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Radical Intermediates in the Catalytic Oxidation of Hydrocarbons by Bacterial and Human Cytochrome P450 Enzymes†

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Abstract

Cytochromes P450_{cam} and P450_{BM3} oxidize α - and β -thujone into multiple products, including 7-hydroxy- α - (or β -)thujone, 7,8-dehydro- α - (or β -)thujone, 4-hydroxy- α - (or β -)thujone, 2-hydroxy- α - (or β -)thujone, 5-hydroxy-5-isopropyl-2-methyl-2-cyclohexen-1-one, 4,10-dehydrothujone, and carvacrol. Quantitative analysis of the 4-hydroxylated isomers and the ring opened product indicates that the hydroxylation proceeds via a radical mechanism with a radical recombination rate ranging from $0.7 \pm 0.3 \times 10^{10} \text{ s}^{-1}$ to $12.5 \pm 3 \times 10^{10} \text{ s}^{-1}$ for trapping of the carbon radical by the iron-bound hydroxyl radical equivalent. 7-[²H]- α -Thujone has been synthesized and used to amplify C-4 hydroxylation in situations where uninformative C-7 hydroxylation is the dominant reaction. The involvement of a carbon radical intermediate is confirmed by the observation of inversion of stereochemistry of the methyl-substituted C-4 carbon during the hydroxylation. With an L244A mutation that slightly increases the P450_{cam} active site volume, this inversion is observed in up to 40% of the C-4 hydroxylated products. The oxidation of α -thujone by human CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 occurs with up to 80% C-4 methyl inversion, in agreement with a dominant radical hydroxylation mechanism. Three minor desaturation products are produced, at least one of them via a cationic pathway. The cation involved is proposed to form by electron abstraction from a radical intermediate. The absence of a solvent deuterium isotope effect on product distribution in the P450_{cam} reaction precludes a significant role for the P450 ferric hydroperoxide intermediate in substrate hydroxylation. The results indicate that carbon hydroxylation is catalyzed exclusively by a P450 ferryl species via radical intermediates whose detailed properties are substrate- and enzyme-dependent.

The regio- and stereoselective hydroxylation of hydrocarbons by cytochrome P450 enzymes is a chemically demanding but physiologically important reaction. Hydroxylation is required, for example, for the conversion of cholesterol into a panoply of mammalian sterol hormones and of arachidonic acid into vasodilator and vasoconstrictor signaling molecules (1,2). The mechanism of carbon hydroxylation has been thought for three decades to involve hydrogen abstraction by the catalytic ferryl species followed by recombination of the resulting substrate carbon radical with the equivalent of an iron-bound hydroxyl radical (3,4). The ferryl species involved in this reaction is generated by reductive activation of molecular oxygen at the iron

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SUPPORTING INFORMATION AVAILABLE. Tables S1, S2, and S3, Figure S1-S9 show GC-MS of thujone metabolite standards, ¹H NMR and GC-MS of 7[²H]- α -thujone, identification of metabolites formed in the reaction of P450_{cam} with α -thujone, identification of carvacrol and ROP formed in the reaction of P450_{cam} with 7[²H]- α -thujone, ion chromatogram region of 4OH α T, 2OH α T, and 4OH β T formed in the reaction of P450_{cam} L244A mutant with α -thujone, identification of metabolites formed in the reaction of CYP3A4 with α -thujone, ion chromatogram region of 4OH α T and 4OH β T in the reactions of α T with CYP1A2, -2C9, -2C19, -2D6, and -2E1.

center of the prosthetic heme group (5). Although the distribution of electrons in this catalytic ferryl species remains experimentally ambiguous, it is generally formulated as a peroxidase Compound I-like intermediate, with the heme iron in the Fe(IV) state and the second oxidation equivalent stored either as a porphyrin or protein radical (6). The radical rebound mechanism described above is supported by a variety of data, including the large magnitude of the intramolecular isotope effect when the abstracted hydrogen is replaced by a deuterium (3,4, 7), the observation of allylic and skeletal rearrangements during hydroxylation reactions (8-10), and density function computational analyses (6). However, one line of evidence, determination of the carbon radical lifetime by radical clock substrates, has given conflicting results. The first such study employing bicyclo[2.1.0]pentane as the probe suggested a rate of $1.4 \times 10^{10} \text{ s}^{-1}$ for the rate of recombination of the carbon radical and iron bound hydroxyl radical (11,12). A rate of this order has also been supported in recent studies of radical clocks based on norcarane, thujone, and cyclopropyl fatty acids (13-16). However, studies of a series of clocks calibrated to rearrange at faster and faster rates did not give the expected increases in the proportion of rearranged to unrearranged products (17-20). This perceived shortcoming in the radical recombination mechanism (17,20), and studies with probes that discriminate between radical and cationic intermediates, led to the proposal that the ferric hydroperoxide precursor of the ferryl species might itself insert into the C-H bond, yielding a cationic rather than radical intermediate (21-23).

In order to address these conflicting findings, we recently introduced and developed α - and β -thujone as novel two-zone radical clocks (14,24). These clocks not only have a classical cyclopropyl substituent attached to the reacting carbon but also a methyl-substituted carbon that can undergo inversion. Thus, the nature of the carbon intermediate formed in the reaction can be probed simultaneously by the extent of cyclopropyl ring opening and C-4 inversion. Furthermore, a cationic rather than radical intermediate gives distinguishable products. In the earlier studies, the oxidation of α - and β -thujone by P450_{cam} (CYP101) and P450_{BM3} (CYP102) was investigated using gas chromatography with flame ionization detection to quantitate the reaction products (14). The results of those studies fully supported the operation of a radical rebound mechanism. We have now refined the assay methodology by shifting to a GC-MS detection system and employing a wider array of synthetic standards. The results have enabled both a more precise determination of the rebound rate for the P450_{cam} and P450_{BM3}-dependent oxidations as well as detection of carvacrol and the 4,10-desaturated product, both of which derive from a cationic intermediate. In a further exploration of the influence of the active site on the radical rebound process, we have (a) examined the oxidation of α -thujone by the P450_{cam} L244A mutant, which has a slightly larger active site, (b) determined the product profiles in the reactions catalyzed by six human P450 enzymes with relatively large active sites, (c) analyzed the effect on the P450_{cam} reaction of changing the pH of the medium, and (d) carried out the reaction in highly deuterated medium to test for involvement of the Fe-OOH intermediate as a hydroxylating species. The results confirm the predominant radical nature of the catalytic processes involved in hydrocarbon hydroxylation and argue against a direct hydroxylating role for the Fe-OOH intermediate.

EXPERIMENTAL SECTION

General Materials

Six recombinant human P450 enzymes, CYP1A2, -2C9, -2C19, -2D6, -2E1, and -3A4 together with recombinant cytochrome P450 reductase were purchased from Biocatalytics Inc. (Pasadena, CA). α -Thujone ($\geq 96\%$) and all the other reagents, unless otherwise mentioned, were from Sigma-Aldrich. The only impurity found in α -thujone was β -thujone ($\leq 4\%$), which can be removed to $\leq 1\%$ by flash column chromatography on silica gel (hexanes/CH₂Cl₂, 30/1-10/1). Both commercial α -thujone ($\geq 96\%$) and the purified α -thujone ($\geq 99\%$) were used

for enzyme assays. β -Thujone was purified to $\geq 99\%$ (α -thujone $\leq 1\%$) by flash column chromatography on silica gel (hexanes/ CH_2Cl_2 , 30/1-10/1). Yeast extract, tryptone, 2YT medium, sodium chloride, EDTA, and organic solvents were obtained from Fisher Scientific (Fair Lawn, NJ). Isopropyl- β -D-thiogalactopyranoside was from FisherBiotech (Fair Lawn, NJ), and ampicillin from Promega (Madison, WI). All of the chemicals were used without purification unless otherwise indicated. Solid-phase extraction of metabolites was carried out with Bond-Elut cartridges C18 (200 mg, 3 mL) (Varian). Cartridges were conditioned sequentially with 2 mL each of methanol, ethyl acetate, and water. The samples were then transferred to the cartridges and slowly drawn through. Cartridges were then washed with 2 mL of water and thoroughly dried for 1 h with nitrogen at 15 psi. The metabolites were eluted using two 1-mL aliquots of ethyl acetate.

