

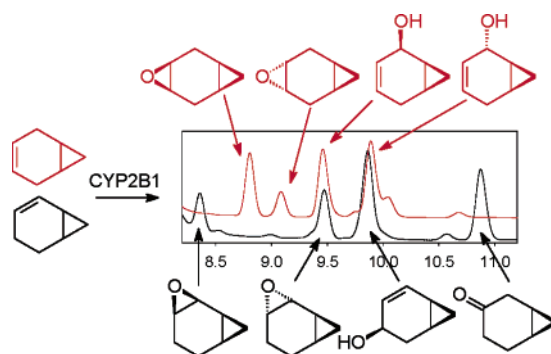
Products from Enzyme-Catalyzed Oxidations of Norcarenes

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Recent studies revealed that norcarane (bicyclo[4.1.0]heptane) is oxidized to 2-norcarene (bicyclo[4.1.0]hept-2-ene) and 3-norcarene (bicyclo[4.1.0]hept-3-ene) by iron-containing enzymes and that secondary oxidation products from the norcarenes complicate mechanistic probe studies employing norcarane as the substrate (Newcomb, M.; Chandrasena, R. E. P.; Lansakara-P., D. S. P.; Kim, H.-Y.; Lippard, S. J.; Beauvais, L. G.; Murray, L. J.; Izzo, V.; Hollenberg, P. F.; Coon, M. J. *J. Org. Chem.* **2007**, *72*, 1121–1127). In the present work, the product profiles from the oxidations of 2-norcarene and 3-norcarene by several enzymes were determined. Most of the products were identified by GC and GC–mass spectral comparison to authentic samples produced independently; in some cases, stereochemical assignments were made or confirmed by 2D NMR analysis of the products. The enzymes studied in this work were four cytochrome P450 enzymes, CYP2B1, CYPΔ2E1, CYPΔ2E1 T303A, and CYPΔ2B4, and three diiron-containing enzymes, soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* (Bath), toluene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1, and phenol hydroxylase (PH) from *Pseudomonas stutzeri* OX1. The oxidation products from the norcarenes identified in this work are 2-norcaranone, 3-norcaranone, *syn*- and *anti*-2-norcarene oxide, *syn*- and *anti*-3-norcarene oxide, *syn*- and *anti*-4-hydroxy-2-norcarene, *syn*- and *anti*-hydroxy-3-norcarene, 2-oxo-3-norcarene, 4-oxo-2-norcarene, and cyclohepta-3,5-dienol. Two additional, unidentified oxidation products were observed in low yields in the oxidations. In matched oxidations, 3-norcarene was a better substrate than 2-norcarene in terms of turnover by factors of 1.5–15 for the enzymes studied here. The oxidation products found in enzyme-catalyzed oxidations of the norcarenes are useful for understanding the complex product mixtures obtained in norcarane oxidations.

Introduction

Mechanistic probes have been used for years to reveal details about reaction mechanisms in chemistry and biology. The

concept of a mechanistic probe study is that a short-lived intermediate can be revealed by a characteristic rearrangement of a probe substrate that is observed in the reaction products. One compound used as a probe substrate for studies of enzyme-catalyzed oxidations is norcarane, bicyclo[4.1.0]heptane (**1**). During the course of probe studies with norcarane in our laboratories,¹ we observed a large number of minor oxidation

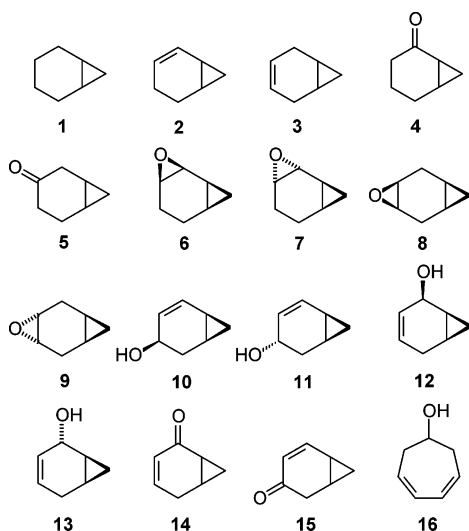
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CHART 1



