Radical Rebound Mechanism in Cytochrome P450-Catalyzed Hydroxylation of the Multifaceted Radical Clocks $\alpha$- and $\beta$-Thujone†

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Running title: Thujone Radical Clock Timing of P450 Hydroxylations

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Footnotes

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Abbreviations: Pd, putidaredoxin; PdR, putidaredoxin reductase.
ABSTRACT

α-Thujone (1α) and β-thujone (1β) were used to investigate the mechanism of hydrocarbon hydroxylation by cytochromes P450cam (CYP101) and P450BM3 (CYP102). The thujones are hydroxylated by these enzymes at various positions, but oxidation at C4 gives rise to both rearranged and unarranged hydroxylation products. Rearranged products result from the formation of a radical intermediate that can undergo either inversion of stereochemistry or ring opening of the adjacent cyclopropane ring. Both of these rearrangements, as well as a C4 desaturation reaction, are observed. The ring opening clock gives oxygen rebound rates that range from $0.2 \times 10^{10}$ to $2.8 \times 10^{10}$ s$^{-1}$ for the different substrate and enzyme combinations. The C4-inversion reaction provides independent confirmation of a radical intermediate. The phenol product expected if a C4 cationic rather than radical intermediate is formed is not detected. The results are consistent with a two-state process and provide support for a radical rebound but not hydroperoxide insertion mechanism for P450 hydroxylation.
Cytochrome P450 enzymes catalyze a diversity of oxidations, including reactions such as the hydroxylation of hydrocarbons with high intrinsic activation barriers. A number of experimental observations, including (a) high intrinsic isotope effects for the hydroxylation reaction (1-3), (b) loss of stereochemistry at the hydroxylated center in some substrates (1, 4, 5), and (c) occasional allylic transposition of the site of hydroxylation (6, 7), support the proposal that the reaction proceeds via a free radical oxygen rebound mechanism (1). In this mechanism, the ferryl species [formally Fe(V)=O] formed by cytochrome P450-catalyzed activation of molecular oxygen abstracts a hydrogen from the substrate to give a carbon radical intermediate (R·) that recombines with the formal equivalent of an iron-bound hydroxyl radical [Fe(IV)-OH] to give the final alcohol product (Fig. 1) (8, 9).

Early experiments with radical clock substrates provided persuasive support for an oxygen rebound mechanism. In the first study of this kind, the oxidation of bicyclo[2.1.0]pentane was found to give a ratio of rearranged and unrearranged products that implicated a recombination rate of \(~10^{10}\) s⁻¹, and thus a lifetime of 50 ps, for the carbon radical intermediate (10, 11). Subsequent experiments with related radical clock substrates confirmed this initial result for secondary carbon radicals and suggested a somewhat faster rate for primary carbon radicals (12). However, as faster and faster radical clocks were examined, conflicting results were obtained, in that the calculated rates for the recombination reaction required by the data were so rapid (in the order of \(10^{12}-10^{13}\) s⁻¹) that the existence of a true carbon radical intermediate became questionable (13-15). Rates of this magnitude are more consistent with a transition state rather than an actual intermediate. Furthermore, the subsequent development of probes that can distinguish between radical
and cation intermediates suggested that the intermediate might be predominantly a cation rather than a radical, and therefore that the oxidizing species might be something altogether different from the ferryl species. One alternative hydroxylating species that has been proposed based on these studies is the ferric hydroperoxide complex [Fe(III)-OOH] preceding the ferryl species in the catalytic activation of oxygen by cytochrome P450 (13-16). However, Density Functional Theory calculations predict that this hydroperoxide complex should be a poor hydroxylating agent and only inferential evidence has been advanced for its involvement (17).

