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Cooperative Effects on Radical Recombination in CYP3A4-Catalyzed Oxidation of the Radical Clock β-Thujone

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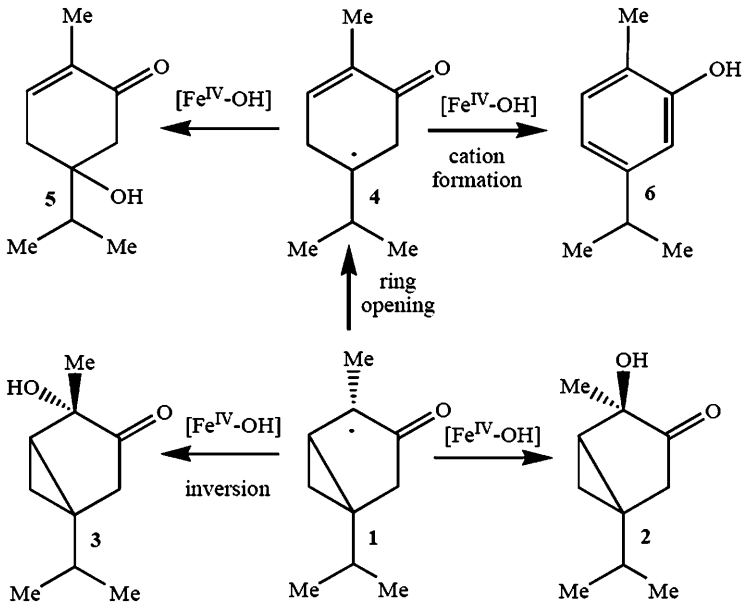
Oxidation of hydrocarbons by cytochrome P450 enzymes is commonly thought to involve hydrogen abstraction by a ferryl species comparable to that of peroxidase Compound I (Cpd I), followed by recombination of the resulting carbon radical with the equivalent of an iron-bound hydroxyl radical.[1,2] This radical rebound mechanism, which was first proposed in 1978, is supported by a variety of experimental results, including: 1) rearrangement and inversion reactions prior to the radical recombination step, 2) the large magnitude (up to $k_H/k_D \approx 13$) of the intrinsic isotope effect for hydrogen abstraction, and 3) computational modeling of the reaction pathway. However, radical clock substrates, in which the radical undergoes a rearrangement at a known rate prior to radical recombination, have provided conflicting evidence on the radical lifetime. Although several radical clocks support a radical recombination mechanism, the so-called ultrafast radical clocks yield radical lifetime estimates that are more consistent with a transition state than an actual intermediate.[3] This discrepancy has led to the postulate that hydroxylation might involve either a concerted insertion into the C–H bond or multiple oxidizing species. An alternative explanation is provided by computational studies that invoke a reaction manifold with a radical intermediate that exists in two different spin states.[4] A further possible explanation is provided by the observation that ultrafast radical clocks generally involve primary radical rearrangements, whereas slower radical clocks generally involve secondary radical rearrangements. The recombination rates of primary and secondary radicals could be differentially susceptible to modulation by interactions with the active site and the iron-oxo species. However, there is little direct evidence that the radical complex exists in two different spin states, or that the radical recombination rates can be influenced by the active-site environment.

CYP3A4 is responsible for a majority of all P450 catalyzed drug oxidations in humans. One of the salient features of CYP3A4 catalysis is that it is subject to homotropic and heterotropic cooperativity (allosterism) in that one substrate molecule can alter the oxidation rate and/or regioselectivity of another.[5] The molecular basis of P450 allosterism remains incompletely understood. Currently, most results fall into three models: multisubstrate binding, peripheral effector binding and conformational heterogeneity.[6] Progesterone has specifically been shown to function as an allosteric modulator of its own CYP3A4 catalyzed oxidation as well as that of other substrates.[7,8] Given that allosterism tunes the CYP active-site environment, its effect on the reaction of a radical-clock substrate might provide valuable mechanistic information associated with the CYP mechanism. In this communication, we report that the oxidation of a radical clock, β-thujone (**1**, shown as its C4 radical in Scheme 1), by CYP3A4 is subject to the concentration-dependent, heterotropic, cooperative effect of progesterone binding on the manifold of



Scheme 1. Reaction manifold showing the intermediate generated by C4 hydrogen abstraction from β-thujone and the products that derive from this initial intermediate.

products that derive from C4 oxidation. The results are consistent with acceleration by progesterone of the radical recombination that traps the C4 radical with concomitant suppression of all the rearrangement pathways.