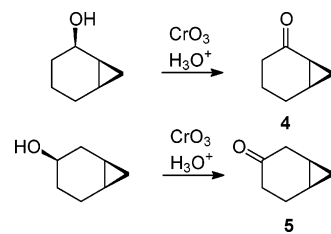
products that appeared to be derived from norcarane as judged by GC retention times and mass spectra. Eventually, we realized that, in addition to expected alcohol products, the enzymes were oxidizing norcarane to both 2-norcaradiene (**2**) and 3-norcaradiene (**3**) and that these alkenes were further oxidized to give small amounts of secondary oxidation products.² Details of the norcarane oxidation studies are reported in the accompanying paper.³ In this paper, we report studies of the oxidations of both 2-norcaradiene and 3-norcaradiene by several iron-containing enzymes. Most of the products from the oxidations of these substrates were synthesized independently, providing firm identifications. The results of this work are used in the accompanying paper³ to aid in identification of the many products from enzyme-catalyzed oxidations of norcarane.

Results and Discussion

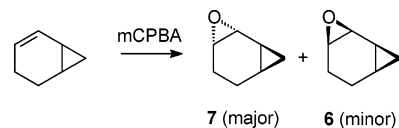
Products from Norcaradiene Oxidations. Iron-containing enzymes oxidize norcarenes to give a complex mixture of products. Chart 1 shows a collection of oxidation products that we have characterized. Compounds **4–13** and **16** are singly oxidized products of the norcarenes, whereas ketones **14** and **15** are doubly oxidized products. Most of the compounds in Chart 1 were synthesized independently so that the products from the enzyme-catalyzed oxidations could be identified by GC and GC–mass spectral comparisons. In some cases, the stereochemical assignments in the compounds were not certain, and we confirmed the stereochemistry with NMR experiments. In addition to the compounds shown in Chart 1, two other minor products were detected in the enzyme-catalyzed oxidation reactions of **2** and **3** but were not fully characterized.

Ketones **4** and **5** were prepared by oxidation of the corresponding alcohols (Scheme 1), as previously reported by Chan and Rickborn.⁴

SCHEME 1



SCHEME 2



The *anti*-epoxide **7** was previously reported to be formed by reaction of 2-norcaradiene with peroxyacetic acid,⁵ and we prepared compound **7** by reaction of 2-norcaradiene with *m*-chloroperoxybenzoic acid (mCPBA) (Scheme 2). After chromatographic purification, compound **7** was judged to be >95% pure by NMR spectroscopy. The structure of **7** was confirmed by 1D NOE difference experiments that showed the enhancements in Figure 1. The chemical shift assignments for H_{7-endo} and H_{7-exo} were made on the basis of the coupling constants between these two protons and the H₆ proton, which are also shown in Figure 1. These coupling constants are similar to those for the analogous protons in norcaradiene.⁶

When the crude product from the preparative reaction for **7** was analyzed by GC, we observed a minor product in ca. 6% relative yield with a shorter retention time than that of **7** on a low-polarity GC column. The mass spectrum of the minor product was the same as that of epoxide **7**. The same product was detected in enzyme-catalyzed oxidations of 2-norcaradiene. We conclude that the minor product is the *syn*-epoxide **6** (Scheme 2).

The diastereomeric epoxides **8** and **9** were reported previously.⁷ These compounds were formed in comparable amounts by the reaction of 3-norcaradiene with mCPBA, and they were separated by chromatography. Reactions of the isolated epoxides with LiNEt₂ gave the known allylic alcohols **10** and **11**, respectively (Scheme 3).⁷

Oxidation of 3-norcaradiene with chromium trioxide⁸ gave the known α,β -unsaturated ketone **14** as the major product and the previously unreported isomer **15** as a minor product (Scheme 4). The reaction of ketone **14** with DIBALH gave the allylic alcohols **12** and **13**, both new compounds, and the previously known⁹ alcohol **16** (Scheme 4).

