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The Syntheses of 1R- and 1S-5-Methylenylcamphor and Their Epoxidation by Cytochrome P-450-CAM#

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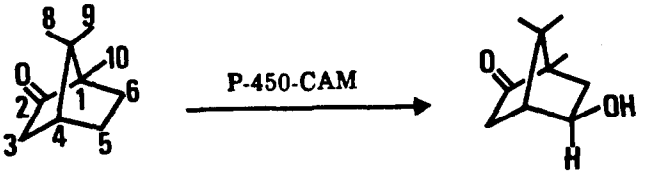
ABSTRACT- The syntheses of 1R- and 1S-5-methylenylcamphor, camphor analogues in which the two methylene hydrogens at C-5 have been replaced with an exocyclic methylene group, are described. The stereospecific epoxidations of both olefins by *Pseudomonas putida* cytochrome P-450-CAM to give the *exo*-epoxides are reported. The turnover rates for the epoxidation of the 1R and 1S olefins are ten- and three-fold slower, respectively, than the rates of hydroxylation of 1R- and 1S-camphor.

INTRODUCTION

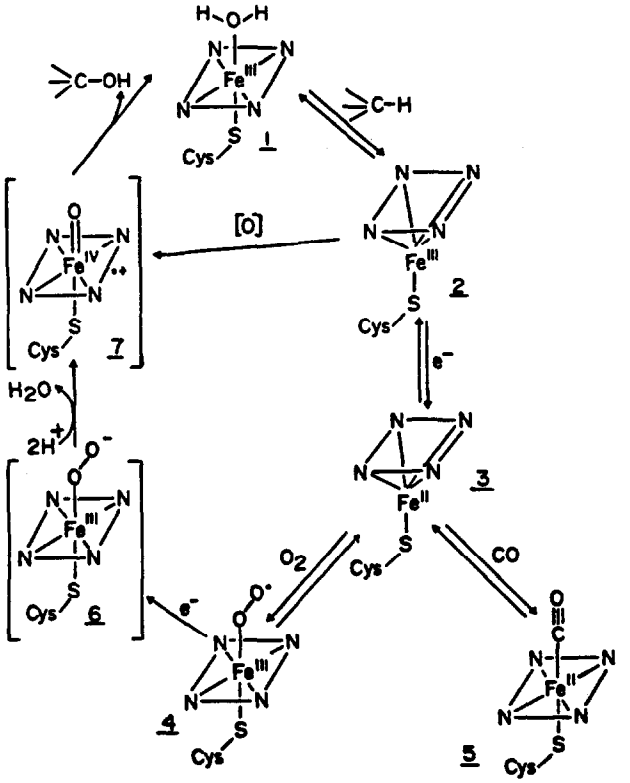
The cytochromes P-450 are a family of heme *b*-containing mono-oxygenases that catalyze the activation of molecular oxygen for incorporation into organic substrates (1-7). Much of our present knowledge of the structure and mechanism of P-450 comes from investigations of bacterial P-450-CAM, a soluble enzyme isolated from *Pseudomonas putida* grown on camphor. P-450-CAM catalyzes the regio- and stereo-specific hydroxylation of 1R-(+)-camphor to form the 5-alcohol (eq 1) as the only product. P-450-CAM is the only P-450 for which a crystal structure is available (8) and therefore serves as the model for the active sites of mammalian P-450's, all of which are membrane bound. Although 1-R-camphor is clearly the physiological substrate, P-450-CAM is also able to hydroxylate camphor analogues such as norcamphor (9), thiocamphor (10), adamantanone (11), adamantane (11), camphane (10), 5-*exo*-bromocamphor (12), 5-*endo*-hydroxycamphor (5), pericyclocamphor (5), and 5,5-difluorocamphor (13) as well as 1S-camphor (14) and to epoxidize 5,6-dehydrocamphor (15). Recently, it also has been shown to hydroxylate the benzylic positions of 4-methyl-1-tetralone (16) and ethylbenzene (17), to epoxidize styrene and ß-methylstyrene (18,19) and to hydroxylate nicotine (20).

Dedicated to Professor Carl Djerassi on the occasion of his seventieth birthday.



