6

Substrate Oxidation by Cytochrome P450 Enzymes

Paul R. Ortiz de Montellano and James J. De Voss

1. Introduction

The cytochromes P450 are catalytic hemoproteins in which the heme iron atom is coordinated to a proximal cysteine thiolate. This thiolate ligand is responsible for the characteristic Soret absorption maximum of the Fe^{II}–CO complex at ~450 nm and is critical for P450 catalysis[1]. Early site-specific mutagenesis studies with CYP1A2 and $P450_{cam}$ suggested that replacement of the cysteine thiolate by a histidine ligand gave inactive protein[2, 3]. Detailed studies of the $P450_{cam}$ Cys357His mutant have recently confirmed that this mutant enzyme has an almost undetectable catalytic activity[4, 5]. The low camphor-oxidizing activity is paralleled by a low rate of reduction of the iron, an elevated autooxidation rate, and an observable peroxidase activity[4]. The thiolate ligand is thus clearly critical for $P450_{cam}$ function, although the relative contributions of the electronic vs structural perturbations of the mutation to the low catalytic activity remain unclear. These results agree with the results of experiments with thiolate ligated metalloporphyrin model systems[6, 7] and of computational analyses of the role of the thiolate (see Chapter 2)[8, 9].

The heme iron ligand on the distal side is a water molecule in all the available crystal structures of substrate-free P450 enzymes, including $P450_{cam}$ (CYP101)[10], $P450_{BM-3}$ (CYP102)[11, 12], $P450_{terp}$ (CYP108)[13], $P450_{eryF}$ (CYP107A1)[14], $P450_{nor}$ (CYP55A1)[15], *Sulfolobus solfataricus* CYP119[16], *Streptomyces coelicolor* CYP154C1[17], *Mycobacterium tuberculosis* CYP52[18], *Sorangium cellulosum* P450epoK[19], and the mammalian CYP2C5 (see Chapter 3)[20]. Although the thiolate ligand is always present, the distal water ligand appears to be absent in some mammalian enzymes, either because the water does not bind in those structures or because it is displaced by an endogenous ligand[21].

The cytochrome P450 catalytic cycle is initiated by the binding of a substrate, usually with concomitant displacement of the distal water ligand. The ferric heme is then reduced to the ferrous state using electrons provided by suitable electron donor proteins (see Chapter 4). In cytochrome $P450_{cam}$ and many other P450 enzymes, substrate binding is widely believed to be a prerequisite for the transfer of the first electron to the iron, but in some enzymes electron transfer can occur without the prior binding of a substrate[21]. Reduction of the iron is followed by binding of oxygen to give the ferrous dioxy complex. Transfer of a second electron to this complex produces the ferric peroxy anion ($PorFe^{III}$–OO^-, where Por = porphyrin) or, after protonation, the ferric hydroperoxo complex (Por^{III}–OOH) (Figure 6.1). Heterolytic cleavage of

Paul R. Ortiz de Montellano • Department of Pharmaceutical Chemistry, University of California, San Francisco, CA. **James J. De Voss** • Department of Chemistry, University of Queensland, Brisbane, QLD Australia.

Cytochrome P450: Structure, Mechanism, and Biochemistry, 3e, edited by Paul R. Ortiz de Montellano Kluwer Academic / Plenum Publishers, New York, 2005.

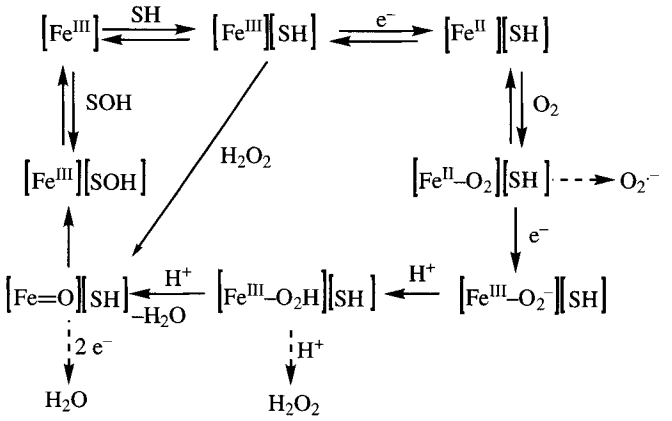


Figure 6.1. The general catalytic cycle of cytochrome P450 enzymes. The [Fe^{III}] stands for the resting ferric state of P450, and SH for a substrate molecule. The shunt pathway utilizing H_2O_2 is shown as are three sites for the uncoupling of the enzyme to give, respectively, $O_2^{\bullet -}$, H_2O_2, or H_2O.

the dioxygen bond in this peroxo intermediate extrudes a molecule of water and forms the putative ferryl oxidizing species (Figure 6.1). Hydrogen bonding of the distal ferric hydroperoxo oxygen, directly or via a water molecule, to a highly conserved threonine facilitates this heterolytic cleavage (see Chapter 5)[22–24]. The ferryl species is thought to be responsible for most P450-catalyzed oxidations, although the ferric peroxo anion and the ferric hydroperoxo complex have been invoked as oxidizing species (see below). It is usually, but not always, possible to circumvent the requirement for activation of molecular oxygen in a so-called "shunt" pathway by employing H_2O_2 or some other peroxide as a co-substrate (Figure 6.1). However, the oxidizing species thus obtained is apparently not identical to that obtained by normal oxygen activation. Thus, peroxides cannot replace molecular oxygen activation in some reactions, they often give product distributions that differ significantly from those obtained by molecular oxygen activation[25–30], and they cause a more rapid degradation of the prosthetic heme group[31].

The P450 oxidation stoichiometry requires one molecule of oxygen and two electrons from NAD(P)H to add one oxygen atom to a substrate. If the ratio of reduced pyridine nucleotide (or oxygen) consumed to product formed is greater than one, the enzyme is said to be uncoupled. Uncoupling occurs when (a) the ferrous dioxy complex reverts to the ferric state by dissociation of superoxide, (b) a molecule of H_2O_2 dissociates from the ferric hydroperoxide complex, or (c) two electrons are used to reduce the ferryl species to a molecule of water before it can be used in a reaction with the substrate (Figure 6.1). The parameters that govern uncoupling at each of the three stages are unclear, but factors that contribute to uncoupling appear to be the degree of uncontrolled water access to the active site, the extent to which the substrate can reside at unproductive distances from the ferryl species, and the presence or absence of sufficiently reactive sites on the substrate molecule[22, 32–34]. The catalytic efficiency of a P450 enzyme can be seriously impaired by uncoupling, as evidenced by the contrast between nearly quantitative coupling in the oxidation of camphor by $P450_{cam}$ and a process that is more than 95% uncoupled when the same enzyme oxidizes styrene[35]. A higher degree of intrinsic uncoupling is often observed in mammalian P450 enzymes, some of which can be suppressed by interaction of the P450 enzyme with reduced cytochrome b_5[36, 37].

2. Activation of Molecular Oxygen

Cryogenic X-ray crystallographic, EPR, ENDOR, and spectroscopic studies have convincingly identified several intermediates in the P450

catalytic cycle (see Chapter 5)[38–40]. These include the ferric, ferrous, ferrous dioxo, and ferric hydroperoxo complexes of $P450_{cam}$. Crystallographic evidence has also been reported for the ferryl species[38], but this intermediate has not been detected by other sensitive cryogenic approaches and its attribution to the ferryl species remains open to question. In low-temperature EPR, ENDOR, and spectroscopic studies, the ferric hydroperoxide intermediate disappears as the hydroxylated camphor product appears without the observation of any intermediate species[39, 40]. All the intermediates in oxygen activation by P450 have thus been observed except for the critical ferryl species, which remains elusive and undefined.

As already mentioned, the activation of molecular oxygen can often be circumvented if peroxides are used as activated oxygen donors. Efforts to identify the reactive oxygen species in these peroxide-supported reactions have been pursued for many years[41–47]. The species that has been spectroscopically detected in these reactions has the spectroscopic signature of a ferryl intermediate[47], but evidence is lacking that this intermediate is the same as that produced by the activation of molecular oxygen. To the contrary, the reactions with peroxides have been shown to produce EPR signals tentatively attributed to tyrosine radicals[41, 45, 46], but no such radicals have been observed under normal turnover conditions. Furthermore, as noted earlier, the peroxide-mediated reactions do not always faithfully reproduce the normal reactions.

Two additional intermediates, the ferric peroxy anion and ferric hydroperoxo complex, have been proposed to substitute for the ferryl as the actual oxidizing species in at least some P450 reactions. The role of the ferric peroxy anion in some reactions is supported by good evidence and is discussed in the section on carbon–carbon bond cleavage reactions (see Section 8), but the proposed role of the ferric hydroperoxide in electrophilic double bond and heteroatom oxidations is discussed here.

The current interest in the ferric hydroperoxo complex as a P450-oxidizing species derives largely from the work by Vaz *et al.*, who observed that mutation of the conserved threonine (Thr303) in CYP2E1 to an alanine decreased the allylic hydroxylation of cyclohexene, *cis*-2-butene, and *trans*-2-butene, but increased the epoxidation of the same three substrates plus styrene[48]. To rationalize this observation, the authors argued that hydroxylation is mediated exclusively by the ferryl whereas epoxidation can be mediated by both the ferryl and ferric hydroperoxide intermediates. Thus, impairing formation of the ferryl species by removing the catalytic threonine would decrease hydroxylation but have little effect upon epoxidation. However, in contrast to the results with the CYP2E1 T303A mutant, the corresponding T302A mutant of CYP2B4 exhibited both decreased hydroxylation and epoxidation rates. This discrepancy does not necessarily contradict the hypothesis, as it could reflect differential changes in the active sites of the two proteins in addition to elimination of the hydrogen bond that facilitates ferryl formation. In a more recent study in which Thr252, the catalytic threonine of $P450_{cam}$, was mutated to an alanine, it was found that camphor hydroxylation was suppressed, but the epoxidation of an olefinic camphor analogue could still be observed[49]. However, the epoxidation reaction occurred at a much slower rate (<20%) despite the expectation that the steady-state level of the ferric hydroperoxide should be elevated. This finding is consistent with the prediction by computational studies that the ferric hydroperoxo complex should be a very poor olefin-oxidizing agent[50]. These results argue that in the wild-type proteins, the ferric hydroperoxide makes no more than a small contribution to epoxidation, and none to hydroxylation.

In a second study, the *N*-oxidation of amines by CYP2B4 and its T302A mutant supported by either NADPH-cytochrome P450 reductase or H_2O_2 was investigated[51]. In contrast to what would be expected if the ferric hydroperoxide were a primary catalytic species, the rates of *N*-demethylation and *N*-oxidation of *N*,*N*-dimethylaniline were both decreased in the mutant. However, as these activities were also decreased when H_2O_2 or phenyliodosobenzene was used in a shunt reaction, little can be said from these results relative to the role of the ferryl vs ferric hydroperoxide species in these reactions. The oxidation of *para*-substituted phenols via an *ipso*-substitution mechanism using the CYP2E1 T303A and CYP2B4 T302A mutants has also given contradictory results (Figure 6.2)[52]. The T303A CYP2E1 mutation increased the rates of *ipso*-substitution with 10 *para* substituents ranging from a chloride to a *tert*-butyl group but did not increase or decrease the rate of reaction of *para*-fluorophenol, by far the most active of the investigated substrates

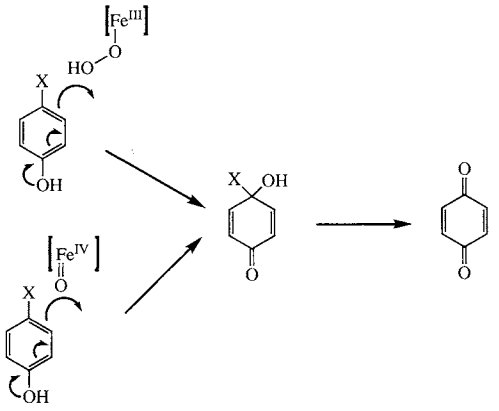


Figure 6.2. Hypothetical *ipso*-substitution mechanism involving the ferric hydroperoxo complex and ferryl species as the potential oxidizing species[52].

for this reaction. Furthermore, although the increase in the reaction rate correlated well with the substituent electronegativity for wild-type CYP2E1, the reaction with the mutant gave a biphasic correlation that included a region in which the reactivity was shown to *decrease* with increasing electron withdrawal[52]. CYP2B4 exhibited only a low activity in this reaction and this activity was not greatly changed when the conserved threonine was mutated to an alanine. It has finally also been proposed that the ferric hydroperoxo complex may play a role in the hydroxylation of saturated hydrocarbons[53–55]. As discussed in Section 3, these studies indicate that a second (or altered) oxidant contributes to the oxidation in threonine mutant enzymes, and suggest that a minor fraction of the reaction products are formed via nonobligate cationic intermediates, but do not specifically implicate the ferric hydroperoxo species in hydrocarbon hydroxylation reactions. It may be relevant to the observation of two oxidizing species that hydrogen bonding to the thiolate ligand, which is very sensitive to structure, has been found in calculations to govern the distribution of unpaired electron density between the porphyrin and protein[56].

In sum, all the reaction intermediates with the exception of the ferryl species have been clearly detected and identified in the catalytic cycle of at least one P450 enzyme. The two instances in which it has been proposed that the ferryl species was detected have shortcomings, one because the finding is not reproduced with other detection techniques, and two because the ferryl produced with peroxides may not be identical to the reactive species formed by oxygen activation. Although it has not been reliably detected as a normal intermediate, the ferryl species is nevertheless almost certainly responsible for the majority of the chemistry supported by P450 enzymes. The circumstantial and contradictory evidence so far available does not provide strong support for significant involvement of the ferric hydroperoxide species in normal P450-catalyzed reactions. Although mutation of the conserved threonine appears in some instances to cause the apparent intervention of a second differentiable oxidizing species, the evidence does not actually indicate the nature of this second species. Computational comparison of the ferric hydroperoxo and ferryl reactivities suggests that the ferric hydroperoxo complex is a poor oxidizing agent unlikely to contribute significantly to P450 catalysis (see Chapter 2)[57, 58].