The stereochemical assignments for allylic alcohols **12** and **13** were made from coupling constant data, the results of 1D NOE difference experiments, and the results of molecular modeling studies. Table 1 summarizes the data. For both isomers, H_{endo} and H_{exo} at C7 were assigned from the coupling constants between these protons and the bridgehead proton at C1. Molecular mechanics modeling of the two isomers indicated that the distances between the endo and exo protons on C7 and

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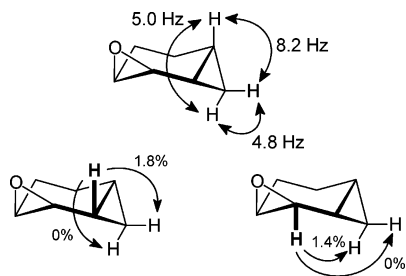
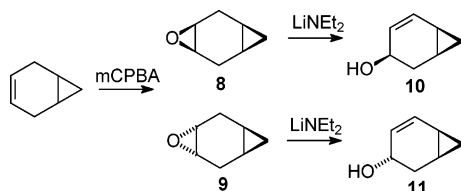
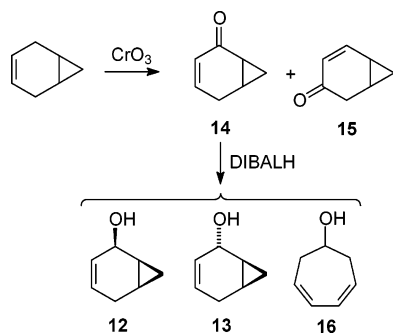


FIGURE 1. Selected coupling constants (top) and NOE enhancements (bottom) for epoxide **7**. The NOE values are for irradiation at H_{C1} and H_{C2} .

SCHEME 3



SCHEME 4



the proton on C2 were shorter for isomer **12** than the respective distances for isomer **13**. A series of NOE experiments were performed where the C7 protons on each isomer were selectively irradiated and the absorbance enhancements for the protons on C2 were measured. The NOE enhancements of the H_{C2} signal were larger for irradiation of both of the C7 protons in isomer **12**, consistent with expectations from the molecular modeling results.

For enzyme-catalyzed oxidation reactions of the norcarenes, the products were quantitated by flame ionization GC and identified by GC–mass spectral analysis. GC retention times for compounds **4–16** are shown schematically in Figure 2A and 2B. Figure 2B also shows a portion of a typical GC result for a P450 enzyme-catalyzed oxidation of 3-norcarenene. The GC analyses were obtained with a low-polarity column, which gave the best separations. Mass spectral fragmentation patterns for these compounds are in the Supporting Information for the accompanying paper.³

As is apparent in Figure 2, several of the oxidation products from the norcarenes have similar GC retention times. Products **10** and **13** were not resolved under our GC conditions, and the similar retention times for some products resulted in overlapping peaks. In principle, the composition of merged peaks can be determined from GC–mass spectral analysis, but this task is not necessarily simple nor reliable because mass spectra of most

TABLE 1. Data Used for Structural Assignments of Isomers **12** and **13**

property or feature	12	13
H_{C1} – H_{C7endo} coupling constant (Hz)	5.0	5.0
H_{C1} – H_{C7exo} coupling constant (Hz)	8.0	8.7
H_{C7exo} – H_{C7endo} coupling constant (Hz)	4.5	4.8
H_{C2} – H_{C7endo} distance (Å) ^a	2.90	3.83
H_{C2} – H_{C7exo} distance (Å) ^a	3.66	4.34
H_{C7endo} NOE enhancement at H_{C2} (%)	2.85	1.52
H_{C7exo} NOE enhancement at H_{C2} (%)	2.50	0

^a Distance between protons in the minimum energy structure calculated by MM2 modeling.