Recent work with norcarane, spiro[2.5]octane, and cyclopropyl fatty acid radical clocks concurs with the earlier studies implicating a radical mechanism and suggests radical lifetimes that range from 16 to 50 ps (18, 19), although a different interpretation has been independently advanced for the norcarane result (20). Trace amounts of cationic rearrangement products were detected in the norcarane but not in the cyclopropyl fatty acid studies. Analysis of these results suggested that differences in the measured radical lifetimes might reflect steric or electronic differences, as secondary radicals generally give slower apparent recombination rates than primary radicals (18).

Computational methods brought to bear on this mechanistic conundrum have provided insight into the possible origin of the discrepancies among the experimental results (21-25). In particular, Density Function Theory calculations suggest a two-state reactivity paradigm in which hydrogen abstraction yields either a doublet or quartet radical species, depending on which of the two possible spin states of the ferryl complex actually abstracts the hydrogen. The calculations show that the low spin species recombines without a measurable barrier to give the alcohol, whereas the quartet species must overcome an
energy barrier and is thus susceptible to competing reactions, such as rearrangements, prior to recombination to give the alcohol. The proportion of the two spin states is sensitive to the environment, so that different extents of doublet and quartet reactions are possible with different enzymes and different substrates. In the case of radical clock substrates, the apparent radical recombination rate will be artificially elevated by the extent of the doublet reaction, as the unrearranged product formed by this pathway will be added to that formed by the quartet reaction, the only one of the two reactions that is in fact relevant to determination of the recombination rate (25). The measured recombination rates thus represent the maximum possible rather than necessarily the actual rate, as it assumes that all the reaction proceeds via the quartet state.

The metabolism and biology of α- and β-thujone (1α and 1β, respectively) have been scrutinized because of the putative role of α-thujone in the physiological action of absinthe. Previous studies of α-thujone, which interacts with the GABA type A receptor, have shown that it is quickly metabolized in vitro by mouse liver microsomes (26). The principal product is 7-hydroxy-α-thujone (8), but five other compounds were found as minor metabolites: 4-hydroxy-α-thujone (3α), 4-hydroxy-β-thujone (3β), two other hydroxythujones and 7,8-dehydro-α-thujone (12) (26-28) (Fig. 2 and 5). From a mechanistic point of view, α- and β-thujone are interesting because hydroxylation at C4 should generate, respectively, the radicals 2α and 2β (or simply 2, if C-4 becomes planar) that can undergo both hydroxylation with inversion of stereochemistry and cyclopropyl ring opening to give a monocyclic alcohol product (Fig. 2). Formation of the two possible 4-hydroxy isomers in the mouse metabolism studies indicates that the
stereochemical inversion is feasible, but the reaction was not investigated and the ring opened product was not reported. In preliminary work, we have chemically generated the C4 radicals expected from of α- and β-thujone and have investigated their chemistry (29). These studies indicate that when \(2\alpha\) and \(2\beta\) are chemically generated they undergo cyclopropyl ring opening at rates of \(4.4 \times 10^7\) and \(1.0 \times 10^8\) s\(^{-1}\), respectively (29). The stereochemistry of radical quenching in these chemical studies established that the C4 radical readily undergoes inversion, but quenching of the radical is largely controlled by steric effects that result in delivery of the quenching hydrogen atom almost exclusively from the α-side regardless of whether the radical is initially generated on the α- or β-side. Furthermore, the chemically generated thujone C4 cation was shown to rapidly rearrange to carvacrol, a phenolic product not observed in the free radical reactions (29).