3. Hydrocarbon Hydroxylation

Proposals on the mechanism of hydrocarbon hydroxylation have become increasingly complex and sophisticated over the past decade. The most widely accepted mechanism involving hydrogen atom abstraction by the ferryl oxygen followed by rebound recombination of the resulting carbon radical with the iron-bound oxygen, first clearly stated in 1978[59], has more recently been challenged, primarily on the basis of work with radical clock probes. As already discussed, the high-valent oxidizing species responsible for most, if not all, cytochrome P450 substrate oxidations is likely to be the iron(IV)oxo porphyrin radical cation ($Por^{+\bullet}Fe^{IV}{=}O$). Recent calculations support this formulation[60, 61], although they suggest that the radical density may reside to a greater or lesser extent on the thiolate iron ligand or other protein residues (see Chapter 2). In the conventional oxygen rebound mechanism, the $Por^{+\bullet}Fe^{IV}{=}O$ species abstracts a hydrogen atom from a carbon of the substrate, producing a $PorFe^{IV}$–OH species and a carbon radical. The Fe^{IV}–OH species, which can also be viewed as a complex of Fe^{III} with a hydroxyl radical, then undergoes a recombination step in which the hydroxyl radical equivalent and carbon radical combine to produce the hydroxylated product. The discrete radical intermediate

proposed in this mechanism readily explains the repeated experimental observation of high intrinsic isotope effects (often >10)[59, 62, 63], partial scrambling of substrate stereochemistry[59, 64, 65], and incidence of allylic rearrangements in P450-catalyzed hydroxylations[64, 65]. Scrambling of stereochemistry has been seen in many situations and includes the early observation that $P450_{cam}$, removes either a 5-*exo* or 5-*endo* hydrogen from camphor but transfers the oxygen exclusively to the 5-*exo* position to yield the 5-*exo*-hydroxy product[65]. Allylic rearrangements, indicative of a delocalized intermediate, have been observed with 3,4,5,6-tetrachlorocyclohexene and other cyclohexenes[66, 68], linoleic acid[67], and a variety of other compounds (Figure 6.3). In the same vein, the recently observed cleavage of a carbon–carbon bond in the P450-catalyzed oxidation of marmesin is most plausibly rationalized by a mechanism involving a carbon radical intermediate (Figure 6.4)[69].

Since 1987[70], major efforts have been made to use radical clocks to estimate the rate of the oxygen rebound step and the lifetime of the radical intermediate in the hydroxylation reaction. In radical clocks, a strained—usually cyclopropyl—ring is directly bonded to the carbon that is the proposed site of the radical intermediate. The ring strain inherent in a cyclopropyl carbinyl (or related) radical leads to a rapid and essentially irreversible rearrangement to the corresponding homoallylic radical. In the case of P450 hydroxylation, the hydroxyl group can be delivered either to the cyclopropyl carbinyl or rearranged homoallylic radical, and the ratio of the two resulting products is determined by the relative magnitudes of the rate constants for radical quenching (k_t, Figure 6.5) and rearrangement (k_r, Figure 6.5). As the intrinsic rearrangement rate can be independently measured in nonbiological experiments, one can calculate both the lifetime of the radical and the rate of radical recombination from the ratio of unrearranged to rearranged products. The first experiments with substrates containing simple, unsubstituted cyclopropyl rings ($k_r = 1.3 \times 10^8\ s^{-1}$) only gave unrearranged products[71–73]. However, bicyclo[2.1.0] pentane, which gives a radical that rearranges much faster ($k_r = 2.4 \times 10^9\ s^{-1}$) due to the additional strain in the system[74], was converted by cytochrome P450 into a mixture of rearranged and unrearranged products from which a rebound rate of $1.4 \times 10^{10}\ s^{-1}$ could be calculated[70, 74].

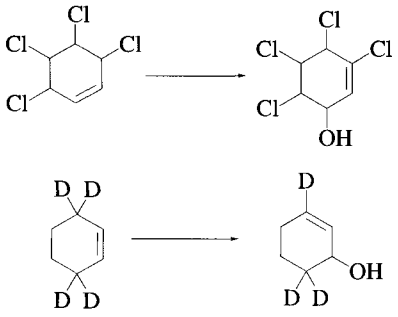


Figure 6.3. Allylic rearrangements observed in the hydroxylation by cytochrome P450 of two substituted cyclohexenes.

Radical clocks of increasing sophistication were subsequently employed to define better the radical intermediate in hydrocarbon hydroxylation[74–79]. Addition of substituents to the cyclopropyl ring increases the rate of the ring-opening reaction. For example, the rearrangement rate for a 2-aryl substituted cyclopropyl carbinyl radical in solution is approximately 1,000-times faster than that of the parent compound without the 2-aryl group[76]. Experiments with these faster radical clocks should have increased the proportion of rearranged products and thus led to a more accurate value for the lifetime of the radical. Contrary to expectation, the measured rate of the radical recombination step appeared to increase in parallel with the rate of the probe rearrangement, resulting in a lower rather than higher proportion of the rearranged

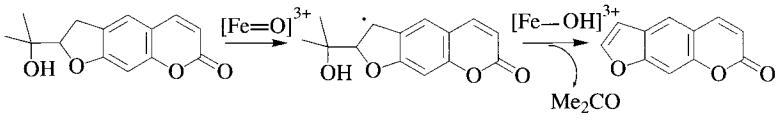


Figure 6.4. Mechanism proposed for the unusual P450-catalyzed carbon–carbon bond fragmentation observed during the biosynthesis of psoralen[69].

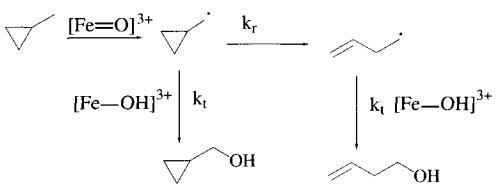


Figure 6.5. The principle of radical clock probes of the cytochrome P450 mechanism based on the methylcyclopropyl radical rearrangement.

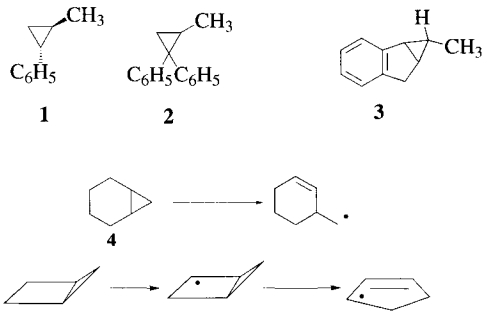


Figure 6.6. Examples of radical clock probes of the cytochrome P450 mechanism, including the radical rearrangements typical of norcarane and bicyclo[2.1.0]pentane.

product[76, 78]. When the very small amount of rearranged product was used to calculate the radical rebound rate and radical lifetime, values were obtained that challenged the existence of a discrete radical intermediate. Thus, the 2-phenyl and 2,2-diphenyl substituted probes **1** and **2** (Figure 6.6) used by Atkinson and Ingold yielded a radical rebound rate of $2\text{–}7 \times 10^{12}\ s^{-1}$ [76], values that approach the limiting rate constant of approximately $6 \times 10^{12}\ s^{-1}$ at 37°C imposed by transition state theory. At these rates, the existence of a discrete radical intermediate is reduced to a question of semantics. The observation of similar high rates with other radical clocks, the demonstration that even large substrates undergo significant motion within P450 active sites, and the observation of intramolecular isotope effects with some of the probes, suggests that the rearrangement is not being suppressed by interaction of the probe with the protein structure[77]. Indeed, analysis of the products formed from the structurally rigid probe **3** gave the unbelievably high rebound rate of $1.4 \times 10^{13}\ s^{-1}$ [78]. These results argued that a radical intermediate was not mandatory in hydrocarbon hydroxylation. However, recent experimental work has again complicated the radical clock results[80, 81]. The oxidation of norcarane (**4**) by four P450 enzymes gave a radical rebound rate of $\sim 10^{10}\ s^{-1}$, a rate very close to that of $1.4 \times 10^{10}\ s^{-1}$ from the original experiments with bicyclo[2.1.0] pentane (Figure 6.6)[70, 74]. Similar experiments with spiro[2,4]octane, a related structure that rearranges much more slowly than norcarane, did not, as expected, detectably yield rearranged products[80].

The discrepancies among the radical clocks, which give credible radical lifetimes in the case of simple cyclopropylmethyl and bicyclic probes, but impossibly short lifetimes with the phenylcyclopropylcarbinyl probes, suggests that the hydroxylation reaction may be more complex than predicted by a simple radical rebound mechanism. Norcarane and bicyclo[2.1.0]pentane differ from most of the radical clocks examined to date in that the radical is located on a secondary rather than primary carbon. This led to the proposal that the extent of rearrangement might depend on both the initial hydrogen abstraction transition state and the subsequent radical recombination transition state, and that the shift from one to the other might be easier for methyl probes because of the tighter transition state due to the higher C–H bond strength and smaller size of the methyl group[80]. However, the finding that four (2-phenyl)cyclopropylalkyl probes, in which the radical also resides on a secondary (or tertiary) carbon, give very high recombination rates is at odds with this explanation[54]. An attractive solution for this dilemma is provided by the two-state reactivity theory, which postulates a competition between two parallel reaction pathways (see Chapter 2). One of these pathways is equivalent to a concerted oxygen insertion and the other akin to a conventional radical recombination mechanism, *and the dominant pathway in any given situation is substrate and environment dependent.* A substrate-dependent reaction mechanism readily rationalizes the differences in the results obtained with the different radical clock probes. Reevaluation of the radical clock results and additional experimental and theoretical work are required to satisfactorily reconcile the contradictory results provided by the probes.

The conclusion from some of the radical clock data that P450 hydroxylation might not proceed

via a radical intermediate led Newcomb to propose that oxygen might be inserted into the C–H via a concerted, nonradical mechanism[75, 79]. According to this proposal, the hydroxylation traverses a bifurcated transition state that allows some of the probes to leak into a radical rearrangement manifold, thus explaining the observation of rearranged products. A shortcoming of this rationale is that it must explain a large diversity of reaction outcomes, including radical clock rearrangements, allylic transpositions, and stereochemical scrambling, by postulating a range of electronically and structurally different bifurcated transition states. Furthermore, even more complex structural rearrangements have been reported that are most consistent with the intervention of a radical intermediate. For example, dieldrin **5** is converted into the intramolecularly bridged ketone **6** and Dolphin has demonstrated that this product is directly produced when **5** is oxidized by a highly halogenated iron porphyrin (Figure 6.7)[82]. The alcohol **7** is also produced metabolically *in vivo* and *in vitro*, but no chemical or biochemical conditions have yet been found that will convert it to the ketone. The most reasonable explanation for the formation of the ketone product involves hydrogen abstraction to give a carbon radical, cyclization of this radical driven by the formation of a chlorine stabilized radical, recombination with the iron-bound hydroxy radical to give the alcohol, and elimination of HCl to give the ketone (Figure 6.7).

A mechanism that reconciles the differences in the radical clock results has been postulated by Shaik on the basis of theoretical calculations. This mechanistic hypothesis, which elegantly combines an essentially concerted insertion and a nonconcerted radical reaction in a single pathway[61, 83–86], is at once complex, intriguing, and satisfying. As discussed in detail in Chapter 2, the oxidizing species is an Fe^{IV}=O porphyrin radical cation that is present as two approximately equienergetic electromers, one in a doublet spin state and the other in a quartet spin state[60, 61]. Intuitively, this small difference can be viewed as arising from the combination of two electrons with unpaired spins in the d orbitals of the iron and a third unpaired electron in the a_{2u} orbital of the porphyrin (Figure 6.8). The quartet and singlet species abstract a hydrogen atom from the hydrocarbon through nearly identical transition states, which readily explains the measured isotope effects and the similarities in the reactivities of the P450 enzymes and the *t*-BuO• radical[87, 88]. The transition states lead to complexes in which the alkyl radical is weakly coordinated to the iron-bound hydroxyl. These complexes, which can be in a doublet or quartet spin state and are again of nearly the same energy, derive from the corresponding doublet and quartet states of the ferryl species. The species in the doublet spin state can collapse to the hydroxylated product in a virtually barrierless (i.e., essentially concerted), nonsynchronous reaction with no true intermediate. The porphyrin cation acts to accept an electron and

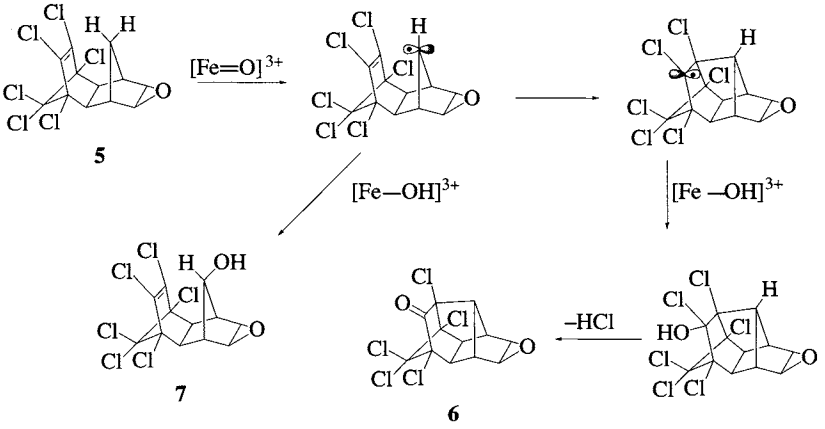


Figure 6.7. Unusual reactions observed in the oxidation of dieldrin.

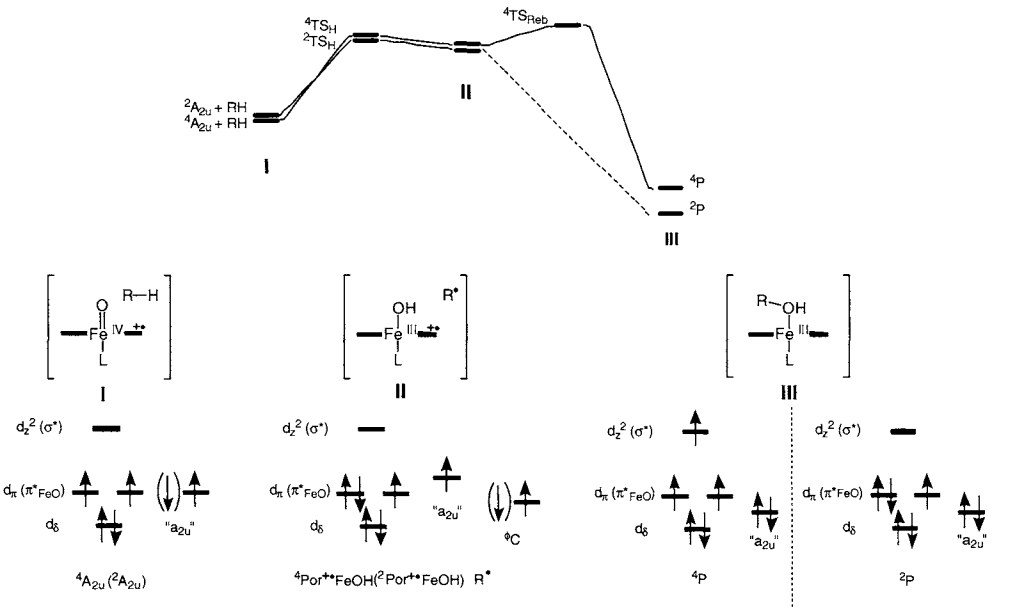


Figure 6.8. The ferryl radical cation–substrate complex (**I**) has two unpaired electrons in the iron d_π orbitals and one in the a_{2u} porphyrin orbital. This may give rise to either a quartet ($^4A_{2u}$) state if all spins are unpaired or, if the spin of the electron in the a_{2u} orbital is inverted, the complex is in a doublet ($^2A_{2u}$) state. (Another possible pair of doublet/quartet configurations has a filled a_{2u} orbital and a single electron in $p\pi$[S] orbital.) In the first step, a hydrogen atom is abstracted from the substrate and an electron is transferred to either the iron, reducing it to the Fe^{III} state (**II**), or to the porphyrin, quenching the radical cation, the former being suggested by calculations. Each of these "intermediates" (**II**) can be in a quartet or doublet state, depending on the electron pairing between the carbon radical R• and the electrons in the iron porphyrin system. Low-spin **II** collapses to a low-spin product (**III**) in a rebound reaction that is virtually barrierless while high-spin **II** traverses a significant barrier to arrive at high-spin **III**. Thus, only high-spin **II** is believed to behave as a true intermediate (radical). The energy diagram corresponding to the transformation is shown above. This scheme is based on the work of Shaik and coworkers.

the thiolate ligand increases its interaction with the iron atom and both of these processes facilitate the effectively concerted nature of the transformation. In contrast, the quartet species must traverse a significant energy barrier to form the product, thus allowing the formation of true intermediate radicals and the consequent reactions (rearrangements) that this implies. The calculated barrier to product formation in this pathway appears to arise from the fact that in the recombination reaction, to maintain the quartet spin state, one electron must reside in a relatively high energy (σ^*) d_z^2 iron orbital and this leads to a loss of bonding across the S–Fe–O axis. In general terms, the above mechanism is a specific example of the two-state reactivity paradigm recently proposed as an important feature in understanding organometallic reactivity (see Chapter 2)[85].