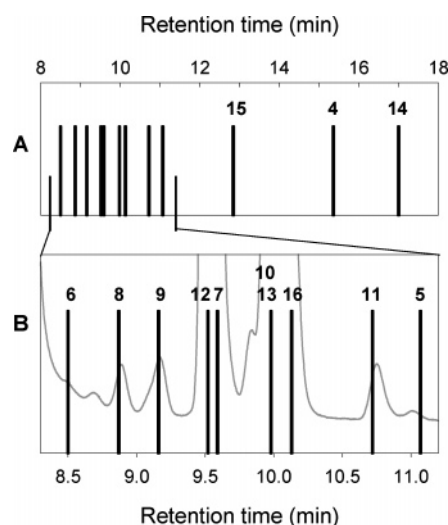


FIGURE 2. (A) GC retention times for compounds **4–16** on a low-polarity column (5% phenylsilicone) at 80 °C. (B) Expansion of the congested region of the trace in (A) with the GC trace of products from the oxidation of 3-norcarenene by CYPΔ2E1 (gray line) superimposed. The compound numbers are shown above the lines that indicate the retention times for each compound. Allylic alcohols **10** and **13** had the same retention times to within 0.01 min.

of the oxidation products are similar. For the norcarenene oxidations discussed later, we report the sum of products **10** and **13**.

Although we prepared many possible oxidation products from 2- and 3-norcarenene, other oxidation products were detected in some of the enzyme-catalyzed oxidation reactions of these substrates. One of the unidentified products appears to have a molecular ion at $m/z = 124$ (Figure 3A), which is consistent with incorporation of two oxygen atoms and loss of four protons from the substrate to give a highly oxidized species with the formula $C_7H_8O_2$. This product is assigned as unknown compound **A**. It coeluted with compound **16** and was detected in product mixtures from oxidations of both 2-norcarenene and 3-norcarenene. In some reactions, unknown **A** apparently was formed in yields comparable to those of some of the primary oxidation products from the norcarenenes.

Another unknown oxidation product was apparent in the reactions of 2-norcarenene. Epoxide **9** should not be formed from the oxidation of 2-norcarenene, but we observed a product with a GC retention time the same as that of epoxide **9** in the product mixtures from most of the oxidations of 2-norcarenene. This product, labeled as unknown compound **B**, not only has the same retention time as compound **9** but also has a similar mass spectral fragmentation pattern. Figure 3B shows the fragmentation pattern for authentic epoxide **9**, and Figure 3C shows the

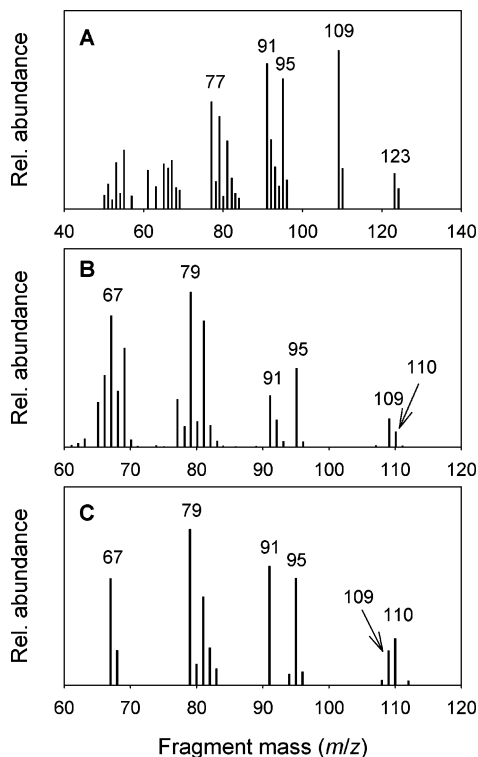


FIGURE 3. (A) Mass spectral fragmentation pattern for unknown product **A**. (B) Mass spectral fragmentation pattern for authentic product **9**. (C) Mass spectral fragmentation pattern observed for unknown product **B**, which eluted at the retention time expected for epoxide **9**, from the product mixture obtained in the CYPΔ2E1-catalyzed oxidation of 2-norcarene.

fragmentation pattern observed at the same retention time as that of compound **9** from the oxidation of 2-norcarene catalyzed by CYPΔ2E1. The differences in the mass spectral fragmentation patterns are subtle. In authentic **9**, the fragment with $m/z = 91$ is smaller than that with $m/z = 95$, and the fragment with $m/z = 109$ is greater than that with $m/z = 110$. In unknown **B**, the relative intensities for these two pairs are reversed. The similarity of these fragmentation patterns illustrates the great difficulty one has in attempting to identify a product by the use of a few fragment peaks obtained from single-ion monitoring (SIM) analysis of the GC trace. If SIM analysis with limited ion channels was used for the unknown product **B** (Figure 3C), one might mistakenly identify that unknown compound as product **9**.

