Published in final edited form as:

J Org Chem. 2007 February 16; 72(4): 1121–1127.

Desaturase Reactions Complicate the Use of Norcarane as a Mechanistic Probe. Unraveling the Mixture of Twenty-Plus Products Formed in Enzyme-Catalyzed Oxidations of Norcarane

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Abstract

Norcarane, bicyclo[4.1.0]heptane, has been widely used as a mechanistic probe in studies of oxidations catalyzed by several iron-containing enzymes. We report here that in addition to oxygenated products, norcarane is also oxidized by iron-containing enzymes in desaturase reactions that give 2-norcarene and 3-norcarene. Furthermore, secondary products from further oxidation reactions of the norcarenes are produced in yields that are comparable to those of the minor products from oxidation of the norcarane. We studied oxidations catalyzed by a representative spectrum of iron-containing enzymes including four cytochrome P450 enzymes, CYP2B1, CYPΔ2B4, CYPΔ2E1, and CYPΔ2E1 T303A, and three diiron enzymes, soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* (Bath), toluene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1, and phenol hydroxylase (PH) from *Pseudomonas stutzeri* OX1. 2-Norcarene and 3-norcarene and their oxidation products were found in all reaction mixtures, accounting for up to half of the oxidation products in some cases. In total, more than 20 oxidation products were identified from the enzyme-catalyzed reactions of norcarane. The putative radical-derived product from oxidation of norcarane, 3-hydroxymethylcyclohexene (**21**), and putative cation-derived product from oxidation of norcarane, cyclohept-3-enol (**22**), co-elute with other oxidation products on low polarity GC columns. The yields of product **21** found in this study are smaller than those previously reported for the same or similar enzymes in studies where the products from norcarene oxidations were ignored, and, therefore, the limiting values for lifetimes of radical intermediates produced in the enzyme-catalyzed oxidation reactions are shorter than previously reported.

Mechanistic probes have been used for many 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. Because transient species are not followed in real time, the existence of an intermediate can only be inferred, and the validity of conclusions based on the use of probes depends on a thorough understanding of potentially complex chemistry.

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Norcarane (**1**) has been applied as a probe in studies of many enzyme-catalyzed oxidation reactions.^{1–10} In most of these works, small amounts of a putative radical-derived product and a putative cation-derived product were detected, providing evidence that radicals and cations were formed as intermediates to some extent in the oxidation reactions. The evidence for radical-derived products in norcarane studies and the conclusions that radical intermediates existed is essentially unique. Most probes that were used in studies of oxidizing enzymes give the same skeletal rearrangement from radical and cation intermediates, and one cannot conclude what type of transient was involved when rearranged products are found. The evidence for radical-derived products in norcarane studies and the conclusions that radical intermediates existed is essentially unique, in disagreement with experimental results conducted with a variety of radical clock substrate probes.¹¹ Moreover, results from “hypersensitive” radical probes,¹² including probes that can differentiate between radicals and cations, indicated that no true radicals were formed in the oxidation reactions, as discussed later. A point of concern in most of the norcarane probe studies was that quite small amounts of rearranged products were found, in yields comparable to those of a myriad of unidentified compounds that appeared to be derived from the norcarane substrate. Eight oxidation products from norcarane were known, but the mixtures appeared to contain many more products with similar molecular weights and GC retention properties.

In this work, we studied oxidation reactions of norcarane by seven iron-containing enzymes with careful attention to the identities of minor products. In addition to oxygenated products, norcarane was oxidized to 2-norcarene and 3-norcarene by all of the enzymes studied, and the norcarenes were efficiently oxidized by all enzymes.⁹ The result is that more than 20 primary and secondary oxidation products from norcarane can be identified in the product mixtures. With authentic samples of various oxidation products from 2-norcarene and 3-norcarene available,¹³ we found that the radical- and cation-derived rearrangement products from norcarane co-elute with norcarene oxidation products on low polarity GC columns. We conclude that the already small yields attributed to these rearranged products probably were *overestimated* in earlier studies, possibly by an order of magnitude or more.