Our discussion of the P450 mechanism to this point has been based on the assumption that the reactive intermediate is a ferryl species. However, the possibility has been raised that the ferric hydroperoxide rather than the ferryl species might be the active agent in carbon hydroxylation[53, 55, 89]. A direct role for the Fe^{III}–OOH intermediate in substrate oxidation was postulated to explain the formation of ring opened products in the oxidation of *trans*-1-methyl-2-(4-trifluoromethyl)phenylcyclopropane (**8**) by CYP2B1 (Figure 6.9)[53]. Radical and cationic pathways could both explain the formation of these ring-opened products, but the authors favored the cationic pathway and proposed that the cationic products arose from an oxidation mediated directly by the Fe^{III}–OOH intermediate. Subsequent studies focused on oxidation of the

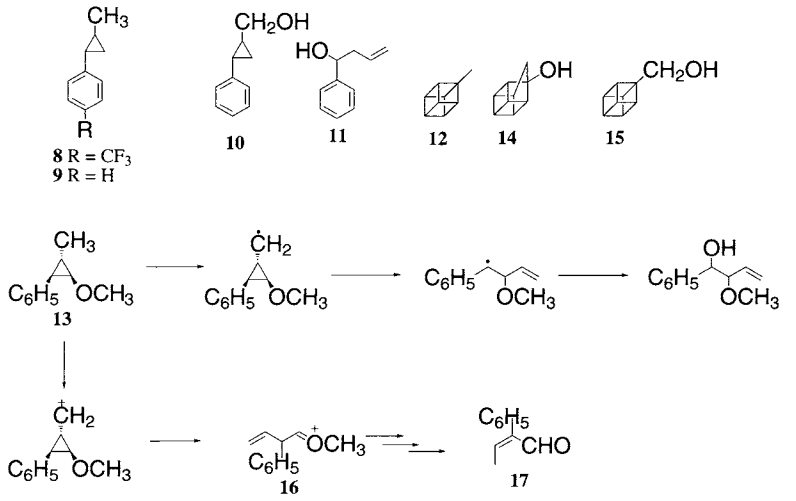


Figure 6.9. Probes of the P450 mechanism, showing the two rearrangement pathways for one of the probes intended to distinguish between radical and cation intermediates.

radical clock probes **8** and **9** by the CYP2B4 T302A mutant, in which the threonine mutant thought to facilitate the formation of the ferryl species by hydrogen bonding to the Fe^{III}–OOH complex was replaced by an alanine. CYP2B4 and its T302A mutant oxidized **9** to products obtained by reaction with the methyl group (including unrearranged **10** and rearranged **11**) and the phenyl ring. Although the same ratio of ring opened to ring closed products was obtained with the wild-type and mutant enzymes, the ratio of phenyl to methyl oxidation products was higher with the T302A mutant. This shift in product profile was interpreted as evidence for the involvement in the mutant enzyme of a second oxiding species that preferentially oxidized the phenyl group. Substitution of the phenyl group in **8** with an electron-withdrawing CF_3 group, which disfavors electrophilic attack on the aromatic ring, resulted in a change in the ratio of ring opened to ring closed products. This change was also interpreted as resulting from an alteration in the nature of the oxiding species, specifically, as evidence for involvement of the Fe^{III}–OOH intermediate in methyl group hydroxylation. However, different explanations are possible for the nature of the two oxidizing species. For example, differences may exist in the proportions of the low spin (LS) and high spin (HS) ferryl species produced by the wild-type and mutant enzymes in the Shaik two-state reactivity model (see Chapter 2), or the ferryl species may be perturbed by differential hydrogen bonding or other environmental factors, giving rise to distinguishable ferryl reactivities. It is important to note, of course, that evidence for a second oxidizing species in the absence of the threonine that is required for normal oxygen activation does not necessarily mean that the second oxidizing species is relevant to catalysis in the presence of the threonine.

In fact, cryogenic ENDOR studies with $P450_{cam}$ provide direct evidence that the ferric hydroperoxide is not involved in camphor hydroxylation[39]. In this work, the ferric hydroperoxide complex of $P450_{cam}$ was prepared at 77 K by radiolytic reduction of the camphor-bound ferrous dioxygen complex. EPR and ENDOR experiments then demonstrated that the hydroperoxide complex was smoothly and quantitatively converted on warming to ~200 K to a complex of the enzyme with the normal 5-*exo*-hydroxycamphor product. The hydroxyl of the 5-*exo*-hydroxycamphor was coordinated to the iron atom in the product formed immediately upon warming, as expected for insertion of a ferryl oxygen into a C–H bond. If the ferric hydroperoxide had been the oxidizing species, the initially formed hydroxycamphor product would incorporate the distal oxygen of the peroxide and the proximal oxygen would have remained bound

to the iron. Coordination of the product hydroxyl to the iron would therefore require displacement of the water ligand, an unlikely exchange reaction at 200 K. Furthermore, ENDOR studies demonstrated that the C-5 hydrogen abstracted from the camphor was bound to the hydroxyl oxygen of the product, in accord with a ferryl insertion mechanism but not with oxidation by the ferric hydroperoxide.

The formation of products from **12** and **13** suggestive of a cationic intermediate is relevant to arguments for participation of the Fe^{III}–OOH species in C–H oxidation, as insertion of the terminal hydroperoxide "HO^+" into a C–H bond would give a protonated alcohol that readily explains the observation of (carbo)cationic rearrangements. Minor amounts of such rearrangement products are obtained in the oxidation of methylcubane **12** and the methylcyclopropane **13** (Figure 6.9)[89]. Formation of small amounts of homocubyl alcohol **14** along with alcohol **15** upon oxidation of the methyl group of **12** provides evidence for cationic intermediates as the cation but not radical derived from the methyl group of **12** rearranges to **14**. However, one shortcoming of **12** as a mechanistic probe, as pointed out by the authors, is that the cationic rearrangement product is readily identified but the products of the radical pathway are too unstable to detect. This is not a shortcoming in the case of **13**. Ring opening of the cyclopropylcarbinyl radical derived from this probe to the benzylic radical gives rise to structurally related products, while the corresponding cation is cleaved in the opposite direction to give first oxonium ion **16** and then aldehyde **17** (Figure 6.9). These radical and carbocationic ring-opening reactions occur with very high regioselectivity: in the case of the cation reaction, the indicated ring opening is favored by >1000 : 1 relative to the direction of ring opening observed with the radical. The traces of aldehyde **17** observed in the oxidation of **13** by a P450 enzyme thus provide credible evidence for at least a minor pathway involving a cationic intermediate. A corollary, however, is that cations cannot be mandatory intermediates in normal hydrocarbon hydroxylation, otherwise, much higher amounts of the rearranged products **12** and **13** would be expected. The cationic intermediate thus diverges from the normal hydroxylation at some branch point in the reaction trajectory.

Two mechanisms have been considered for the formation of cationic intermediates. In the first of these, electron transfer from the radical intermediate in the conventional oxygen rebound mechanism occurs more rapidly than oxygen transfer and produces a cationic intermediate. Direct electron transfer from a substrate to the P450-oxidizing species is proposed to occur in the oxidation of electron-rich nitrogen atoms (see Section 4), and a precedent exists for such electron transfer even in the case of hydrocarbon hydroxylation. Thus, the product formed in the P450-catalyzed oxidation of quadricyclane **18**, a hydrocarbon with a low oxidation potential, is most consistent with electron transfer to give a radical cation that is subsequently trapped by the iron-bound oxygen (Figure 6.10)[90]. An alternative explanation for the formation of cation rearrangement products is that the oxidation is mediated by the P450 Fe^{III}–OOH complex. As already noted, insertion of the hydroperoxide oxygen into a C–H bond would produce the protonated alcohol that could either undergo deprotonation to give the alcohol or ionization to give the carbocation. In this mechanism, the carbocation is not an intermediate in the hydroxylation reaction but rather a result of decomposition of the initial product. The observation that the proportion of cationic products formed in the oxidation of **12** and **13** increases when the catalytic threonine is mutated to an alanine can be taken as evidence for this mechanism[89], but can also be rationalized by environmental perturbation of a ferryl oxidizing species. In any case, the significance of results with the mutants to catalysis by the native enzyme is questionable. A further important observation is that the extent of cationic rearrangement does not parallel the stability of the cation, as shown by studies of a series of 1-aryl-2-alkyl-cyclopropane probes with either a phenyl or *para*-trifluoromethylphenyl aryl group and an ethyl, propyl, or isopropyl alkyl group[55]. Although ring opened

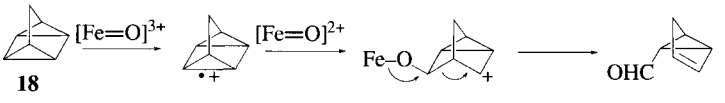


Figure 6.10. The cytochrome P450-catalyzed oxidation of quadricyclane involving an initial electron abstraction step.

products were observed with these probes, substitution with cation-stabilizing groups yielded *less* rather than more rearrangement products, contrary to expectation if a protonated alcohol were the initial oxidation product[55]. This result is not consistent with initial formation of a protonated alcohol product, and therefore does not support the involvement of an Fe^{III}–OOH species in hydrocarbon oxidation. In contrast, the two-state radical recombination model (see Chapter 2) predicts that better electron donors such as a methine hydrogen will favor oxidation through the LS manifold which proceeds without the formation of a true radical intermediate, precluding further oxidation of the carbon to a cation.

In sum, the evidence in hand is most consistent with Shaik's two-state reactivity model for hydrocarbon hydroxylation. Both the LS and HS electromers of the ferryl species first abstract a hydrogen atom. The LS species then follows a barrierless, effectively concerted, pathway to give the unrearranged product, while the presence of a significant energy barrier in the pathway for the HS species leads to the formation of a true radical intermediate. Radical formation readily explains reaction characteristics such as high kinetic deuterium isotope effects, stereochemical scrambling, and structural rearrangement, while the existence of two parallel pathways allows the reactivity pattern to vary both with the substrate and the enzyme. Carbocationic species are not obligate hydroxylation intermediates, as a much higher extent of cationic rearrangements would be expected if they were. Small amounts of cationic products, however, apparently can be formed by a pathway that diverges from that responsible for normal hydroxylation, possibly by a mechanism that involves electron transfer from a radical intermediate to the active oxidizing species.

4. Heteroatom Oxidation and Dealkylation

Heteroatom oxidation can be viewed as part of the hydrocarbon hydroxylation continuum if the reaction outcome is the introduction of a hydroxyl group onto the carbon attached to the heteroatom, an outcome that is usually followed by elimination of the heteroatom with concomitant formation of a carbonyl group. *O*-dealkylation, *N*-dealkylation, *S*-dealkylation, and oxidative dehalogenation are all examples of such processes. However, although in appearance the outcome may be initially similar, that is, carbon hydroxylation, the mechanisms of hydroxylation of a simple C–H bond and a C–H bond adjacent to a heteroatom need not be the same. Whereas carbon hydroxylation involves hydrogen abstraction from the carbon to give a transient carbon radical, hydroxylation adjacent to a heteroatom may proceed via initial electron abstraction from the heteroatom to give the radical cation, deprotonation of the adjacent carbon, and recombination of the resulting carbon radical with the iron-bound hydroxyl radical (Figure 6.11). Such a mechanism is particularly feasible in the case of atoms such as nitrogen that are electron rich and easily oxidized. Of course, if the oxygen rebound reaction is faster than deprotonation of the adjacent carbon, the oxidation will simply result in oxidation of the heteroatom, as in conversion of a trialkylamine to a trialkylamine *N*-oxide or a dialkyl sulfide to a sulfoxide. The reaction outcome, and the mechanism of the reaction (i.e., electron abstraction vs C–H oxygen insertion) would thus be expected to depend on the ease of oxidation of the heteroatom and the relative energies of the various reaction pathways.

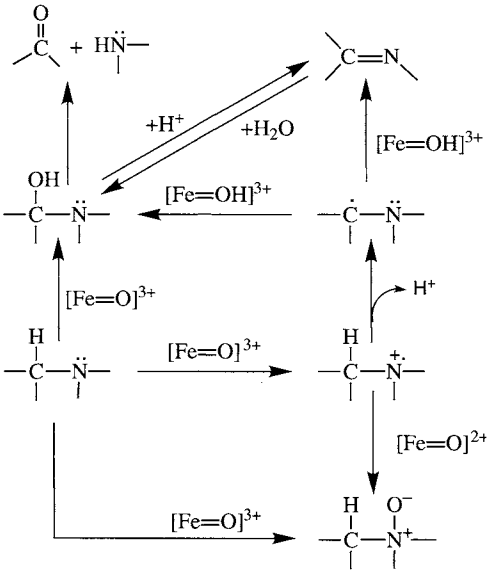


Figure 6.11. The reaction manifold for the oxidation of an amine or related nitrogen compound by cytochrome P450.