(1)

The P-450 reaction cycle involves four well-characterized intermediates (**1** - **4**, Scheme 1) (1-3). The low-spin ferric resting state (**1**) is converted to the high-spin ferric enzyme (**2**) upon substrate binding. Reduction then leads to the high-spin ferrous derivative (**3**) which can bind O_2 to yield the oxy-ferrous state (**4**) or CO to form the ferrous-CO adduct (**5**). Addition of the second electron has been proposed to yield a ferric peroxide complex (**6**) followed by rapid cleavage of the peroxide O-O bond upon proton addition to give water and an oxo-ferryl porphyrin radical intermediate (**7**). Oxygen transfer then produces the organic product and regenerates the ferric enzyme (**1**). For P-450-CAM, the two required electrons are derived from NADH via a pair of electron transfer proteins, the flavoprotein putidaredoxin reductase (PdR) and the iron-sulfur protein putidaredoxin (Pd). No intermediates have yet been detected between **4** and **1** because the second electron transfer is rate limiting (21).

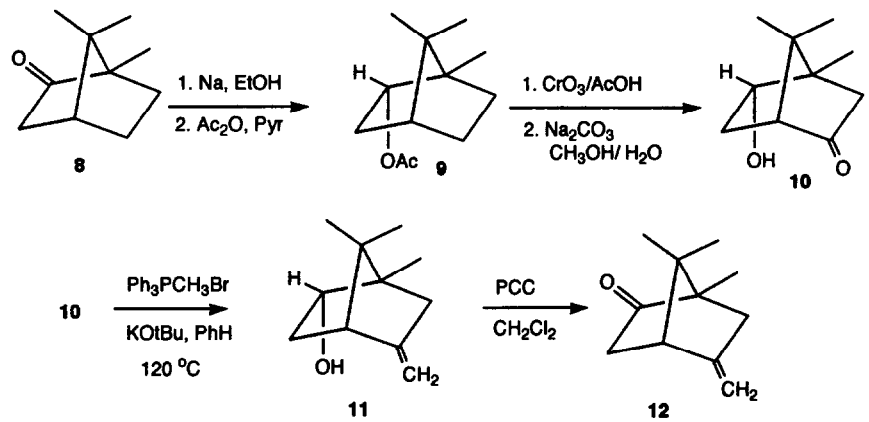


Scheme 1, **1**, **2**, and **7** are neutral; the charge on **3** - **5** is -1, on **6** is -2

A plausible way to build up observable concentrations of the reactive intermediates involved in dioxygen activation would be to slow down enzyme turnover through the use of carefully constructed camphor analogues in which the site of hydroxylation (C-5) is blocked or modified. We describe herein the syntheses of both enantiomers of such a camphor analogue in which C-5 has been modified to contain an exocyclic methylene unit. The reactivity of these substrates with the fully reconstituted NADH/PdR/Pd/P-450-CAM system has been investigated and compared to that of both 1R- and 1S-camphor. Our efforts to probe the mechanism of action of P-450-CAM using slow substrates compliments ongoing studies by Sligar (22) and Ishimura (23) using active site mutants of the enzyme.

RESULTS

Syntheses of 1R- and 1S-5-Methylenylcamphor (**12**) - The syntheses of both enantiomers of 5-methylenylcamphor are described in Scheme 2. The preparation of the 1R olefin started from 1R-camphor which was converted to the ketoalcohol **10** by the procedure of Mälkönen (24). In the 1S series, 1S-bornyl acetate was converted to **10** by the method of Money (25). With each enantiomer of **10**, treatment with the anion of methyl triphenylphosphonium bromide resulted in formation of the exocyclic olefin **11**. Standard pyridinium chlorochromate oxidation of **11** gave the desired product, **12**.



