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The Mechanism of Inhibition of Cytochrome P450IIE1 by Dihydrocapsaicin

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Dihydrocapsaicin **(2)** is a noncompetitive inhibitor of cytochrome P450IIE1. This was demonstrated by studying the inhibition of *p*-nitrophenol oxidation. The covalent binding of **2** to cytochrome P450IIE1 was examined by incubating tritiated **2** with rat liver microsomes and gel electrophoresis of the resulting bound cytochrome P450 isozymes. Autoradiograms of these gels displayed a single band at approximately 48 kDa. The mechanism whereby **2** became covalently bonded to cytochrome P450IIE1 was explored by studying the oxidation of **2** by electrochemical, chemical, and enzymatic methods. Oxidation of **2** by all methods generally resulted in the formation of the 5,5′-dimer of **2, 4,** which likely forms by dimerization of the phenoxy radical **7.** It is suggested that **2** is oxidized to **7** by cytochrome P450IIE1 and **7** then covalently bonds to cytochrome P450IIE1 thereby inactivating it.

INTRODUCTION

There has been increasing recognition of the importance of radical intermediates and processes in biological systems. For example, an area of particular interest is the importance of radical species in carcinogenesis as they have been suggested as intermediates in the activation of xenobiotics as well as in suppressing the activation process (*1*). Many antioxidants possess antimutagenic and/or anticarcinogenic activity (*2*). An important group of such antioxidants are phenols since many foods contain naturally occurring and/or synthetic phenols (*3*). The mechanism(s) by which phenols inhibit mutagenesis or carcinogenesis is not understood and there is a need for further study in these aspects of phenol metabolism.

We have studied the phenols capsaicin **(1)** and dihydrocapsaicin **(2)** (*4*), which are best known as the pungent ingredients in red peppers (*Capsicum frutresceus*) and are the major capsaicinoids (85–90%) present, in regard to their potential antioxidant activity. Capsaicin affects the cardiovascular system (*5*) and the central nervous system (*6*) and is both a promoter and an inhibitor of substance P (*7*). More recently **1** and **2** have been investigated as possible mutagens (*8, 9*) and carcinogens (*10*). Compound **2** has also been studied for its anticarcinogenic activity. In particular, Bickers *et al.* (*11*) have suggested that **2** may possess anticarcinogenic activity as it inhibits polyaromatic hydrocarbon (PAH) metabolism and DNA binding of PAH metabolites.

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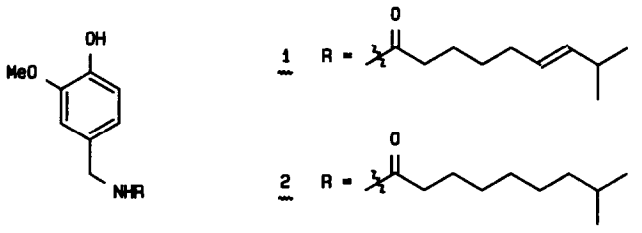


As part of the carcinogenesis studies of **1** and **2,** we became interested in how they were metabolized by cytochrome P450 isozymes. These investigations revealed that **2** was effective at inhibiting cytochrome P450IIE1 (P450j)-mediated metabolism. These studies also suggested that **2** was a mechanism-based inhibitor of cytochrome P450IIE1. We have explored the reactivity of **2** under oxidizing conditions toward the end of elucidating reactive intermediates which may form when **2** is metabolized by cytochrome P450IIE1. These investigations have demonstrated that **2** is oxidized to a phenoxy radical which then inactivates cytochrome P450IIE1 by covalently bonding to it.

RESULTS

Inhibition of p-nitrophenol oxidation. Dihydrocapsaicin inhibited the conversion of *p*-nitrophenol to *p*-nitrocatechol. These data, K_m and V_{max} values, are shown in Table 1. When 4-*O*-methyldihydrocapsaicin **(3)** was used as substrate, the inhibition was greatly attenuated relative to that observed for **2.**

TABLE 1

Inhibition of *p*-Nitrophenol Hydroxylase by Dihydrocapsaicin and Methyldihydrocapsaicin

		Activity (nmol/mg protein/min)	
p-Nitrophenol (μM)	Control[a]	**2** 50 μM	**3** 50 μM
25	0.096 ± 0.013 (3)[b]	0.022 ± 0.001 (2)[c]	0.076 ± 0.025 (3)
50	0.160 ± 0.020 (2)	0.023 (1)	0.135 ± 0.002 (3)
100	0.192 ± 0.04 (2)	0.116 ± 0.042 (2)	0.249 ± 0.048 (2)
150	0.321 (1)	0.128 (1)	—
200	0.418 (1)	0.294 (1)	0.314 (1)
K_m^{app}[d]	142	1531	199
V_{max}[d]	0.637	1.20	0.681

[a] Control sample includes an equal volume of methanol to **2** or **3.**

[b] Values represent means ± standard error with number of samples indicated in parentheses.