In view of the high electronegativity of oxygen and, therefore, the high energy required to remove one of its electrons, it is not surprising that the oxidation of a dialkyl ether occurs by direct reaction of the oxidizing species with the C–H bond, although the distribution of electron density in the hydroxylation transition state is likely to be perturbed by the vicinal oxygen atom. There is no evidence for electron abstraction from the oxygen to give an oxygen radical cation, and also none for transfer of the ferryl oxygen to an ether or alkoxy oxygen to give a 1,1-disubstituted zwitterionic peroxo species. Thus, the *O*-dealkylation of ethers with at least one C–H bond next to the oxygen is most appropriately treated as an extension of carbon hydroxylation. In the absence of a vicinal C–H bond, ether functions are resistant to P450-catalyzed oxidation.

The oxidation of nitrogen compounds gives more diverse products than that of oxygen compounds, and the attendant mechanisms are more varied and controversial. As a result of the lower electronegativity of nitrogen relative to oxygen, oxidation of a nitrogen center can result in hydroxylation of the adjacent carbon (and thus *N*-dealkylation), oxidation of the nitrogen electron pair to an *N*-oxide, or insertion of an oxygen into an N–H bond. The key mechanistic question in the P450-catalyzed oxidation of a nitrogen function is whether it proceeds via initial electron abstraction to give the nitrogen radical cation, followed by either collapse to give the *N*-oxide or deprotonation of the vicinal carbon to give a carbon radical that combines with the iron-bound oxygen to give the alcohol. Oxidation of an N–H bond to a hydroxylamine by a mechanism analogous to that for carbon hydroxylation is also possible. To the extent that the nitrogen radical cation mechanism is operative, *N*-oxide formation and *N*-dealkylation represent the divergent partitioning of a common intermediate (Figure 6.11). The alternative is that nitrogen oxidation and carbon hydroxylation are independent reactions, one involving reaction of the ferryl oxygen with the nitrogen electron pair, and the other a more or less classical hydroxylation of the vicinal carbon.

The ability of P450 enzymes to oxidize nitrogen atoms to radical cations via an initial electron abstraction is supported by a number of experimental results. The finding that the 4-alkyl group of 3,5-(*bis*)carbethoxy-2,6-dimethyl-4-alkyl-1,4-dihydropyridines is transferred to a nitrogen of the prosthetic heme group almost certainly requires initial oxidation of the dihydropyridine nitrogen to a radical cation (see Chapter 7)[91]. This heme alkylation reaction occurs upon oxidation of the dihydropyridine within the P450 active site. However, in incubations with liver microsomes, the dihydropyridine can also be oxidized by trace metals in the solution. This adventitious oxidation releases the 4-alkyl group as a spin-trappable free radical that obscures whatever radical release, if any, occurs in the enzyme-catalyzed reaction[92, 93]. No nitrogen radicals have been observed by EPR in P450 systems that are free of medium-dependent peroxidative reactions except perhaps for the reported observation of nitrogen radicals in the CYP2B1-catalyzed oxidation of *para*-substituted dimethylanilines supported by iodosobenzene rather than NADPH-cytochrome P450 reductase[93]. It is possible that radical formation is detected in this system due to a faster rate of substrate oxidation with phenyliodosobenzene than P450 reductase, but the possibility also exists that the radicals stem from an abnormal process supported by the artificial oxidizing agent.

Differences in the deuterium isotope effects for the oxidation of carbons adjacent to nitrogen vs oxygen suggest that the two reactions are mediated by different mechanisms. The intramolecular isotope effect for *O*-deethylation of deuterium substituted 7-ethoxycoumarin is ~13 and for *O*-demethylation of 4-nitroanisole with a trideuterio methyl group is 10[94, 95]. In contrast, an isotope effect of 2–3 is obtained for the *N*-dealkylation of *N*-methyl-*N*-trideuteriomethylaniline[62, 94]. The intrinsic isotope effects for *O*-dealkylation thus approach those for normal carbon hydroxylation reactions, but the isotope effects for *N*-dealkylation are much lower.

The intramolecular isotope effects observed for *N*-demethylation of *para*-substituted *N*-methyl-*N*-trideuteriomethylanilines increased from $k_H/k_D = 2.0$ to 3.3 in traversing the range from the most electron withdrawing (NO_2) to the most electron-donating (CH_3O) substituent[96]. Similar values were obtained in an earlier study[92]. The *N*-dealkylation rates are also increased by electron-donating substituents[96], in accord with the finding that the rates of oxidation of 12 *para*-substituted *N,N*-dimethylanilines can be fit to the equation $\log V_{max} = 0.41\pi - 1.02\sigma - 0.023MR$

+ 1.72 ($r = 0.953$)[97], where π is the partition coefficient, σ, the Hammett electronic factor, and MR, the molecular refractivity. A strong enhancement of the reaction by electron-donating substituents is indicated by the negative sign and magnitude of the cofactor of the electronic parameter. These results are consistent with a mechanism involving a nitrogen radical cation.

The oxidizing species of cytochrome P450 is thought to have some resemblance to the well-characterized ferryl species of horseradish peroxide (HRP). It is therefore relevant that the rates of reduction of the HRP Compound I by *para*-substituted *N,N*-dimethylanilines and *N,N*-di(trideuteriomethyl)anilines correlate with the oxidation potentials of the anilines[98, 99], and that no kinetic isotope effects are observed in these reactions[99]. Earlier studies measuring the rates of product formation rather than the reduction of the ferryl species led to the conclusion that dimethylaniline *N*-demethylation by HRP is subject to large isotope effects[94, 100]. However, in the case of HRP, product formation involves a disproportionation reaction subsequent to radical cation formation that is subject to a large isotope effect. The more recent results of Goto *et al.*[99] are most consistent with a single electron transfer (SET) mechanism for the *N*-dealkylation mediated by HRP. A similar dependence of the *N*-dealkylation reaction on the substrate oxidation potential was observed in the reactions mediated by $TMP^{+}\bullet Fe^{IV}{=}O$ (TMP = 5,10,15,20-tetramesitylporphyrin dianion), but with this P450 model system, kinetic isotope effects of 3.9 (p-CH_3O) to 6.2 (p-NO_2) and intramolecular isotope effect of 1.3–5.9 for the corresponding *N*-methyl-*N*-trideuteriomethylanilines were observed[99]. The authors argue that isotope effects are observed in this instance due to a competition between back electron transfer from the partially reduced $TMPFe^{IV}{=}O$ species to the nitrogen radical cation and oxygen transfer to the nitrogen radical cation. The implication of a SET mechanism in both the HRP and P450 model reactions agrees with earlier findings on the rates of oxidation of dimethylaniline by HRP, CYP2B1, and two metalloporphyrin systems. Unfortunately, similar spectroscopic rate studies cannot be carried out with P450 itself because the analogous "Compound I" form of cytochrome P450 is not sufficiently stable.

Support for a nitrogen radical cation mechanism in P450-catalyzed *N*-dealkylation reactions is provided by the fact that *N*-demethylation is usually favored over *N*-deethylation. For example, *N*-demethylation is favored over *N*-deethylation by a factor of 16:1 in the CYP2B1-catalyzed oxidation of *N*-methyl-*N*-ethylaniline[94]. Electronic factors would favor deethylation if a direct hydroxylation of the carbon adjacent to the nitrogen were involved because the incipient radical would be better stabilized by hyperconjugation. In contrast, demethylation should be favored if the reaction involves deprotonation of an initially formed nitrogen radical cation because a methyl is more acidic than an ethyl methylene. However, these arguments are not unambiguous because the electronic differences in the two reactions may be masked by the differences in the steric effects of a methyl and an ethyl group. Thus, steric effects possibly account for the observation that *N*-demethylation of *N*-methyl-*N*-alkyl-4-chlorobenzamides is favored over *N*-deethylation by a factor of 2.2, as this reaction (see below) is thought to involve direct oxygen insertion into the C–H bond[101]. Despite this caveat, the observation of intramolecular isotope effects ≤ 2.0 in the *N*-dealkylation of *N,N*-dimethylaniline by CYP2B1, chloroperoxidase, and metalloporphyrin models, but of isotope effects >8 in the corresponding reactions catalyzed by hemoglobin, HRP, and prostaglandin H synthase, provides independent evidence that the iron-bound oxygen removes a proton from the carbon adjacent to the nitrogen radical cation in the first but not the second set of proteins (Figure 6.12)[102]. The pK_a values of the protons next to the trimethylamine and dimethylaniline

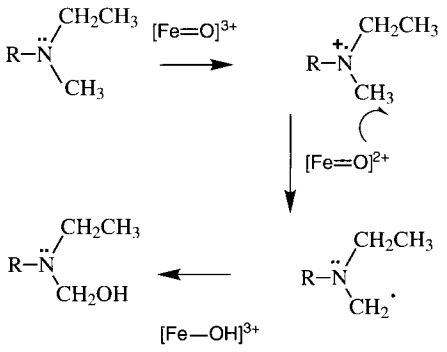


Figure 6.12. The nitrogen radical cation pathway proposed for a P450-catalyzed *N*-dealkylation reaction.

nitrogen radical cations have been estimated to be, respectively, ~15 and 9[102, 103].

It has been proposed that the isotope effects for the P450-catalyzed oxidations of hydrocarbons and alkylamines are similar to those observed in the reactions of the same substrates with the *tert*-butoxyl radical[87, 104]. The finding that the measured kinetic isotope effects for the hydrogen abstraction from benzylic methyl groups fall on the same line as those for the *N*-demethylation of 4-substituted dimethylanilines has, therefore, been advanced as evidence that *N*-dealkylations occur via a hydrogen abstraction (HAT) rather than SET mechanism, in contrast to the evidence for a SET mechanism provided by the already discussed isotope effect and rate data. Recent studies of the rates and isotope effects in the reactions of deuterated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridines with the *tert*-butoxyl radical suggest, however, that the *tert*-butoxyl radical may not fully mimic the enzymatic oxidizing species, as the *tert*-butoxyl radical did not discriminate between C–H bonds that differed in bond strength by as much as 10 kcal /mol^{-1} [105]. A correlation of reaction rates with bond dissociation energies for a range of alkylamine C–H bonds indicated that entropy factors make a significant contribution to the rate constant. The poor correlation between the absolute rates and C–H bond strengths is caused by differences in the entropy required to align the C–H bond to be broken with the electron pair on the adjacent nitrogen. A correlation between isotope effects and bond strength may nevertheless be observed if the entropy factor is similar for a series of substrates and thus cancels out, but caution must be exercised in interpreting such correlations.

Cyclopropylamines can, in principle, be used to probe the mechanism of nitrogen oxidation because a cyclopropyl substituent on a nitrogen radical cation can undergo ring-opening reactions analogous to those of a cyclopropyl attached to a carbon radical. The inactivation of P450 enzymes by cyclopropyl amines was postulated in early studies to involve formation of the nitrogen radical cation, ring cleavage to give an iminium carbon radical species, and alkylation of the heme group[106, 107]. The observation of a correlation between the one-electron oxidation potentials and the rates of P450 inactivation by a series of nitrogen-, oxygen-, and halide-substituted cyclopropanes offers circumstantial support for an electron abstraction mechanism[108]. Hanzlik has recently examined the oxidation of cyclopropylamine probes by HRP, an enzyme that demethylates *N*,*N*-dimethyl- and *N*-methyl,*N*-isopropylaniline in the presence of H_2O_2 and O_2[109]. *N*-cyclopropyl,*N*-methylaniline (**19**) was oxidized to both *N*-methylaniline and a cyclized product that derives from a radical-based ring opening of the cyclopropyl group (Figure 6.13). However, cyclopropanone was obtained as the product in the P450-catalyzed oxidation of *N*-methyl, *N*-(1-alkylcyclopropyl)aniline[110]. No trace of radical cation products such as those obtained when the same substrate was oxidized by HRP were detected. The results suggest that direct oxygen insertion into the cyclopropyl group occurs faster than oxidation of

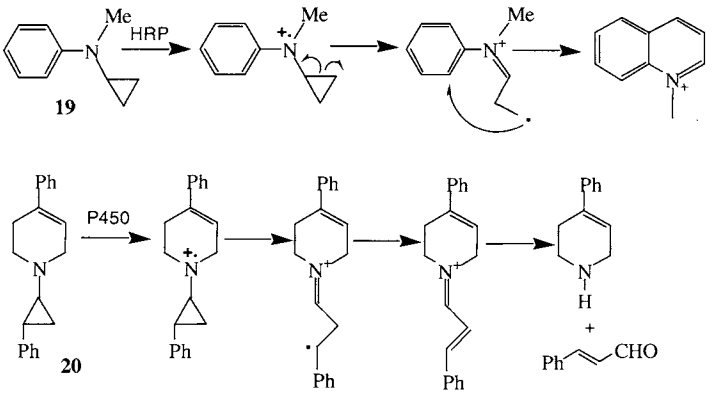


Figure 6.13. Probes designed to investigate whether a nitrogen radical cation is involved in P450-catalyzed *N*-dealkylation reactions.

the nitrogen, and thus is more consistent with a HAT than a SET mechanism.

4-Phenyl-*trans*-1-(2-phenylcyclopropyl)-1,2,3,6-tetrahydropyridine (**20**) is oxidized by rat liver microsomes to cinnamaldehyde and *N*-dealkylated tetrahydropyridine in addition to conventional metabolites (Figure 6.13). The first two metabolites have been postulated to be formed via the nitrogen radical cation, cyclopropyl ring opening, electron abstraction, proton elimination to form the double bond, and hydrolysis of the iminium link to release the aldehyde[111].

The oxidation potential for an amide nitrogen is higher than that of an amine due to the electron-withdrawing effect of the carbonyl group. The P450-catalyzed *N*-dealkylation of amides with a deuterated and undeuterated *N*-methyl substituent, $RCON(CH_3)(CD_3)$, are subject to intramolecular isotope effects of 4–7[112, 113]. The corresponding isotope effect for the *N*-dealkylation catalyzed by a model porphyrin was 5.6[114], a much higher value than that observed for the electrochemical reaction that proceeds via a nitrogen radical cation intermediate[115]. These results suggest that amide *N*-dealkylation occurs by direct carbon hydroxylation as a result of the higher oxidation potential of the amide nitrogen.