Enzyme-Catalyzed Oxidations of Norcarenes. We studied the oxidations of 2-norcarene and 3-norcarene with a representative spectrum of iron-containing enzymes that included four mammalian hepatic cytochrome P450 enzymes and three diiron enzymes. The P450 enzymes, which were expressed in *Escherichia coli* and purified, were CYP2B1,¹⁰ CYPΔ2B4,¹¹ CYPΔ2E1,¹² and CYPΔ2E1 T303A.¹² The latter three P450s contain short deletions at the N-terminal ends of the enzymes. CYP2B1 (rat) and CYP2B4 (rabbit) are induced in animals by phenobarbital treatment. CYP2E1 is a constitutive and ethanol-inducible P450

linked to alcohol-related liver disease. In the CYPΔ2E1 T303A mutant, a highly conserved threonine located near the active site of the enzyme was replaced with alanine; this conserved Thr is thought to be involved in the proton delivery system in the activation of the P450 enzymes.¹² The diiron enzymes studied were the soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* (Bath), one of the more extensively studied diiron enzymes,^{13–15} toluene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1, and phenol hydroxylase (PH) from *Pseudomonas stutzeri* OX1. As discussed in the accompanying paper,³ results for norcarene oxidations by the iron-containing enzymes studied in this work and closely related enzymes are available.

The substrates 2-norcarene (**2**) and 3-norcarene (**3**) were prepared by literature methods involving cyclopropanation of 1,3-cyclohexadiene and 1,4-cyclohexadiene, respectively.^{5,16} Purity of the substrate is an issue when quantification of small amounts of products are necessary, and we used preparative GC as the final step in the purification of **2** and **3**. The products used in the enzyme studies contained no detectable impurities by NMR spectroscopy and were 99.7% homogeneous (**2**) and 99.3% homogeneous (**3**) by analytical GC.

Enzyme-catalyzed oxidations of the norcarenes involved three or four individual experiments with each enzyme and substrate. The norcarenes are good substrates for most of the enzymes studied here. For the sMMO enzyme, turnover was limited, probably because the active site is optimized for the oxidation of methane, and 10 nmol of sMMO was used in each experiment. The oxidation products were extracted into methylene chloride, and the product mixtures were analyzed by GC for yield quantifications and by GC–mass spectroscopy for identification. Table 2 contains the results. Figure 4 shows representative GC traces for oxidations by a P450 enzyme and two diiron enzymes.

From the total amounts of oxidation products in the final column, 3-norcarene was determined to be a better substrate than 2-norcarene for all of the enzymes studied. For CYPΔ2B4, five times as much product was formed from 3-norcarene as from 2-norcarene. For the diiron enzymes, about an order of magnitude more product was formed in the oxidations of 3-norcarene than in the oxidations of 2-norcarene.

Allylic alcohols **10–13** were the major products formed with all of the P450 enzymes, although ketone **5** was formed in considerable yields in the P450 oxidations of 2-norcarene. For the diiron enzyme sMMO, allylic alcohols were the major product from 3-norcarene, whereas ketone **5** was the major product from 2-norcarene. Interestingly, the diiron enzymes ToMO and PH gave high yields of epoxide **9** in oxidation reactions of 3-norcarene but low yields of the epoxides from 2-norcarene. We noted previously that unknown product **B** overlaps with epoxide **9** on the GC, but mass spectral analyses confirmed that epoxide **9** was the major component obtained in the oxidations of 3-norcarene.