Results and Discussion

Norcarane (**1**) and the oxidation products discussed in this work are shown in Chart 1. 2-Norcarene (**2**) and 3-norcarene (**3**) and products **4–16** from oxidations of these compounds are described in the accompanying paper,¹³ which reports synthetic details for the preparations of authentic samples, structural assignments for **4–16**, and the products formed by oxidation of the norcarenes. In this work, we used the same numbering system for **1–16** as in the accompanying report.¹³ Two unknown compounds were detected in oxidations of the norcarenes,¹³ and one of those products, with apparent formula $C_7H_8O_2$ (**A**), also was detected in the oxidations of norcarane. Authentic samples of known compounds **17–22** also were prepared.^{5,14–19}

Enzymes

For a representative spectrum of iron-containing enzymes, we studied the reactions catalyzed by four cytochrome P450 enzymes (P450s) and three diiron enzymes. All of the enzymes were purified and reconstituted for the oxidation reactions.

The P450s studied were mammalian hepatic enzymes that were expressed in *E. coli*. CYP2B1 is a rat P450 induced by phenobarbital treatment.²⁰ CYPΔ2B4 is the rabbit P450 induced by phenobarbital treatment;²¹ the expressed enzyme has an 18 amino acid deletion at the N-terminal end that does not appear to affect its function.²¹ CYPΔ2E1 and CYPΔ2E1 T303A are the ethanol-inducible P450 enzyme and its mutant, both of which also contain an 18 amino

acid deletion at the N-terminal end.²² This collection of P450 enzymes has been studied in reactions with various mechanistic probes.^{5,21–34}

The diiron enzymes studied were soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* (Bath), toluene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1, and phenol hydroxylase (PH) from *Pseudomonas stutzeri* OX1. The sMMO from *M. capsulatus* (Bath) has been studied with several mechanistic probes,^{5,11,27,35–37} as has a related sMMO from *Methylosinus trichosporium* OB3b.^{3,11,38–41} Mechanistic studies of the ToMO and PH enzymes from *P. stutzeri* OX1, have not been reported previously, but oxidation of norcarane catalyzed by a related toluene 4-monooxygenase was reported.⁷

Desaturase reactions of norcarane

Small amounts of impurities including norcarenes can be detected in distilled samples of norcarane. In order to avoid confusion from the use of slightly contaminated substrate, we treated distilled norcarane with mCPBA to oxidize traces of olefins and isolated the final sample by preparative GC. Analysis of the purified sample by analytical GC indicated that norcarane was at least 99.96% homogeneous, and no impurities of 2-norcarene or 3-norcarene could be detected to the limit of our instrumental sensitivity. Figure 1 shows an analytical GC trace of the norcarane used in the enzyme studies.

Following enzyme-catalyzed oxidation reactions of norcarane, the reaction mixtures were extracted with methylene chloride, an internal standard was added, and the product mixtures were analyzed by GC without concentrating the samples. Both 2-norcarene and 3-norcarene were found in the product mixtures, as illustrated in Figure 1, which shows typical GC results. The yields of 2-norcarene and 3-norcarene in terms of nmol of products found are listed in Table 1, which also includes the total yields of other oxidation products discussed later. The norcarenes were found in high yields relative to other oxidation products, amounting to nearly half of the total amount of oxidation products in some cases. The initial reactions contained ca. 1500 nmol of norcarane, and the yields of norcarenes relative to initial substrate ranged from 0.1% for ToMO to 2% for CYPΔ2B4. In all cases, the norcarene yields were greater than the yields of any minor primary oxygenated product of norcarane. Importantly, the norcarene yields were 1 or 3 orders of magnitude greater than the yields of alcohols **21** and **22** (see below), which are the “probe” rearrangement products implicating a radical and cationic intermediate, respectively.

Oxidations of norcarane to give norcarenes are examples of “desaturase” reactions, which are well characterized reactions of iron-containing enzymes. Perhaps the best known desaturase enzyme is stearyl-ACP Δ⁹ desaturase, the soluble plant enzyme that oxidizes stearyl-ACP to oleoyl-ACP, which contains a diiron cluster similar to that in sMMO enzymes.⁴² Desaturase, or dehydrogenation, reactions have been reported for many iron-containing enzymes including an sMMO⁴³ and cytochrome P450 enzymes.^{44,45}