P450-catalyzed oxygen transfer to amines to give the *N*-oxide or *N*-hydroxyl product is generally considered to involve nitrogen radical cation formation followed by recombination with the ferryl oxygen (Figure 6.11)[116, 117]. As these reactions are less amenable to direct investigation with mechanistic probes, the postulate of a radical cation mechanism rests largely on the evidence for such intermediates in *N*-dealkylation reactions. However, the reported absence of a systematic relationship between the electronic properties of substituents and the rates of oxidation of anilines and dimethylanilines to hydroxylamines and *N*-oxides, respectively, provides no support for such a mechanism[117, 118]. One as yet unproven explanation for the absence of a correlation is that the stability of the *N*-oxide–iron complex makes dissociation of the *N*-oxide partially rate limiting[117]. Hlavica has also proposed that *N*-oxide formation is mediated by the P450-ferric hydroperoxide rather than ferryl species based, in part, on the observation that the oxidation of *N*,*N*-dimethylaniline to the corresponding *N*-oxide mediated by CYP2B4 is both inhibited by superoxide dismutase and supported by H_2O_2[119]. However, these criteria do not differentiate between the ferric hydroperoxide and ferryl species, as one is the precursor of the other. In the absence of more direct evidence, it is not possible to determine whether *N*-oxide and hydroxylamine formation proceeds by a mechanism other than reaction with the ferryl species to give a transient nitrogen radical cation intermediate.

The conversion of thioethers to sulfoxides or *S*-dealkylated products, as noted for the oxidation of amines, could involve the formation of a transient sulfur radical cation or direct oxygen transfer to either the sulfur or the adjacent carbon. If any function can be oxidized by direct reaction with the P450 ferric hydroperoxide species, it would appear to be a thioether sulfur. The ratio of *S*-dealkylation to sulfoxidation products was reported in early work to correlate well with the acidity of the protons adjacent to the sulfur[120]. Furthermore, electron-donating groups modestly accelerate the rate of formation of sulfoxides from substituted thioanisoles (Hammett $\rho^+ = -0.16$), and of the sulfoxides to the corresponding sulfones (Hammett $\rho^+ = -0.2$)[121, 122]. In an intramolecular competition experiment, it has been found that the thioether of thianthrene-5-oxide is oxidized in preference to the symmetry-related thioether sulfoxide function, confirming the expected higher reactivity of the sulfide than sulfoxide[123]. Unfortunately, although these results indicate that sulfoxidation occurs most readily at electron-rich sulfur atoms, the magnitudes of the effects are such that they cannot be used to unambiguously differentiate between radical cation and oxygen transfer mechanisms for sulfur oxidation.

Bacciochi *et al.* have shown that a radical cation localized on the trimethoxy-substituted phenyl ring is generated when a thioether, with a 2-(3,4,5-trimethoxyphenyl)ethyl on one side and a phenyl group on the other, is chemically oxidized. They have then shown that liver microsomes exclusively oxidize a thioether with a 3,4,5-trimethoxyphenyl group on one of the sulfur-bearing carbons to a sulfoxide rather than to the products expected from formation of the trimethoxyphenyl radical cation[124]. In view of the finding that chemical oxidation yields the trimethoxyphenyl radical cation, they conclude that the sulfoxide is formed by direct oxygen transfer from the P450 to the sulfur because oxygen rebound to the

sulfur should be slow if the unpaired electron is not localized on the sulfur. In contrast, HRP gives both sulfoxidation and radical cation cleavage products, but only the sulfoxide is formed with chloroperoxidase[125]. However, these studies all assume that the ferryl oxidation is equivalent to a chemical oxidation in favoring the trimethoxyphenyl ring over the sulfur. It is possible that the P450-oxidizing species oxidizes the sulfur to the radical cation and recombines with it faster than the electron can be transferred to the trimethoxyphenyl ring. A similar caveat tempers the conclusions that can be drawn from the finding that phenyl cyclopropyl sulfide is oxidized by *Mortierella isabellina* to the sulfoxide without opening of the cyclopropyl ring[126].

In sum, the course of heteroatom oxidation appears to be sensitive to the oxidation potential of the heteroatom, the acidity of hydrogens on the adjacent carbon, and steric factors. The bulk of the evidence suggests that oxidation of the nitrogen in amines generally involves electron abstraction followed primarily by *N*-dealkylation if a labile proton is present, or nitrogen oxidation if it is not. As the nitrogen oxidation potential increases, there is a shift toward direct insertion into the C–H bond, as is thought to occur in the *N*-dealkylation of amides. *O*-dealkylation reactions are mediated by direct insertion of the oxygen into the vicinal C–H bond, as electron abstraction from the oxygen is too difficult due to the high oxygen oxidation potential. Transfer of the P450 ferryl oxygen to an oxygen atom to give a peroxide is not known, presumably for the same reason. The mechanisms of sulfur oxidation remain more uncertain, but the limited evidence suggests that sulfur dealkylation may occur via direct insertion into the vicinal C–H bond, as found for *O*-dealkylation, in a reaction that diverges from that responsible for sulfoxidation.

5. Olefin and Acetylene Oxidation

No critical experimental advances have been made in the past decade toward a fuller understanding of the mechanisms of P450-catalyzed epoxidation reactions, although new insights into the process are emerging from computational studies. The finding that P450-catalyzed olefin epoxidations invariably proceed with retention of the olefin stereochemistry, as illustrated by results on the epoxidation of *cis*-stilbene[127], oleic acid[128], and *trans*-[1-^{2}H]-1-octene[129] supports the view that the reaction occurs by a concerted mechanism. However, retention of stereochemistry does not preclude a nonconcerted mechanism, a point clearly made by the fact that the stereochemistry is retained in most carbon hydroxylation reactions even though they are mediated by a stepwise radical mechanism. Early isotope effect studies also provided evidence for a nonconcerted, or at least asynchronous, reaction mechanism[130]. Thus, substitution of a deuterium on the internal but not terminal carbon of the exocyclic double bond of *p*-methyl- and *p*-phenylstyrene led to the observation of an inverse secondary isotope effect k_H/k_D = 0.93 in the epoxidation reaction. Similar isotope effects would be expected at both carbons if the two carbon–oxygen bonds were formed simultaneously. However, differential secondary isotope effects can be observed if one carbon–oxygen bond is formed to a significantly greater extent than the other in an asynchronous epoxidation transition state. This is clearly shown by the finding that olefin epoxidation by *meta*-chloroperbenzoic acid, a well-established concerted reaction, also gives differential secondary isotope effects, in this instance, the isotope effect being seen on the terminal but not internal carbon[131].

Acetylenes, like olefins, have oxidizable π-bonds, although it is harder to oxidize an acetylenic than an olefinic π-bond because the triple bond is shorter and stronger. Nevertheless, cytochrome P450 readily oxidizes terminal acetylenic bonds to give ketene products in which the terminal hydrogen of the acetylene has migrated quantitatively to the internal acetylenic carbon (Figure 6.14)[132, 133]. The oxirene that would result from "epoxidation" of the triple bond has not been detected and may not form, as oxirenes are highly unstable moieties. If formed, they would be expected to rearrange to the observed products. The finding of a substantial kinetic isotope effect on formation of the arylacetic acid metabolites when the terminal hydrogen of the arylacetylene is replaced by a deuterium indicates that the hydrogen migration occurs in the rate-determining step of the catalytic process[133, 134]. This finding, and the observation that the same products are formed with similar isotope effects in the oxidation of aryl acetylenes by *m*-chloroperbenzoic acid[132, 134],

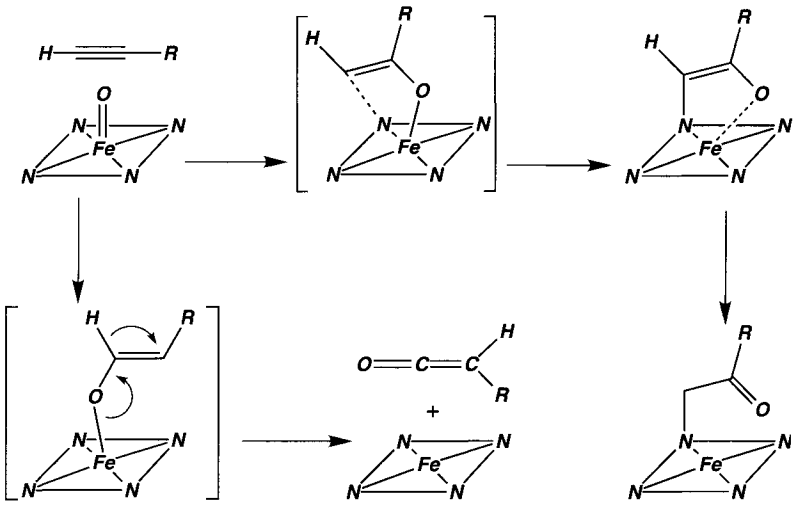


Figure 6.14. Schematic mechanism for the oxidation of a terminal acetylene by cytochrome P450, showing that addition at the terminal end of the triple bond leads to a ketene product, whereas addition to the internal carbon results in alkylation of a nitrogen of the heme group.

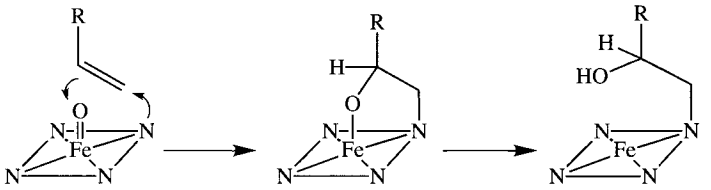


Figure 6.15. Alkylation of a nitrogen of the heme during the P450-catalyzed oxidation of a terminal double bond.

suggest that the hydrogen migrates to the vicinal carbon as the oxygen is transferred to the terminal carbon in a concerted reaction process. The demonstration that 2-biphenylpropionic acid is a minor product in the CYP1A1- and CYP1A2-catalyzed oxidation of 4-(1-propynyl)biphenyl indicates that it is also possible to oxidize a triple bond with the migration of an alkyl group rather than a hydrogen[135].

Two types of evidence, again based on relatively early experiments, provide serious support for the availability of a nonconcerted olefin epoxidation pathway. Thus, the observation that terminal olefins, including ethylene gas, are not only oxidized to the corresponding epoxides but simultaneously alkylate one of the prosthetic heme nitrogen atoms is incompatible with a concerted epoxidation mechanism (Figure 6.15) (see Chapter 7)[136]. Although the structure of the adducts is that which would result from addition of the porphyrin nitrogen to the terminal carbon of the epoxide, control experiments have clearly established that epoxides do not alkylate the heme group[137]. Furthermore, the stereochemistry of the *N*-alkylated products is opposite to that expected from backside attack of the nitrogen on the epoxide[138]. The heme alkylation must, therefore, occur prior to formation of the epoxide metabolite. Given the requirement for catalytic turnover of the enzyme[137] and the fact that the resulting *N*-alkyl group has incorporated an atom from molecular oxygen[138, 139], it is clear that the heme is alkylated by a reactive intermediate generated during the olefin epoxidation reaction (Figure 6.15). The reactive species that alkylates the heme must be a precursor of the epoxide product, or the result of a parallel but divergent olefin oxidation pathway. The partitioning of the common intermediate, or of the flow of oxidation equivalents into two parallel, but

distinct, pathways is defined by the partition ratio between epoxidation and heme alkylation. This partition ratio ranges from values as low as 1–2 (i.e., nearly every oxidation leads to heme alkylation) to values as high as several hundred (i.e., heme alkylation competes poorly with epoxide formation).

Prosthetic heme alkylation is also observed during the oxidation of terminal acetylenes by cytochrome P450 (Figure 6.14). As found in inactivation by olefins, the terminal carbon of the acetylene is bound to a nitrogen of the P450 heme group and an atom derived from molecular oxygen to the internal carbon[129, 140]. The oxygen is present as a carbonyl group due to tautomerization of the enol that would be formed by simple addition of a hydroxyl group to the internal acetylenic carbon. In the case of acetylene oxidation, a clear distinction is possible between the reaction pathway that produces the ketene metabolites and that which yields the *N*-2-ketoalkyl adducts because metabolite formation involves delivery of the oxygen to the terminal carbon, but *N*-alkylation delivery to the internal carbon. In the case of acetylenes, enzyme inactivation can also occur by a different mechanism subsequent to metabolite formation because the initial ketene product can acylate nucleophilic protein residues before it is hydrolyzed to a stable carboxylic acid (see Chapter 7)[141, 142]. The partition ratios for metabolite formation vs heme alkylation are usually smaller for acetylene than for olefin oxidation.

The second type of evidence that strongly argues for the availability of a nonconcerted epoxidation pathway is provided by the finding that carbonyl products are directly formed during the oxidation of a few olefins. As a case in point, the oxidation of trichloroethylene yields both trichloroethylene oxide and trichloroacetaldehyde. As control experiments indicated that trichloroethylene oxide did not rearrange to trichloroacetaldehyde under the incubation conditions, the aldehyde apparently arose by an oxidation pathway distinct from that which generated the epoxide (Figure 6.16)[143, 144]. Similar results were obtained for the oxidation of 1,1-dichloroethylene to the epoxide vs monochloro- and dichloroacetic acids—again the epoxide did not appear in control experiments to rearrange to the acids under physiological conditions[145]. Direct formation of a carbonyl product during the oxidation of an olefin has been observed in a few other situations, notably in the formation of 1-phenyl-1-butanone and 1-phenyl-2-butanone as minor products of the oxidation of *trans*-1-phenylbutene[145], and of 2-phenylacetaldehyde in the oxidation of styrene[146]. Under physiological conditions, these carbonyl products do not appear to be formed by rearrangement of the epoxides. The carbonyl products are consistent with the formation of a carbocation intermediate, possibly through leakage from the normal epoxidation pathway into an alternative pathway within the overall reaction manifold. This could occur, for example, if a competition exists between closing the second epoxide bond and electron transfer from a radical-like carbon intermediate to the iron to give the cation. However, the search for products indicative of radical intermediates in olefin epoxidation reactions has so far been fruitless. Thus, no cyclopropane ring-opened products were observed in the oxidation of *trans*-1-phenyl-2-vinylcyclopropane[147].

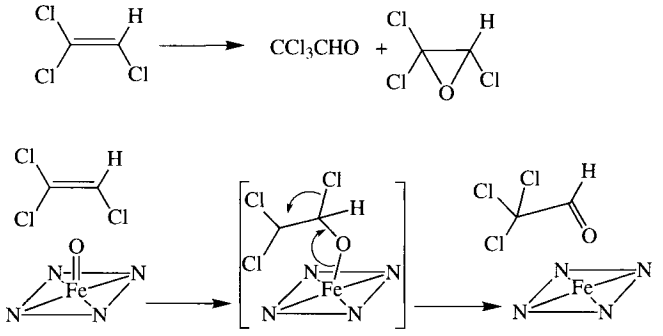


Figure 6.16. The oxidation of some halogenated olefins has been shown to yield the corresponding epoxides and rearrangement products that appear to arise via a reaction path that does not include the epoxide as an intermediate.