GC-MS Analyses of Thujones and their Metabolites

Analyses were performed on an Agilent 6850 gas chromatograph coupled to an Agilent 5973 Network Mass Selective Detector in the flame ionization mode. Helium was used as the carrier gas. The GC was equipped with an HP-5MS cross-linked 5% PH ME Siloxane capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The temperature program was 70 $^\circ\text{C}$ for 1 min and then 70-280 $^\circ\text{C}$ at 15 $^\circ\text{C}/\text{min}$ or 70 $^\circ\text{C}$ for 5 min, 70-160 $^\circ\text{C}$ at 5 $^\circ\text{C}/\text{min}$, and then 160-280 $^\circ\text{C}$ at 20 $^\circ\text{C}/\text{min}$. The temperatures were 250 $^\circ\text{C}$ for the injection port and 280 $^\circ\text{C}$ for the detector. A sample of 3 μL was injected in a 10:1 split ratio onto the column. Identification of the thujones and their metabolites involved comparison with authentic standards by cochromatography and mass spectrometric fragmentation patterns both as the parent compounds and, where appropriate, as the trimethylsilyl (TMS) derivatives. Product yields were determined by digital integration of the total GC ion current using the resident HP ChemStation software.

Synthesis

β -Thujone (βT)¹ (25), 4,10-dehydrothujone (410DHT), 4-hydroxy- α -thujone (4OH αT), 4-hydroxy- β -thujone (4OH βT), (2*R*)-2-hydroxy- α -thujone (2OH αT), 7-hydroxy- α -thujone (7OH αT), 7,8-dehydro- α -thujone (78DH αT) (26), and 5-hydroxy-5-isopropyl-2-methyl-2-cyclohexen-1-one (ROP) (27) were prepared according to the indicated published procedures. The purity of all products was assessed by ¹H NMR and GC-MS.

Preparation of 7-[²H]- α -Thujone

To a stirred solution of 7-hydroxy- α -thujone (23 mg, 0.14 mmol) and Et_3SiD (100 μL) in dry CH_2Cl_2 (6 mL) at -10 $^\circ\text{C}$ was added a solution of BF_3 etherate (48%, 100 μL). The reaction was continued with stirring at -5 $^\circ\text{C}$ for 1 h and was then quenched by adding saturated Na_2CO_3 (1 mL). The CH_2Cl_2 phase was separated and dried over anhydrous Na_2SO_4 , filtered and concentrated on a rotary evaporator. The residue was chromatographed on silica gel (hexanes/ CH_2Cl_2 , 30/1-10/1) to give the desired compound as a colorless oil (GC-MS purity 99.0%): ¹H NMR (CDCl_3 , 400 MHz) δ ppm 2.56 (d, $J = 18.8$ Hz, 1H), 2.23 (q, $J = 7.5$ Hz, 1H), 2.08 (d, $J = 19.2$ Hz, 1H), 1.17 (d, $J = 7.6$ Hz, 3H), 1.09 (dd, $J = 8, 4$ Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.77 (t, $J = 6$ Hz, 1H), 0.13 (t, $J = 5$ Hz, 1H); GC-MS (FID) 153 (M^+), 138, 125, 111, 96, 81, 68.

¹Abbreviations: βT , β -thujone; 410DHT, 4,10-dehydrothujone; 4OH αT , 4-hydroxy- α -thujone; 4OH βT , 4-hydroxy- β -thujone; 2OH αT , (2*R*)-2-hydroxy- α -thujone; 7OH αT , 7-hydroxy- α -thujone; 78DH αT , 7,8-dehydro- α -thujone; ROP, 5-hydroxy-5-isopropyl-2-methyl-2-cyclohexen-1-one; carv, carvacrol.

Preparation of Enzymes

P450_{cam} (CYP101), P450_{cam} L244A mutant (28), P450_{BM3} (CYP102) (13), putidaredoxin, and putidaredoxin reductase (28) were expressed heterologously in *Escherichia coli* and were purified as previously reported (28). The purified P450_{cam} or P450_{cam} L244A mutant containing camphor was flash frozen over dry ice and stored at -70°C . The camphor present in the protein sample was removed prior to use by two passages through a PD-10 size exclusion column eluted with 100 mM potassium phosphate buffer at pH 7.4 (37°C).

Enzyme Incubations

Unless otherwise indicated, the buffer was 100 mM potassium phosphate at pH 7.4 (37°C). In the case of P450_{cam} and the P450_{cam} L244A mutant, the enzyme (2 μM), putidaredoxin (10 μM), putidaredoxin reductase (2 μM), substrate (1 mM), superoxide dismutase (350 unit/mL), and catalase (100 $\mu\text{g}/\text{mL}$) were dissolved in the buffer (2 mL). The reaction mixture was preincubated at 37°C for 3 min and the reaction was initiated by the addition of NADH (1 mM). In the case of P450_{BM3}, the enzyme (2 μM), substrate (1 mM), and superoxide dismutase (350 unit/mL) were dissolved in buffer. The reaction was initiated by the addition of NADPH (1 mM). All of the substrates were added from a stock solution (100 mM in MeOH). Control reactions were carried out without enzymes, and separately, without NADPH or NADH. All of the reaction mixtures were incubated at 37°C for 1 h and quenched by adding ethyl acetate. The aqueous phase was saturated with NaCl and extracted with ethyl acetate (2 mL \times 2). Alternatively, the reaction mixture was extracted by Bond-Elut C18 cartridges as described above. The organic extract was dried over anhydrous Na_2SO_4 and evaporated to 50-100 μL under a stream of nitrogen at room temperature for GC-MS analysis of a 3.0 μL aliquot. TMS ethers, formed by adding MSTFA (50 μL) to the extract residue and heating for 30 min at 80°C , were analyzed with a 1.0 μL aliquot by GC-MS.

For the incubations with human enzymes, the incubation procedure was slightly modified from the assay protocol provided by Biocatalytics, Inc. The reaction buffer mixture provided by the vendor was dissolved in deionized water. To the solution was added α -thujone (100 mM in MeOH). After incubation at 37°C for 3 min, the reaction was initiated by addition of the enzyme dissolved in deionized water. The final reaction volume was 1 mL, containing enzyme (1 μM) and substrate (1 mM). The reaction mixtures were incubated at 37°C for 1 h before being extracted with Bond-Elut cartridges C18.

P450_{cam} Incubations in $[\text{}^2\text{H}]_2\text{O}$

The buffer was 100 mM phosphate buffer in $[\text{}^2\text{H}]_2\text{O}$, pH 7.4 (37°C). The camphor present in the P450_{cam} sample was removed prior to use by two passages through a PD-10 column eluted with $[\text{}^2\text{H}]_2\text{O}$ buffer. The H_2O in putidaredoxin and putidaredoxin reductase was also exchanged with $[\text{}^2\text{H}]_2\text{O}$ by passage through a PD-10 column. α -Thujone (1 mM) was added from a stock solution (100 mM in CH_3CN), superoxide dismutase (375 unit/mL), catalase (100 $\mu\text{g}/\text{mL}$), and NADH were added in $[\text{}^2\text{H}]_2\text{O}$ stock solution. The reaction and the reaction mixture extraction were performed according to the procedures described above.

Determination of Extraction Factors and GC-MS Response Factors for Thujone Metabolites

To determine the extraction factors of thujone metabolites, a mixture of α -thujone and authentic metabolite standards in MeOH (50 μL) was added to the buffer (2 mL) and vortexed before being extracted with ethyl acetate (2 mL \times 2) or Bond-Elut C18 cartridges. The organic extract was dried over anhydrous Na_2SO_4 , evaporated under a stream of nitrogen at room temperature, and reconstituted to 50 μL . The samples before and after the extraction were analyzed by GC-MS. To determine the GC-MS response factors of thujone metabolites, solutions (0.1-4 mM in EtOAc) of each authentic metabolite were prepared and analyzed by GC-MS via a 3 μL

aliquot. The GC-MS integration area was correlated to the corresponding concentration by a 1st order equation.

RESULTS

Extraction Factors and GC-MS Response Factors of Thujone Metabolites

The recovery factors for thujone metabolites from the incubation solution were experimentally determined. A mixture of the metabolite standards was dissolved in the incubation buffer and was then extracted with ethyl acetate or Bond-Elut C18 cartridges. The concentration and percentage of each metabolite were determined by GC-MS before and after the extraction (Table S1). The recovery factor was >65% for each metabolite by ethyl acetate extraction and >80% by solid phase extraction. The percentage of each metabolite was essentially the same before and after the extraction, indicating that there was no bias for any component in the extraction process.