**Experimental Section**

*General materials.* Unless otherwise mentioned, all reagents were from Sigma-Aldrich (St. Louis, MO). Organic solvents, 4-toluenesulfonic acid, yeast extract, 2YT, sodium chloride, potassium chloride and Tris-Cl were purchased from Fisher Scientific (Fair Lawn, NJ). α-Thujone, terpinen-4-ol and (-)-terpinen-4-ol were purchased from Acros Organics. All the chemicals were used without further purification. The UV-visible spectra were recorded on a Hewlett-Packard 8452A diode array spectrometer or a Cary 1E Varian UV-visible spectrometer. GC and GC-MS analyses were performed on a Hewlett Packard Model 5890 gas chromatograph with a J&W DB-17 or DB-1 50%-phenylmethylpolysiloxane capillary column.
Synthesis. β-Thujone (1β), 4,10-dehydrothujone (11), 4-hydroxy-α-thujone (3α), 4-hydroxy-β-thujone (3β), (4S)-(+)4-hydroxy-p-menth-1-en-6-one (5) and (4R)-(−)-4-hydroxy-p-menth-1-en-6-one (6) were prepared according to published procedures (30). As shown by gas chromatography, the β-thujone sample contains 2-3% α-thujone, but this was taken into account in the calculations for the enzymatic studies. The purity of all the intermediates and products was confirmed by 1H NMR and GC-MS.

Enzymes and binding assays. P450<sub>cam</sub> (CYP101), putidaredoxin (Pd), and putidaredoxin reductase (PdR) from <i>Pseudomonas putida</i> were expressed heterologously in <i>E. coli</i> and were purified as previously reported (31). The purified proteins containing camphor were flash-frozen over dry ice and stored at −70 °C. P450<sub>BM3</sub> was kindly provided by Dr. Karine Auclair. The K<sub>s</sub> values for the association of α-thujone and β-thujone with the two different P450 enzymes were determined by UV spectroscopy. The camphor present in the P450<sub>cam</sub> sample was removed using a PD-10 column with 50 mM Tris at pH 7.4 containing 200 mM KCl. The binding assays were performed immediately after P450<sub>cam</sub> was buffer-exchanged (P450<sub>cam</sub> is unstable to freezing/thawing in the absence of camphor). The increase in the absorbance of the high-spin species at 391 nm and the decrease in the absorbance of the low-spin species at 416 nm were monitored upon addition of α-thujone. In the case of β-thujone with P450<sub>cam</sub>, the increase in the absorbance at 411 nm and the decrease in the absorbance at 416 nm were monitored instead, as the typical increase at 390 nm was not observed.

Enzyme incubations. Unless otherwise indicated, the buffer was 100 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.4. In the case of P450<sub>cam</sub>, the incubations contained P450<sub>cam</sub> (0.5 µM), Pd (5 µM),
PdR (5 μM), α-thujone (2000 μM), superoxide dismutase (2 μM), and catalase (100 μg/mL) in the reaction buffer. The reaction was initiated by addition of NADH (2000 μM). In the case of P450BM3, the final incubation contained P450BM3 (2 μM), superoxide dismutase (2 μM), α-thujone (2000 μM), and NADPH (2000 μM). Control reactions were carried out without enzyme and, separately, without NADPH/NADH. All the reaction mixtures (500 μL) were incubated at 37 °C in 1.5 mL Eppendorf tubes for 1 h before being extracted with CH2Cl2 (250 μL x 2). The organic layer was dried over anhydrous Na2SO4, concentrated to 100 μL, and analyzed by GC and/or GC/MS.

Results

Binding of α- and β-thujone to P450cam and P450BM3. The binding affinities of α and β-thujone to P450cam and P450BM3 were measured by UV spectroscopy. In most cases, the binding of a substrate to a P450 enzyme displaces the distal water ligand from the heme iron atom. This event is observed in the ultraviolet spectrum as a decrease in the intensity of the water-ligated absorption maximum at 416 nm and a concomitant increase in the band at 391 nm of the substrate-bound enzyme without a coordinated water molecule (32, 33). These changes are observed when α-thujone is bound to P450cam and P450BM3, and when β-thujone is bound to P450BM3. However, when β-thujone binds to P450cam, the increase at 391 nm is not observed (Fig. 3). Instead the absorption maximum at 416 simply shifts to 412 nm. Spectroscopic binding constants (Ks) were calculated from the changes in the difference between the absorption at 391 and 416 nm except for the case of β-thujone and P450cam, for which the changes in the absorption bands at 412 and 416 nm were used (Table 1). These results indicate that P450cam has a slightly higher affinity for α- than β-
thujone, whereas the affinity of P450BM-3 for α-thujone is significantly (5- or 6-fold) higher than for β-thujone. However, both compounds bind to both enzymes with affinities comparable to those for other non-native substrates.