The seemingly contradictory evidence for concurrent concerted and nonconcerted epoxidation pathways can be satisfyingly rationalized by the two-state reactivity paradigm advocated by Shaik and colleagues (see Chapter 2). These investigators have shown by density functional theoretical calculations that the P450 ferryl porphyrin radical cation can exist in doublet and quartet spin states that are quite close in energy. The calculations suggest that ethylene epoxidation by both the doublet and quartet oxidizing species involves addition of the oxygen to one carbon of the olefin, leaving the other carbon of the olefin with an unpaired electron (Figure 6.17)[148]. This radical complex can again exist in both doublet and quartet states, although the system is more complex in that each of the two states has two equilibrating electromers that differ in whether the electron contributed by the olefin is used to neutralize the porphyrin radical cation or to reduce the iron to the Fe^{III} state (see Figure 6.8 and Chapter 2). The critical difference between the two states is that there is no barrier to closure of the doublet state to the epoxide, so that epoxidation occurs via an almost concerted trajectory with retention of the olefin stereochemistry even though a true, concerted mechanism is not predicted by the calculations[149]. In contrast, a barrier of 2.3 kcal mol^{-1} is found for closure to the epoxide of the quartet intermediate with the configuration $PorFe^{IV}$–O–$CH_2CH_2^{\bullet}$, and a barrier of 7.2 kcal mol^{-1} for the quartet intermediate with the $Por^{+\bullet}Fe^{III}$–O–$CH_2CH_2^{\bullet}$ structure[148]. These energy barriers are sufficiently high to allow alternative reactions to compete with epoxide ring closure and provide a ready explanation for the experimental observation that the heme undergoes nitrogen alkylation in the epoxidation of some olefins[150]. The partitioning between epoxidation and heme alkylation is largely determined in this model by the proportion of the doublet and quartet transition-state complexes. The critical feature of the two-state epoxidation mechanism is the presence of a doublet and a quartet transition state of sufficiently close energies that the oxidation reaction can proceed via either of the two transition states. This computational model rationalizes the experimental data available on olefin oxidation in a satisfying and comprehensive manner, although the model is difficult to test experimentally.

The discussion of epoxidation has been framed in terms of a ferryl catalytic species. It has been proposed that the ferric hydroperoxo intermediate may contribute to olefin epoxidation[48], but as discussed in Section 2, the support for this postulate is contradictory. Although it appears that the ferric hydroperoxo intermediate can oxidize double bonds at a low rate, the data strongly suggests that the ferric hydroperoxide

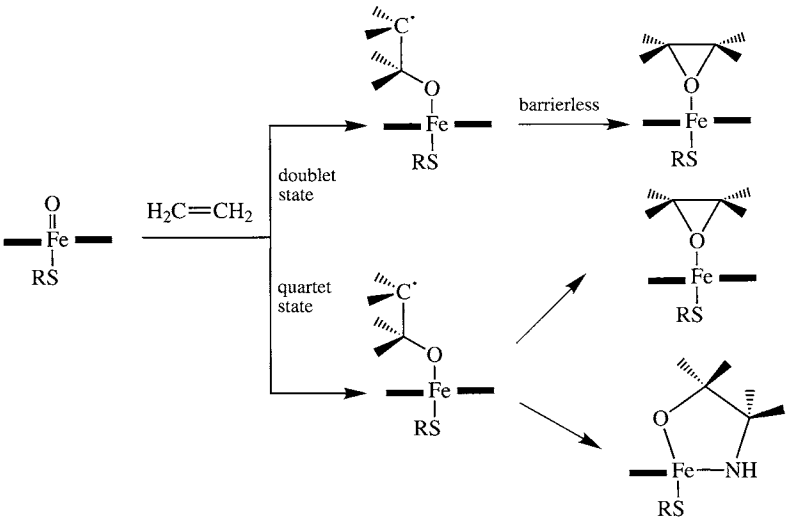


Figure 6.17. Explanation of the dual pathways of olefin oxidation resulting in epoxide formation and heme alkylation in terms of the two-state hypothesis of P450 catalysis extensively described in Chapter 2.

makes little contribution to oxidations catalyzed by the wild-type proteins.

Although the stereochemical evidence suggests that olefin oxidation occurs by a concerted mechanism, it is clear from the observation of heme alkylation and rearranged products that nonconcerted oxidation pathways are also operative. The oxidation of terminal acetylenes to ketenes by addition of the oxygen to the unsubstituted carbon and to species that alkylate the heme group by addition to the internal carbon also suggests that multiple oxidation pathways are possible. The puzzle that is posed by these mechanistic dichotomies may find a solution in the recently formulated hypothesis of two-state reactivity, in which two energetically similar transition states are obtained, one of which is in a doublet spin state and reacts essentially as if the reaction were concerted, and the second of which is in a quartet spin state and can give rise to products characteristic of nonconcerted reactions (see Chapter 2).

6. Oxidation of Aromatic Rings

The oxidation of an aromatic ring by cytochrome P450 invariably involves oxidation of one of the π-bonds rather than direct insertion of the oxygen into one of the aromatic ring C–H bonds. Thus, benzene oxide has been specifically identified as a product of the oxidation of benzene by liver microsomes[151]. However, benzene oxide and the similarly unstable epoxides expected from the oxidation of other aromatic rings readily undergo heterolytic cleavage of one of the epoxide C–O bonds. This bond cleavage is followed by migration of a hydride from the carbon retaining the oxygen to the adjacent carbocation to give a ketone intermediate. Tautomerization of this ketone yields a phenol product. This sequence of steps is the so-called "NIH-shift" (Figure 6.18)[152]. Key evidence for this mechanism is provided by the finding that the hydrogen atom (H*) at the position that is oxidized migrates to the adjacent carbon, where it is partially retained in the final phenol product. Partial retention of the migrating hydrogen reflects nonstereospecific elimination of one of the two hydrogens in the tautomerization step. This mechanism is widely applicable, but ferryl oxygen transfer to aromatic rings can also proceed via a transient intermediate that undergoes the NIH shift or eliminates a substituent on the tetrahedral carbon before the epoxide is actually formed (*vide infra*). The migrating atom in the NIH shift is usually a hydrogen, but other moieties, notably a halide or an alkyl group, can also shift to the adjacent carbon as the result of the hydroxylation event[152, 153].

The rate of the hydroxylation reaction is not very sensitive to deuterium substitution because the deuterium-sensitive tautomerization step occurs after the rate-limiting enzymatic oxidation. The observation of a small inverse secondary isotope effect (0.83–0.94) for ring hydroxylation of *ortho*- and *para*-xylene is consistent with rate-limiting addition of the ferryl oxygen to the π-bond, as the transition state for the addition reaction requires partial rehybridization of the carbon from the sp^2- to the sp^3-state[154]. The observation of an inverse isotope effect does not support a mechanism in which the rate-determining step is oxidation of the aromatic ring to a π-cation radical, as the secondary isotope effect for such a process should be negligible. The oxidation of cyclopropylbenzene to 1-phenylcyclopropanol and cyclopropylphenols without detectable opening of the cyclopropyl ring also argues against the

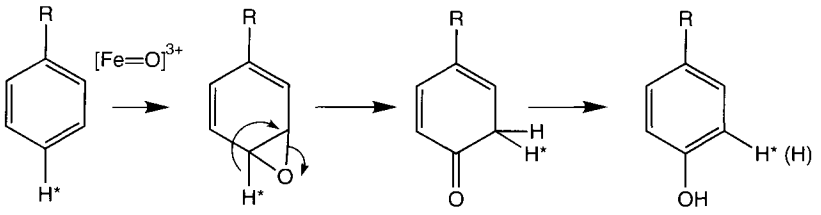


Figure 6.18. The NIH shift involving initial formation of an epoxide metabolite in the oxidation of the aromatic ring by cytochrome P450. The starred hydrogen shows that the hydrogen undergoes a 1,2-shift and then is partially lost in the final tautomerization step.

involvement of a radical cation intermediate in the oxidation of small, unactivated aromatic rings[155].

In some instances, particularly in hydroxylations *meta* to a halide substituent, the hydrogen on the hydroxylated carbon is quantitatively lost (i.e., there is no NIH shift), and a small deuterium kinetic isotope effect is observed[156, 157]. These hydroxylations could result from direct oxygen insertion into the C–H bond, as in a true "hydroxylation" mechanism, but they are more likely to result from oxidation of the aromatic ring without the formation of a discrete epoxide intermediate. Isotope effect studies with deuterated benzenes bearing a variety of substituents have shed some light on this process[158, 159]. A small, normal isotope effect is observed for *meta*-hydroxylation when deuterium is located *meta*- to the halogen in chlorobenzene (k_H/k_D = 1.1–1.3), but a small, inverse isotope effect (k_H/k_D = ~0.95) is observed for *ortho*- and *para*-hydroxylation when the deuterium is at those positions[159]. Simultaneous formation of the two epoxide bonds in a concerted process should be subject to a small, normal isotope effect when either of the two oxidized carbons bears a deuterium atom, although asynchronous formation of the two bonds could give rise to different isotope effects at the two sites. In the limiting situation in which one carbon–oxygen bond is completely formed first, formation of this bond could be followed either by closure to the epoxide or by an *ipso*-substitution mechanism that obviates the epoxide intermediate (*vide infra*).

The cytochrome P450-catalyzed oxidation of pentafluorochlorobenzene to tetrafluorochlorophenol has been proposed to involve ferryl oxygen addition to the fluorine-substituted carbon *para* to the chloride, followed by electron donation from the chloride to eliminate the fluorine as a fluoride ion (Figure 6.19). The resulting positively charged chloronium intermediate can then be reduced to the phenol, possibly by cytochrome P450 reductase, or can undergo hydrolysis to the tetrafluoroquinone[160]. This mechanism is supported by ^{19}F-NMR evidence for the release of fluoride ion and by molecular orbital calculations. A correlation of molecular orbital calculations with the regiochemistry of the oxidation of 1-fluorobenzene, 1,2-difluorobenzene, 1,3-difluorobenzene, 1,2,3-trifluorobenzene, and 1,2,4-trifluorobenzene suggests that the reaction is initiated by direct attack of the electrophilic ferryl oxygen on the aromatic π-system rather than by an initial electron abstraction[161]. More refined local density approximation calculations for the oxidation of benzene and *mono*-fluorobenzene suggest that epoxidation is disfavored vs a direct NIH shift from a tetrahedral oxygen-addition intermediate, and that hydroxylation *para* to the fluorine is favored[162]. In addition to the electronic effect of the halide, a steric interference is observed in the ability of the

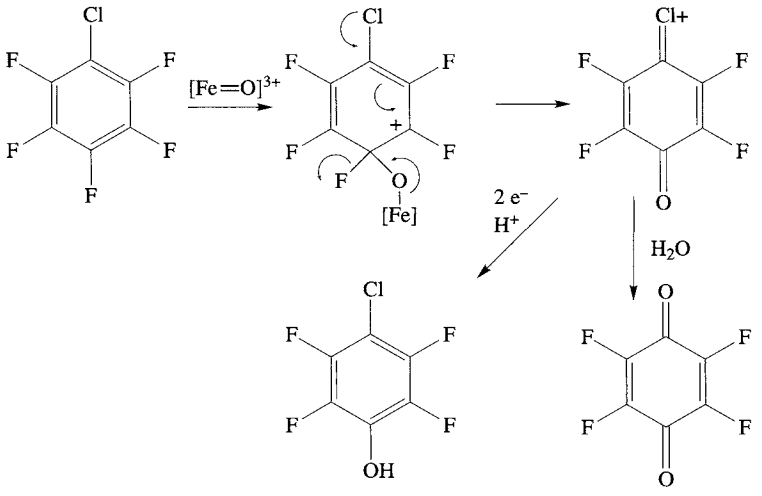


Figure 6.19. Oxidation of a polyhalogenated ring via an *ipso* mechanism that does not involve formation of an epoxide metabolite but rather an addition–elimination reaction that directly yields a quinone product.

enzyme to hydroxylate *ortho* to the halide when it is a bromine or iodine but not fluorine or chlorine[163].

The oxidation by rat liver microsomes of phenols bearing a *para*-$OPhNO_2$, -NO_2, -CN, -CH_2OH, -$COCH_3$, -COPh, -CO_2H, -F, -Cl, or -Br substituent eliminates the *para*-substituent and forms the hydroquinone (Figure 6.20)[164, 165]. Studies with $^{18}O_2$ show that an atom of molecular oxygen is incorporated into one of the two quinone carbonyl groups. These results, and the finding that the *p*-nitrophenoxy group is not eliminated when the phenol hydroxyl is replaced by a methyl ether, suggest that the phenoxy radical generated by one-electron oxidation of the phenol undergoes *ipso*-recombination with the ferryl oxygen to give a tetrahedral intermediate. Direct elimination of the *para*-substituent then gives the quinone. However, it has also been reported that 4-iodoanisole is oxidized to 4-methoxyphenol without the incorporation of label from $H_2{}^{18}O$ or ^2H_2O[166]. This finding argues that the phenol hydroxyl is not absolutely required for the reaction, so the addition can occur via *ipso*-addition without prior formation of the phenoxy radical. In accord with an *ipso*-mechanism, the substituent is eliminated from 4-halophenols as a halide anion, a *para*-CH_2OH group as formaldehyde, and a PhCO-substituent as benzoic acid[164,165].

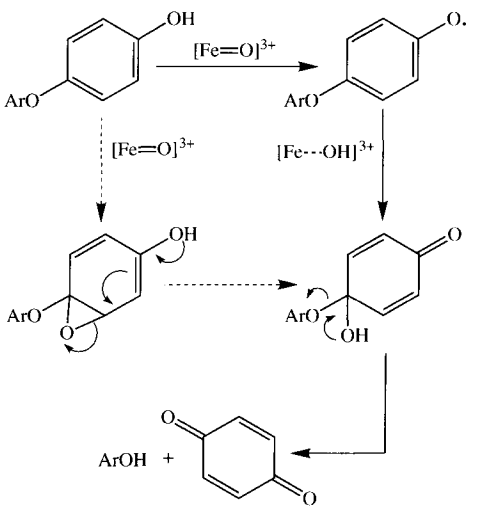


Figure 6.20. Two possible pathways for the direct P450-catalyzed oxidation of a *p*-aryloxy phenol to the quinone, one involving initial formation of a phenoxy radical and the other of an epoxide.

Furthermore, the methyl in 4-methylphenol is not a viable leaving group and this compound is simply oxidized to 4-hydroxy-4-methyl-2,5-cyclohexadiene-1-one[165]. It is to be noted that the regiochemical results do not rule out epoxide formation, as electron donation from the phenolic hydroxyl group would regiospecifically open the epoxide to the same products, but epoxide formation appears an unlikely explanation for the collective results.