To determine the response factor of each metabolite on GC-MS, metabolite standards of various concentrations were injected into the GC-MS in a fixed volume (3 μ L). Fig. S1 shows that all of the compounds responded proportionately to their concentration. All the compounds except carvacrol have a factor of around 4 between their GC-MS response and their actual concentration, which reflects their similar chemical structures. The factor for the aromatic compound carvacrol is about 2-fold higher than that for its non-aromatic analogues. This result indicates that the real concentration of each metabolite in the solution is properly represented by its proportional response on GC-MS except that of carvacrol, which should be divided by a factor of 2 in comparison to other metabolites.

Oxidation of α -Thujone by Bacterial Enzymes

We previously demonstrated the utility of thujone stereoisomers as P450 diagnostic substrates (14), but report here methodological improvements that update those results. α - and β -thujone were incubated with P450_{cam} and P450_{BM3}. To suppress possible substrate oxidation or P450 degradation by hydrogen peroxide or superoxide, both of which are formed through uncoupled turnover of P450 enzymes, catalase and superoxide dismutase were included in the reaction mixture. Control reactions did not show any oxidation products in the absence of either the P450 enzyme or NADH/NADPH.

The reaction of α -thujone with P450_{BM3} yielded 8% of 7-hydroxy- α -thujone, 0.3% of 7,8-dehydro- α -thujone, 4.2% of 2-hydroxy- α -thujone, 80.2% of 4-hydroxy- α -thujone (the major product), 4.7% of 4-hydroxy- β -thujone, and 0.6% of 4,10-dehydro-thujone (Scheme 2, Table 1). The ring-opened product from the radical intermediate, 5-hydroxy-5-isopropyl-2-methyl-2-cyclohexen-1-one, was produced in 0.1% yield. As shown previously by chemical experiments (24), carvacrol is the principal product expected in solution from a 4-cationic intermediate. This compound was not detected in our previous work due to the low sensitivity of the flame ionization detector for this compound, but after switching to GC-MS it was detected here in 0.4% yield. The observation of carvacrol in the incubation mixture was further confirmed by comparing a TMS-derivatized sample with a TMS-derivatized authentic sample. The overall conversion of α -thujone by P450_{BM3} under these conditions was 32%. The stereochemical inversion ratio at C-4 was 17/1 (4-hydroxy- α -thujone/4-hydroxy- β -thujone) (Table 2).

The reaction of α -thujone with P450_{cam} afforded the same products but in different proportions: 75.9% of 7-hydroxy- α -thujone, 13.6% of 7,8-dehydro- α -thujone, 0.3% of 2-hydroxy- α -thujone, 6.1% of 4-hydroxy- α -thujone, 0.4% of 4-hydroxy- β -thujone, 0.02% of the ring-opened product, 0.1% of 4,10-dehydrothujone, and 0.15% of carvacrol. The conversion was 85% based on the starting α -thujone detected in the reaction mixture. The stereochemical

inversion ratio was 15/1. In contrast to the reaction of α -thujone with P450_{BM3}, the reaction of α -thujone with P450_{cam} occurred mainly at C-7 instead of C-4. The ratio between the total C-4 and C-7 oxidation products was 10/1 for P450_{BM3} and 1/13 for P450_{cam}, respectively.

Oxidation of β -Thujone by Bacterial Enzymes

The reaction of β -thujone with P450_{BM3} yielded 13.3% of 7-hydroxy- β -thujone, 0.5% of 7,8-dehydro- β -thujone, 8.6% of 2-hydroxy- β -thujone, 0.6% of 4-hydroxy- α -thujone, 62.1% of 4-hydroxy- β -thujone, 0.05% of the ring-opened product, 0.14% of 4,10-dehydrothujone, and 0.1% of carvacrol (Scheme 2, Table 1). The overall conversion of β -thujone by P450_{BM3} under these conditions was 42%. The stereochemical inversion ratio was 1/104 (Table 2).

The reaction of β -thujone with P450_{cam} yielded 2.4% of 7-hydroxy- β -thujone, 0.5% of 7,8-dehydro- β -thujone, 0.1% of 2-hydroxy- β -thujone, 0.09% of 4-hydroxy- α -thujone, 95.3% of 4-hydroxy- β -thujone, 0.1% of the ring-opened product(s), 0.1% of 4,10-dehydrothujone, and 0.2% of carvacrol. The overall conversion of β -thujone by P450_{cam} under these conditions was 48% (Table 1). The stereochemical inversion ratio was 1/1059 (Table 2). The much lower stereochemical inversion in the oxidation of β -thujone than in that of α -thujone is consistent with the stereoselective reduction of chemically-generated thujone radicals by thiophenol (24), a reaction that greatly favors addition of the hydrogen from the α -face.

Oxidation of 7[²H]- α -Thujone by P450_{cam}

In order to shift metabolism from the C-7 to the C-4 position in enzymes such as P450_{cam}, thus amplifying the signals due to oxidation of the carbon of interest through metabolic switching, 7[²H]- α -thujone was synthesized from 7-hydroxy- α -thujone by trifluoroborane-catalyzed deuterium triethylsilane reduction (Scheme 1). When TFA was used instead of BF₃, the trifluoroacetate of 7-hydroxythujone was formed but not reduced under the reaction conditions.

The reaction of 7[²H]- α -thujone with P450_{cam} afforded 31.4% of 7-hydroxy- α -thujone, 8.6% of 7,8-dehydro- α -thujone, 1.6% of 2-hydroxy- α -thujone, 33.8% of 4-hydroxy- α -thujone, 1.3% of 4-hydroxy- β -thujone, 0.04% of the ring-opened product, 1% of 4,10-dehydrothujone, and 0.8% of carvacrol. The formation of carvacrol and the ring-opened product from the radical intermediate were further confirmed by the corresponding molecular ions, which were one unit higher than those of the undeuterated samples, and by the corresponding fragmentations in their mass spectra. The conversion was 70% based on the starting 7[²H]- α -thujone detected in the reaction mixture. In contrast to the reaction of α -thujone with P450_{cam}, the reaction of 7[²H]- α -thujone with P450_{cam} shifted significantly from the C-7 to the C-4 position, as expected from the operation of a deuterium isotope effect on the hydroxylation reaction at C-7. The ratio of C-4 to C-7 oxidation products was 1/1 for 7[²H]- α -thujone, 13-fold higher at C-4 than the 1/13 ratio obtained with undeuterated α -thujone. The ring-opened product from the radical intermediate increased about 2-fold in the oxidation of 7[²H]- α -thujone compared to its formation from non-deuterated α -thujone (Table 3). Furthermore, the stereochemical inversion ratio (26/1) exhibited about the same shift relative to that for the oxidation of undeuterated α -thujone (15/1) (Table 2).

Oxidation of α -Thujone and 7[²H]- α -Thujone by the P450_{cam} L244A Mutant

The reaction of α -thujone with the P450_{cam} mutant L244A was investigated to explore the influence of a small increase in the dimensions of an otherwise essentially identical active site cavity on the radical clock reaction. The reaction afforded 83% of 7-hydroxy- α -thujone, 9.7% of 7,8-dehydro- α -thujone, 2.4% of 2-hydroxy- α -thujone, 2.1% of 4-hydroxy- α -thujone, 1.1% of 4-hydroxy- β -thujone, 0.02% of the ring-opened product, 0.06% of 4,10-dehydrothujone, and 0.16% of carvacrol (Table 3). While the C-7 oxidation product, 7-hydroxy- α -thujone, was the major product in both the L244A and wild-type oxidations, the product distributions from

oxidation at the C-4 and C-2 positions were somewhat different between these two enzymes. Compared to the wild-type P450_{cam}, the L244A mutant produced more C-2 oxidation product (2.4% vs 0.3%), and the C-4 stereochemical inversion in the L244A oxidation gave a 2/1 stereoisomer ratio, a value 7.5-fold higher than the 15/1 ratio observed in the wild-type oxidation.