Scheme 2, only the 1R enantiomer is shown

Binding of Substrates to Cytochrome P-450-CAM - The substrate-free state of cytochrome P-450-CAM consists of a low-spin six-coordinate, aquo-ligated ferric heme iron (**1**). Addition of each substrate leads to a shift in the Soret peak of the UV-visible absorption spectrum from 417 to 391 nm as is typical for conversion to the high-spin ferric enzyme (**2**). The apparent dissociation constants (K_d) for the substrate complexes are listed in Table 1. For the complexes with 1R-camphor and 1R-5-methylenylcamphor, the Soret peak at 391 nm is highly symmetrical as expected for complete

Table 1. Binding Dissociation Constants, NADH Oxidation Rates and Reaction Stoichiometries for the 1R and 1S Enantiomers of Camphor and 5-Methylenylcamphor with Cytochrome P-450-CAM.*

	Camphor (8)		5-Methylenylcamphor (12)	
	1R	1S	1R	1S
Dissociation Constants, μM	1.6 ± 0.3	2.6 ± 0.3	1.2 ± 0.1	5.8 ± 0.1
NADH Oxidation Rate§	2075 ± 77†	1450 ± 13	212 ± 15	446 ± 10
Stoichiometries				
NADH Oxidized, nmoles	281 ± 6	279 ± 5	261 ± 12	261 ± 6
Product Formed, nmoles	272 ± 6	249 ± 11	135 ± 15	239 ± 10
Percent Uncoupling	3.3 ± 0.5	11 ± 4	48 ± 5	9 ± 2

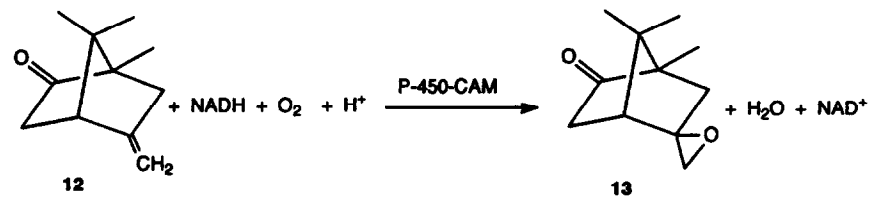
* The dissociation constants were determined at 4°C; the rates and stoichiometries were measured at room temperature. See the Experimental Section for additional information.

§ Units: nmole NADH oxidized/nmole cytochrome P-450-CAM/min.

† Lit., 2040 Min^{-1}; Gunsalus and co-workers (26).

conversion to the high-spin state (**2**). However, the spectra of both 1S-substrate complexes have noticeable shoulders at 417 nm indicative of incomplete conversion to the high-spin state. The K_d values for the two 1R substrates are nearly identical while the values for the 1S substrates are somewhat larger.

Epoxidation of 5-Methylenylcamphor and Hydroxylation of 1S-Camphor - Reaction of 1R- and 1S-5-methylenylcamphor (**12**) with O_2 in the presence of the NADH/PdR/Pd/P-450-CAM system leads to formation of predominantly (>90%) one product with a molecular weight (M^+ = 180) consistent with the addition of one oxygen atom (Scheme 3). Identical mass spectra are obtained for the product derived from each enantiomer (Figure 1). In the 1S case, sufficient product was isolated for the determination of its structure through an extensive NMR study, including high-field proton (Figure 2) and carbon spectra as well as several two dimensional experiments. The presence of a minor impurity is indicated by the additional resonances in the methyl region (0.8 - 1.2 ppm) of the ^1H spectrum. Because the concentration of this impurity was less than 5%, its identity was not established.