[c] Statistically different from control value $P < 0.01$.

[d] Calculations of K_m and V_{max} are from five different concentrations of substrate.

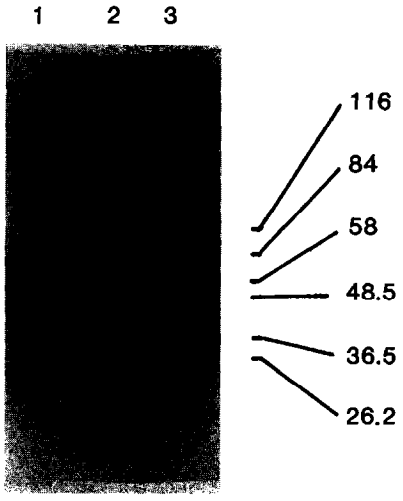


Fig. 1. SDS–gel of rat liver microsomes and [^{3}H]-**2**.

Cytochrome P450 binding of **2**. Tritiated [^{3}H-6,7]dihydrocapsaicin (*12*) was incubated with rat liver microsomes for 15 min at 37°C with NADPH. The microsomes were then electrophoresed after solubilizing and denaturing. Development of the autoradiogram (Fig. 1) revealed, at low concentrations, one band at approximately 50 kDa. At higher concentrations additional bands were observed in the range of 42 kDa.

Specificity of binding of **2** *to cytochrome P450IIE1*. Tritiated [^{3}H-6,7]dihydrocapsaicin was incubated with rat liver microsomes for 10 min at 37°C with and without NADPH. The cytochrome P450IIE1 was then purified by antibody affinity chromatography. Only incubations which contained NADPH resulted in binding of **2** to cytochrome P450IIE1 (Table 2). The incubation contained approximately 0.38–0.53 nmol of cytochrome P450IIE1 (6 mg microsomal proteins, 0.8 nmol cytochrome P450/mg microsomal protein yields 4.8 nmol total cytochrome

TABLE 2

Binding of [^{3}H-6,7]-**2** to Cytochrome P450IIE1 by Antibody Affinity Chromatography[a]

Sample	Eluted (cpm)	Baseline (cpm)	Difference (cpm)
Control[b]	—	—	145×10^4
−NADPH	1.7×10^4	1.5×10^4	0.2×10^4
+NADPH	7.6×10^4	1.5×10^4	6.1×10^4

[a] An antibody against cytochrome P450IIE1 was used.

[b] Value for a sample of [^{3}H-6,7]dihydrocapsaicin not exposed to microsomal protein.

P450 and, in uninduced rats, 8–11% is cytochrome P450IIE1 (*13, 14*)), complete saturation of the cytochrome P450IIE1 would have required the [^{3}H-6,7]dihydrocapsaicin to have a specific activity of 60.7 Ci/mol. The specific activity of [^{3}H-6,7]dihydrocapsaicin was 3.2×10^4 Ci/mol, thus 0.19% saturation of cytochrome P450IIE1.

Cyclic voltammetry. The cyclic voltammetry (CV) data for **2, 3,** and the 5,5′-dimer of **2, 4,** are presented in Table 3. In acetonitrile or methanol, **2** displays an irreversible oxidation wave corresponding to a one-electron oxidation as determined by comparison with 1,4-dimethoxybenzene (*15*). In addition, a reduction wave corresponding to the reduction of protium was observed. The CV data of **2** in methylene chloride, with or without added fluorosulfonic acid, displayed only one irreversible oxidation wave. The observed peak potential, however, was dependent upon whether or not acid was present. The addition of acid shifted the observed peak potential by 154 mV. Finally, **3** displayed only one oxidation wave, and, like **2**, this wave corresponded to a one-electron oxidation (by comparison to 1,4-dimethoxybenzene). In addition, the peak potential of **3** in methylene chloride/fluorosulfonic acid was only slightly shifted relative to the potential observed for its oxidation in methylene chloride alone.

