol of [^3H]-2-ethynylnaphthalene/mol of $P\text{-}450_{\beta NF\text{-}B}$.

Discussion

The results of these studies indicate that the interaction of 2-ethynylnaphthalene with the 2-naphthylamine N-oxidation activity of hepatic microsomes from isosafrole-pretreated rats exhibits the characteristics of mechanism-based inactivation (*30*) as outlined in Scheme I, where E is the enzyme ($P\text{-}450_{ISF\text{-}G}$), I is 2-ethynylnaphthalene, and P is the stable product (2-naphthylacetic acid, as indicated from experiments on the metabolism of [^3H]-2-ethynylnaphthalene). For a range of 2-ethynylnaphthalene concentrations, the loss of activity was time-dependent and exhibited pseudo-first-order kinetics. Inactivation required NADPH and was saturable, consistent with the view that

Scheme I



oxidative metabolism of 2-ethynylnaphthalene, involving complexation with the enzyme, is a prerequisite for the inactivation process. The values determined for the $k_{inactivation}$ and the K_I (0.23 min^{-1} and 7.7 μM, respectively) suggest the high potency of this inactivator of the enzymatic activity. These results are similar to those from earlier studies of the mechanism-based inactivation of the benzo[*a*]pyrene hydroxylase activity by 1-ethynylpyrene and by methyl-1-pyrenylacetylene, where the pyrenyl group is hypothesized to position these inhibitors in the active site of cytochrome $P\text{-}450_{\beta NF\text{-}B}$ (*3*, *4*). Comparison of rates of inactivation of microsomal and purified P-450 by mechanism-based inhibitors available in the literature is made in Table III. Some of the rates are estimates based only upon the fraction of catalytic activity remaining

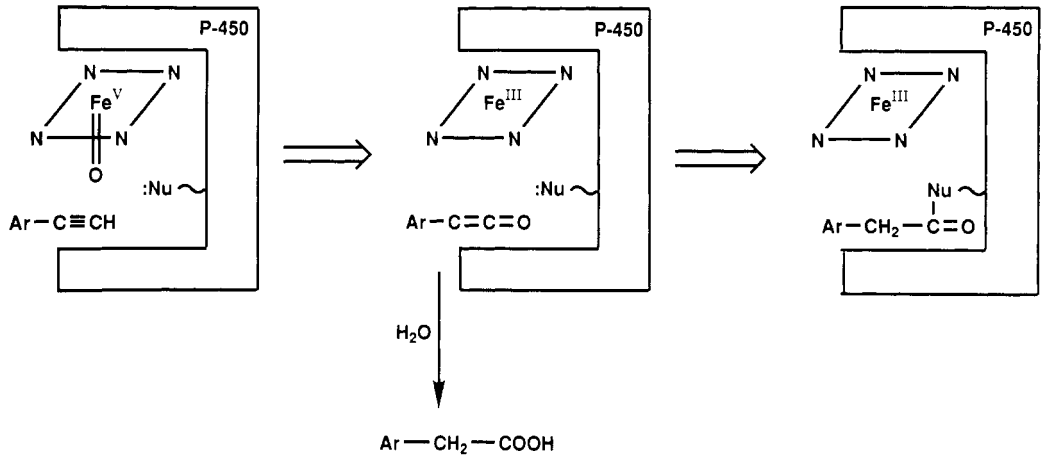


Figure 4. Proposed mechanism of inactivation of P-450 by 2-ethynylnaphthalene.

after a certain period of time (i.e., 1-ethynylnaphthalene and 2-ethynylfluorene results from Table I of this report). The list includes a series of olefins, cyclopropylamines, and dihalides known to inactivate P-450s (*31*) as well as other acetylenes. When comparisons have been made between microsomal and purified P-450s with regard to rates of inactivation, the rates are generally greater in the case of the purified P-450s (*32*), in part because correction is being made for the other P-450s that are not inactivated. The results presented in Table II for 2-ethynylnaphthalene may be used to calculate a minimal $k_{inactivation}$ of 0.46 min^{-1} for purified $P\text{-}450_{ISF\text{-}G}$. Thus, the rates of inactivation by 2-ethynylnaphthalene compare favorably with those for several other well-known P-450 inhibitors, particularly, the acetylenes.