Evidence for aniline oxidation without the formation of an epoxide intermediate is provided by the demonstration that an atom of molecular oxygen is incorporated into the quinone oxygen when *p*-ethoxyacetanilide (phenacetin) is oxidized to *N*-acetyl-*p*-benzoquinoneimine[167,168]. This finding requires that the reaction proceeds with cleavage of the bond between the oxygen and the aryl carbon rather than via a conventional *O*-dealkylation mechanism. The most probable mechanism for this reaction is P450-mediated hydrogen abstraction from the nitrogen to give a radical that undergoes *ipso*-recombination with the ferryl oxygen at the carbon bearing the ethoxy moiety (Figure 6.21)[169]. The concurrent formation of 2-hydroxyphenacetin is consistent with this mechanism because high unpaired electron density would be present in the aniline radical on both the *ortho*- and *para*-carbons[170]. The formation of quinoneimines accompanied by the elimination of fluoride anion in the oxidation of 4-fluoroanilines can be explained by a similar mechanism[171].

The oxidation of phenols via HAT from the hydroxyl group (or sequential electron transfer and deprotonation) is supported by data on the oxidation of estradiol and estrone. In accord with a key role for the phenolic hydroxyl group, the predominant *ortho*-hydroxylation of estradiol does not occur when the phenolic hydroxyl is replaced by a methyl ether[172]. Early experiments established that 2-hydroxylation of estradiol occurs without a detectable NIH shift[173]. More recent work has shown that, whereas estrone is converted to both 2- and 4-hydroxyestrone by CYP3A4, conjugation of an additional aromatic ring, as in equilenin and 2-naphthol, leads exclusively to 4-hydroxylation of estrone and 1-hydroxylation of 2-naphthol. In both these reactions, the site that is exclusively hydroxylated is that expected to carry the greatest share of the unpaired electron density if the initial step is

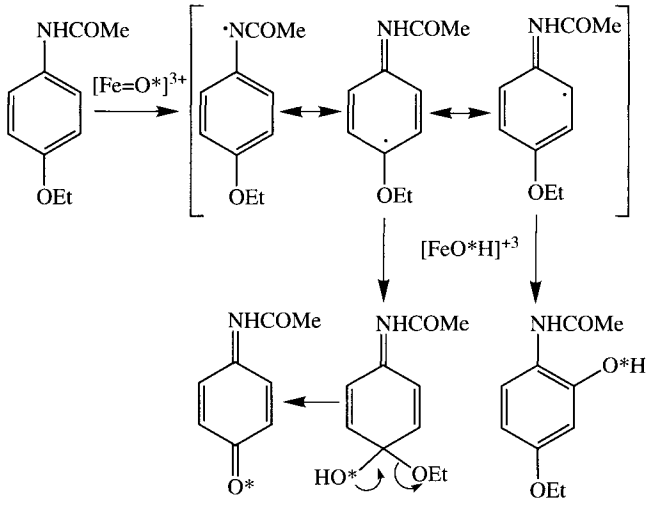


Figure 6.21. Possible mechanisms for the oxidation of phenacetin.

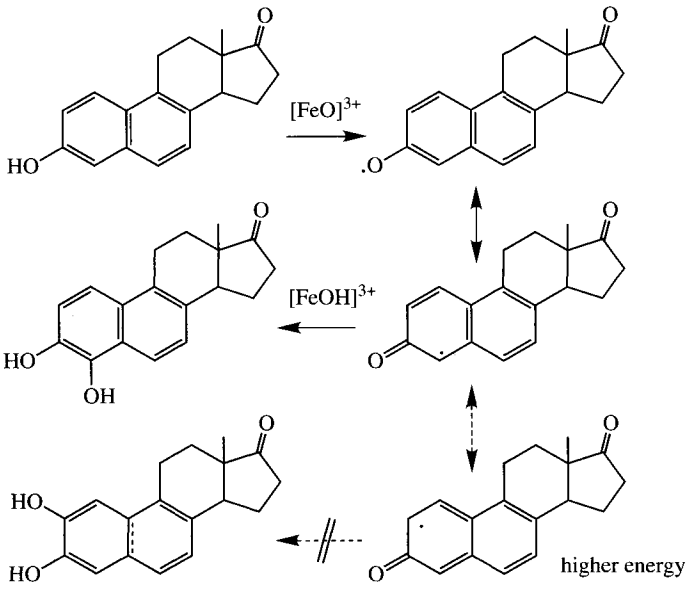


Figure 6.22. The additional aromatic ring in equilenin leads to a change in regiochemistry of hydroxylation of the phenol ring relative to that observed in the oxidation of estrone, possibly due to a change in the localization of the unpaired electron density from the 2- to the 4-position.

formation of the phenoxy radical, followed by recombination with the iron-bound hydroxyl radical (Figure 6.22)[174].

The cytochrome P450-catalyzed formation of phenol radicals is clearly required for the cross-linking of phenol rings catalyzed by a variety of plant cytochrome P450 enzymes, including the enzyme from *Berberis stolonifera* that catalyzes the biosynthesis of dibenzylisoquinoline alkaloids (Figure 6.23)[175, 176], and the enzymes that convert reticuline to salutaridine[177, 178], and autumnaline to isoandrocymbine in colchicine biosynthesis[179].

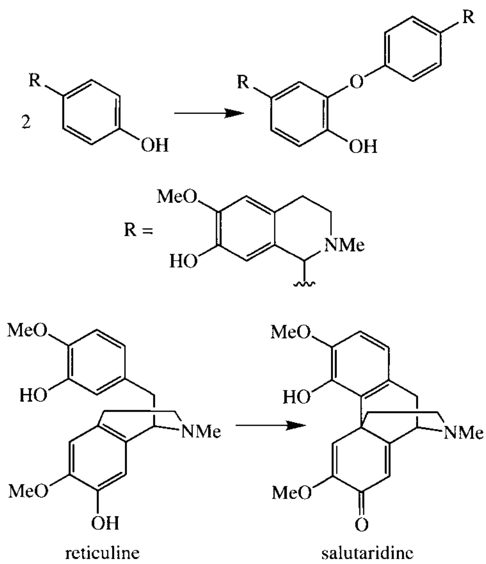


Figure 6.23. The formation of phenoxy radicals is indicated by the coupling products formed in the P450-catalyzed biosynthesis of some alkaloids.

Another interesting example is provided by the therapeutically important antibiotic vancomycin (Figure 6.24), which consists of a crosslinked heptapeptide backbone glycosylated with a disaccharide residue. The phenolic coupling that occurs between the aromatic side chains of the heptapeptide core is believed to be mediated by a P450 enzyme. The vancomycin biosynthetic cluster encodes three highly related P450 enzymes[180] that are suggested by gene knockout studies to be involved in the coupling of residue 4 with resides 2 and 6 via C–O bonds and of residues 5 and 7 via a C–C bond[181]. One of these enzymes ($P450_{OxyB}$) has been cloned and overexpressed in *Escherichia coli* and an X-ray crystal structure obtained. It has a relatively open active site, consistent with a large substrate, but whether the substrate is the free heptapeptide or one bound to a peptidyl carrier domain is unclear[182]. Balhimycin, chloroeremomycin[182, 183], and complestatin[184] are antibiotics structurally related to vancomycin in which analogous C–C and C–O bond formation is believed to be P450 mediated. These reactions, like the

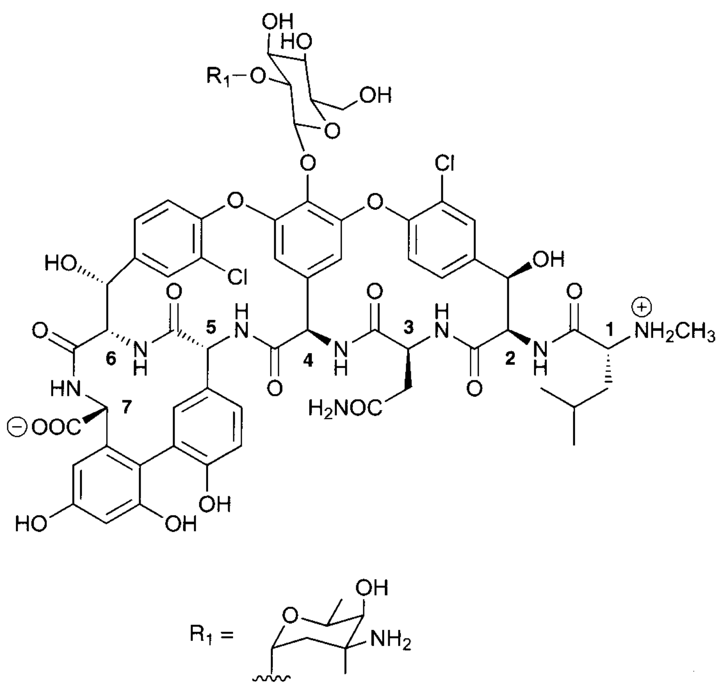


Figure 6.24. The biosynthesis of vancomycin involves a phenoxy radical crosslinking step that is catalyzed by a cytochrome P450 enzyme.

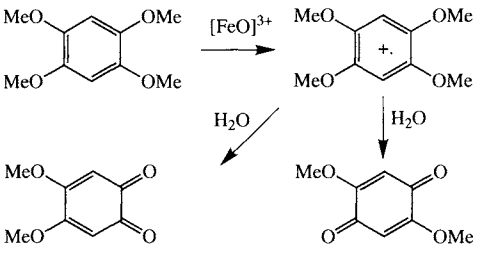


Figure 6.25. The oxidation of a highly oxidizable aromatic probe substituted with multiple electron-donating substituents yields a detectable radical cation product.

peroxidative crosslinking of tyrosine residues, require the coupling of two phenoxy radicals presumably generated by proton-coupled electron transfer from each phenol to the ferryl species. Concurrent formation of the two phenoxy radicals within the confines of the P450 active site should suffice to generate the observed crosslinked products.

The clearest available evidence that aromatic rings can be oxidized to radical cations by cytochrome P450, at least when the ring is substituted with multiple electron-donating groups, is provided by the reported oxidation of 1,2,4,5-tetramethoxybenzene to a radical cation by CYP1A2 (Figure 6.25)[185]. The radical cation was detected by absorption spectroscopy and spin-trapping EPR. The stable metabolites formed in the reaction were 2,5-dimethoxy-1,4-benzoquinone and 4,5-dimethoxy-1,2-benzoquinone, the products expected from hydrolysis of the radical cation. A negligible deuterium isotope effect was observed on formation of the radical cation or products derived from it even though a large isotope effect was seen for simple *O*-dealkylation. The evidence for other aromatic radical cations is less conclusive. In an interesting set of experiments, the products formed in the incubation of 9-methylanthracene and related compounds with the following systems were determined: (a) CYP2B1 in the presence of either NADPH-cytochrome P450 reductase or PhIO, (b) HRP in the presence of H_2O_2 or EtOOH, and (c) a model system consisting of iron tetraphenylporphine and PhIO[186]. Apart from the uninformative 1,2- and 3,4-diols that are formed exclusively with the P450 system, the relevant products were 9-hydroxymethylanthracene, 10-methyl-10-hydroxy-9-anthrone, and anthraquinone[186]. The 9-hydroxymethyl product results from a straightforward carbon hydroxylation, but more complex reactions are required to rationalize the other two products. The incorporation of label from both $H_2{}^{18}O$ and $^{18}O_2$, but not $H_2{}^{18}O_2$, into the ring-oxidized products can best be rationalized by the formation of a radical cation species that combines with water and/or molecular oxygen to give the observed products. However, with cytochrome P450, ^{18}O-label was incorporated only from $^{18}O_2$ and not $H_2{}^{18}O$. The absence of label from water in the products from the P450 system, in view of its incorporation with HRP, is inconsistent with diffusion of a radical cation out of the enzyme active site. Thus, if a radical cation is formed, it occurs as a highly transient intermediate that is immediately trapped by the ferryl oxygen to give the observed products. The results are reminiscent of the report by Ohe, Mashino, and Hirobe that (a) a hydroxymethyl group is eliminated as formaldehyde when the ferryl oxygen adds in an *ipso*-manner to the substituted carbon in a 4-substituted phenol, leading to formation of the 1,4-quinone, and (b) when the substituent is a methyl, the reaction results in addition of the hydroxyl group to the substituted ring carbon with concomitant oxidation of the phenol group to a keto function (Figure 6.20)[164, 165]. As reported by Rizk and Hanzlik, a methoxy group also makes possible this kind of reaction[166]. Thus, mechanisms based on *ipso*-addition of the ferryl oxygen to the aromatic ring are likely to account for the products formed from 9-methylanthracene. A scheme based on that proposed by Anzenbacher *et al.* (Figure 6.26)[186], or a variant of it, readily explains the observed results without requiring a radical cation intermediate. The results do not, however, preclude the existence of nondiffusible radical cations as transient intermediates.

Cavalieri and coworkers, following earlier investigators[187], have championed the hypothesis that the covalent binding of polycyclic aromatic hydrocarbons to DNA is due to radical cations formed from them by the action of cytochrome P450 and/or peroxidase enzymes[188, 189]. They have reported that polycyclic aromatic hydrocarbons with ionization potentials below 7.35 eV can be oxidized to radical cations by peroxidases[190, 191]. Furthermore, the formation of a benzo[a]pyrene-DNA adduct consistent with oxidation of the

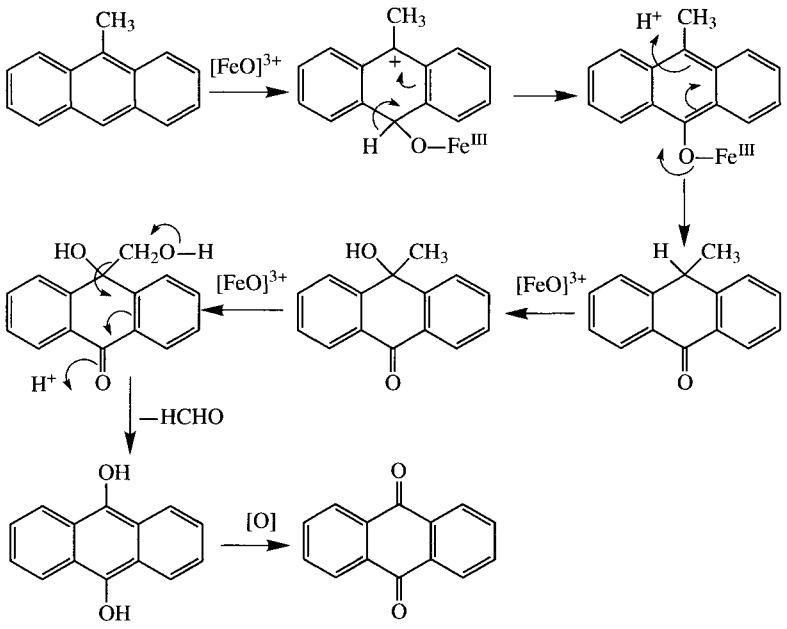


Figure 6.26. Reactions proposed to explain the oxidation of 9-methylanthracene by cytochrome P450.[186]

hydrocarbon to a radical cation by rat liver microsomes and rat skin[192, 193], and the presence of cytochrome P450 in the nuclear membrane[194], support the proposal that cytochrome P450 enzymes may also oxidize polycyclic aromatic hydrocarbons to radical cations[188]. The oxidation of 6-fluorobenzo[a]pyrene by liver microsomes to 6-hydroxybenzo[a]pyrene has been interpreted as evidence for cytochrome P450-catalyzed radical cation formation, although the reaction could arise, as postulated for polyhalogenated benzenes[160], by direct addition of the activated oxygen to the aromatic system[195]. Addition of the ferryl oxygen in such a mechanism would be expected to occur at the 6-position as that is the position most sensitive to electrophilic attack. The evidence for the formation of a diffusible radical cation in the normal (as opposed to peroxide-dependent) cytochrome P450-catalyzed oxidation of polycyclic aromatic hydrocarbons remains inconclusive.