Preparative electrochemical oxidation of dihydrocapsaicin. The electrochemical oxidation of **2** was conducted in methanol under neutral and basic conditions and in phosphate buffer. The principal product was the 5,5′-dimer of dihydrocapsaicin, **4,** under each set of electrolysis conditions.

Chemical oxidation of dihydrocapsaicin. Chemical oxidation of **2** with potassium ferricyanide lead to the formation of **4.** This was the only material that could

TABLE 3

Peak Oxidation Potentials of **2, 3,** and **4**[a]

Compound	Solvent[b]	Ep_f^{ox} (V)
2	MeCN	1.314
2	MeOH	0.971
2	CH_2Cl_2	1.456
2	CH_2Cl_2/FSA[c]	1.610
3	MeCN	1.497
3	MeOH	1.431
3	CH_2Cl_2	1.451
3	CH_2Cl_2/FSA	1.450
4	MeCN	1.361

[a] CV data obtained in 1 mM solutions of **2, 3,** or **4.** Pt working electrode, SCE reference electrode, 0.1 M tetra-*n*-butylammonium perchlorate as supporting electrolyte, ambient temperature, scan rate 200 mV/s under argon.

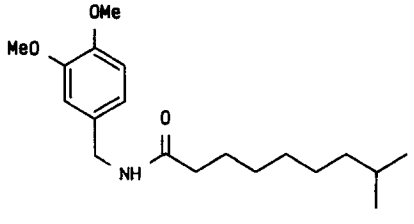
[b] MeCN, acetonitrile; MeOH, methanol; CH_2Cl_2, methylene chloride; FSA, fluorosulfonic acid.

[c] CH_2Cl_2/TFA, 9/1.

be isolated from the reaction mixture. In contrast, oxidation of **2** with lead dioxide or nickel peroxide yielded the amide **5a.** The presumed by-product, **6,** has eluded our efforts to isolate, observe, trap, or synthesize despite considerable effort to do so. It is likely that **6** polymerizes, and this may be the cause of our inability to isolate it.

Visible spectra of horseradish peroxidase (HRP) and dihydrocapsaicin. The visible spectrum of HRP in phosphate buffer displayed a maximum at 403 nm (HRP (Fe(III))). When treated with hydrogen peroxide, a new maximum is observed at 410 nm (HRP compound I) (*16*). Addition of 1 eq of **2** (based on added hydrogen peroxide) lead to a rapid decrease in the absorption at 410 nm and the appearance of a maximum absorbance at 418 nm (HRP compound II). Addition of a second equivalent of **2** resulted in the regeneration of the original HRP (Fe(III)) spectrum. When **3** was used as the substrate instead of **2,** the changes in the visible spectrum described for **2** were not observed.

Horseradish peroxidase-mediated oxidation of dihydrocapsaicin. Incubation of **2** with HRP in phosphate buffer/DMF yielded primarily **4** (*17*). In addition, **5b,** a product derived from what is formally hydrolysis of **2,** was also isolated. Whether or not **5b** is formed by hydrolysis of **2** or **5a,** or through some other process, is not clear (*18*).



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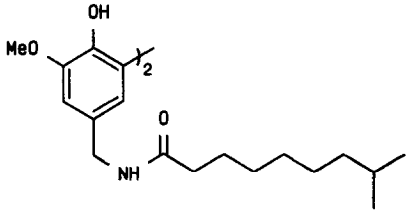

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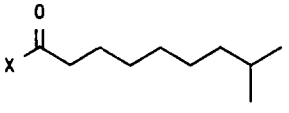
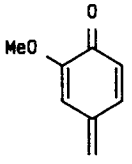