In comparison to the amounts of substrate norcarane in the samples, approximately 1.5 mmol, the norcarenes represented a minor component of substrates, but the ratios of norcarane to norcarenes at the end of the reactions can be misleading. Comparisons of enzyme turnovers for oxidations of norcarane in this work with those for oxidations of 2- and 3-norcarene with the same enzymes¹³ indicate that norcarenes are more efficiently oxidized than norcarane. For example, for sMMO, the product yield for oxidation of 3-norcarene¹³ was more than six times as great as that for oxidation of norcarane, which included some products from oxidation of norcarenes formed in the desaturase reactions. Another possibly important point is that because the norcarenes are formed in the active site of the enzymes, they might suffer a second oxidation in competition with escape from the active site. If a second oxidation reaction were only 1% as fast as escape from the active site, then the amounts of norcarene oxidation products would

exceed the amounts of the minor products from oxidation of norcarane in most cases. A second oxidation of substrate in competition with product release is a concern for the P450 enzymes, where the oxidation reaction is only initiated when substrate is present in the active site.

Analytical protocol for oxidation products

Previous studies did not recognize the production of norcarenes in desaturase reactions of norcarane, and the products from oxidations of norcarenes were ignored in developing analytical protocols. With an understanding that norcarene oxidation products are formed in the enzyme-catalyzed oxidation reactions and the availability of authentic samples of the oxidation products in Chart 1,¹³ we studied methods for identifying the radical-derived product, 3-hydroxymethylcyclohexene (**21**), and cation-derived product, 3-cycloheptenol (**22**). Our results indicate that recognition of the norcarene oxidation products is critically important for the analyses.

Products from enzyme-catalyzed oxidations of norcarane are formed in small amounts. Typically, the products were quantified by GC and identified by GC-mass spectrometry using single ion monitoring (SIM) with three to five ion channels monitored. In regard to the probe rearranged products, radical-derived alcohol **21** and cation-derived alcohol **22**, SIM identification is not robust because the mass spectra of many closely eluting products are similar. The radical-derived product **21** presents an especially serious problem because its mass spectrum is dominated by two large fragment ions that are found in most other mass spectra, and it elutes on a low polarity GC column with a retention time similar to those of products **10**, **13**, **16**, and **A**. These other products, all of which are formed in oxidations of norcarenes,¹³ contain fragment ions in their mass spectra that match the major ions in the mass spectrum of **21**.

Figure 2 shows examples of mass spectra from reaction mixtures and from samples that illustrate the problem in compound identification. In the mass spectral analysis of the product mixtures, "SCAN" mode with all ion channels monitored could not be employed for minor products (100 pmol or smaller yields) because the background signals from materials ascribed to the enzyme mixtures were too great. SIM (selective ion monitoring) mode effectively suppressed the background signals, but SIM mode with only a few ion channels cannot provide an unambiguous identification of the products. SIM mode analysis with many channels open gave the results in Figure 2, which are typical. The top two spectra in Figure 2 are SIM mode mass spectra from oxidation reactions of norcarane catalyzed by two enzymes, where the data collection is at the appropriate elution time for radical-derived product **21**. These two mass spectra look similar, but they do not resemble the mass spectrum of any compound that has a similar retention time, i.e. compounds **21**, **16**, or **A**.

The mass spectra of **21**, **16**, and **A** collected in SIM mode using the same channels as for the enzyme product mixtures are shown in the three bottom panels in Figure 2. From the mass channels 94, 95, 109, and 110, one concludes that a mixture of compounds was present in the enzyme products. The mixture might contain comparable amounts of **21**, **16** and **A**, but radical-derived product **21** clearly cannot be the only component because the mass spectrum of **21** contains small amounts of fragments at $m/z = 67$, 68, and 95 and effectively zero intensities at $m/z = 109$ and 110.

Standard methods for GC quantification of yields also were not robust for some of the minor norcarane oxidation products because complete GC separation of some of the products was not possible, and this applied to radical-derived product **21**. Authentic samples and a mixture containing radical-derived product 3-hydroxymethylcyclohexene (**21**), cyclohepta-3,5-dienol (**16**) and *syn*-2-norcaranol (**17**) in an approximate 1:1:50 ratio illustrate the difficulty (Table 2). On a low polarity 5% phenyl silicone column (DB-5), the elution times for products **21** and

16 were indistinguishable when the GC oven temperature was at 70 °C or greater. At lower oven temperatures, the relative retention time for product **21** increased more than those for **17** and **18**. Thus, **21** could be separated from **16** at 50 or 60 °C oven temperature, but it now overlapped with product **17** at those temperatures. Because product **17** was the major product formed in enzyme-catalyzed oxidations of norcarane, its signals would overwhelm those from a small amount of **21** if the analyses were conducted at low GC oven temperatures, thus precluding “low temperature” quantification for **21**. At higher column temperatures, quantification of **21** remains difficult because **21** and **16** co-elute and **16** was shown to be a major product from oxidations of 3-norcarene by P450 and sMMO enzymes.¹³