We have previously shown that α- and β-thujones function as two-zone radical clocks in that C4 oxidation triggers two concurrent timing reactions, one which involves ring opening of the cyclopropyl ring and the other inversion of the stereochemistry of the C4 methyl group (Scheme 1).[9] The α- and β-thujone, C4 radical, ring-opening reaction rates are 4.4×10^7

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and $1.0\times10^8\ s^{-1}$, respectively, both of which are consistent with the lifetimes of radical intermediates. This is also consistent with the observation of a significant extent of methyl inversion in these reactions. In addition to hydroxylated products, this reaction manifold gives rise to two minor desaturated products, one with a C4=C10 double bond and another (**6**, carvacrol), in which the ring aromatizes to give a phenol product. The formation of these two products was attributed to a second oxidation of the C4 radical intermediate. The latter was previously shown to be generated from a cationic intermediate.[10]

In this study, the CYP3A4 reaction of α- or β-thujone (100 μM) was carried out in the presence of concentrations of progesterone rising from 0 to 150 μM. The incubation mixture contained purified, recombinant, human CYP3A4, NADPH-cytochrome P450 reductase, and cytochrome b_5 in a 1:2:1 ratio, respectively, all in a reconstituted lipid environment.[11] The identified metabolites include 7-hydroxyl-α(or β)-thujone, 7,8-dehydro-α-thujone, 2-hydroxyl-α(or β)-thujone, 4-hydroxyl-α-thujone (**3**), 4-hydroxyl-β-thujone (**2**), carvacrol (**6**) and the ring-opened product **5**.[9] These are all the major metabolites formed in the reaction and account for ≈95% and ≈90% of the total yield for α- and β-thujone, respectively. 4,10-Dehydrothujone was previously identified in the reactions of the thujones with P450cam and P450M3, but not with mammalian P450 enzymes.[9] This metabolite was found to conjugate with glutathione in the CYP3A4 solution, and the conjugate could not be extracted with the hydrophobic metabolites (data not shown). It was reported recently for norcarane that desaturation products and their secondary metabolites might compromise its use in measuring radical lifetimes.[12,13] This was not observed in the CYP3A4 oxidation of thujones, for which no secondary metabolites of the desaturated products were detected at a significant level (>1%; Figure S1 in the Supporting Information).

The distribution and yields of the metabolites in the CYP3A4 oxidation of β-thujone in the presence of various concentrations of progesterone are summarized in Tables 1 and S1. Not unexpectedly, a decrease from 15.4 to 11.7% in the proportion of 7-hydroxylation of β-thujone is observed as the progesterone concentration increases from 0 to 150 μM. A smaller progesterone-dependent decrease is also seen in 2-hydroxylation and the desaturation of the isopropyl group. These conventional metabolic shifts reflect repositioning of the substrate prior to the oxidation step and provide no information on the reaction mechanism. However, the alterations in the product distributions that stem from the oxidation of C4, the carbon that is pivotal for the radical-clock mechanisms of β-thujone (Scheme 1), are more informative. As shown in Figure 1, the proportion of 4-hydroxy-β-thujone (**2**), the unrearranged product, increases systematically from 65.6 to 78.3% as the progesterone concentration increases from 0 to 150 μM. In contrast, there is a corresponding systematic decrease in the yield of 4-hydroxy-α-thujone (**3**), in which the methyl stereochemistry is inverted (from 10.7% to 6%), metabolite **5**, in which the cyclopropyl ring is opened (from 0.61% to 0.24%), and carvacrol (**6**), the aromatic product produced by cyclopropyl-ring opening and cation formation (from 3.5% to 1.5%). A direct correlation exists between the decrease in the combined rearrangement products (**3**+**5**+**6**) and the increase in the unrearranged

Table 1. Allosteric effects of progesterone on the CYP3A4 metabolism of β-thujone at C4.[a,b]

PG [μM]	4OHβT (**2**)	4OHαT (**3**)	ROP (**5**)	Carv. (**6**)	k_1[c] [$\times10^{10}\ s^{-1}$]
0	65.6 (1.00)	10.7 (1.00)	0.61 (1.00)	3.5 (1.00)	1.3
5	68.9 (1.05)	9.9 (0.92)	0.54 (0.88)	3.3 (0.94)	1.5
25	72.8 (1.11)	8.4 (0.78)	0.4 (0.66)	2.5 (0.71)	2.0
50	74.2 (1.13)	7.5 (0.70)	0.39 (0.64)	2.2 (0.63)	2.1
80	75.8 (1.16)	7.0 (0.65)	0.33 (0.53)	1.9 (0.54	2.5
100	77.2 (1.18)	6.6 (0.62)	0.28 (0.46)	1.7 (0.49)	3.0
150	78.3 (1.19)	6.0 (0.56)	0.24 (0.39)	1.5 (0.43)	3.5