5a X = NH_2

5b X = OH



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DISCUSSION

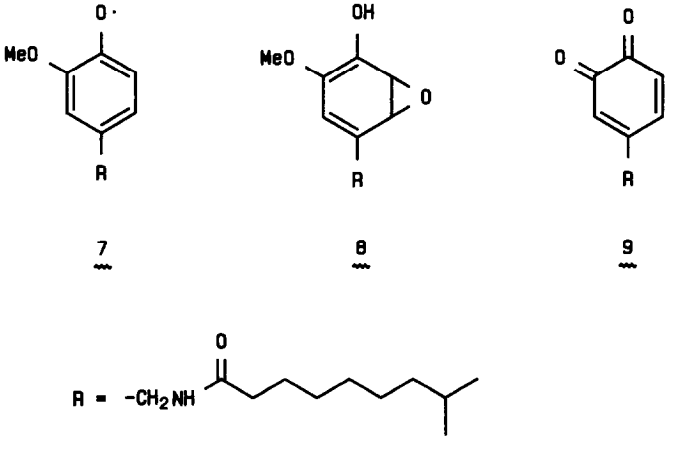
When dihydrocapsaicin was incubated with mixtures of *p*-nitrophenol and rat liver microsomes a significant decrease in the rate of formation of *p*-nitrocatechol was observed. *p*-Nitrophenol is oxidatively metabolized by cytochrome P450IIE1 to the corresponding catechol (*19*). The inhibition suggested that **2** was selectively inhibiting cytochrome P450IIE1 activity. Furthermore, a Lineweaver–Burk plot

of the inhibition data indicated that **2** was a mechanism-based inhibitor since the *y*-intercept of this plot was nearly zero, unlike data from analogous experiments with BHT, a competitive inhibitor of cytochrome P450IIE1 (*20*).

To demonstrate that **2** inhibited cytochrome P450IIE1 in a mechanism-based fashion, tritiated **2** was incubated with microsomes which were then solubilized and electrophoresed. At low concentrations of **2,** the resulting gel displayed one band having a molecular weight of approximately 50 kDa, corresponding to cytochrome P450IIE1 (Fig. 1) (*21*). At higher concentrations, additional bands appeared around 42 kDa. However, this molecular weight is below the range of cytochrome P450 isozymes (*21*).

The specificity for cytochrome P450IIE1 was further investigated with an antibody specific for cytochrome P450IIE1. Incubation mixtures of tritiated **2** with rat liver microsomes, with and without added NADPH, were examined by antibody affinity chromatography. By this method we found that **2** had specifically bound to the cytochrome P450IIE1 in the incubation mixtures. Binding did, however, require NADPH to be present suggesting that the binding of **2** to cytochrome P450IIE1 required prior metabolic activation of **2** (Table 2).

The inhibition and binding data indicate that **2** inhibited cytochrome P450IIE1-mediated metabolism by covalently binding to, and thereby inactivating, the enzyme. It was therefore of interest to explore the mechanism of this process. In this regard we envisioned three intermediates derived from **2** which could covalently bond to the enzyme and which could also be formed by the oxidative metabolism of **2.** These three intermediates are **7–9** and each intermediate would be a reactive electrophile capable of covalently bonding to cytochrome P450. There are literature precedents for the metabolic formation of **7–9** (*22–24*).