N-Oxidation of 2-naphthylamine has been shown to be catalyzed by only one of eight rat liver P-450s surveyed, namely, $P\text{-}450_{ISF\text{-}G}$ (*6*). $P\text{-}450_{ISF\text{-}G}$ is the major enzyme induced by isosafrole (*8*, *33*), and it has been found to be very efficient in catalyzing of the N-oxidation of several primary aromatic amines (*1*). Because of the structural similarity between 2-naphthylamine and 2-ethynylnaphthalene, it seems likely that the 2-ethynyl derivative is a selective inactivator of $P\text{-}450_{ISF\text{-}G}$. Our initial studies with purified cytochrome $P\text{-}450_{ISF\text{-}G}$ in a reconstituted system have demonstrated that 2-ethynylnaphthalene inhibits the enzyme and that this effect increases on prior incubation; and 2-ethynylnaphthalene is a more potent inhibitor of purified $P\text{-}450_{ISF\text{-}G}$ than of $P\text{-}450_{\beta NF\text{-}B}$. In this regard, further studies on the inhibition of other purified cytochromes P-450 by 2-ethynylnaphthalene may be useful to assess its selectivity as a mechanism-based inactivator.

2-Ethynylnaphthalene is also found to be a more potent inhibitor of $P\text{-}450_{ISF\text{-}G}$ than is 1-ethynylnaphthalene. This result is consistent with the finding that 1-naphthylamine is oxidized by P-450s to ring-hydroxylated products but is not hydroxylated on the amine nitrogen (*6*). Additionally, 2-naphthylamine is ring-hydroxylated by both $P\text{-}450_{ISF\text{-}G}$ and $P\text{-}450_{\beta NF\text{-}B}$ but N-hydroxylated only by $P\text{-}450_{ISF\text{-}G}$. Thus, the 2-amino group appears to be in a position such that N-hydroxylation can occur in the active site of $P\text{-}450_{ISF\text{-}G}$, but the 1-amino group is not. Some further insight into the structure–activity relationship has been gained in preliminary studies with *N,N*-dimethyl-1-naphthylamine. Although, 1-naphthylamine is not N-hydroxylated by any of the rat liver P-450s (*6*), the *N,N*-dimethyl derivative is readily demethylated [0.6 nmol of product formed/(min·nmol of P-450) in liver microsomes prepared from untreated rats]. Furthermore, both purified $P\text{-}450_{ISF\text{-}G}$ and $P\text{-}450_{\beta NF\text{-}B}$ catalyze the reaction at rates of 1–2 min^{-1}. Thus, it would appear that several P-450s may be able to abstract electrons from the 1- and 2-amino positions, but only in the case of $P\text{-}450_{ISF\text{-}G}$ is the juxtaposition of the substrate controlled in such a way as to allow oxygen rebound to the electron-deficient nitrogen intermediate. This specificity can be considered in light of current knowledge concerning the similarity of and distinctions between active sites of P-450s and peroxidases (*34*, *35*). That is, low-potential, one-electron abstractions by peroxidases can occur via the formal Fe^{IV}=O entity or the porphyrin edge, but only in P-450 when the position of the nitrogen is appropriate can oxygen transfer occur. In $P\text{-}450_{ISF\text{-}G}$, the oxygen rebound to the amine nitrogen of 2-naphthylamine may be controlled by an active site directed process where the positive charge on the nitrogen is further enhanced, possibly by interaction with a nucleophilic amino acid during the catalytic process (*6*). The inactivation and covalent binding by 2-ethynylnaphthalene to $P\text{-}450_{ISF\text{-}G}$ could involve reaction with the same nucleophilic constituent.