Oxidation of α-thujone. α- and β-Thujone were incubated with P450cam in the presence of Pd, PdR, and NADH. Superoxide dismutase and catalase were included in the incubation to consume any superoxide or H₂O₂ generated in the incubation by uncoupled oxygen reduction. The reaction of P450cam with α-thujone (Fig. 4) yielded 76% 7-hydroxy-α-thujone (8) as the major product, 4.5% 4-hydroxy-α-thujone (3α), 0.3% 4-hydroxy-β-thujone (3β), 0.1% 4,10-dehydrothujone (7), and 0.1% (4S)-(+)4-hydroxy-p-menth-1-en-6-one and/or (4R)-(−)-4-hydroxy-p-menth-1-en-6-one (5/6), as well as other hydroxylated or dehydrogenated products (19%) (Fig. 5 and Table 2). The overall conversion of α-thujone to products was 97%. In contrast, the reaction of P450BM3 with α-thujone yielded 77% 4-hydroxy-α-thujone (3α), 2.1% 4-hydroxy-β-thujone (3β), 13% 7-hydroxy-α-thujone (8), 0.6% of the rearranged products 5/6, and 6% of other products (Table 2). In this case, the overall conversion of α-thujone was 7%. No carvacrol (18) was detected as a metabolite in either reaction despite a specific search for this compound with the help of an authentic standard.

Oxidation of β-thujone. The reaction of P450cam with β-thujone yielded 90% 4-hydroxy-β-thujone (3β), 0.6% 4-hydroxy-α-thujone (3α), 0.2% 4,10-dehydrothujone (7), 0.3% of rearranged products 5/6, and 6% of 7-hydroxy-β-thujone (13) (Fig. 6 and Table 2). The conversion of β-thujone is 72%. Likewise, the reaction of P450BM3 with β-thujone yielded 51% 4-hydroxy-β-thujone (3β), 1.9% 4-hydroxy-α-thujone (3α), 0.3% 4,10-
dehydrothujone (7), 0.4% of rearranged products 5/6, and 21% of 7-hydroxy-β-thujone (13), with other hydroxylated products accounting for the rest (Table 2). The conversion yield was 14% in this case. No carvacrol was detected as a metabolite in either incubation.