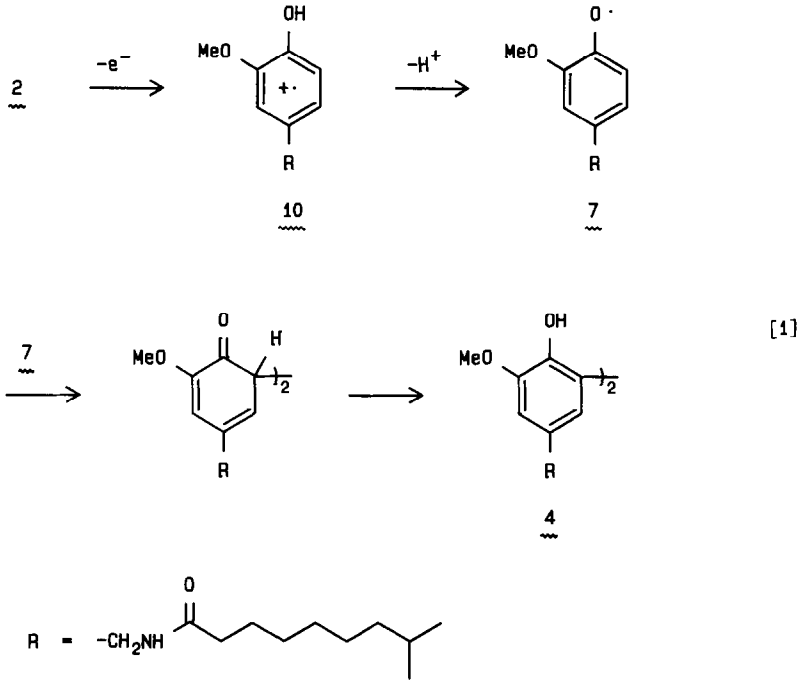


7 **8** **9**

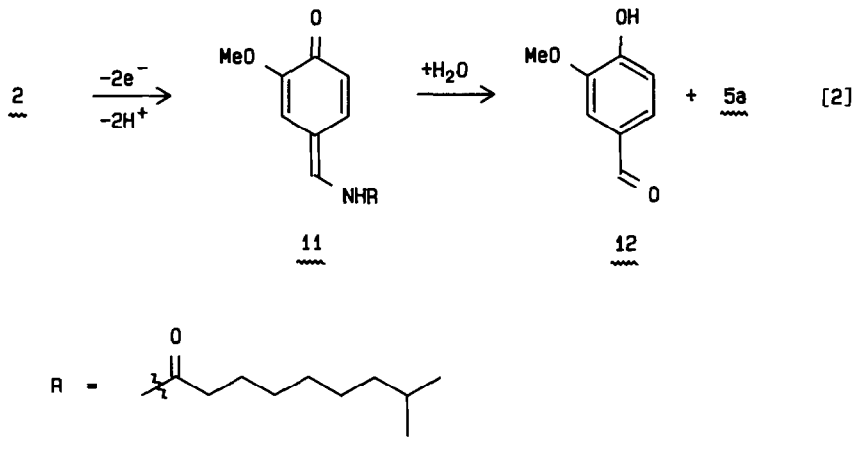
To explore this, the oxidation of **2** was investigated. CV was used to determine if **2** is oxidized by a one- or two-electron transfer process. CV studies conducted in acetonitrile or methanol showed that **2** is oxidized by a one-electron transfer process. This was determined by comparison of peak heights (current) with that from 1,4-dimethoxybenzene since the CV of **2** is irreversible (*15*). When the oxidation wave was repetitively cycled through without agitating the solution, a second

wave appeared and grew in intensity, indicating the formation of a product derived from **2.** It was subsequently determined that this wave was due to **4,** the 5-5′-dimer of **2,** and **4** is a radical coupling product derived from one-electron oxidation/deprotonation of **2** (*25*). Finally, the behavior of the peak oxidation potentials observed for **2,** in the presence of the acid relative to those observed under neutral conditions, also supports a one-electron oxidation process (*26*).

Preparative electrochemical oxidation of **2** further supports the intermediacy of **7.** Electrochemical oxidation of **2** in absolute methanol or aqueous phosphate buffer (pH = 7.4) resulted in the formation of **4,** which was the sole product isolated from the electrolysis mixture. Its formation occurred with the net consumption of approximately 1 f/mol and, based on structurally related compounds, formed by coupling of the phenoxy radical **7** (*27*):



Oxidation of **2** with potassium ferricyanide (*28*) or potassium persulfate (*29*) resulted in the formation of the dimer **4,** although its formation was accompanied by **5b** and some tarry material. However, oxidation with nickel peroxide (*30*) or lead dioxide (*31*) did not result in dimer formation. Instead, **5a** was the principal product. This product could be formed by two-electron oxidation to yield **11,** which could hydrolyze to **5a** and vanillin **(12)** (Eq. [2]). However, inspection of the reaction mixtures revealed no trace of **12.** We suspect that, with these oxidants, further oxidation of the aromatic ring or of the ring substituents occurred, as has been observed with other substrates. It is clear that **2** could be oxidized by a two-electron process, but a one-electron oxidation and deprotonation, especially in protic solvents, should be a more facile process (*32*). Finally, the capsaicin derivative **3** was not affected by any of these oxidants.



The electrochemical and chemical oxidation of **2** clearly showed that it was prone to one-electron oxidation rather than two-electron oxidation and that a phenoxy radical intermediate was formed. It became important to determine if **2** could be similarly oxidized enzymatically. For this purpose HRP was used as it models the one-electron oxidation component of cytochrome P450 (*33*). In some respects, using HRP as a model for the enzymatic oxidation of **2** was inappropriate, as it is known that this enzyme oxidizes phenols by a single electron process. However, if HRP did not oxidize **2**, then this would suggest that **2** is not prone to an enzymatic one-electron oxidation process.

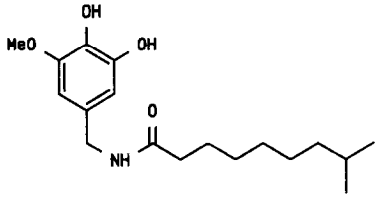
Initial studies with HRP and **2** by visible spectroscopy indicated that **2** was a substrate for HRP. The visible spectrum of HRP (resting state) has a maximum at 403 nm. When hydrogen peroxide is added, a new maximum appears at 410 nm. When one equivalent (based on added peroxide) of a suitable substrate is added, a new maximum appears at 418 nm. When the addition of the substrate is continued, the original HRP spectrum will be observed after a second equivalent of substrate has been added (*34*). These changes were seen when **2**, but not **3**, was used as the substrate.