Oxidation of 7[²H]- α -thujone by the P450_{cam} L244A mutant afforded 41.5% of 7-hydroxy- α -thujone, 7% of 7,8-dehydro- α -thujone, 11.9% of 2-hydroxy- α -thujone, 10.8% of 4-hydroxy- α -thujone, 6.9% of 4-hydroxy- β -thujone, 0.04% of the ring-opened product(s), 0.3% of 4,10-dehydrothujone, and 0.8% of carvacrol (Table 3). The conversion was 69% based on the starting 7[²H]- α -thujone detected in the reaction mixture. The ratio between the C-4 and C-7 oxidation products is 1/2.5 for 7[²H]- α -thujone, 11-fold higher than the ratio of 1/27 for undeuterated α -thujone (Table 2). The ring-opened product from the radical intermediate increased 2-fold in the oxidation of 7[²H]- α -thujone compared to its formation from undeuterated α -thujone, but the C-4 stereochemical inversion ratio was little altered.

Effect of pH on α -Thujone Metabolite Distribution

To examine the effect of pH on the product distribution, the reaction of P450_{cam} with α -thujone was conducted under four pH conditions ranging from pH 6.5 to pH 8.0 (100 mM phosphate buffer). The metabolite distributions under these conditions are summarized in Table S2. Only minor differences were observed within the indicated pH range. The conversion yield at pH 7.4 (80.8%) was slightly higher than at other pH conditions. A nominal change was also observed in the C4 product distribution. The yield of 4OH β T increased 26% from pH 6.5 to pH 8.0 and, correspondingly, the yield of 4OH α T decreased 7% and the yield of ROP decreased 29%. One olefinic product, 410DHT, suffered a 40% decrease in yield, and the cation product, carvacrol, an 8% increase in yield.

[²H]₂O Effect on α -Thujone Metabolite Distribution

The reaction of P450_{cam} with α -thujone in a highly deuterated medium (>99% [²H]₂O) was investigated to explore the role of the ferric hydroperoxide (Fe^{III}-OOH) precursor of the ferryl (Fe^{IV}=O) intermediate as a hydroxylating species. The metabolite distributions under H₂O and [²H]₂O conditions are summarized in Table S3. No significant differences were observed as a result of carrying out the reaction in a deuterated medium. The very small changes observed probably stem from the difference between pD and pH (pD = pH + 0.41) (29).

Oxidation of α -Thujone by Human P450 Enzymes

Six human enzymes were examined as catalysts of α -thujone to further explore the extent to which active site structure influences the radical recombination and dehydrogenation reactions. All six human enzymes catalyzed the oxidation of α -thujone, but only CYP2D6 and CYP3A4 gave sufficient conversion of the substrate to metabolites within a 1-h incubation time to enable a detailed analysis. The other four enzymes, CYP1A2, -2C9, -2C19, and -2E1, gave only ~1% conversion under these experimental conditions and the data was only suitable for identification of the major metabolites (Table 4).

CYP3A4 oxidized α -thujone to 49.6% of 7-hydroxy- α -thujone (the major product), 3.8% of 7,8-dehydro- α -thujone, 2.2% of 2-hydroxy- α -thujone, 25.8% of 4-hydroxy- α -thujone, 7.4% of 4-hydroxy- β -thujone, and 3.3% of carvacrol (Table 4). The product of ring opening of the C-4 radical intermediate, 5-hydroxy-5-isopropyl-2-methyl-2-cyclohexen-1-one, accounted for 0.3% of the product yield. 4,10-Dehydrothujone was not detected in the CYP3A4 reaction. The stereochemical inversion ratio was 3.6/1 (Table 5). The ratio between the C-4 and C-7 oxidation products was 1/1.3.

The oxidation of α -thujone by CYP2D6 yielded 58.7% of 7-hydroxy- α -thujone, 9.1% of 7,8-dehydro- α -thujone, 17.8% of 4-hydroxy- α -thujone, 7.1% of 4-hydroxy- β -thujone, and 4.3% of carvacrol (Table 4). The ring-opened product was produced in 0.26% yield. 4,10-Dehydrothujone, which was observed in the reactions of the bacterial enzymes, was not detected in the reaction of CYP2D6. 2-Hydroxy- α -thujone was also not detected. The stereochemical inversion ratio was 2.5/1 (Table 5). The ratio between the C-4 and C-7 oxidation products was 1/2.7.

The only two products detected in the oxidation of α -thujone with CYP1A2 due to the relatively low turnover of the substrates were 4-hydroxy- α -thujone (21.7%) and 4-hydroxy- β -thujone (78.4%). A 1-h reaction of α -thujone with CYP2C9 produced 18.1% of 7-hydroxy- α -thujone, 19.0% of 4-hydroxy- α -thujone, and 62.9% of 4-hydroxy- β -thujone. The stereochemical inversion ratios for these two proteins were 1/3.6 and was 1/3.3, respectively. Similarly, the oxidation of α -thujone by CYP2C19 produced 14.1% of 7-hydroxy- α -thujone, 19.5% of 4-hydroxy- α -thujone, and 66.5% of 4-hydroxy- β -thujone (Table 4). The stereochemical inversion ratio was 1/3.4 (Table 5). The ratio between the C-4 and C-7 oxidation products was 6.1/1. Finally, a 1-h reaction of α -thujone with CYP2E1 yielded 15.5% of 7-hydroxy- α -thujone, 19.4% of 4-hydroxy- α -thujone, and 65.1% of 4-hydroxy- β -thujone. The stereochemical inversion ratio was 1/3.4 and the ratio between the C-4 and C-7 oxidation products was 5.5/1.

CYP1A2 thus primarily oxidized α -thujone at C-4 to give the two 4-hydroxythujone isomers whereas CYP2C9, CYP2C19, and CYP2E1 primarily catalyzed similar C-4 oxidation but also produced a significant amount of the 7-hydroxy- α -thujone metabolite. The radical recombination rates, C-4 methyl stereochemistry ratio, C-4 to C-7 product ratio, and ratio of C-4 radical to cation products in the oxidation of α -thujones by human P450s, as available, are listed in Table 4.

DISCUSSION

Analysis of the products formed on oxidation of the radical clock probes α - and β -thujone by P450_{cam} and P450_{BM3} has resulted in identification of several minor products in addition to those previously reported (14). The new products are 2-hydroxy- α -thujone, 2-hydroxy- β -thujone, 7,8-dehydro- α -thujone, 7,8-dehydro- β -thujone, and carvacrol (Scheme 2). The 2-hydroxythujone and the 7,8-dehydrothujone isomers were detected in earlier studies of the metabolism of α - and β -thujone by mammalian liver (26,30,31), but were not specifically identified in the previous work with purified bacterial enzymes (14). As indicated by the "Other" column in Table 1, a few small unidentified peaks remain in the gas chromatogram, but the percent of the metabolites represented by these products has greatly decreased compared to the earlier study: from 6 to 1.5% for P450_{BM3} and α -thujone, 25 to 14.6% for P450_{BM-3} and β -thujone, 19 to 3.5% for P450_{cam} and α -thujone, and 3 to 1.2% for P450_{cam} and β -thujone.

P450_{cam} oxidizes α -thujone primarily at the 7 position, producing 7-hydroxy- α -thujone (75.9%) and 7-dehydro- α -thujone (13.6%). Oxidation at C-4 accounts for 6.8%, oxidation at C-2 for 0.3%, and unidentified minor products for 3.5% (Table 1). Interestingly, oxidation of the opposite end of the substrate dominates in the reaction of P450_{cam} with β -thujone (Table 1). In this instance, C-7 oxidation accounts for only 2.9%, oxidation at C-4 for slightly under 96%, and oxidation at C-2 for 0.1%. Inversion of the stereochemistry of C-4, which bears a single methyl group, thus causes the thujone to completely invert its preferred orientation in the P450_{cam} active site.

Cytochrome P450_{BM3}, in contrast, is not sensitive to the stereochemistry of the 4-methyl group. Thus, 4-hydroxylation is the predominant reaction in the oxidation of both α - and β -thujones (Table 1). However, the P450_{BM3} reaction is less regioselective, as there is a considerable

amount of oxidation at C-7 (α -thujone, 8.3%, β -thujone 13.8%) and C-2 (α -thujone 4.2%, β -thujone 8.6%). The oxidation of α -thujone by P450_{BM3} occurs 8.3% at C-7, 86% at C-4, and 4.2% at C-2, and that of β -thujone 13.8% at C-7, 63% at C-4, and 8.6% at C-2.