**Discussion**

P450\textsubscript{cam} and P450\textsubscript{BM-3} are well-characterized bacterial enzymes for which crystal structures have been determined with and without ligands bound in the active site cavity (34-37). As such, they are highly attractive enzymes for the investigation of mechanistic questions. We have therefore employed these two enzymes in conjunction with the new radical clock probes α- and β-thujone to investigate the mechanism of hydrocarbon hydroxylation. As shown in Fig. 3 and Table 1, both of these probes bind with good affinity to P450\textsubscript{cam} and P450\textsubscript{BM-3}. The spectroscopic changes observed on addition of α-thujone to both enzymes are those expected for binding of the organic ligand within the active site with concomitant displacement of the distal water ligand from the heme iron atom (32, 33). Similar spectroscopic changes are observed on binding of β-thujone to P450\textsubscript{BM-3}, but its binding to P450\textsubscript{cam} gives rise to an anomalous spectroscopic transition in which the maximum of the resting state of the enzyme at 416 nm is slightly shifted to 412 nm rather than to the usual 390 nm. This spectroscopic shift, which corresponds to a so-called reverse Type I spectrum (32), suggests that a perturbed sixth iron ligand is retained in the complex. The carbonyl group of the β-thujone may replace the water as a ligand to the iron, or the water ligand may be retained but is perturbed by the presence of β-thujone. This perturbation could result from changes in active site polarity or in the hydrogen bonding pattern of the water ligand.
The two thujone probes not only bind tightly to P450\textsubscript{cam} and P450\textsubscript{BM3}, but are also accepted by these enzymes as substrates. Incubation of P450\textsubscript{cam} with the thujone isomers gives mixtures of products. P450\textsubscript{cam} primarily hydroxylates \(\alpha\)-thujone on the tertiary carbon of the isopropyl group to give 8 (76%), but largely hydroxylates \(\beta\)-thujone at C4 to give the unrearranged alcohol 3\(\beta\) (90%). P450\textsubscript{BM3}, in contrast, hydroxylates \(\alpha\)-thujone at the C4 position to give the unrearranged alcohol 3\(\alpha\) (77%), and \(\beta\)-thujone at both the C4 position to give the unrearranged alcohol 3\(\beta\) (51%) and the tertiary carbon of the isopropyl group to give 13 (21%). In all cases the preferred site of oxidation is a tertiary C-H bond, in accord with the inverse correlation between bond strength and susceptibility to oxidation (38-41). The selectivity for C4 versus C7 oxidation must reflect differences in the binding orientations of the thujones within the active sites of the two enzymes. Indeed, evidence for different orientations is provided by the spectroscopic data discussed above, which suggests that the binding of \(\beta\)-thujone to P450\textsubscript{cam} differs from both that of its binding to P450\textsubscript{BM3} and of the binding of \(\alpha\)-thujone to either enzyme.

Previous studies of the metabolism of \(\alpha\)-thujone in mice have shown that the principal metabolite in that species, as found here for P450\textsubscript{cam} but not P450\textsubscript{BM3}, is the 7-hydroxy product 8 (26, 28). The same is true in the metabolism by rat and human liver microsomes, except that the 4-hydroxy metabolite 3\(\alpha\) and the 7,8-dehydro product 12 are also significant metabolites. Metabolism of \(\beta\)-thujone by the mouse, rat, and human primarily gives the 7- and 4-hydroxy metabolites, in agreement with the finding here that the principal route of oxidation of this compound by P450\textsubscript{cam} and P450\textsubscript{BM3} is 4-hydroxylation to give 3\(\beta\) (Table 1).
More importantly, the two rearrangement reactions observed in the hydroxylation of α-thujone by P450$_{cam}$ generate the minor products $3\beta$ (0.3\%) and $5/6$ (0.1\%). Both of these rearrangement products stem from oxidation of the substrate at C4. In addition, two other minor metabolites arise from oxidation at C4, the unrearranged alcohol $3\alpha$ (4.5\%) and the dehydrogenation product $7$ (0.1\%). Although C4 is not the major site of the oxidation reaction, the products of oxidation of this carbon provide information on the mechanism of the hydroxylation reaction. All three products can be rationalized by initial hydrogen abstraction by the enzyme ferryl species to give the C4 α-thujone radical $2\alpha$. Recombination of this radical with the iron-bound hydroxyl species yields the unrearranged alcohol $3\alpha$, the major product of the reaction at this site. However, to the extent that inversion of the C4 radical stereochemistry competes with radical recombination, the rearranged alcohol $3\beta$ is also obtained (Fig. 2). The cyclopropyl group can undergo ring opening in competition with the C4 inversion reaction, resulting in formation of the monocyclic products $5$ and/or $6$. Finally, formation of the desaturated product $7$ can be rationalized either by conversion of the C4 radical to a cation concomitant with abstraction of a proton from the methyl group, or by abstraction of the vicinal methyl hydrogen atom by the ferric hydroxide species obtained after the first hydrogen abstraction. Studies of the isotope effects associated with P450-catalyzed dehydrogenation reactions indicate that the initial step is usually hydrogen abstraction from the carbon with the weaker C-H bond, followed by loss of a hydrogen from an adjacent carbon to give the double bond (42-44). The first abstraction is associated with a large isotope effect and the second only with a weak one. A recent computational study has concluded that P450-catalyzed desaturations proceed via a cationic intermediate and are favored by steric hindrance to recombination of
the initial carbon radical and iron-bound oxygen (45). Interestingly, a previous attempt to
demonstrate the involvement of a radical intermediate in a non-P450 desaturation reaction
by placing a cyclopropyl ring on the carbon that is oxidized was unsuccessful, presumably
because the cyclopropyl ring opening was too slow in that system to compete with the
desaturation process (46).