When **2** was incubated with HRP on a preparative scale compounds **4** and **5b** were isolated. The source of **5b** is unclear and it could be a hydrolysis product of **2** or be formed by some other process. Only starting material was recovered in incubations of HRP and **3**.

Oxidation of **2** by electrochemical methods, one-electron chemical oxidants, and HRP resulted in the formation of **4**. This product clearly forms by dimerization of the phenoxy radical **7** and this intermediate must form under all three sets of reaction conditions. The interaction of **2** with cytochrome P450s, and in particular with cytochrome P450IIE1, is likely to proceed in an analogous fashion. If so, **7** would be formed at the active site and, once formed, could then covalently bind to the active site or, alternatively, be oxygenated. Our results with cytochrome P450IIE1 suggest that the former process is strongly favored.

Brief mention should be made of the possible intermediacy of **8** and **9**. The benzoepoxide **8** has been previously proposed as a metabolic intermediate based on the isolation of **13** (*35*). This product, however, could also form by oxygenation

of **7.** In addition, the effect of O-methylation of **2** observed tends to weigh in favor of **7** and against **13.** Quinone intermediates such as **9** (and **11**) are also potential intermediates and could also covalently bond to cytochrome P450IIE1. However, the oxidation of **2** to a quinone should be a much more difficult process than that to the phenoxy radical **7.** In this regard, metabolism studies previously conducted with **2** support this as the isolated metabolites do not provide any evidence for the formation of quinones (*36, 37*).



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In conclusion, our data do not unambiguously show that **7** was the reactive intermediate responsible for the inactivation of cytochrome P450IIE1. They do not prove that **8** and **9** were not formed by other cytochrome P450 isozymes. Any of the intermediates **7–9** could be formed by cytochrome P450IIE1 and covalently bind to it, since all are electrophilic species. However, previous studies and the present investigation indicate that **7** is the most probable intermediate responsible for the inactivation of cytochrome P450IIE1. These studies suggest a mechanism whereby **2** inactivates cytochrome P450IIE1 (*38*). Thus the oxidation of **2** in the active site of cytochrome P450IIE1 results in the formation of the phenoxy radical **7.** In turn, **7** could react with oxygen, with a nucleophile within the heme cavity, or with the porphyrin ring. The inhibition and binding data suggest that oxygenation of **2** is not a facile process. The latter two reactions, however, would inactivate the enzyme and result in binding of **2** to the enzyme. We cannot distinguish between these two processes but we believe that reaction with the porphyrin ring is more likely to occur, as similar reactions have been previously observed for other aryl radicals (*38*).

EXPERIMENTAL

General. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian XL-300. Chemical shifts are reported downfield from tetramethylsilane on the δ scale. Infrared spectra were recorded on a Mattson Alpha Centaria FT-IR controlled by an AT&T PC 7300. Mass spectra were measured at 70 eV on an AEI-MS9. Ultraviolet and visible spectra were obtained on a Varian DMS 300 spectrophotometer. Capsaicin **(1),** dihydrocapsaicin **(2),** and *O*-4-methyl dihydrocapsaicin **(3)** were prepared as previously described (purity >99%) (*38*). Other reagents were purchased from Aldrich Chemical Co. and used without further purification unless otherwise noted. HRP (Type II) was purchased from Sigma (St. Louis, MO). All solvents were uv grade. Solvents for electrochemical studies were further treated to remove residual water. MeOH was dried by distillation from

$Mg(OMe)_2$, MeCN by distillation from CaH_2, and DMF by distillation from BaO. Tetra-*n*-butylammonium perchlorate (Alfa) was recrystallized from aqueous ethanol (50%). All new compounds were fully characterized. GC and HPLC data were obtained under previously described conditions (*39*).

Animals. Six-week-old male Sprague–Dawley rats were obtained from Sasco (Omaha, NE). They were freely maintained on food and water and were housed under standard conditions (22 ± 3°C, 40 ± 5% relative humidity, 12-h light–dark cycle).