The use of thujones as mechanistic probes depends on their oxidation at C-4, so the high degree of oxidation at C-7 rather than C-4 in the reaction of P450_{cam} with α -thujone diminishes the sensitivity of this compound as a radical clock substrate for this enzyme. To overcome this defect, we synthesized 7-deuterated α -thujone in the expectation that a primary isotope effect on 7-oxidation would shift the oxidation process to the mechanistically more informative C-4 site. This was, indeed, the result, although the isotope effect appears to be partially masked because the C-7 metabolites decreased from 89.5% to 40%, a net deuterium isotope effect of 2.2 (Table 3). Nevertheless, this isotope effect on the major pathway produced a large increase in the mechanistically interesting minor metabolites. Thus, C-4 oxidation increased 5.5-fold and C-2 hydroxylation 5-fold. Thus, the 7-deuterated probe makes it possible to enhance the proportion of the oxidation at the mechanistically informative C-4 position in situations where C-7 hydroxylation competes too effectively with C-4 hydroxylation.

The radical recombination rates for hydroxylation at C-4 can be calculated from the data in Table 1 using the equation below, given the rate of 4.4×10^7 for ring opening of the thujone C-4 radical determined by our earlier calibration studies (24):

$$k_1 = \left[\frac{4\text{OH}\alpha\text{T} + 4\text{OH}\beta\text{T}}{\text{ROP}} \right] \left[\times 4.4 \times 10^7 \text{ s}^{-1} \right]$$

The recombination rates calculated for the various combinations of enzyme and substrate are given in Table 2. Thus, the radical recombination rate for P450_{BM3} with α -thujone is $(3.7 \pm 1.4) \times 10^{10} \text{ s}^{-1}$ and with β -thujone $(12.5 \pm 3.0) \times 10^{10} \text{ s}^{-1}$. The corresponding recombination rate for P450_{cam} with α -thujone is $(2.0 \pm 0.6) \times 10^{10} \text{ s}^{-1}$, and with β -thujone $(9.5 \pm 1.2) \times 10^{10} \text{ s}^{-1}$. These revised rates differ somewhat from those previously reported: P450_{BM3} with α -thujone $0.6 \times 10^{10} \text{ s}^{-1}$ and with β -thujone $1.6 \times 10^{10} \text{ s}^{-1}$; and P450_{cam} with α -thujone $0.2 \times 10^{10} \text{ s}^{-1}$ and with β -thujone $2.8 \times 10^{10} \text{ s}^{-1}$ (14). The new rates are higher by a factor of from 3.4 (P450_{cam} with β -thujone) to 10 (P450_{cam} with α -thujone). These differences reflect changes in the product ratios due to the improved GC-MS methodology. More importantly, the 7-deuterated probe makes it possible to enhance the proportion of the oxidation by P450_{cam} at the mechanistically informative C-4 position and thus to improve the accuracy of the measurements. All the rates nevertheless remain within the range expected for radical intermediate reactions and support the reaction mechanism in Scheme 3.

The radical recombination rates show substantial substrate- and enzyme-dependent variation. The rates determined from the oxidation of α -thujone by P450_{cam} and P450_{BM3} thus differ by a factor of 1.9, and the rates from the oxidation of α - and β -thujone by P450_{cam} by a factor of 4.8. This is consistent with the accumulated evidence employing a range of probes that, within limits, the measured recombination rate is not constant but is substrate and enzyme dependent. The differences in the recombination rates could reflect true differences in recombination rates or, according to the two-transition state paradigm championed by Shaik (6), a change in the proportions of the reaction that occur from the high- and low-spin transition state pathways of the ferry species.

To explore further the substrate and enzyme dependence of the recombination rates, we have investigated the oxidation of α -thujone and 7-[²H]- α -thujone by the L244A mutant of P450_{cam}. In the L244A mutant, three atoms are removed from a protein side-chain that lines one side of the active site pocket. Previous studies of the substrate specificity of the L244A mutant have shown that it possesses a diminished catalytic activity but accepts larger substrates than the wild-type enzyme (32). A crystal structure of the L244A mutant is not available, but

modeling of the active site suggests that the cavity is modestly expanded but otherwise little altered. A comparison of the oxidation of the two probe substrates by wild-type P450_{cam} and its L244A mutant reveals interesting differences. The ratio of C-7 to C-4 metabolism remains essentially unaltered, with 92.7% (versus 89.5% for wild-type) of the reaction in the L244A mutant occurring at C-7 and 3.4% (versus 6.8% in wild-type) at C-4 (Table 3). However, 2-hydroxylation greatly increases from 0.3 to 2.4% (an 8-fold change). The 7-deuterated probe again causes a shift from C-7 to C-2 and C-4 oxidation, with a 1.9-fold (versus 2.2-fold for wild-type) decrease in C-7 oxidation, a 5-fold (versus 5.3-fold for wild-type) increase in C-2 oxidation, and a 5.5-fold (versus 5.4-fold in wild-type) increase in C-4 oxidation (Table 1). The isotope effect shifts are therefore similar for the wild-type and mutant.

The most interesting difference in the L244A mutant is in the extent of inversion of stereochemistry of the methyl-substituted C-4 carbon. The ratio of 4-hydroxylated thujone isomers in the P450_{BM3}-catalyzed oxidation of α -thujone was 17:1 and of β -thujone 1:104 (Table 2). The corresponding value for the oxidation of α -thujone by P450_{cam} was 15:1, and for β -thujone 1:1059, indicating that very little inversion occurred. However, in the case of the L244A mutant with α -thujone, the ratio of inverted to non-inverted 4-hydroxythujone metabolites was 2:1 (i.e., 33% inversion), and for the 7-deuterated analogue was about the same (38%). Thus, the L244A mutant allows a much greater rate of inversion of the methyl-substituted C-4 center during hydroxylation at this position. The original calibration studies indicated that trapping of a C-4 radical by hydrogen atom transfer from thiophenol gave essentially the same mixture of isomers regardless of whether the radical was generated with the methyl in the α - or β -orientation (24). In both cases, the α - to β -ratio was \sim 6:100, indicating that the hydrogen trapping reaction occurred from an equilibrium isomeric mixture and greatly favored delivery of the hydrogen to C-4 from the sterically less hindered α -face. This preference is clearly abrogated by the radical recombination reaction of the P450_{cam} L244A mutant, as an equal amount of delivery of the hydroxyl group to the α - and β -faces is observed despite the fact that the hydrogen must be abstracted from the β -face. Thus, the increased volume or flexibility of the L244A active site allows a greater degree of substrate rotation prior to transfer of the hydroxyl group to the carbon.

The radical recombination rates for C-4 hydroxylation of α -thujone by CYP2D6 and CYP3A4 were 0.34 and $0.48 \times 10^{10} \text{ s}^{-1}$, respectively (Table 5). These values are somewhat smaller than the values obtained for oxidation of the same substrate by P450_{cam} ($2 \times 10^{10} \text{ s}^{-1}$) and P450_{BM3} ($3.7 \times 10^{10} \text{ s}^{-1}$). Radical recombination rates could not be calculated for CYP1A2, CYP2C9, CYP2C19, and CYP 2E1 due to limiting amounts of enzyme and low turnover rates for α -thujone. However, in all cases it has been possible to assess the extent to which the methyl-substituted C-4 center undergoes inversion of configuration during the hydroxylation reaction (Table 5). For all six human enzymes the extent of inversion is greater than for P450_{cam} or P450_{BM3}. Indeed, with all the human enzymes other than CYP2D6 and CYP3A4, the 4-hydroxylated product with inverted stereochemistry is the dominant product. With CYP2D6 and CYP3A4, the major product is that with the original methyl stereochemistry, but even here methyl-substituted C-4 inversion occurs to a much larger extent than is seen with wild-type P450_{cam} and P450_{BM3} (Table 1). Interestingly, the L244A P450_{cam} mutant more closely resembles the mammalian enzymes in this regard, as inversion of the methyl stereochemistry is observed in 33% of the C-4 hydroxylated metabolite. These results are consistent with the intervention of a radical recombination mechanism with a radical species that has a lifetime comparable to, if somewhat longer than, those observed for the wild-type bacterial enzymes. The larger extent of methyl inversion may reflect the expected “looseness” of fit of α -thujone in the larger active sites of CYP2D6, CYP3A4, and the P450_{cam} L244A mutant, although the data do not specifically require this. The high degree of methyl inversion seen with the other human enzymes does provide direct evidence that the major (or exclusive) pathway of carbon hydroxylation proceeds through the carbon radical species. A cationic intermediate is

inconsistent with this finding because the cation has been shown to rearrange efficiently to the aromatic product carvacrol (14).