The ratio of the C4-derived products depends on the relative rates of the reactions that
channel the C4 radical into the four different products. In previous chemical studies, we
have calculated that opening of the cyclopropyl ring in the α-thujone C4 radical occurs at a
rate of 4.4 x 10⁷ s⁻¹ (29). From this value and the ratio of the ring opened products 5/6 to
the unrearranged alcohol isomers 3α plus 3β, it is possible to calculate a value (k₁) for the
radical recombination reaction of the α-thujone radical 2α (or 2) according to the following
equation:

\[
k₁ = \left[ \frac{3α + 3β}{5/6} \right] \times 4.4 \times 10⁷ \text{ s}^{-1}
\]

The value thus calculated is \( k₁ = 0.2 \times 10^{10} \text{ s}^{-1} \) (Table 1).

It is not possible to determine the recombination rate independently using the rate of
methyl group inversion as the “clock” because our chemical studies showed that there is an
inherent bias in solution for quenching of the radical from the less stereochemically
hindered α-face. A similar bias is observed in the reactions of the C4 enolate with
alkylating agents (29). In the chemical studies, the ratio of α- to β-products is determined
primarily by the differential rates of hydrogen atom transfer to the stereoisomeric radicals.
This stereochemical bias precluded calibration of the rate of the inversion reaction and, indeed, left open the possibility that the C4 radical is planar rather than a pair of inverting tetrahedral structures. From the present results we can say that the α-thujone C4 radical undergoes inversion at approximately the same rate as the ring opening reaction because we obtain approximately as much 3β as 5/6, but we cannot obtain an independently determined radical recombination rate. It is remarkable in this context that recombination to give 3α competes very effectively with formation of 3β in the oxidation of 1α, as chemical quenching of 2α and 2β with thiophenol resulted almost exclusively in delivery of the hydrogen atom to the α-face regardless of which radical conformation was initially generated. The hydroxylated product predicted by the chemical results would be 3β which is found as the minor of the two products Thus, despite the inherent steric preference for delivery of the quenching moiety from the α-face indicated by the chemical studies, delivery to the β-face in the enzymatic hydroxylation reaction is an effective process. The same result is obtained in the oxidation of α-thujone by P450BM3, for which the 3α:3β product ratio is approximately 6:1. This may reflect the much faster rate of the oxygen rebound than hydrogen transfer reaction, or an ability of the active site to overcome the intrinsic sterochemical bias by binding and orienting the substrate.

If the cyclopropyl ring opening reaction is used as an internal clock, it appears that dehydrogenation of the C4 radical occurs at approximately the same rate as opening of the cyclopropyl ring, as the dehydrogenated product 7 is formed in approximately the same amounts as the ring-opened products 5/6 in the oxidation of both α- and β-thujone by P450cam and P450BM3 (Table 1).
The oxidation of β-thujone by P450<sub>cam</sub> occurs primarily at C4, yielding 90% of the unisomerized alcohol 3β. In addition, small amounts of the inverted alcohol 3α and of the ring opened products 5/6 are also obtained (Table 2). Analysis of the data for the P450<sub>cam</sub>–catalyzed oxidation of β-thujone as done for the α-thujone oxidation, using the rate of 1.0 x 10<sup>8</sup> s<sup>-1</sup> for ring opening of the radical 2β (29), gives a recombination rate of 2.8 x 10<sup>10</sup> s<sup>-1</sup>. Similar analysis of the oxidation of α-thujone by P450<sub>BM3</sub> results in a calculated rate of 0.6 x 10<sup>10</sup> s<sup>-1</sup> for the recombination rate (Table 1). The corresponding rate determined from the data for the reaction of P450<sub>BM3</sub> with β-thujone is 1.6 x 10<sup>10</sup> s<sup>-1</sup>. These values are consistent with values previously reported for secondary radicals (10, 11, 18, 19). The variation among them presumably reflects the differences in the catalytic environment in the various substrate-enzyme complexes.