12 **13**

Scheme 3

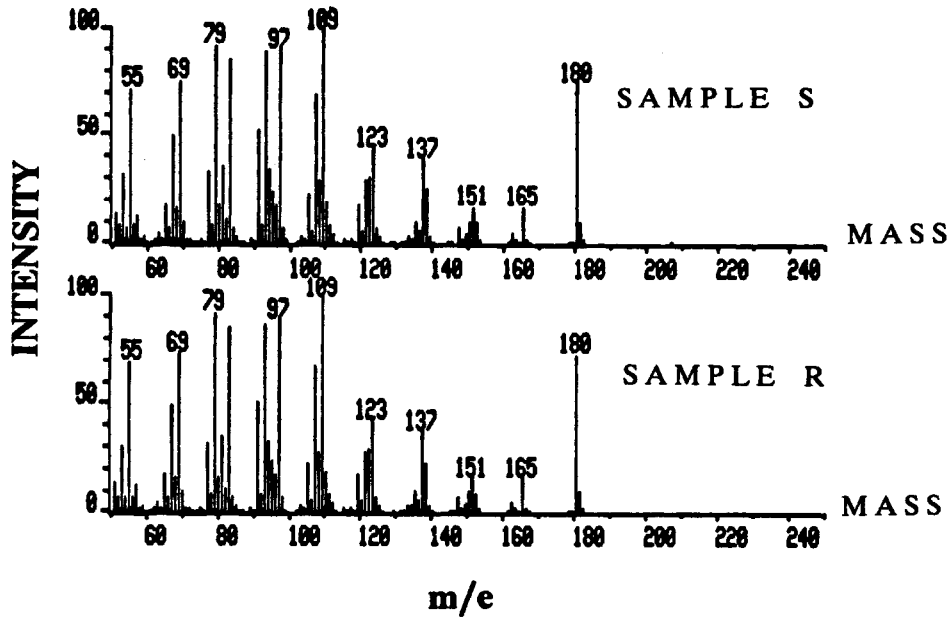


Figure 1. Electron impact mass spectra of the major products produced by oxygenation of 1R- (Sample R, bottom) and 1S- (Sample S, top) 5-methylenylcamphor (**12**) by cytochrome P-450-CAM.

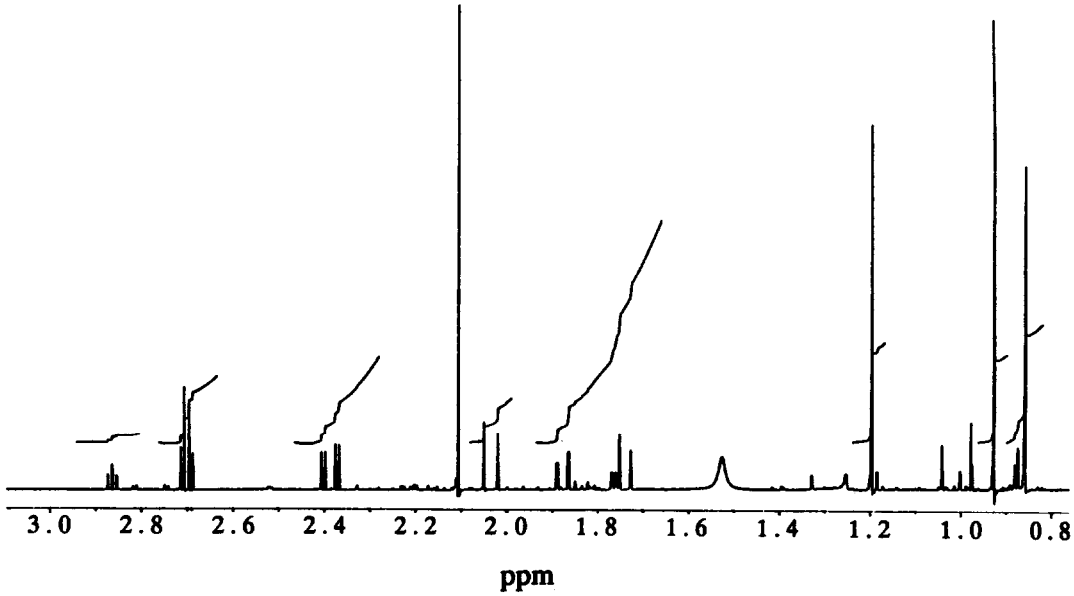


Figure 2. The 600 MHz proton NMR spectrum of the 5,11-epoxide produced by oxygenation of 1S-5-methylenylcamphor (**12**) by cytochrome P-450-CAM in d_6-acetone.

The NMR data obtained for the 1S product demonstrate that it is the 5,11-epoxide with the epoxide oxygen having the *exo* orientation (13). The assignments of the ^1H NMR spectrum are listed in Table 2. The downfield shift in the position of the resonance for the 9-methyl protons from 0.93 in camphor to 1.19 ppm provides the first evidence that the 5,11-epoxide has the *exo* orientation. A similar shift to 1.23 is seen for the resonance of the 9-methyl protons in 5-hydroxycamphor when the hydroxyl oxygen has the *exo* orientation (13); the resonance for the 9-methyl proton in 5-*endo*-hydroxycamphor is at 0.99 ppm (13). Extensive COSY and NOESY experiments provide further evidence for the *exo* orientation of the epoxide. In particular, the NOESY spectrum showed an interaction between the two epoxide protons in **13** and the respective *endo* protons on carbons 3 and 6 as would be expected only if the epoxide oxygen has the *exo* orientation.