The inactivation of the enzyme activity by 2-ethynylnaphthalene is assumed to proceed by the formation of a reactive ketene or oxirene-related species as proposed by Ortiz de Montellano et al. (*29*, *36*, *37*) for microsomal P-450 inactivation by terminal alkynes (Figure 4). This process can result in an alkylation of a nucleophilic amino acid (vide supra) or of the heme nitrogen, the latter of which is known to destroy the P-450 chromophore. In this regard, derivatization of the porphyrin can only be partially responsible for the covalent binding and inactivation caused by 2-ethynylnaphthalene as described in this study. An approximate 15% loss of P-450 content associated with >95% inactivation of enzymatic activity cannot account for the $P\text{-}450_{ISF\text{-}G}$ content, estimated at 40% (*8*, *33*), in liver microsomes prepared from isosafrole-treated rats. Accordingly, we propose that alkylation of an amino residue(s) in the active site most likely contributes to the inactivation described. Loss of cytochrome P-450 activity without parallel loss of P-450 content has also been observed with the rationally designed alkynes, 1-ethynylpyrene (*3*), methyl-1-pyrenylacetylene (*4*), and 11-dodecynoic acid (*5*), as well as with certain dichloromethyl compounds (*38*).

In conclusion, 2-ethynylnaphthalene has been identified as a potent mechanism-based inactivator of cytochrome P-450 activity. Since this activity is presumed to be catalyzed selectively by $P\text{-}450_{ISF\text{-}G}$, which plays a critical role in the conversion of primary aromatic amines to ultimate carcinogenic metabolites, 2-ethynylnaphthalene may prove useful as a tool for studying the active site modification

of P-450$_{ISF-G}$, as well as the inhibition of arylamine carcinogenesis in rats.

Registry No. 1-Ethynylnaphthalene, 15727-65-8; 1-acetonaphthone, 941-98-0; 2-ethynylnaphthalene, 2949-26-0; 2-acetonaphthone, 93-08-3; 2-acetyl[*ring*-G-^{3}H]naphthalene, 123333-46-0; 2-ethynyl[*ring*-G-^{3}H]naphthalene, 123333-47-1; 2-ethynylfluorene, 57700-19-3; 2-naphthylamine, 91-59-8; P-450, 9035-51-2.

References

(1) Kadlubar, F. F., and Hammons, G. J. (1987) The role of cytochrome P-450 in the metabolism of chemical carcinogens. In *Mammalian Cytochrome P-450* (Guengerich, F. P., Ed.) Vol. II, pp. 81–130, CRC Press, Boca Raton, FL.

(2) Ortiz de Montellano, P. R., Reich, N. O. (1986) Inhibition of cytochrome P-450 enzymes. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 273–314, Plenum Press, New York.

(3) Gan, L.-S., Acebo, A. L., and Alworth, W. L. (1984) 1-Ethynylpyrene, a suicide inhibitor of cytochrome P-450 dependent benzo[*a*]pyrene hydroxylase activity in liver microsomes. *Biochemistry* **23**, 3827–3836.

(4) Gan, L.-S., Lu, J.-Y., Hershkowitz, D. M., and Alworth, W. L. (1985) Effects of acetylenic and olefinic pyrenes upon cytochrome P-450 dependent benzo[*a*]pyrene hydroxylase activity in liver microsomes. *Biochem. Biophys. Res. Commun.* **129**, 591–596.

(5) Ortiz de Montellano, P. R., and Reich, N. O. (1984) Specific inactivation of hepatic fatty acid hydroxylases by acetylenic fatty acids. *J. Biol. Chem.* **259**, 4136–4141.

(6) Hammons, G. J., Guengerich, F. P., Weis, C. C., Beland, F. A., and Kadlubar, F. F. (1985) Metabolic oxidation of carcinogenic arylamines by rat, dog, and human hepatic microsomes and by purified flavin-containing and cytochrome P-450 monooxygenases. *Cancer Res.* **45**, 3578–3585.

(7) Guengerich, F. P. (1978) Separation and purification of multiple forms of microsomal cytochrome P-450. Partial characterization of three apparently homogeneous cytochromes P-450 prepared from livers of phenobarbital- and 3-methylcholanthrene-treated rats. *J. Biol. Chem.* **253**, 7931–7939.