[a] The yield of the product is given as a percent of the total products. In the parenthesis is the relative amount of the product at a given progesterone concentration relative to the yield, which was set at 1.0 for each metabolite, in the absence of progesterone. The plot in Figure 2 is based on these relative yields. The abbreviations are: PG, progesterone; 4OHβT, 4-hydroxy-β-thujone (**2**); 4OHαT, 4-hydroxy-α-thujone (**3**); ROP, ring-opened product **5**; Carv, carvacrol (**6**). [b] The results are the average values from a set of three incubations. [c] The value of $1.0\times10^8\ s^{-1}$ for the rate of the cyclopropyl-ring opening of β-thujone was employed in calculating the recombination rates; $k_1=(1.0\times10^8)\times([4HO\beta T]+[4HO\alpha T])/[ROP]$.

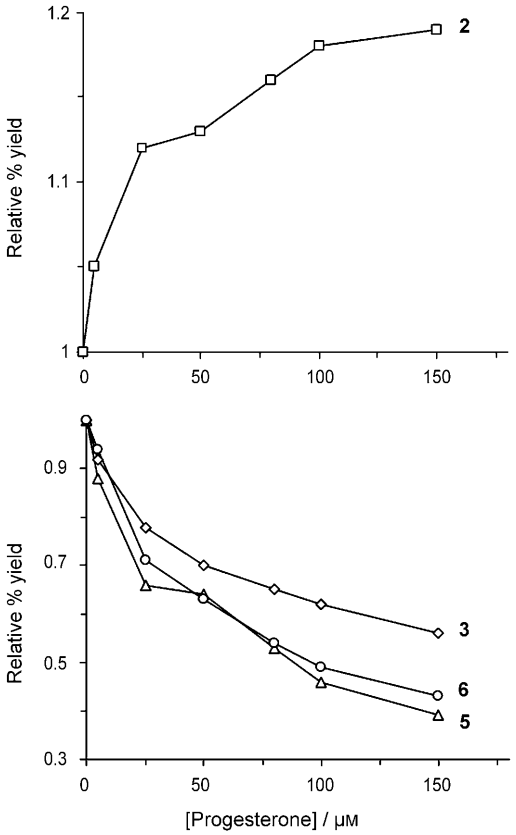

Figure 1. Relative yields [%] of 4-hydroxylated β-thujone (**2**), 4-hydroxylated α-thujone (**3**), cyclopropyl ring-opened thujone alcohol (**5**) and the aromatized product carvacrol (**6**) formed during the oxidation of β-thujone by CYP3A4 in response to increasing progesterone concentrations.

alcohol (**2**, Figure 2). This shows that the loss in the rearranged products is quantitatively correlated with the increase in formation of the unrearranged alcohol (**2**). While it has been well

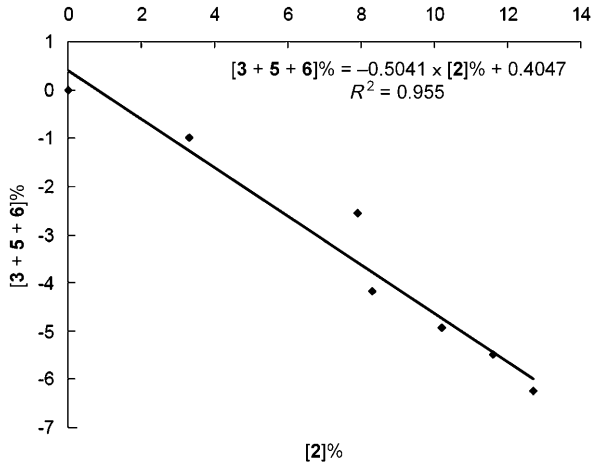

Figure 2. Increase in the yield of the unrearranged compound **2** versus the combined yields of the rearranged products **3**+**5**+**6**; the graph shows a direct correlation between the increase in concentration of **2** at the expense of the rearranged products. The data points, from left to right, correspond to the following progesterone concentrations: 0, 5, 25, 50, 80, 100 and 150 μM.

documented that allosterism alters the reaction rates and regioselectivity of CYP oxidation on the different reaction sites of a substrate, to our knowledge, this is the first report of allosteric modulation of the product distribution profile derived from a single reaction site. This differs from a conventional metabolic shift in that it can only happen after the hydrogen abstraction reaction. As all these reactions stem from initial C4 oxidation, this result demonstrates that the changes in the product profile depend on progesterone allosteric effects associated with or subsequent to the C4 hydrogen abstraction reaction.