The oxidation patterns observed for the norcarenes undoubtedly reflect substrate binding preferences in the active site superimposed on the high reactivities expected for the double

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TABLE 2. Product Yields from Oxidations of 2-Norcaradiene and 3-Norcaradiene^a

enzyme ^b	4	5	6	7	8	9+B	10+13	11	12	14	15	16+A	total
2-norcaradiene													
CYPΔ2E1	ND ^c	5.62	0.72	0.72	ND ^c	1.95	26.90	1.35	3.04	4.21	4.35	2.71	51.57
CYPΔ2E1 T303A	ND ^c	3.89	1.47	1.44	ND ^c	0.17	8.84	1.03	3.72	6.01	6.19	0.26	33.02
CYP2B1	0.08	7.11	2.94	3.65	ND ^c	0.32	8.35	0.62	0.54	0.18	0.14	0.91	24.84
CYPΔ2B4	0.15	14.46	3.53	5.02	ND ^c	0.47	25.38	1.37	1.11	0.27	0.24	2.06	54.06
sMMO	0.08	9.56	<0.08	<0.05	ND ^c	ND ^c	2.03	1.08	0.09	0.13	0.15	0.62	13.74
ToMO	0.03	2.20	<0.01	0.09	ND ^c	0.19	0.12	0.30	0.04	0.17	0.09	ND ^c	3.23
PH	0.01	0.74	<0.01	1.54	ND ^c	0.03	0.05	0.03	0.06	0.07	0.06	0.03	2.62
3-norcaradiene													
CYPΔ2E1	ND ^c	ND ^c	ND ^c	ND ^c	0.58	1.26	17.02	1.26	46.08	4.36	3.48	7.26	81.30
CYPΔ2E1 T303A	ND ^c	ND ^c	ND ^c	ND ^c	12.29	4.86	8.59	1.79	30.72	6.78	7.20	2.52	74.75
CYP2B1	ND ^c	0.21	ND ^c	ND ^c	13.90	5.85	23.16	1.72	19.30	0.52	0.41	4.13	69.20
CYPΔ2B4	ND ^c	0.87	ND ^c	ND ^c	34.77	11.00	75.11	7.98	82.63	1.07	0.69	16.86	230.98
sMMO	0.15	0.96	ND ^c	ND ^c	<0.15	1.66	50.4	0.55	44.48	1.84	0.17	17.74	117.95
ToMO	0.04	0.55	ND ^c	0.05	1.03	41.61	1.19	0.55	0.50	0.38	0.33	0.61	46.84
PH	0.03	ND ^c	ND ^c	0.06	0.39	18.86	0.20	0.08	0.09	0.13	0.12	0.05	20.01

^a Product yields in nmol; averages of three or four experiments. ^b CYP = cytochrome P450, sMMO = soluble methane monooxygenase from *M. capsulatus* (Bath), ToMO = toluene monooxygenase from *P. stutzeri* OX1, PH = phenol hydroxylase from *P. stutzeri* OX1; oxidation reactions employed 0.5 nmol of P450 enzyme, 1.0 nmol of ToMO or PH, or 10 nmol of sMMO. ^c ND = not detected.

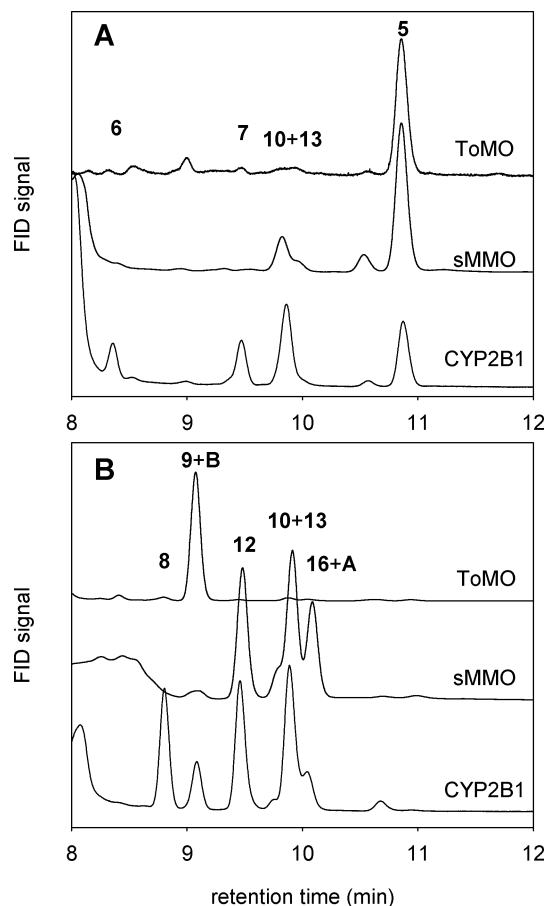


FIGURE 4. Portions of GC traces of products from the oxidation of 2-norcaradiene (A) and 3-norcaradiene (B) with toluene monooxygenase from *P. stutzeri* OX1 (ToMO), soluble methane monooxygenase from *M. capsulatus* (Bath) (sMMO), and cytochrome P450 2B1 (CYP2B1). The identities of some of the products are indicated with the compound numbers.