The differential retention time versus temperature behavior found for the radical-derived product **21** and other alcohols with similar GC elution times is interesting. It was also observed for a mixture containing product **21**, *syn*-bicyclo[4.1.0]hept-4-en-3-ol (**10**), and *syn*-2-norcaranol (**17**) in a ca. 1:2:200 mixture (**21**:**10**:**17**) (Figure 3). At an oven temperature of 70 °C, the peak for radical product **21** was separated from that of **10** but was a shoulder on the peak from **17**. At higher oven temperatures, product **21** separated from **17** but overlapped with product **10**. As a result of the variable relative retention time behavior, the radical derived product **21** can co-elute with different products as a function of column oven temperature, but we could not separate it from all other products under any circumstances. In order to obtain accurate yields of this product, one needs to determine the yield of the mixture of co-eluted products and the percentage of product **21** in the mixture.

By using the mass spectra available from authentic samples, we could identify many of the products in the mixtures formed by enzyme-catalyzed oxidations of norcarane. Figure 4 shows an example of a GC trace where peak areas were obtained via flame ionization detection (FID). Peaks associated with identifiable compounds are labeled with the appropriate compound numbers. We emphasize that identification of compounds was only possible with mass spectral analysis, which was accomplished with a small bore capillary DB-5 column, and we note that some peaks arise from co-elution of multiple compounds.

Product yields from norcarane oxidations

The yields of oxygenated products from enzyme-catalyzed oxidations of norcarane were determined by GC and GC-mass spectral analysis of the product mixtures after concentration. The results are listed in Table 3. We identified 18 oxygenated products for which authentic samples were available in addition to the two norcarenes produced in the desaturase reactions. One additional product detected in the norcarane oxidations had the same GC retention time and mass spectrum as an unknown product observed in the norcarene oxidations; that product is labeled **A** in Table 3 and in the accompanying paper.¹³ Even with the identification of more than 20 oxidation products from norcarane, the product analyses were not complete. The presence of other, unknown oxidation products was suggested by the complexity of the GC traces, such as that shown in Figure 4.

The total yields in nmols of oxygen-containing oxidation products for the P450 enzymes^{4,5} and sMMO⁵ are similar to those reported for the same and other enzymes in comparable studies when one adjusts for the amounts of enzymes used. The major oxygenated products are 2-norcaranols **17** and **18**, as previously reported, and as expected based on the reduced bond dissociation energy (BDE) of approximately 3 kcal/mol for a C-H bond adjacent to a cyclopropyl group.⁴⁶ One unique aspect of our results is that large amounts of 2-norcarene and 3-norcarene were found, comprising 10% to 47% of the oxidation products, and these products were not previously noted. The identification of secondary oxidation products from oxidations of the norcarenes is also novel; peaks for those products were previously observed in GC analyses, but the compounds were not identified.

The mechanistic probe aspects of norcarane arise because formation of a radical or a cation at C2 result predominantly in different fragmentation pathways^{18,47} that are expected to give, ultimately, alcohol **21** from a radical and alcohol **22** from a cation (Scheme 1). The detection of these products provides qualitative support for formation of the intermediates, and, because the rate constant for rearrangement of a cyclopropylcarbinyl radical can be estimated accurately, the percentage of alcohol **21** formed can be used to estimate the lifetime of a radical transient.

Unfortunately, the amounts of product **21** appear to have been overestimated in previous studies. In Table 3, we listed the amounts of products that elute in mixtures that might contain alcohol **21**, but inspection of the mass spectra of those peaks shows that **21** was not the major component in the mixtures as illustrated in Figure 2. From these observations, we conclude that product **21** cannot be 50% of any product mixture and might be 10% or less. Using that range, the yields of the mixtures that might contain radical-derived product **21**, and a rate constant for radical ring opening of ca. $2 \times 10^8 \text{ s}^{-1}$,^{2,48} we can set a limit for the lifetime of a putative radical intermediate at less than 25 picoseconds (ps) and possibly as small at 0.5 ps. The lower limit is too short a lifetime for a true radical intermediate, and it is similar to the values found in enzyme-catalyzed reactions of several hypersensitive radical probes.^{12,24–26,31,35–37}

Previous studies of enzyme-catalyzed oxidations of norcarane concluded that relatively large amounts of radical-derived product **21** were formed, and we noted the conundrum regarding “radical” lifetimes estimated from those results and studies using “faster” probe substrates.