The oxidation of α-thujone, in contrast to β-thujone, shows a progesterone concentration-dependent increase in 2-hydroxylation compensated by a decrease in 7-hydroxylation, but no systematic changes are observed in the products from C4 oxidation (Table S2 in the Supporting Information). While the formation of 4-hydroxy-β-thujone (**2**) and carvacrol slightly decreases with increasing progesterone concentration, the trends in the formation of the other two C4 products are ambiguous.

The results from the oxidation of β-thujone indicate that the alteration of the CYP3A4 active-site cavity caused by the simultaneous binding of progesterone decreases the apparent lifetime of radical **1**, which is generated from β-thujone by the activated P450 species. The apparent radical recombination rate changes from $1.3\times10^{10}\ s^{-1}$ in the absence of progesterone to $3.5\times10^{10}\ s^{-1}$ in the presence of 150 μM progesterone (Table 1). These numbers represent the ratio of the C4 products **2**, **3** and **5** over the ring-opened product **5**, multiplied by the rate of the ring-opening reaction.[9] The apparent change in the radical lifetime can be rationalized if the binding of progesterone either: 1) restricts the motion of the β-thujone radical in a manner that favors radical recombination, which effectively accelerates the trapping of the unrearranged radical, or 2) reduces the rates of the rearrangements themselves. In either case, the decrease in the formation of all the rearrangement products results in an apparent change in radical lifetime. If the observed radical lifetime reflects the ratio between high-spin and low-spin Cpd I species, as suggested by theory,[4] this result would indicate that the low-spin Cpd I species with a lower energy barrier increases in response to progesterone allosterism. However, the observed apparent radical-rebound rate change is more likely to result from allosterically induced physical changes in the enzyme active site.

The observation of products derived from cationic intermediates has been used as an indicator of the involvement of alternative oxidizing species in the P450 catalytic cycle, one of which involves a direct insertion of the hydroxyl cation into the C–H bond.[3] In the present study, the cationic product, carvacrol (**6**), decreases in a trend very similar to that of the radical rearrangement products **5** and **3** as a result of the progesterone allosteric effect (Figure 1). The coordinated changes we observed among the four C4 products strongly suggest that the cationic product carvacrol shares the same precursor as the other radical products and is a secondary product of the C4 radical. This is consistent with our previous observation that the C4 product distribution profile was not altered by a highly-deuterated medium.[9]

In conclusion, we have observed that progesterone exerts heterotropic cooperative effects on the C4-derived oxidation product profile in the CYP3A4-catalyzed oxidation of β-thujone. Our results demonstrate that 1) all the C4-derived products stem from a common radical precursor, and 2) the rate of radical recombination, which gives the unrearranged hydroxylated product, is accelerated by the allosteric effector. This is the first demonstration that the apparent timing of a radical clock can be specifically altered by modulation of the active-site topology or other properties by an allosteric effector.

Experimental Section

Chemicals: Synthesis of β-thujone (≥99%) and the metabolite standards have been reported previously.[9] L-α-Dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), L-α-diloleoyl-*sn*-glycero-3-phosphocholine (DOPC), and L-α-dilauroyl-*sn*-glycero-3-phosphoserine (DLPS) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). These lipids were used to reconstitute the membranes required for the CYP3A4 reaction.[11]

Enzymes: Human CYP3A4, rat cytochrome P450 reductase (CPR) and rat cytochrome b_5 were expressed and purified according to published protocols.[14–16] Optical spectra were recorded by using a CARY UV-visible scanning spectrophotometer (Varian) in potassium phosphate buffer (100 mM, pH 7.4) at 20 °C.

GC-MS analyses of thujones and their metabolites: The GC-MS instrument and methods used to analyze thujones and their metabolites were previously described.[9]

Enzyme incubations: The CYP3A4 reconstitution system was prepared according to a published protocol.[11] The CYP3A4/cytochrome P450 reductase/cytochrome b_5 ratio was 1:2:1. α- or β-thujone (100 μM in CH_3CN) and the corresponding amount of proges-

terone in methanol (5 μL) were added to the reconstituted enzyme solution. The mixture was incubated at 37 °C for 3 min, and the reaction was then initiated by the addition of NADPH (1 mM). The final reaction volume was 1 mL, and contained CYP3A4 (1 μM) and substrate (100 μM). The reaction mixtures were incubated at 37 °C for 1 h and quenched by the addition of ice-cold ethyl acetate (2 mL). The aqueous phase was saturated with NaCl and further extracted with ethyl acetate (2 mL). Separate control reactions were carried out either without enzymes or NADPH. The combined organic extracts were dried over anhydrous Na_2SO_4, filtered and concentrated to 50–100 μL under a stream of nitrogen at room temperature for the GC-MS analysis of a 3.0 μL aliquot.

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Keywords: allosterism · cytochrome P450 · ferryl species · radical reactions · reaction mechanisms

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