bonds and the allylic hydrogen atoms. Allylic C–H bonds have bond dissociation energies (BDE's) that are about 12 kcal/mol less than those of analogous C–H bonds in saturated systems,^{17,18} and for the C–H bonds in 3-norcaradiene, the BDE will be smaller due to the adjacent cyclopropyl group.¹⁹

The product distributions for norcaradiene oxidations found in this work are useful for understanding and identifying the many products found in enzyme-catalyzed oxidations of norcaradiene, which result in formation of norcaradiene by desaturase-type reactions.³ One of the more important observations is that the yields of products 16 and A were relatively large for the P450 and sMMO enzymes. Those two products coelute with the radical-derived rearranged alcohol from the oxidations of norcaradiene in GC analyses and interfere with quantification of the latter, as discussed in the accompanying paper.³

Experimental Section

General. The preparations of 2-norcaradiene, 3-norcaradiene, and authentic samples of oxidation products are described in the Supporting Information. NMR spectra were recorded in CDCl₃ at 300 or 500 MHz. Proton correlation was resolved with bidimensional COSY experiments using the sequence cosygpqf, and stereochemistry was determined by monodimensional NOE difference experiments. 2-Norcaradiene and 3-norcaradiene were purified by preparative GC (10% SE-52 on 60/80 Chromosorb W, 1/4 in. × 8 ft column) at 100 °C (isothermal), and the purities of the samples were determined by GC (DB-5, 40 °C) using flame ionization detection. All yields and purities of other authentic samples were determined by GC (DB-5) using flame ionization detection and by GC–MS (HP-5MS 5% phenylmethylsiloxane column) with electron impact (EI) ionization. Details of the sample preparations are in the Supporting Information. Mass spectra are in the Supporting Information for the accompanying article.³

Cytochrome P450-Catalyzed Oxidations. Cytochrome P450 2B1,¹⁰ P450 Δ2B4,¹¹ P450 Δ2E1,¹² and P450 Δ2E1 T303A¹² enzymes and P450 reductase¹⁰ were expressed in *E. coli* and purified as previously described. A stock solution of substrate was prepared from substrates 2 or 3 (2 mg in 100 μL of methanol) and DLPC (2 mg) in 1.0 mL of 50 mM phosphate buffer, pH 7.4; the mixture was sonicated and stored at 0 °C. A stock solution of NADPH was prepared by addition of 15 mg of NADPH to 375 μL of the same buffer, and this solution was also stored at 0 °C. Reaction mixtures were prepared by mixing 0.5 nmol of enzyme, 1.0 nmol of P450

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reductase, and 30 μL of DLPC at 0 $^{\circ}\text{C}$ and were allowed to stand for 10 min. The buffer was added to the mixture to bring the total volume to 1.89 mL, and the mixture was allowed to stand at 0 $^{\circ}\text{C}$. After 10 min, 10 μL of substrate stock solution was added, and the reaction mixture was allowed to equilibrate at 26 $^{\circ}\text{C}$ for 5 min. The reaction was initiated by adding 100 μL of the NADPH stock solution. Reactions were maintained at 26 $^{\circ}\text{C}$ for 30 min and then quenched by addition of 1 mL of CH_2Cl_2 . The organic phase was separated, and the aqueous phase was extracted with CH_2Cl_2 (2×1 mL). The combined organic layer was dried with MgSO_4 and filtered. An internal standard of 1-phenyl-1-propanol was added, and the mixture was concentrated under a stream of nitrogen. The mixture was analyzed by GC (0.32 mm \times 30 m capillary column, 5% phenylsilicone-bonded phase) and GC–mass spectroscopy (0.25 mm \times 30 m, capillary column, 5% phenylsilicone-bonded phase).