¹¹ The present work appears to resolve the conflicting conclusions. The amounts of product **21** reported in earlier studies probably were in error due to incomplete characterization of the minor oxidation products that overlap with **21** in GC analyses.

Another possible source of error in earlier works involves the purity of the norcarane samples employed. We found that distilled samples of norcarane were contaminated with small amounts of 2-norcarene, which we removed by treatment of the distilled sample with mCPBA followed by preparative GC collection. The introduction of impurities of norcarenes in the norcarane sample at the outset of the study would result in even greater yields of products that interfere with accurate quantification of radical product **21**, and the present work demonstrates that very small amounts of norcarenes can give measurable amounts of “secondary” oxidation products. The extent of complications in earlier probe studies from contaminated norcarane cannot be evaluated because the purity of the substrate was not reported, and probably not determined, at the level of parts per thousand contaminants. It is important that any future study employing norcarane as a probe should demonstrate by high performance GC that the norcarane sample is not contaminated with norcarenes.

The desaturase reactions catalyzed by the iron-containing enzymes complicate studies with norcarane, leading to formation of considerable amounts of norcarenes that are subsequently oxidized to products that interfere with the identification and quantification of minor products from norcarane oxidations. The efficiency of the norcarane desaturase reactions is surprising, and one should expect that any mechanistic probe with a reactive CH functionality adjacent to the readily oxidized position will react readily in a desaturase reaction. Thus, any such probe might give relatively large amounts of secondary oxidation products that complicate mechanistic studies. Included are most conceivable probes, of course, but in the subset of probe substrates containing a methyl group adjacent to a cyclopropyl ring, the methyl group should be oxidized with minimal desaturase activity due to the large increase in strain energy that would result from an *sp*²-hybridized carbon atom in a cyclopropyl ring. When the probe is capable of reacting in a desaturase reaction, care should be taken to identify as many products

as possible, including secondary oxidation products from the “desaturated”, or dehydrogenated, probe to avoid confusion about the identities and yields of the products.

Experimental Section

Norcarane (1) was prepared as described to the point of isolation of the distilled product,⁴⁹ which was ca. 99% pure. The mixture was treated with mCPBA to oxidize a trace of olefinic materials and purified by preparative GC (1/4 inch × 8 ft column, 10% SE-52 on 60/80 Chromosorb W). The isolated sample was shown to be >99.96% homogeneous by analytical GC.

The preparations of samples of 2-norcarene (**2**), 3-norcarene (**3**) and oxygenated products **4–16** were reported in the accompanying paper.¹³ Literature methods were employed for the preparation of products **17–22**.^{5,14–19}

Enzyme-catalyzed oxidation reactions were conducted as described in the accompanying paper¹³ using norcarane (1.5 mmol) as the substrate. Following the reaction, an internal standard of 1-phenyl-1-propanol was added to the product solution in CH₂Cl₂, and the mixture was analyzed by GC (0.32 mm × 30 m, DB-5 column) to determine yields of 2-norcarene and 3-norcarene. The reaction mixture was then concentrated under nitrogen, and the concentrated sample was analyzed by GC and GC-mass spectrometry on DB-5 columns. The results given in Table 3 are averages of 2–4 reactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NIH grants GM-48722 (M.N.), GM-32134 (S.J.L.), CA-16954 (P.F.H.), and DK-10339 (M.J.C.).

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Figure 1.

Portions of GC traces (40 °C, DB-5 column) of norcarane before reactions and after reactions with three enzymes. The expansions are 100× the amplitude of the lower traces. Under the GC conditions used for these analyses, 3-norcarene and 2-norcarene elute with retention times of 10.1 and 10.3 minutes, respectively. CYP = cytochrome P450, ToMO = toluene monooxygenase.

Figure 2. SIM mode GC spectra obtained by monitoring 12 ion channels. The CYP Δ 2B4 and sMMO spectra were measured for the product mixture at the GC elution time where products **21** and **16** and unknown **A** co-elute. In each spectrum, the $m/z = 95$ signal is marked with an arrow for calibration.