Generation of the thujone C4 cation by non-enzymatic means shows that it rearranges almost exclusively to the phenol carvacrol (Fig. 7) (29). Only a tiny trace (1-2%) of a peak with the retention time of 5/6 and no trace of 7 were found in these chemical studies. It is therefore significant that no trace of carvacrol is found in the incubations of the P450 enzymes with either α- or β-thujone. This indicates that the cationic intermediate is not detectably formed. Interestingly, a trace of the unsaturated product 7 is found in our incubations (Table 1). Computational studies suggest that desaturation products are formed via the cation (45). However, they also indicate that the desaturation reaction occurs within a very tight complex of the iron-hydroxyl intermediate and the carbon radical generated by the initial hydrogen abstraction (45). If the cation is actually formed, this intimate ion pair mechanism may channel the cation towards the desaturated product, in contrast to its fate in solution (29). The absence of carvacrol, and the formation of only a trace of the desaturated
product, argue against a significant role for the cationic species in this P450 oxidation. This result is inconsistent with a mechanism that invokes oxidation of the C-H bond by the iron hydroperoxy complex, as the cation would be expected to be a central intermediate in such a reaction (15).

In sum, α- and β-thujone are oxidized at C4 by P450 enzymes to give, *inter alia*, products in which (a) the stereochemistry of the oxidized center is inverted, (b) the adjacent cyclopropyl ring undergoes ring opening, or (c) a double bond is introduced by a dehydrogenation reaction. Furthermore, the ratio of the ring opened products to α-hydroxylated thujones (3α β) indicates that the reaction proceeds via a radical mechanism with a radical recombination rate that ranges from 0.2 x 10^{10} to 2.8 x 10^{10} s^{-1}. Apart from unavoidable experimental error, this variation reflects the dependence of the oxygen rebound rate on the specific substrate and enzyme. Theoretical calculations suggest that the measured rate is at least partially determined by the ratio of the two spin states of the ferryl oxidizing species (25). In the extreme situation, where the low spin state only gives unrearranged product and the high spin state only rearranged product, the ratio of rearranged to unrearranged product would be determined by the ratio of the two spin states. The radical clock measurements truly measure the recombination rate if only the high spin state is significantly populated, but as the proportion of the low spin state increases in importance it will elevate the amount of unrearranged product that is formed by a process that does not compete with rearrangement. This results in an artificially high calculated radical recombination rate. Thus, the rates reported here are the maximum possible and some variation in rates is to be expected as the enzyme active site and substrate are changed. The simultaneous observation of inversion of stereochemistry, a reaction that is
faster than ring opening, without the formation of carvacrol via a cation pathway strongly
supports the proposal of a free radical rather than OH\(^+\) insertion hydroxylation mechanism.
References


Table 1. Spectroscopic binding constants and radical recombination rates in the oxidation of α- and β-thujones by P450<sub>cam</sub> and P450<sub>BM3</sub>