Table 2. Proton NMR Chemical Shifts and Observed Coupling Constants for the 1S-Epoxide.

Proton(s)	Average Chemical Shift (ppm)	Coupling Constant (Hz)
epoxide	2.7	4.7 (geminal)
3-*exo*	2.4	18.4 ($J_{exo\text{-}endo}$) 5.1 ($J_{3\text{-}4}$)
3-*endo*	2.02	18.3 ($J_{exo\text{-}endo}$)
6-*exo*	1.88	14.8 ($J_{exo\text{-}endo}$)
4	1.76	5.1 ($J_{3\text{-}4}$)
6-*endo*	1.74	14.8 ($J_{exo\text{-}endo}$)
9-methyl	1.19	
10-methyl	0.93	
8-methyl	0.85	

Reaction of 1S-camphor with O_2 in the presence of the NADH/PdR/Pd/P-450-CAM system leads to formation of 1S-5-hydroxycamphor and a small amount (~2.2%) of the 2,5-diketone. The gas chromatographic retention time and mass spectrum of the 1S alcohol exactly match those of 1R-5-hydroxycamphor (data not shown). As previously reported by Gunsalus and co-workers (14), the NADH oxidation rate for 1S-camphor is about 70% that of 1R-camphor.

NADH Consumption and Product Quantification - The rates of NADH oxidation in the metabolism of 1R- and 1S-camphor and of 1R- and 1S-5-methylenylcamphor and the stoichiometries of NADH consumption and organic product formation are listed in Table 1. The rates of NADH oxidation reflect the consumption of electrons by the system under conditions required to give a maximum turnover number. Under somewhat different conditions designed to facilitate quantitation of both the organic product and NADH uptake (ten-fold higher P-450-CAM and two and one half-fold higher NADH concentrations), it is also possible to measure the extent to which electron and oxygen transfer are coupled. Because these reactions were limited by O_2 concentration, the amount of NADH consumed

was nearly the same with all substrates. The amount of organic product formed varied considerably and the extent of uncoupling observed with each substrate is also listed in Table 1.

DISCUSSION

1R- and 1S-5-methylenylcamphor (**12**), cytochrome P-450-CAM substrate analogues having the normal site of hydroxylation replaced by an exocyclic olefin, have been synthesized from 1R-camphor (**8**) and 1S-bornyl acetate (**9**), respectively (Scheme 2). The key synthetic steps are the remote chromic acid oxygenation of C-5 of **8** and the subsequent Wittig reaction carried out on **11**. These two substrate analogues as well as 1S-camphor all bind tightly to P-450-CAM, although the two 1S substrates have two- to four-fold diminished binding affinity. The 1R olefin, like 1R-camphor, converts the low-spin resting state of the enzyme (**1**) to the high-spin state (**2**). The UV-visible absorption spectra of the adducts of the ferric enzyme with the 1S olefin and 1S-camphor, however, are not completely shifted to the spectrum of the high-spin form. The spin state conversion that occurs upon substrate binding to ferric P-450-CAM results from the displacement of the bound water sixth ligand of the low-spin state yielding a five-coordinate high-spin derivative. The lack of complete conversion to the high-spin state upon addition of the two 1S substrates indicates that the binding of the "wrong" enantiomers is non-optimal and does not lead to complete displacement of water from the substrate binding site (27). Similar observations have been reported by Sligar and co-workers for the binding of camphor analogues such as 1R-norcamphor to P-450-CAM and of 1R-camphor to mutants of P-450-CAM with amino acid modifications at the substrate binding site (28).

As shown in Scheme 3, the reaction of P-450-CAM and the olefinic camphor analogues with O_2 leads to formation of the 5,11-epoxide product having the *exo* orientation for the epoxide oxygen (**13**). Product identification is based on the combination of mass spectral and NMR evidence presented above. The stereospecific production of only one epoxide product contrasts with the formation of two epoxides from each of the styrenes studied by Loew, Ortiz de Montellano and their co-workers (18,19). The stereospecificity observed in the present case and the tight binding affinity of the olefin substrates for the ferric enzyme suggest that the substrate binds in a single predominant position that is poised for oxygen atom addition to the *exo* face of the substrate. *Exo* oxygen addition was observed in the epoxidation of dehydrocamphor (15) and in the hydroxylation of 5,5-difluorocamphor to the 9-alcohol (13). Sligar and co-workers have also observed that while either *exo* or *endo* hydrogen atom at C-5 can be abstracted in the hydroxylation of camphor, oxygen addition always occurs from the *exo* face (29).