In addition to 2-, 4-, and 7-hydroxylation, the oxidation of α - and β -thujone produces three desaturated metabolites: α -(or β)-7,8-dehydrothujone, 4,10-dehydrothujone, and carvacrol. The 7,8-dehydrogenation presumably reflects formation of a cation at C-7 by electron transfer or proton-coupled electron transfer from the initial C-7 radical to the enzyme. In agreement with this, substitution of a deuterium at C-7 decreases both 7-hydroxylation and 7-desaturation with approximately the same isotope effect on both reactions. This finding is consistent with other P450-mediated desaturations, which have been shown to exhibit a significant isotope effect when deuterium is located at the carbon from which the hydrogen is abstracted by the catalytic species, but not for deuterium at the carbon from which the hydrogen is lost as a proton (33-36). Thus, desaturation triggered by abstraction of a hydrogen from the C-8 methyl group is ruled out, in accord with the apparent absence of a C-8 hydroxylated metabolite.

4,10-Desaturation was observed previously with P450_{cam} and P450_{BM3} (14). A mechanism similar to that proposed for 7,8-desaturation was proposed for this reaction in which hydroxyl radical transfer to the C-4 radical and ring opening of the radical clock to give the rearranged (ROP) product occur as competitive processes. In the previous studies, carvacrol was not detected despite a specific search for its presence using an authentic standard. In the present studies in which GC-MS rather than GC-FID was used as the analytical method, it has been possible to detect and quantitate this product. Carvacrol formation is significant because chemical studies demonstrated that carvacrol is quantitatively formed when the C-4 cation is generated in solution (14). Thus, carvacrol formation and possibly 4,10-dehydrogenation provide a measure of the extent of C-4 cation formation.

In all cases, radical recombination to give 4-hydroxylated thujones dominates the C-4 reaction, but small amounts of cation and desaturation products are obtained. For the purpose of quantitation, the 4,10-desaturated product is assumed here to arise via cation formation rather than by a second hydrogen atom abstraction by the ferryl species. In the oxidation of α -thujone by P450_{BM3}, C-4 radical recombination accounts for 84.9% of the product, whereas carvacrol and 4,10-dehydrothujone account for only 1%. Radical recombination is thus rapid relative to both ring opening and cation formation. The results for oxidation of β -thujone by P450_{BM3} are similar, with C-4 radical recombination accounting for 62.7% of the products and the cation pathway for only 0.24% (Table 1). In the case of α -thujone oxidation by P450_{cam}, recombination accounts for 6.5% and the cation pathway for 0.25% of the total products, and in that of β -thujone 95.4% and 0.3%, respectively. When the 7-deuterated substrate is used, 4-hydroxylation increases to 35.1% and carvacrol plus 4,10-dehydrothujone to 1.8%. With the L244A mutant, the ratio for α -thujone oxidation is 3.2% 4-hydroxylation and 0.22% C-4 cation formation, whereas with 7-[²H]- α -thujone the corresponding values are 17.7% C-4 hydroxylation and 1.1% C-4 cation products. Inspection of these numbers indicates that the ratio of hydroxylation to C-4 cation formation is not constant. The ratios (Table 2) vary from 15:1 for the L244A oxidation of α -thujone to 318:1 for the oxidation of β -thujone by P450_{cam}. The factors that control this 6- to 20-fold difference in partitioning of the C-4 intermediate into radical and cation pathways remain obscure, but could include the presence or absence of basic residues, the polarity of the active site, and the degree to which the C-4 radical is positioned for rebound hydroxy transfer from the heme iron atom. If the 4,10-desaturated product is formed via a C-4 cation, the factors that control whether it yields 4,10-dehydrothujone or carvacrol are likewise unknown. The possibility cannot be ruled out that the 4,10-desaturated product is formed by a second hydrogen abstraction from C-10 by the protonated ferryl species generated in the first hydrogen abstraction rather than via the cation. Nevertheless, the slight decrease in formation of 4-hydroxy- β -thujone paralleled by a corresponding slight increase in formation of the 4,10-dehydro product upon changing the pH

of the P450_{cam}/ α -thujone incubation medium from 6.5 to 8.5 suggests that these two reactions are inversely linked by a process that involves an active site base.

The finding that there is no detectable alteration in the outcome of the oxidation process when the reaction is carried out in highly deuterated medium rather than in normal water provides strong support for a single ferryl oxidizing species and against a direct oxidative role for the ferric hydroperoxide (Fe^{III}-OOH) intermediate. Previous studies have shown that a hydrogen-bonding network exists that delivers protons to the hydroperoxide to facilitate its cleavage to give the ferryl species (Fig. 1) (37,38). This network of hydrogen bonds is critical, as mutation of Thr252 to residues with non-hydrogen bonding side-chains leads to a high degree of uncoupling to produce H₂O₂ at the expense of ferryl formation and substrate oxidation (39-41). A recent crystal structure has provided evidence that the Thr252 hydroxyl serves as a hydrogen bond acceptor (42). Sligar and colleagues have examined the role of the hydrogen bonds in catalysis by determining the solvent deuterium isotope effect on each of the accessible kinetic steps of the reaction (37,38). Their results have established that the second electron transfer, which is linked to protonation of the ferric hydroperoxide intermediate, exhibits a deuterium KSIE of 1.8 and is the only isotopically sensitive step in the catalytic cycle (37, 38). Replacement of the hydrogen bond network with deuterium atoms thus alters the rate of cleavage of the ferric hydroperoxide complex to give the ferryl species. If both the hydroperoxide and ferryl were involved in oxidation reactions and gave different products or product ratios, an alteration in the product profile associated with a shift from hydrogen to deuterium should be observed due to this solvent isotope effect. However, the data indicate that the oxidizing species is the same in deuterated and nondeuterated medium. Assuming that there is no change in the mechanism of oxygen activation by P450_{cam} due to the use of α -thujone rather than camphor, this finding suggests that the ferric hydroperoxide does not play a significant role in the oxidation of the substrate. This result is consistent with the report that mutation of Thr252 both suppresses camphor hydroxylation and greatly decreases epoxidation of a camphor analogue with a double bond (43), which also argues against a significant role for the ferric hydroperoxide species in normal hydrocarbon oxidation.