Preparation of microsomes. Rats were killed by decapitation and the liver was perfused with cold isotonic saline. Liver microsomes were prepared by differential centrifugation of a 20% homogenate (*40*). Protein was measured by the method of Lowry *et al.* (*41*). Enzyme activities were determined after storage of the microsomal fraction at −20°C.

Enzyme activity assay. The cytochrome P450 concentration was measured by the method of Omura and Sato (*42*). *p*-Nitrophenol hydroxylase activity was determined from the 4-nitrocatechol formed after incubation of substrate with microsomes and NADPH at 37°C for 15 min (*19*). **2** and **3** were dissolved in methanol at 10 mM concentration. Control incubations included the same amount of methanol as did the incubations containing substrate.

Polyacrylamide gels. Microsomes (approximately 30 mg protein/ml sodium phosphate buffer, pH 7.2) were solubilized for 1 h by diluting (1/2, v/v) with 10 mM phosphate buffer containing 0.75% (w/v) sodium cholate, 0.15 mM EDTA, 30% (v/v) glycerol, and 0.3% Emulgen 911 at pH 7.2. The samples were first boiled in 1% (w/v) SDS, 1.5% (v/v) glycerol, 0.1% (w/v) bromophenol blue, and 5% (v/v) 2-mercaptoethanol. Electrophoresis was performed at 40 mA through a 4% polyacrylamide stacking gel and 70 mA through a 7% polyacrylamide running gel (*43*). The gel was soaked in DMSO and then in DMSO containing 20% (w/v) PPO which was precipitated by transferring the gel to distilled water. The gel was dried under vacuum and exposed to Kodak RP panoramic film for 4 weeks.

Monoclonal antibody assay. A monoclonal antibody directed against cytochrome P450IIE1, kindly provided by Dr. Heidrick (University of Nebraska Medical Center, Omaha, NE), was bound to a Nalgene affinity chromatography filter unit (Nalge Co., Rochester, NY) by incubating 4 mg of monoclonal antibody in a 0.5 M carbonate buffer, pH 9.3, with the filter unit for 18 h at 25°C. The binding was stabilized by incubating the filter in 0.1% (w/v) sodium borohydride in phosphate-buffered saline (PBS) for 30 to 60 min at room temperature. The filter was then sequentially washed with PBS, 1 M NaCl, 0.1 M glycine (pH 2.3), and finally PBS.

Dihydrocapsaicin binding to cytochrome P450IIE1 was determined by incubation of 1.4×10^7 cpm of [^{3}H]capsaicin (specific activity = 3.2×10^4 Ci/mole) with 6) mg of adult-male rat-liver microsomes in Tris (50 mM), EDTA buffer (1 mM, pH 7.4, total volume 1 ml) at 37°C for 10 min. Incubations were performed both with and without 2 mM NADPH. At the end of the incubation the reaction was stopped by the addition of 0.2 ml glycerol and 0.005 ml Nonidet-P40 detergent to solubilize the proteins. The solution was homogenized for 30 s with a tissue miser to produce a clear solution.

The cytochrome P450IIE1 was purified by dilution of the above samples with PBS (1 : 1) and the resulting solution passed through the antibody affinity chromatography filter unit. The filter unit was then washed with 10 ml PBS so that no detectable OD_{280} was eluting from the filter. The cytochrome P450IIE1 was eluted with 5 ml 0.1 M glycine (pH 2.3), collecting 1.5-ml fractions. The eluted fractions were combined with 10 ml Hydrocount and examined by liquid scintillation chromatography. A baseline value was established by adding [^{3}H]dihydrocapsaicin to a sample of solubilized microsomes which were separated with the affinity filter.

Cyclic voltammetry. CV studies were conducted on a BAS CV-27 voltammograph at room temperature under argon on samples containing 1 mM substrate, 0.1 M tetra-*n*-butylammonium perchlorate as supporting electrolyte, a platinum working electrode (*44*), a platinum counter electrode, and a silver/silver chloride reference electrode. The scan rate was 200 mV/s.

Preparative electrochemical oxidation of **3.** Electrochemical oxidations were conducted in a one compartment cell in methanol using a PAR 173 potentiostat and a PAR 179 coulometer. Substrate concentration was 22 mM, the supporting electrolyte was sodium perchlorate (0.22 M), the working and counter electrodes were platinum, and an SCE was used as the reference electrode. Electrolysis was conducted under argon, at ambient temperature, on stirred solutions and under constant potential conditions. Electrochemical oxidations were allowed to proceed until 1 f/mol had been consumed at which time the current had decayed to nearly background levels.