The rates at which electrons are accepted by P-450-CAM during turnover, the NADH oxidation rates, for the 1R and 1S olefins are ten- and five-fold lower, respectively, than for the physiological substrate 1R-camphor (Table 1; the rate for the 1S olefin is three-fold slower than for 1S-camphor). Curiously, the turnover rate for epoxidation of the 1S camphor olefin is twice as fast as that of the 1R olefin! The rate of NADH oxidation during the epoxidation of dehydrocamphor and hydroxylation of camphor have been found to be identical (15) which shows that there is no inherent slowdown during epoxidation. We have previously reported a three-fold decrease in the turnover number for the hydroxylation of 1R-5,5-difluorocamphor (13) at C-9 by P-450-CAM (30). The epoxidation of styrene

and ß-methylstyrene by P-450-CAM has recently been reported to be five- to nine-fold slower than during hydroxylation of camphor (19,32), although the reaction is substantially uncoupled (see below).

The slowdown in the turnover rate for epoxidation relative to hydroxylation could be indicative of a change in the rate limiting step of the cycle (Scheme 1) from the second electron addition to a later step in the process. This proposal is based on the idea that there is no obvious reason why a particular tight-binding substrate should, by itself, slow down the intrinsic rate at which the second electron is transferred. However, if the subsequent rate of oxygen transfer is slowed down by the presence of a "poor" substrate, then it could present a new bottleneck in the cycle that would slow down the overall rate of electron transfer, i.e., the NADH oxidation rate. A "poor" substrate could be one having the site of hydroxylation blocked with fluorine atoms as in 5,5-difluorocamphor, or a camphor analogue that does not fit as precisely into the substrate binding pocket, perhaps such as the camphor olefins.

The final issue of concern is the coupling of electron and oxygen transfer. Hydroxylation of 1R-camphor by P-450-CAM is almost completely coupled (Table 1) (9). A small amount of uncoupling is seen during hydroxylation of 1S-camphor (~11%) and epoxidation of 1S-methylenylcamphor (~9%). The latter reaction, therefore, runs at about one-fifth the rate at which 1R-camphor is hydroxylated and yet is almost completely coupled. On the other hand, substantial uncoupling (~48%) is seen during the epoxidation of the 1R olefin. The quantification of NADH oxidized and of product formed has been done under conditions where the limiting reagent is O_2 which has a concentration of about 270 μM in aqueous buffer at room temperature. A 1:1 ratio of NADH to O_2 consumption is expected for a tightly coupled mono-oxygenase reaction as with 1R-camphor. Observation of a 1:1 ratio even where there is substantial uncoupling shows that the uncoupling is still a two electron process such as leakage of H_2O_2, perhaps from **6**. The epoxidation of styrenes by P-450-CAM was over 98% uncoupled via H_2O_2 formation (19). The alternative mode of uncoupling would be a four electron oxidase process where both atoms of dioxygen are reduced to water. Uncoupling of that type would require a 2:1 NADH to O_2 consumption ratio and has been observed during the metabolism of 1R-norcamphor by P-450-CAM (9) and of poor substrates by mammalian P-450 (33).

In summary, we have synthesized both enantiomers of 5-methylenylcamphor (**12**) and have investigated their epoxidation by cytochrome P-450-CAM using the fully reconstituted NADH/PdR/Pd/P-450-CAM system. The hydroxylation of 1S-camphor has also been studied for comparison with the oxygenation of the 1S olefin. The epoxidation reaction of both enantiomers of **12** is stereospecific with the 5,11 epoxide having the *exo* orientation of the epoxide oxygen as the product. The rates of epoxidation of the 1R and 1S olefins by P-450-CAM are considerably slower than is the rate of hydroxylation of camphor. Oxygen and electron transfer are almost completely coupled with the 1S olefin substrate while the 1R olefin is about 50% uncoupled, apparently via H_2O_2 leakage. The 1S substrate is therefore a reasonable target for use in attempts to look for intermediates between **4** and **1** because it is a slow substrate and yet is almost fully coupled.