CONCLUSIONS

The radical recombination rates for 4-hydroxylation of α - and β -thujone by P450_{BM3}, P450_{cam}, the L244A P450_{cam} mutant, CYP3A4, and CYP2D6 range from a low of $0.34 \times 10^{10} \text{ s}^{-1}$ to a high of $(12.5 \pm 3) \times 10^{10} \text{ s}^{-1}$, well within the range for transition states of radical recombination reactions. In addition, C-4 hydroxylation of the thujones is accompanied by a small extent of inversion of the C-4 methyl group, but this inversion in the L244A mutant accounts for 33% of the 4-hydroxylated product, implying a major role for the radical species in the reaction. Limited experimental results on human P450 enzymes show that the recombination rates for these enzymes are comparable to those for the bacterial proteins and reveal a particularly high extent of C-4 inversion, a finding strongly supportive of a radical mechanism. The C-4 dehydrogenation products 4,10-dehydrothujone and carvacrol, as well as the 7,8-dehydrothujones arising from C-7 dehydrogenation, can be rationalized by formation of C-4 and C-7 cations via electron transfer from the corresponding radicals to the ferryl species, although the two olefinic products could arise by a second hydrogen atom abstraction by the ferryl species. The observation that no changes occur in the product profiles when the aqueous medium is replaced by highly deuterated medium argues against involvement of the P450_{cam} ferric hydroperoxide intermediate, a species that has been postulated to lead directly to cationic products (20,23), in substrate oxidation. Overall, the results provide persuasive support for the view that carbon hydroxylation reactions by cytochrome P450 enzymes proceed via a radical rebound mechanism as shown in Scheme 3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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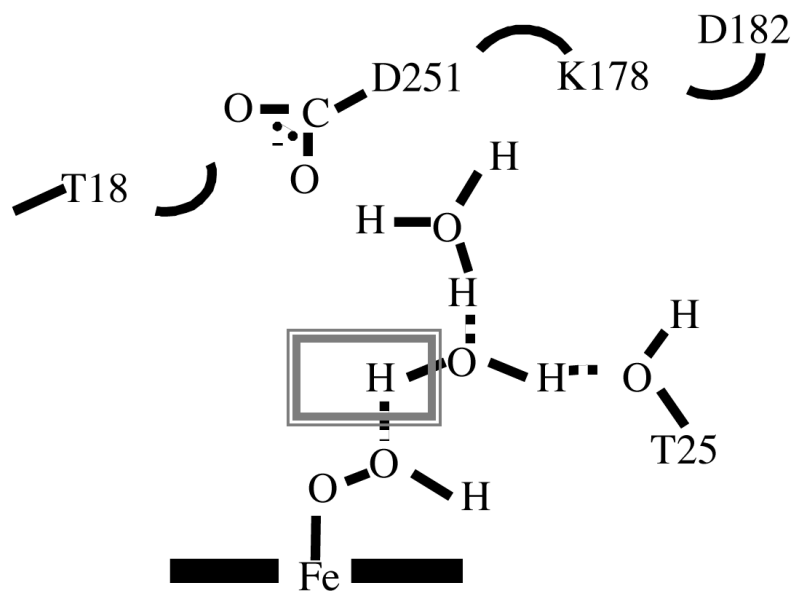
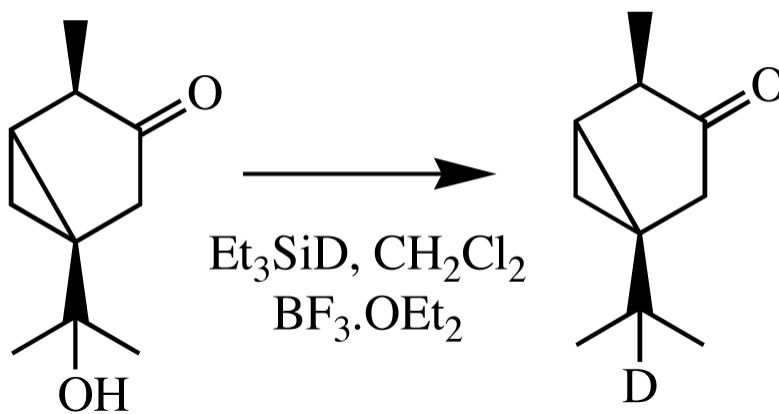
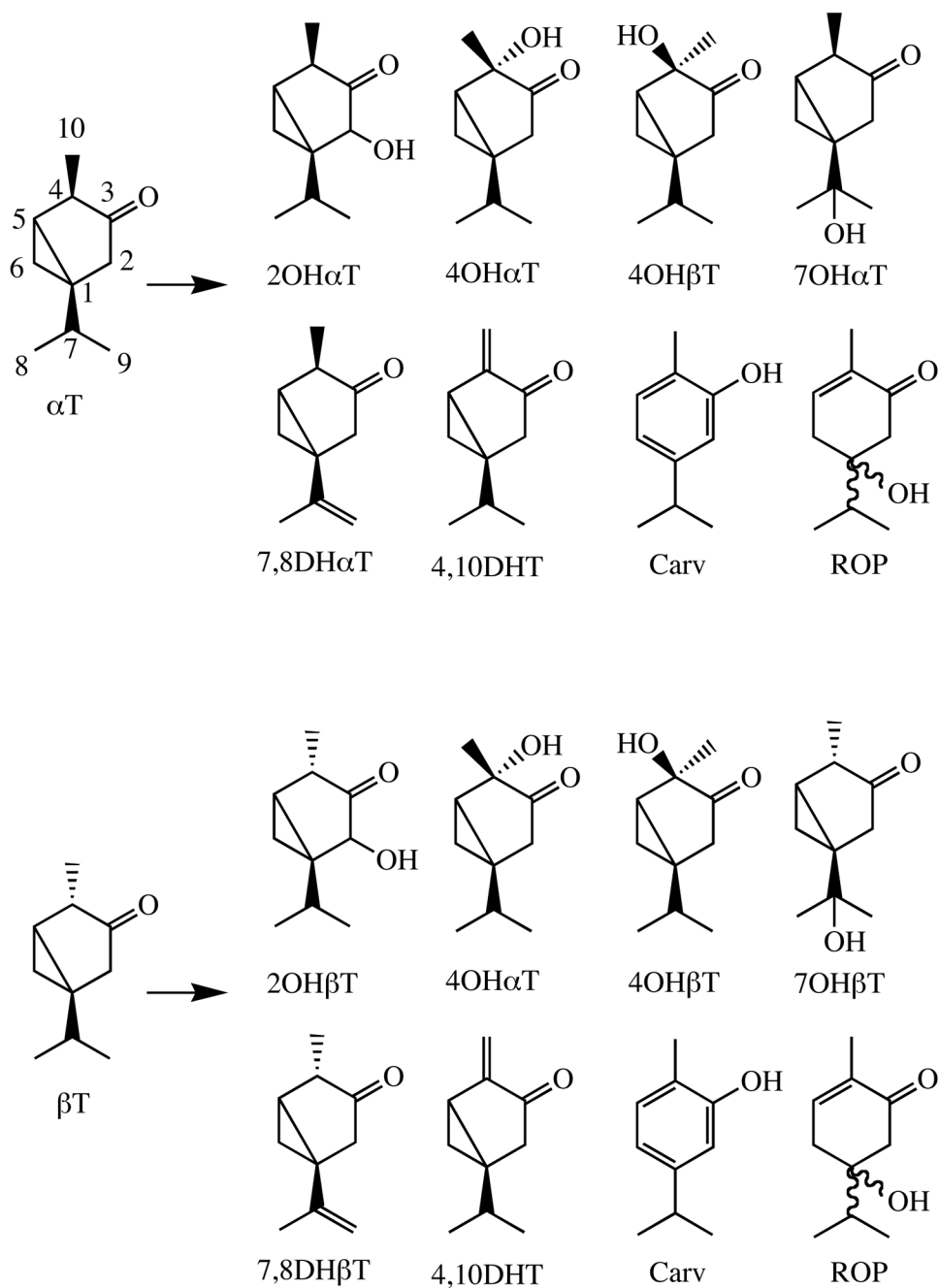


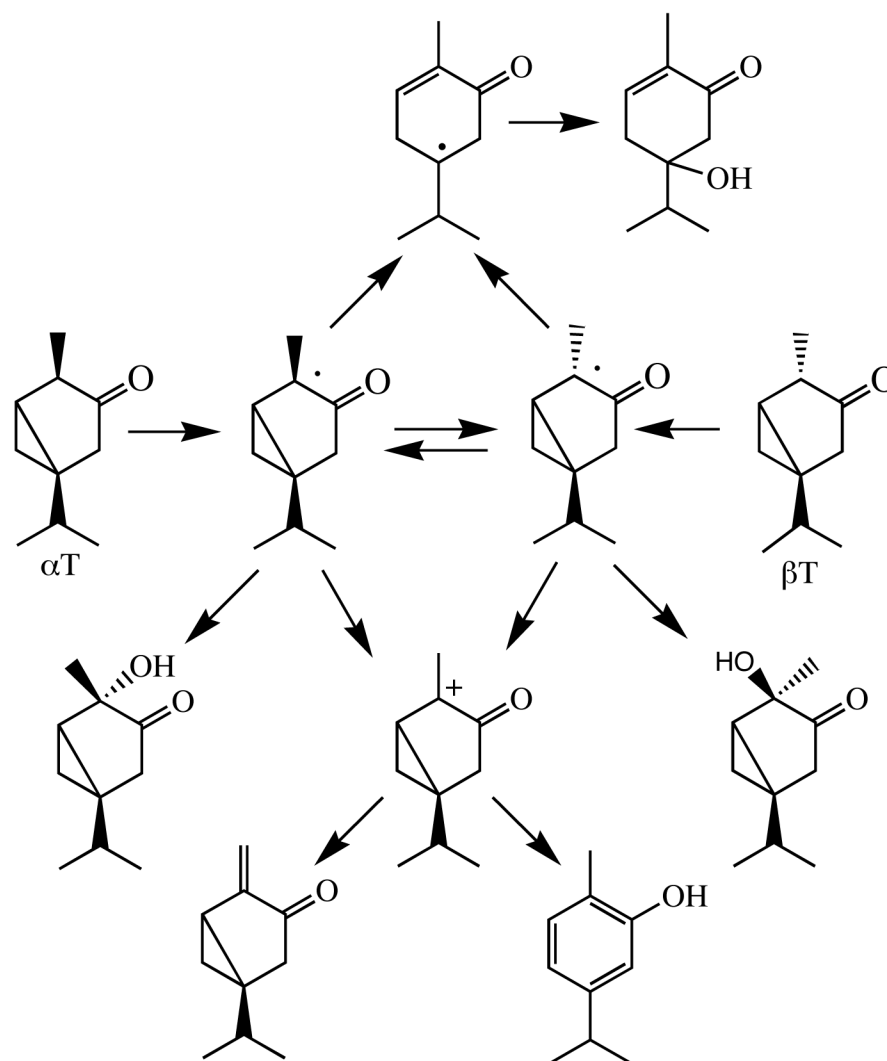
FIGURE 1. Hydrogen bonding network proposed to be involved in conversion of the ferric hydroperoxide complex to the putative ferryl species responsible for substrate oxidation in P450_{cam}. This figure is based on that reported in reference (38).