Soluble Methane Monooxygenase/Oxygenase-Catalyzed Oxidations. The hydroxylase protein (MMOH) was purified from *M. capsulatus* (Bath), as previously described.^{20,21} The regulatory (MMOB) and reductase (MMOR) components were expressed recombinantly in *E. coli* and purified as reported elsewhere.^{22,23} In a typical reaction, a mixture of 10 nmol of MMOH, 20 nmol of MMOB, and 5 nmol of MMOR was diluted to a volume of 400 μL with 25 mM potassium phosphate buffer (pH = 7.0). The enzyme was incubated for 1 min at 45 $^{\circ}\text{C}$, and 10 μL of a solution of substrate (2 mg of substrate in 100 μL of methanol) was added. The oxidation was initiated by the addition of 1.6 μmol of NADH. The mixture was gently shaken at 45 $^{\circ}\text{C}$ for 25 min and then extracted with CH_2Cl_2 (3×2 mL). The reaction mixtures were worked up and analyzed as described above.

Toluene Monooxygenase-Catalyzed Oxidations. Plasmids containing the genes for the toluene/*o*-xylene monooxygenase hydroxylase (ToMOH), the coupling protein (ToMOB), and the Rieske

protein were kindly supplied by Prof. Alberto Di Donato. The proteins were expressed in *E. coli* and purified as described elsewhere.^{24,25} The toluene/*o*-xylene monooxygenase NADH oxidoreductase (ToMOR) was provided by Dr. Valeria Cafaro. The specific activity on phenol was $1.2(1) \times 10^3$ mU/mg of hydroxylase, and the iron content for ToMOH was 4.3(2), as determined by methods reported elsewhere.^{24,25} In a typical reaction, a mixture of 1 nmol of ToMOH, 66.57 nmol of ToMOB, 20 nmol of Rieske protein, and 0.1 nmol of ToMOR was diluted to a volume of 2 mL with 20 mM potassium phosphate buffer (pH = 7.4). The mixture was allowed to stand in an ice bath for 10 min. Substrate (10 μL of a solution of 2 mg of 2-norcarene or 3-norcarene in 100 μL of methanol) was added. The mixture was incubated at 26 $^{\circ}\text{C}$ for 10 min while shaking. The oxidation was initiated by the addition of NADH buffer solution; the final concentration of NADH was 1 mM. The mixture was gently shaken at 26 $^{\circ}\text{C}$ for 30 min and then extracted with CH_2Cl_2 (3×2 mL). The combined organic phase was dried (MgSO_4) and filtered. The mixtures were analyzed by GC and GC–MS, as described previously.

Phenol Hydroxylase-Catalyzed Oxidations. The hydroxylase component PH/H of the phenol hydroxylase complex was purified from *E. coli*, strain JM109 expressing the entire *ph* operon cloned into vector pGEM3Z.²⁶ The hydroxylase moiety was purified with a slight modification of a reported procedure.²⁷ The iron content was 4.2(3) per mol protein, and the specific activity on phenol was 515 (SD of 44) mU/mg of hydroxylase, determined as reported elsewhere.²⁴ The coupling (PHM) and reductase (PHP) components were expressed recombinantly in *E. coli*, strain BL21DE3, and purified as previously described.²⁷ In a typical reaction, a mixture of 1 nmol of PH/H and 2 nmol of PHM was diluted to a volume of 2 mL with 0.1 M Tris–HCl buffer (pH = 7.5). Substrate (10 μL of a solution of 2 mg of 2-norcarene or 3-norcarene in 100 μL of methanol) and 1 mM NADH were added. The mixture was incubated at 25 $^{\circ}\text{C}$ for 5 min while shaking. The oxidation was initiated by the addition of 2 nmol of PHP (oxidoreductase). The mixture was gently shaken at 25 $^{\circ}\text{C}$ for 30 min and then extracted with CH_2Cl_2 (3×2 mL). The combined organic phase was dried (MgSO_4) and filtered. The mixtures were analyzed by GC and GC–MS, as described previously.

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Supporting Information Available: Synthetic details and NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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