EXPERIMENTAL

Materials and General Procedures- Reagents and chemicals were obtained from Aldrich, Sigma and Fisher. An argon atmosphere was employed for all non-aqueous reactions. Anhydrous diethyl ether was obtained by distillation from sodium benzophenone ketyl; Ethyl acetate, methylene chloride, benzene and hexanes were purchased in reagent grade quality and stored over molecular sieves. Thin layer chromatography (TLC) was routinely used to monitor reactions and check for sample homogeneity. Plastic plates precoated 0.25 mm thick with E. Merck silica gel 60 F254 (Brinkmann) were utilized. Visualization was accomplished by UV light or by charring after treatment with 5% phosphomolybdic acid in ethanol. Preparative scale chromatography (95/5 hexanes/ethyl acetate) was performed using E. Merck silica gel (230-240 mesh) from EM Science. Melting points (uncorrected) were determined on a Thomas-Hoover glass capillary melting point apparatus.

Instrumentation - Varian-Cary 210 or 219 spectrophotometers were used for UV-visible absorption spectral analysis. Infrared spectra were recorded on a Perkin Elmer 1600 series FT-IR spectrometer using sodium chloride plates. Electron impact mass spectra and GC-MS data were obtained on a Finnegan 4521 GC/MS/Data system. Molecular masses are given in atomic mass units (amu) followed by percent relative intensity to the most abundant ion. High resolution mass spectrometry (HRMS) was carried out on a VG Instruments 70SQ spectrometer. A Hewlett-Packard Model 5890 gas chromatograph equipped with a flame ionization detector, a Hewlett-Packard 3390A reporting integrator, and a stabilwax column (30 meter, 0.25 mm ID) programmed at 70°C followed by a temperature increase to 225°C at 10°C/min was used for product analysis. Routine NMR spectra were obtained on Bruker AM-300 or AM-500 instruments using $CDCl_3$ as the solvent. Chemical shifts are reported in parts per million downfield from tetramethylsilane (TMS, 0.00) or deuteriochloroform (7.24). Analysis of the 1S-epoxide was carried out in d_6-acetone using a Bruker AMX-600 spectrometer operating at a ^1H frequency of 600.13 MHz and a carbon frequency of 150.91 MHz. NMR experiments were conducted at 303 ± 0.1K. For the ^1H 1-D experiment, a 32K complex data table was collected and zero filled to 64K prior to transformation yielding a 0.09 Hz digital resolution. A 7 microsecond pulse (60°) was used for excitation and a 3 second relaxation delay minimized the saturation of the signals. The data was weighted with a combination of an exponential (-1.5 Hz) and a Gaussian weighting (0.15) function which narrowed the lines to nearly their theoretical limit.

Syntheses of 1R- and 1S-5-Methylenylcamphor (12) - The synthetic sequence followed for the preparation of 1R- and 1S-5-methylenylcamphor (**12**) is displayed in Scheme 2. In the 1R case, the starting material was 1R-camphor (**8**) while in the 1S series, the synthesis began from 1S-bornyl acetate (**9**). In each case, 5-oxoborneol (**10**) was synthesized following published procedures (24,25).

5-Methylenylborneol (**11**) was prepared by a Wittig reaction (34). To a suspension of methyl triphenylphosphonium bromide (2.25 g, 7.00 mmol) in benzene (100 mL) was added potassium *tert*-butoxide (0.80 g, 7.10 mmol) and the mixture heated to reflux for 2 h. Benzene was removed by distillation until the temperature of the remaining slurry reached 100°C. The ketoalcohol **10** (1.00 g, 5.95 mmol) was added and the reaction was heated at 120°C for 2 h, then cooled to room temperature. Hexanes (150 mL) and water (5 mL) were added and the resulting precipitate was filtered and washed

with hexanes (50 mL). The organic phase was dried with $MgSO_4$, filtered, and the volatiles were removed *in vacuo*. Flash chromatography gave a crystalline white solid, 0.56 g (56%): mp 67°C; TLC (80/20 hexanes/ethyl acetate) R_f, 0.70; GC t_R, 10.78 min; ^1H NMR (300 MHz) δ 0.81 (s, 3H), 0.87 (s, 3H), 0.91 (s, 3H), 1.05 (m, 1H), 1.43 (s, 1H), 1.86 (m, 1H), 2.11 (d, 1H), 2.40 (m,1H), 2.53 (m, 1H), 4.08 (m, 1H), 4.59 (s, 1H), 4.79 (s, 1H); IR 2670, 3400, 2990, 1670, 1450, 1060, 890 cm^{-1}.