(8) Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., and Kaminsky, L. S. (1982) Purification and characterization of liver microsomal cytochrome P-450; electrophoretic, spectra, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital and β-naphthoflavone. *Biochemistry* **21**, 6019–6030.

(9) Nebert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalex, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R., and Waterman, M. R. (1989) The P450 superfamily: update on listing of all genes and recommended nomenclature of the chromosomal loci. *DNA* **8**, 1–13.

(10) Guengerich, F. P. (1987) Enzymology of rat liver cytochromes P-450. In *Mammalian Cytochromes P-450* (Guengerich, F. P., Ed.) pp 1–54, CRC Press, Boca Raton, FL.

(11) Guengerich, F. P. (1987) Cytochrome P-450 enzymes and drug metabolism. In *Progress in Drug Metabolism* (Bridges, J. W., Chasseaud, L. F., and Gibson, G. G., Eds.) Vol. 10, Chapter 1, pp 1–54, Taylor and Francis, London.

(12) Willstätter, R., and Kubli, H. (1908) Uber die reduction van nitroverbindungen nach der methode von zinin. *Chem. Ber.* **41**, 1936–1940.

(13) Brill, E. (1961) Diethyl azodicarboxylate oxidation of some carcinogenic arylhydroxylamines to nitroso derivatives. *Experientia (Basel)* **25**, 680.

(14) Wade, A., Symons, A. M., Martin, L., and Purke, D. V. (1979) Metabolic oxidation of the ethynyl group in 4-ethynylbiphenyl. *Biochem. J.* **184**, 509–517.

(15) Still, W. C., Kahn, M., and Mitra, A. (1978) Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* **43**, 2923–2925.

(16) Takahashi, S., Kuroyama, Y., Sonogashira, K., and Hagihara, N. (1980) A convenient synthesis of ethynylarenes and diethynylarenes. *Synthesis* 88 627–630.

(17) Okamoto, Y., Chellappa, K. L., and Kundu, S. K. (1972) Magnetic shielding of acetylenic protons in ethynylarenes. *J. Org. Chem.* **37**, 3185–3187.

(18) Wilcox, C. F., Jr., and Weber, K. A. (1985) A study of the ^{13}C NMR shifts in two tetradehydrocyclodecabiphenylenes and related aryl and biphenylenylacetylenes. *Tetrahedron* **41**, 4425–4436.

(19) Matsumato, M., and Kuroda, K. (1980) A convenient synthesis of 1-bromoolefins and acetylenes by a chain extension of aldehydes. *Tetrahedron Lett.* **21**, 4021–4024.

(20) Dickens, M., Bridges, J. W., Elcombe, C. R., and Netter, K. J. (1978) A novel haemoprotein induced by isosafrole pretreatment in the rat. *Biochem. Biophys. Res. Commun.* **80**, 89–96.

(21) Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751–766.

(22) Yasukochi, Y., and Masters, B. S. S. (1976) Some properties of a detergent-solubilized NADPH–cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**, 5337–5344.

(23) Omura, T., and Sato, R. (1964) The carbon monoxide-binding pigment of the liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**, 2370–2378.

(24) Kitz, R., and Wilson, I. B. (1962) Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J. Biol. Chem.* **237**, 3245–3249.

(25) Waxman, D. J., and Walsh, C. (1982) Phenobarbital-induced rat liver cytochrome P-450: purification and characterization of two closely related isozymic forms. *J. Biol. Chem.* **257**, 10446–10457.

(26) Miller, N. E., and Halpert, J. (1986) Analogues of chloramphenicol as mechanism-based inactivators of rat liver cytochrome P-450: modifications of the propanediol side chain, the *p*-nitro group, and the dichloromethyl moiety. *Mol. Pharmacol.* **29**, 391–398.

(27) Macdonald, T. L., Zirvi, K., Burka, L. T., Peyman, P., and Guengerich, F. P. (1982) Mechanism of cytochrome P-450 inhibition of cyclopropylamines. *J. Am. Chem. Soc.* **104**, 2050–2052.