Figure 3.

GC traces (DB-5 column) for elution of a mixture of **21**, **10**, and **17** in a ca. 1:2:200 ratio, respectively, at varying GC column oven temperatures. The retention times shown are for the 70 °C run, and other traces were adjusted such that the peaks for **10** and **17** overlapped for each trace. The compound numbers show the positions of the maxima for each compound.

Figure 4. Portions of GC traces (30 m $\times$ 0.32 mm DB-5 column) of products from oxidation of norcarane catalyzed by CYP Δ 2E1 T303A. Identifiable compounds are indicated with compound numbers. Product A is an unknown compound observed in the oxidations of norcarenes catalyzed by iron-containing enzymes.

Scheme 1.

Chart 1.

Table 1Yields of Norcarenes from Enzyme-Catalyzed Oxidations of Norcarane.^a

Enzyme (nmol) ^{bc}	2-norcarene	3-norcarene	other products ^d	% norcarenes ^e
CYPΔ2E1 (1.0)	5.3	2.2	33.3	19
CYPΔ2E1 T303A (0.5)	1.5	0.5	14.5	12
CYP2B1 (0.5)	9.9	5.8	94.5	14
CYPΔ2B4 (0.5)	24.7	1.7	31.4	46
CYPΔ2B4 (1.0)	17.2	0.8	72.1	20
sMMO (20)	9.0	1.3	27.1	27
ToMO (1.0)	0.7	0.6	11.3	10
PH (1.0)	1.5	2.1	4.0	47

^a Yields of products in nmols.^b CYP = cytochrome P450, sMMO = soluble methane monooxygenases from *M. capsulatus* (Bath); ToMO = toluene monooxygenase from *P. stutzeri* OX1, PH = phenol hydroxylase from *P. stutzeri* OX1.^c Nanomoles of enzyme used.^d Yields of all other oxidation products.^e Percentage of oxidation products that are norcarenes.

Table 2Observed Retention Times for GC Elutions as a Function of Column Temperature.^a

Temp (°C)	21	16	17
100	7.0	7.0	7.1
90	8.4	8.4	8.6
80	10.7	10.7	10.9
70	14.4	14.4	14.6
60	20.7	20.5	20.8
50	31.6	30.8	31.3

^a Retention times in minutes for elution on a 5% phenyl-silicone bonded-phase column. Compound **21** is 3-hydroxymethylcyclohexene, compound **16** is cyclohepta-3,5-dienol, and compound **17** is *syn*-2-norcaranol.

Table 3
Yields of Oxygenated Products from Oxidations of Norcarane Catalyzed by Iron-Containing Enzymes.^a

Enzyme (nmol) ^{b,c}	21+16 +A	22+12	17	18+19 +11	4	20	5	7	10+13	14	15	8	9
CYP2A2E1 (1.0)	0.05	0.15	7.5	24.3	0.04	0.53	0.26	0.09	0.18	0.09	0.10	d	d
CYP2A2E1 T303A (0.5)	0.08	0.15	5.4	7.6	0.12	0.38	0.43	0.08	0.09	0.09	0.10	d	d
CYP2B1 (1.0)	0.09	0.15	54.2	33.2	1.14	4.25	0.69	0.12	0.26	0.18	0.20	d	d
CYP2A2B4 (0.5)	0.05	0.06	21.4	7.6	0.26	1.30	0.48	0.04	0.16	d	0.02	d	d
CYP2A2B4 (1.0)	0.10	0.12	49.9	17.3	0.46	3.03	0.70	0.10	0.41	d	0.02	d	d
sMMO (20)	0.07	0.29	14.4	9.0	0.77	1.80	0.50	d	0.06	0.20	0.05	d	d
ToMO (1.0)	0.11	0.06	5.5	5.0	0.04	0.14	0.31	d	0.03	0.03	0.02	0.02	0.02
PH (1.0)	0.03	0.05	2.7	0.55	0.04	0.13	0.25	0.07	0.03	0.03	0.04	0.02	0.12

^a Average product yields in nmol for duplicate reactions.

^b CYP = cytochrome P450, sMMO = soluble methane monooxygenase, ToMO = toluene monooxygenase, PH = phenol hydroxylase.

^c Nanomoles of enzyme used in the oxidation reaction.

^d Not detected; yield less than 0.02 nmol.