5-Methylenylcamphor (**12**) was prepared by oxidation of **11** (34). To a suspension of pyridinium chlorochromate (1.55 g, 7.08 mmol) in CH_2Cl_2 (50 mL) was added **11** (0.61 g, 3.93 mmol) in CH_2Cl_2 (15 mL). The resulting mixture became homogeneous and then deposited a black residue. After 2 h, diethyl ether (200 mL) was added and the solvent decanted. The residue was washed with 50 mL of diethyl ether and the combined organic phases were dried over $MgSO_4$, filtered and the solvent removed *in vacuo*. Flash chromatography provided a clear semi-crystalline residue, 0.43 g (83%): TLC (90/10 hexanes/ethyl acetate) R_f, 0.85; GC t_R, 7.34 min; ^1H NMR (300 MHz) δ 0.84 (s, 3H), 0.91 (s, 3H), 0.94 (s, 3H), 1.86 (s, 1H), 1.92 (m, 1H), 2.32 (m, 1H), 2.43 (m, 1H), 2.53 (d, 1H), 4.74 (s, 1H), 4.99 (s, 1H); ^{13}C NMR (125 MHz) δ 8.7, 18.6, 19.0, 37.2, 42.0, 46.4, 52.9, 58.6, 104.8, 151.0, 217.5; IR 2985, 1723, 1645, 1245, 1030 cm^{-1}; MS, *m/z* (relative intensity) 164 (82, M^+), 149 (20), 136 (25), 121 (84), 107 (90), 93 (33), 65 (40), 57 (43). HRMS *m/z* calcd 164.1201, obsd 164.1204.

Protein Purification - Cytochrome P-450-CAM, putidaredoxin (Pd) and putidaredoxin reductase (PdR) were isolated from *Pseudomonas putida* grown on 1R-camphor. P-450-CAM was purified by the method of Peterson and co-workers (35) with minor modifications (31); Pd and PdR were obtained using the procedures of Gunsalus and Wagner (36) with slight alterations (31).

Determination of Dissociation Constants, NADH Oxidation Rates and NADH and Product Stoichiometries - Substrate binding to substrate-free P-450-CAM was quantified as previously described using the Hill equation (14). The overall catalytic activity of P-450-CAM in the presence of the various substrates was determined from the rate of NADH oxidation which was monitored by following the loss of absorbance at 340 nm (ϵ = 6.22 $mM^{-1}cm^{-1}$) as a function of time. The standard assay mixture contained 0.05 μM P-450-CAM, 4.0 μM PdR, 10 μM Pd, 100 mM KCl, 1 mM substrate, 400 μM NADH in 20 mM potassium phosphate buffer, pH 7.40 at 23°C in a 1.0 mL volume. Reactions were initiated by addition of P-450-CAM.

In separate experiments, the quantities of NADH consumed and oxygenated product produced were directly measured. Reaction mixtures were the same as above except as follows: 0.5 μM P-450-CAM, 1 mM NADH. Under these conditions, the extent of reaction was limited by the concentration of O_2. The reaction volume was 1.0 mL and the reaction was initiated by addition of P-450-CAM. NADH consumption was monitored by the change in absorbance at 340 nm as above using a 0.2 cm cuvette. After 15 minutes, a known amount of an internal standard, 1R-3-*endo*-bromocamphor, was added and the products were twice extracted with methylene chloride. The organic extract was concentrated under a slow stream of N_2 and analyzed by gas chromatography. The amount of product was determined from the relative integrated peak area of the internal standard to the product. If electron transfer to P-450-CAM and oxygen transfer to substrate are tightly coupled, there should be a 1:1 stoichiometry of NADH consumed to product formed. The percent uncoupling was calculated based on the difference between NADH consumed and organic product formed.

ACKNOWLEDGMENTS

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