SCHEME 1.
Synthesis of 7[²H]- α -thujone from 7-hydroxy- α -thujone.

**SCHEME 2.**

Structures of the products formed in the oxidation of α - and β -thujone by P450_{cam}, P450_{BM3}, and the P450_{cam} L244A mutant.

**SCHEME 3.**

Scheme proposed for formation of 4-hydroxy- α -thujone, 4-hydroxy- β -thujone, 4,10-dehydrothujone, carvacrol, and the ring opened product in the P450-catalyzed oxidation of α - and β -thujone at C-4.

Table 1
Product Distributions in the Reactions of P450_{BM-3} and P450_{cam} with α -, β -, and 7[²H]- α -Thujone.^a

	7OH α T (7OH β T)	78DH α T (78DH β T)	2OH α T (2OH β T)	4OH α T	4OH β T	ROP	410DHT	Carv	Other	Conv
P450 _{BM3}										
α T	8.0 \pm 0.6	0.3 \pm 0.03	4.2 \pm 0.5	80.2 \pm 2.5	4.7 \pm 0.9	0.1 \pm 0.06	0.6 \pm 0.06	0.4 \pm 0.2	1.5 \pm 0.4	32 \pm 4
β T	13.3 \pm 2.2	0.5 \pm 0.05	8.6 \pm 0.5	0.6 \pm 0.03	62.1 \pm 2.4	0.05 \pm 0.02	0.14 \pm 0.02	0.1 \pm 0.01	14.6 \pm 2.2	42 \pm 4
P450 _{cam}										
α T	75.9 \pm 3.3	13.6 \pm 3.7	0.3 \pm 0.2	6.1 \pm 0.4	0.4 \pm 0.2	0.02 \pm 0.02	0.1 \pm 0.04	0.15 \pm 0.05	3.4 \pm 1	85 \pm 4
β T	2.4 \pm 0.1	0.5 \pm 0.1	0.1 \pm 0.01	0.09 \pm 0.02	95.3 \pm 0.2	0.1 \pm 0.04	0.1 \pm 0.03	0.2 \pm 0.06	1.2 \pm 0.2	48 \pm 3

^aProduct yields were determined by digital integration of the total GC ion current signal using the resident HP ChemStation software.

Table 2
 Radical Recombination Rates, C-4 Methyl Stereochemistry Ratio, Carvacrol to 4,10DHT Ratio, C-4 to C-7 Product Ratio, and Ratio of C-4 Radical to Cation Products in the Oxidation of α -, β -, and 7[²H]- α -Thujones by P450_{cam}, P450_{BM3}, and the P450_{cam} L244A Mutant.

	substrate	k_1^a ($\times 10^6 \text{ s}^{-1}$)	4OH α T/ 4OH β T	carv/ 410DHT	C-4/ C-7	C-4 radical/ C-4 cation
P450 _{BM3}	α T	3.7 \pm 1.4	17/1	0.7/1	10/1	85/1
	β T	12.5 \pm 3.0	1/104	0.7/1	4.6/1	262/1
P450 _{cam}	α T	2.0 \pm 0.6	15/1	1.5/1	1/13	26/1
	β T	9.5 \pm 1.2	1/1059	2/1	33/1	318/1
	7[² H] α T	3.9 \pm 0.9	26/1	0.8/1	1/1	20/1
L244A	α T	0.7 \pm 0.3	2/1	2.7/1	1/27	15/1
	7[² H] α T	2 \pm 0.8	1.6/1	2.7/1	1/2.5	16/1

^aThe value of $4.4 \times 10^7 \text{ s}^{-1}$ for the rate of cyclopropyl ring opening of α -thujone and $1.0 \times 10^8 \text{ s}^{-1}$ for ring opening of β -thujone was employed in calculating the recombination rates.

Table 3

Product Distributions in the Reactions of P450_{cam} and the P450_{cam} L244A Mutant with α -Thujone and 7[²H]- α -Thujone.^a

	7OH α T (7OH β T)	78DH α T (78DH β T)	2OH α T (2OH β T)	4OH α T	4OH β T	ROP	410DHT	Carv	Other	Conv
P450 _{cam} α T	75.9 ± 3.3	13.6 ± 3.7	0.3 ± 0.2	6.1 ± 0.4	0.4 ± 0.2	0.02 ± 0.02	0.1 ± 0.04	0.15 ± 0.05	3.5 ± 1	89 ± 4
7[² H] α T	31.4 ± 1.2	8.6 ± 0.4	1.6 ± 0.5	33.8 ± 4.0	1.3 ± 0.6	0.04 ± 0.02	1.0 ± 0.1	0.8 ± 0.1	21.5 ± 4.6	70 ± 5
L244A α T	83.0 ± 1.8	9.7 ± 1.1	2.4 ± 0.3	2.1 ± 0.2	1.1 ± 0.5	0.02 ± 0.01	0.06 ± 0.02	0.16 ± 0.04	1.4 ± 0.4	72 ± 6
7[² H] α T	41.5 ± 0.3	7.0 ± 0.7	11.9 ± 1.0	10.8 ± 0.3	6.9 ± 1.0	0.04 ± 0.02	0.3 ± 0.02	0.8 ± 0.1	20.7 ± 1.0	69 ± 3

^aProduct yields were determined by digital integration of the total GC ion current signal using the resident HP ChemStation software.

Table 4
Product distribution in the reactions of human P450 enzymes with α -thujone.^a

	7OH α T	78DH α T	2OH α T	4OH α T	4OH β T	ROP	410DHT	Carv	Other	Conv
1A2	-	-	-	21.7 \pm 0.5	78.4 \pm 0.4	-	-	-	-	0.9 \pm 0.3
2C9	18.1 \pm 0.4	-	-	19.0 \pm 1.2	62.9 \pm 1.5	-	-	-	-	1.3 \pm 0.2
2C19	14.1 \pm 0.4	-	-	19.5 \pm 0.7	66.5 \pm 2.1	-	-	-	-	1.5 \pm 0.2
2D6	58.7 \pm 0.3	9.1 \pm 3.3	-	17.8 \pm 3.4	7.1 \pm 1.2	0.26 \pm 0.1	-	4.3 \pm 1.5	5.3 \pm 1.8	11 \pm 2.3
2E1	15.5 \pm 1.6	-	-	19.4 \pm 1.3	65.1 \pm 0.3	-	-	-	-	1.2 \pm 0.1
3A4	49.6 \pm 0.7	3.8 \pm 1.3	2.2 \pm 0.3	25.8 \pm 2.6	7.4 \pm 0.1	0.3 \pm 0.02	-	3.3 \pm 0.6	5.8 \pm 1.1	37.2 \pm 0.8

^aProduct yields were determined by digital integration of the total GC ion current signal using the resident HP ChemStation software.

Table 5

Radical recombination rates, C-4 methyl stereochemistry ratio, carvacrol to 4,10DHT ratio, C-4 to C-7 product ratio, and ratio of C-4 radical to cation products in the oxidation of α -thujone by human P450 enzymes.

	k_r^a ($\times 10^{10} \text{ s}^{-1}$)	4OH α T/ 4OH β T	C-4/ C-7	C-4 radical/ C-4 cation
1A2	-	1/3.6	-	-
2C9	-	1/3.3	4.5/1	-
2C19	-	1/3.4	6.1/1	-
2D6	0.34 \pm 0.09	2.5/1	1/2.7	16.7/1
2E1	-	1/3.4	5.5/1	-
3A4	0.48 \pm 0.06	3.6/1	1/1.3	10.6/1

^aThe value of $4.4 \times 10^7 \text{ s}^{-1}$ for the rate of cyclopropyl ring opening of α -thujone was employed in calculating the recombination rates.