(28) Komives, E. A., and Ortiz de Montellano, P. R. (1987) Mechanism of oxidation of π bonds by cytochrome P-450. Electronic requirements of the transition state in the turnover of phenylacetylenes. *J. Biol. Chem.* **262**, 9793–9802.

(29) CaJacob, C. A., Chan, W. K., Shepard, E., and Ortiz de Montellano, P. R. (1988) The catalytic site of rat hepatic acid ω-hydroxylase. Protein *versus* prosthetic heme alkylation in the ω-hydroxylation of acetylenic fatty acids. *J. Biol. Chem.* **263**, 18640–18649.

(30) Walsh, C. (1982) Suicide substrates: mechanism-based enzyme inactivators. *Tetrahedron* **38**, 871–909.

(31) Ortiz de Montellano, P. R., and Correia, M. A. (1983) Suicidal destruction of cytochrome P-450 during oxidative drug metabolism. *Annu. Rev. Pharmacol. Toxicol.* **23**, 481–503.

(32) Bondon, A., Macdonald, T. L., Harris, T. M., and Guengerich, F. P. (1989) Oxidation of cyclobutylamines by cytochrome P-450: Mechanism-based inactivation, adduct formation, ring expansion, and nitrone formation. *J. Biol. Chem.* **264**, 1988–1997.

(33) Thomas, P. E., Reik, L. M., Ryan, D. E., and Levin, W. (1983) Induction of two immunochemically related rat liver cytochrome P-450 isozymes, cytochromes P-450c and P-450d, by structurally diverse xenobiotics. *J. Biol. Chem.* **258**, 4590–4598.

(34) Marnett, L. J., Weller, P., and Battista, J. R. (1986) Comparison of the peroxidase activity of hemeproteins and cytochrome P-450. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 29–76, Plenum Press, New York.

(35) Ortiz de Montellano, P. R. (1987) Control of the catalytic activity of prosthetic heme by the structure of hemoproteins. *Acc. Chem. Res.* **20**, 289–294.

(36) Ortiz de Montellano, P. R., and Kunze, K. L. (1980) Self-catalyzed inactivation of hepatic cytochrome P-450 by ethynyl substrates. *J. Biol. Chem.* **255**, 5578–5585.

(37) Ortiz de Montellano, P. R., and Komives, E. A. (1985) Branchpoint for heme alkylation and metabolite formation in the oxidation of arylacetylenes by cytochrome P-450. *J. Biol. Chem.* **260**, 3330–3336.

(38) Halpert, J. R., Balfour, C., Miller, N. E., and Kaminsky, L. S. (1986) Dichloromethyl compounds as mechanism-based inactivators of rat liver cytochromes P-450. *Mol. Pharmacol.* **30**, 19–24.

(39) Guengerich, F. P. (1988) Oxidation of 17α-ethynylestradiol by human liver cytochrome P-450. *Mol. Pharmacol.* **33**, 500–508.

(40) Nagahisa, A., Spencer, R. W., and Orme-Johnson, W. H. (1983) Acetylenic mechanism-based inhibitors of cholesterol side chain cleavage by cytochrome P-450$_{SCC}$. *J. Biol. Chem.* **258**, 6721–6723.

(41) Murphy, M. J., Dunbar, D. A., Guengerich, F. P., and Kaminsky, L. S. (1981) Destruction of highly purified cytochromes P-450 associated with metabolism of fluorinated ether anesthetics. *Arch. Biochem. Biophys.* **212**, 360–369.
(42) Guengerich, F. P., and Strickland, T. W. (1977) Metabolism of vinyl chloride: destruction of the heme of highly purified liver microsomal cytochrome P-450 by a metabolite. *Mol. Pharmacol.* **13**, 903–1004.
(43) Loosemore, M. J., Wogan, G. N., and Walsh, C. (1981) Determination of partition ratios for allylisopropylacetamide during suicidal processing by a phenobarbital-induced cytochrome P-450 isozyme from rat liver. *J. Biol. Chem.* **256**, 8705–8712.