The electrolysis reaction mixtures were worked up by first concentration *in vacuo*. The residue was extracted with methylene chloride, and the extracts were then dried ($MgSO_4$) and filtered. The filtrate was then concentrated and chromatographed (low pressure; silica gel; 2/1 to 5/1, EtOAc/hexane) to give **4** (yield 32%).

2,2'-Bis-(E-8-methyl non-6-enamidomethyl)-6-methoxy phenol **(4).** ir ($CHCl_3$) cm^{-1} 3541, 3445 (NH), 3016 (C=CH), 2962 (RH), 1660 (C=O), 1513 (C=C), 1271, 1153, 1036 (C—O, C—N). ^{1}H NMR ($CDCl_3$) δ ppm 0.795 (12H, d, J = 7.5 Hz, $(CH_3)_2$), 1.090 (4H, m), 1.213 (12H, m), 1.443 (4H, m, CH), 1.608 (4H, m), 2.133 (4H, t, J = 7.4 Hz, CH_2C=O), 3.816 (6H, s, CH_3O), 4.290 (4H, d, J = 5.2 Hz, CH_2N), 5.653 (4H, bs, NH, OH), 6.742 (2H, bs, ArH), 6.783 (2H, bs, ArH); ^{13}C NMR ($CDCl_3$) δ ppm 22.63, 25.79, 27.23, 27.94, 29.38, 29.63, 36.88, 38.96, 43.60, 56.21, 110.3, 122.8, 123.9, 130.3, 142.2, 147.4, 173.0; uv (MeOH) λ nm (log ε) 288 (3.75), 245 (3.84). Exact mass calcd for $C_{36}H_{52}N_2O_6$: 612.4139. Found: 612.4144.

Potassium ferricyanide oxidation **2.** To a solution of **2** (100 mg, 0.3 mmol) and Na_2CO_3 (50 mg, 0.41 mol) in water (1 ml) and DMF (*45*) (0.2 ml) was added, in one portion, $K_3Fe(CN)_6$ (0.13 g, 0.40 mmol). The resulting mixture, which was initially red and became green within 30 min, was stirred overnight, concentrated *in vacuo* (0.1 mm Hg), taken up in water (10 ml), and extracted (CH_2Cl_2, 3X 10 ml). The extracts were dried ($MgSO_4$), filtered, and concentrated. The residue was purified by preparative TLC (silica gel; 1/1, hexane/acetone). The material with an R_f of ca. 0.4 was collected and corresponded to **4.**

Nickel or lead peroxide oxidation of **2.** To a solution of **2** (0.1 g, 0.33 mmol) in 1/3, acetone/$CHCl_3$ (4 ml), was added PbO_2 (3.4 g, 7.7 mmol). The resulting solution

was stirred overnight at room temperature, filtered through a filter cell, and concentrated *in vacuo* to yield **5a.** ir ($CHCl_3$) cm^{-1} 3684, 3531, 3412 (N—H), 2990 (C—H), 1682 (C=O), 1423 (C—C), 1210 (C—N). ^1H NMR ($CDCl_3$) δ ppm 0.772 (6H, d J = 6.6 Hz, $(CH_3)_2C$), 0.9–1.3 (10H, m), 1.42 (1H, m, CH), 2.125 (2H, t, J = 7.2 Hz), 5.65 (2H, bd, NH_2), MS *m/z* (relative intensity) 171 (1.7), 128 (11), 72 (36), 59 (100), 43 (10). Exact mass calcd for $C_{10}H_{21}NO$: 171.1623. Found: 171.1628.

HRP incubation of **2.** To a solution of **2** (15 mg, 0.05 mmol), horseradish peroxidase (Type II, 200 units/mg, 2 mg), and disodium EDTA (37.4 mg) in water (80 ml) and dimethylformamide (20 ml) (*45*) was added hydrogen peroxide (30%, 11.4 μl, 0.1 mmol). The incubation was maintained at 25°C with gentle stirring for 30 min. A second portion of hydrogen peroxide was added (11.4 μl), and stirring continued for 0.5 h longer. The incubation mixture was then extracted with methylene chloride (5X 50 ml), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Following preparative thin layer chromatography (silica gel; 1/1, acetone/hexane) **4** (R_f = 0.4), 10 mg, was isolated. A second component was isolated (R_f = 0.8), 2 mg, which was identical to an authentic sample of 8-methyl nonoic acid (*9*).

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