<table>
<thead>
<tr>
<th></th>
<th>K&lt;sub&gt;s&lt;/sub&gt; (µM) at 25 °C</th>
<th>k&lt;sub&gt;i&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>α-Thujone</td>
<td></td>
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<tr>
<td>P450&lt;sub&gt;cam&lt;/sub&gt;</td>
<td>13 ± 1</td>
<td>(0.2 ± 0.1) x 10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>P450&lt;sub&gt;BM3&lt;/sub&gt;</td>
<td>2 ± 0.4</td>
<td>(0.6 ± 0.3) x 10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-Thujone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450&lt;sub&gt;cam&lt;/sub&gt;</td>
<td>17 ± 1</td>
<td>(2.8 ± 0.5) x 10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>P450&lt;sub&gt;BM3&lt;/sub&gt;</td>
<td>13 ± 3</td>
<td>(1.6 ± 0.4) x 10&lt;sup&gt;10&lt;/sup&gt;</td>
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<sup>a</sup>The value of 4.4 x 10<sup>7</sup> s<sup>-1</sup> for the rate of cyclopropyl ring opening of 2α and 1.0 x 10<sup>8</sup> s<sup>-1</sup> for ring opening of 2β was employed in calculating the recombination rates (29). The ratio of 3α + 3β to 5/6 was used as a measure of the ring-opened to unopened products in the calculations.
**Table 2.** Product formation in the reactions of P450\textsubscript{cam} and P450\textsubscript{BM3} with \(\alpha\)- and \(\beta\)-thujone

<table>
<thead>
<tr>
<th>Product (relative yield)</th>
<th>Conversion (%)</th>
<th>TON (%)</th>
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<tbody>
<tr>
<td>(\alpha)</td>
<td>(\beta)</td>
<td>5/6</td>
</tr>
<tr>
<td>P450\textsubscript{cam}</td>
<td>4.5±0.2</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>P450\textsubscript{BM3}</td>
<td>77±0.7</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>P450\textsubscript{cam}</td>
<td>0.6±0.2</td>
<td>90±0.1</td>
</tr>
<tr>
<td>P450\textsubscript{BM3}</td>
<td>1.9±0.3</td>
<td>51±0.6</td>
</tr>
</tbody>
</table>

\(a\) Product yields were determined by digital integration of the total GC ion current signal using the resident HP ChemStation software. Product identity was confirmed by retention time and mass spectrometric fragmentation pattern. Products reported in less than 1% relative yield were assigned by comparing their retention times with authentic standards: 5 and 6 are indistinguishable by GC. TON indicates turnover number. Products designated “nd” were below detectable limits with a good baseline.
Figure Legends

**Fig. 1.** The “oxygen rebound” mechanism of cytochrome P450 catalyzed hydroxylations.

**Fig. 2.** The methyl flipping and ring opening reactions undergone by the thujone C4 radical. The radical recombination rates of the isomeric radicals $2\alpha$ and $2\beta$ with the ferryl oxygen are given by $k_1$ and $k_2$, respectively. The rates of rearrangement of $2\alpha$ and $2\beta$ to 4 are given by $k_{r\alpha}$ and $k_{r\beta}$ respectively.

**Fig. 3.** Spectroscopic changes observed on binding of β-thujone to P450<sub>cam</sub>. The spectra of substrate-free P450<sub>cam</sub> (solid line) and β-thujone-bound P450<sub>cam</sub> (broken line) are shown.

**Fig. 4.** GC traces for the reactions of P450<sub>cam</sub> and P450<sub>BM3</sub> with α- and β-thujone. A trace is shown at the bottom of a solution of authentic standards, identified in the figure by structure number.

**Fig. 5.** Products resulting from formation of a radical intermediate upon P450-catalyzed oxidation of α-thujone.

**Fig. 6.** Products resulting from formation of a radical intermediate upon P450-catalyzed oxidation of β-thujone.

**Fig. 7.** Carvacrol (18), the product expected from formation of a thujone C4 cationic intermediate and the sequence of steps that result in its formation. Carvacrol is not observed in any of the P450 reactions.
Fig. 1.
Fig. 2.
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Radical rebound mechanism in cytochrome P450-catalyzed hydroxylation of the multifaceted radical clocks α- and β-thujone
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