In sum, considerable evidence is now available for the oxidation of aromatic rings not only via the conventional epoxidation pathway, but also by mechanisms that do not involve formation of an epoxide as an intermediate. The non-epoxide mechanisms involve addition of the ferryl oxygen to one carbon of the aromatic ring, producing a tetrahedral intermediate that decays by extrusion of a substituent at that carbon or by electron transfer, resulting in two-electron oxidation of the ring system and/or addition of a second nucleophile from the solution. This type of reaction is particularly favored with aromatic rings such as phenols and anilines that bear electron-donating substituents. The evidence for the formation of radical cations by direct electron abstraction from polycyclic aromatic hydrocarbons remains ambiguous, although the formation of such intermediates is favored by multiple electron-donating substituents.

7. Dehydrogenation Reactions

Cytochrome P450 enzymes catalyze dehydrogenation as well as oxygenation reactions, including the oxidation of saturated to unsaturated hydrocarbons, alcohols to carbonyl compounds, and amines to imines or other unsaturated products. The most extensively investigated of these reactions in terms of mechanism is the desaturation of valproic acid to 2-*n*-propyl-4-pentenoic

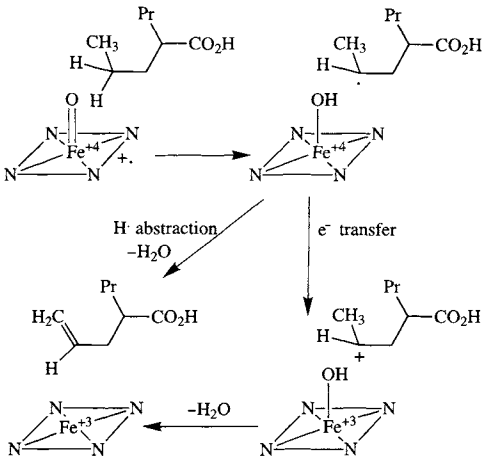


Figure 6.27. Two mechanistic alternatives for the dehydrogenation of valproic acid catalyzed by P450 enzymes.

acid (Figure 6.27)[196–199]. Formation of the $\Delta^{4,5}$-unsaturated product from valproic acid is catalyzed by rat, rabbit, mouse, monkey, and human liver microsomes and by purified CYP2B1, CYP2C9, CYP2A6, CYP3A1, and CYP4B1, but not by CYP3A4 or CYP4A1[196, 197, 200–203]. The $\Delta^{3,4}$ isomer is also formed, in some instances in greater amounts than the $\Delta^{4,5}$ isomer[201]. The 3- and 4-hydroxyvalproic acids are also formed, but these hydroxylated products are not precursors of the unsaturated compounds[196]. Oxidation of the two enantiomers of stereospecifically [3-^{13}C]-labeled valproic acid by cultured hepatocytes shows that the *pro*-(*R*)-side chain is preferentially desaturated[198]. The $\Delta^{2,3}$-unsaturated analogue of valproic acid, 2-*n*-propyl-2(*E*)-pentenoic acid, is also desaturated to give the $\Delta^{2,3}$, $\Delta^{4,5}$-diene, and an asymmetric but related molecule, 2-ethylhexanoic acid, is oxidized to both 2-ethyl-1,6-hexanedioic acid and 2-ethyl-5-hexenoic acid[199, 204].

The intramolecular isotope effects for the oxidation of valproic acid with two deuterium atoms on the C-4 carbon of one of the two propyl side chains by rabbit liver microsomes reveal that 4-hydroxylation (k_H/k_D = 5.05) and $\Delta^{4,5}$-desaturation (k_H/k_D = 5.58) are sensitive to isotopic substitution[197]. In contrast, when the methyl group of one of the side chains is trideuterated, only minor intramolecular isotope effects are observed for 4-hydroxylation (k_H/k_D = 1.09) or $\Delta^{4,5}$-desaturation (k_H/k_D = 1.62). Comparable results have been obtained when the oxidation is mediated by either CYP2B1 or CYP4B1[202]. These results indicate that removal of a hydrogen from C-4 is rate limiting for both 4-hydroxylation and desaturation, whereas loss of a hydrogen from C-5 is not. These results agree well with a mechanism in which removal of a C-4 hydrogen is followed by either oxygen rebound to give the 4-hydroxy product or transfer of a hydrogen from the terminal methyl to the ferryl oxygen to give the olefin product. The hydrogen could be transferred to the ferryl oxygen together with an electron or could be transferred as a proton following transfer of the electron to give a cationic intermediate (Figure 6.27). Analogous mechanisms can be postulated for the finding that CYP3A1 also oxidizes valproic acid to the $\Delta^{3,4}$-unsaturated isomer, except that in this case deuterium isotope experiments suggest that the olefin is obtained equally well by initial oxidation of C-3 (k_H/k_D = 2.00) or C-4 (k_H/k_D = 2.36)[201]. Interestingly, the observation of an isotope effect at only one of the two carbons in an aerobic desaturation process is also found for the nonheme iron-dependent fatty acid desaturases, for which a related mechanism involving a nonheme iron center has been postulated[205].

The ratio of 4-hydroxy to $\Delta^{4,5}$-desaturated metabolites depends on the P450 isoform and is larger for CYP2B1 (37:1) than for CYP4B1 (2:1)[202]. The proportion of the olefin is much higher when the substrate is the $\Delta^{2,3}$-unsaturated valproic acid, a result that is particularly consistent with a mechanism in which the electron is transferred to the ferryl oxygen before the hydrogen[199]. The structural determinants that control whether hydroxylation or desaturation occurs are unknown, but if the Shaik formalism applies (see Chapter 2), it is probable that the desaturation reaction involves the quartet rather than doublet hydroxylation transition state.

The isopropyl group of ezlopitant, which bears a 2-methoxy-5-isopropylbenzylamino group, is oxidized by both CYP3A4 and CYP2D6 to the tertiary alcohol and the desaturated 1-methylvinyl moiety[206]. The alcohol was specifically shown not to be a precursor of the unsaturated product, and a small primary isotope effect was observed when deuterium was placed at the benzylic but not methyl carbons of the isopropyl group. Although not studied in detail, concurrent hydroxylation and

desaturation of an isopropyl group was also observed in the metabolism of α- and β-thujone even though the isopropyl group is not bound to an aromatic or conjugating function[207]. These observations are well accommodated by the mechanistic alternatives proposed for desaturation of valproic acid.

The desaturation of sterols has also been observed. Quantitatively, the most important of these is the P450-mediated Δ^{22}-desaturation in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae*[208]. The enzyme (CYP61) has been purified and shown to specifically catalyze the Δ^{22}-desaturation without forming hydroxylated sterol products[209, 210]. Related Δ^{22}-desaturases are found in other organisms, including mammals[211, 212]. Sterol desaturation also occurs at other positions. Thus, the CYP2A1-catalyzed oxidation of testosterone yields the 7-hydroxylated, 6-hydroxylated, and $\Delta^{6,7}$-desaturated sterols in a 38:1:1 ratio (Figure 6.28)[213, 214]. As might be expected, a primary intermolecular isotope effect is only observed for 6-hydroxylation and $\Delta^{6,7}$-desaturation when the deuterium is at the allylic C-6 position, although an isotope effect is observed for 7-hydroxylation when the deuterium is at C-7[215]. The formation of 17β-hydroxy-4,6-androstadiene-3-one in this reaction presumably occurs via the mechanism discussed above, although the finding that oxidation of C-6, and not C-7, leads to desaturation again suggests that electron transfer from the free-radical intermediate to the iron to give the allylically stabilized cation may contribute to the emergence of the desaturation pathway.

The past decade has shown that hydrocarbon desaturation is not uncommon but, except in cases such as the biosynthesis of ergosterol, it generally accounts for a minor proportion of the metabolic products. The earliest reported example of P450-mediated hydrocarbon desaturation appears to be the conversion of lindane (1,2,3,4,5,6-hexachlorocyclohexane) to 1,2,3,4,5,6-hexachlorocyclohexene[216], but the known hydrocarbon desaturation reactions now include the $\Delta^{6,7}$-desaturation of androstenedione and deoxycorticosterone by adrenal mitochondria[217], the oxidation of dihydronaphthalene to naphthalene and 7,8-dihydrobenzo[a]pyrene to benzo[a]pyrene[218, 219], the conversion of warfarin to dehydrowarfarin[220], the desaturation of lovostatin and simvastatin to the 6-*exo*-methylene

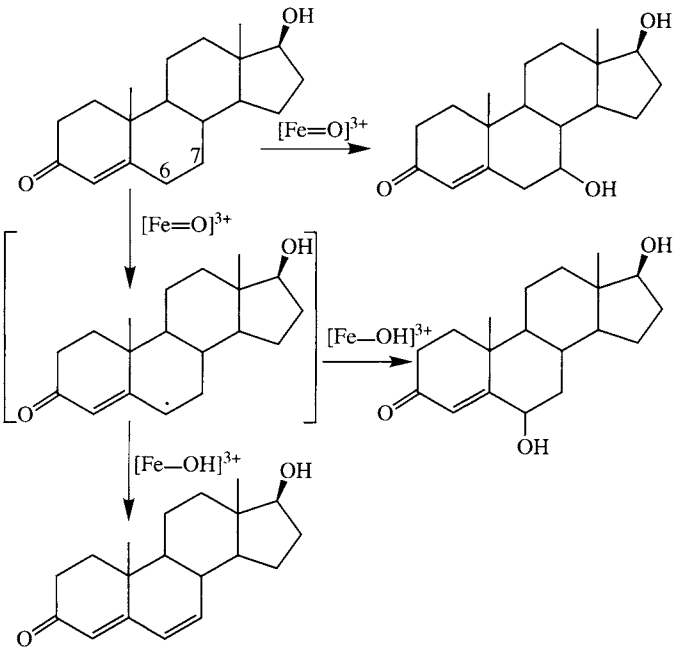


Figure 6.28. Parallel hydroxylation and dehydrogenation of testosterone.

derivatives[221–223], and the formation of 11-dodecenoic acid from lauric acid[224].

The direct desaturation of carbon adjacent to heteroatoms, notably oxygen and nitrogen, has also been observed. These reactions include the desaturation of a tetrahydrofuran ring in the biosynthesis of aflatoxin and sterigmatocystin by a specific P450 enzyme[225], the P450-catalyzed desaturation of flavanones to flavones[226], and the conversion of ethylcarbamate to vinyl carbamate[227]. In some instances, the desaturation may involve the carbon and the heteroatom instead of two carbon atoms. The CYP2B1-catalyzed oxidation of testosterone to androstenedione, which involves oxidation of the 17-hydroxy to a 17-keto function, is possibly such a reaction, because only 5–8% of the keto group oxygen derives from O_2 with testosterone, but 84% with epitestosterone[228]. A similar observation has been made for the P450-catalyzed oxidation of 6-hydroxy- to 6-keto-progesterone by CYP2C13[229]. Thus, either one of the two hydroxyls in a conventional *gem*-diol intermediate is eliminated with high stereoselectivity, or HAT is followed by loss of an electron without actual formation of the *gem*-diol:

$$RR'CHOH + [FeO]^{+3} \rightarrow RR'C \bullet (OH) + [FeOH]^{+3} \rightarrow RR'C{=}O + [Fe]^{+3} + H_2O$$

The dehydrogenation of a carbon next to a nitrogen has been unambiguously demonstrated. Acetaminophen (4-hydroxyacetanilide) is oxidized to the iminoquinone intermediate by a mechanism explicitly shown not to involve hydroxylation of the nitrogen (Figure 6.29)[230]. Other examples are the

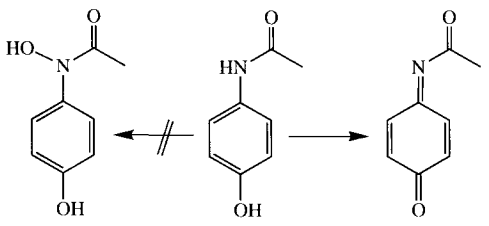


Figure 6.29. Dehydrogenation of acetaminophen to the iminoquinone occurs by a mechanism that does not include the hydroxyamide structure as an actual intermediate, even though the hydroxylamide product is found with related compounds that do not have the *para*-hydroxyl group.

aromatization of 4-alkyl- and 4-aryl-1,4-dihydropyridines[231, 232], the oxidation of 3-methylindole to the highly reactive 3-methyleneindolenine[233], and possibly the conversion of the *N*-ethyl to an *N*-vinyl moiety in the metabolism of tracazolate[234].

8. Carbon–Carbon Bond Cleavage Reactions

The power of P450 enzymes to catalyze oxidative transformations is perhaps nowhere better illustrated than in their ability to catalyze the cleavage of unactivated C–C bonds. Somewhat ironically, these reactions generally form part of biosynthetic pathways and allow organisms to build complex molecules via striking metabolic transformations. However, C–C bond cleavages have also been reported for some degradative/xenobiotic metabolizing enzymes. Additionally, many of these C–C bond cleavage reactions require a sequence of oxidations that are all carried out by the one enzyme. These P450 enzymes thus form a mechanistically fascinating group as they are not only capable of standard oxygen activation and hydroxylation chemistry, but also react through different mechanisms to eventually cleave a C–C bond. The examples below are arranged by the functional group(s) that is (are) adjacent to the C–C bond cleaved, although this group(s) may be introduced by the P450 during the course of oxidation of the original substrate. Excluded from this discussion are reactive compounds specifically designed to undergo cleavage of C–C bonds as mechanistic probes, for example, cyclopropylmethyl containing compounds (Section 3) and the cleavage of C–C bonds as part of the oxidation of an aromatic ring (Section 6).

8.1. Cleavage between Oxygenated Carbons

Diols. One of the best known examples of a P450-catalyzed C–C bond cleavage is carried out by $P450_{scc}$ (CYP11A) which converts cholesterol to pregnenolone and 4-methylpentanal (Figure 6.30). The mechanism by which this P450 effects scission of the C20–C22 bond of cholesterol has been extensively studied. The enzyme is trifunctional, catalyzing three sequential reactions that each consumes

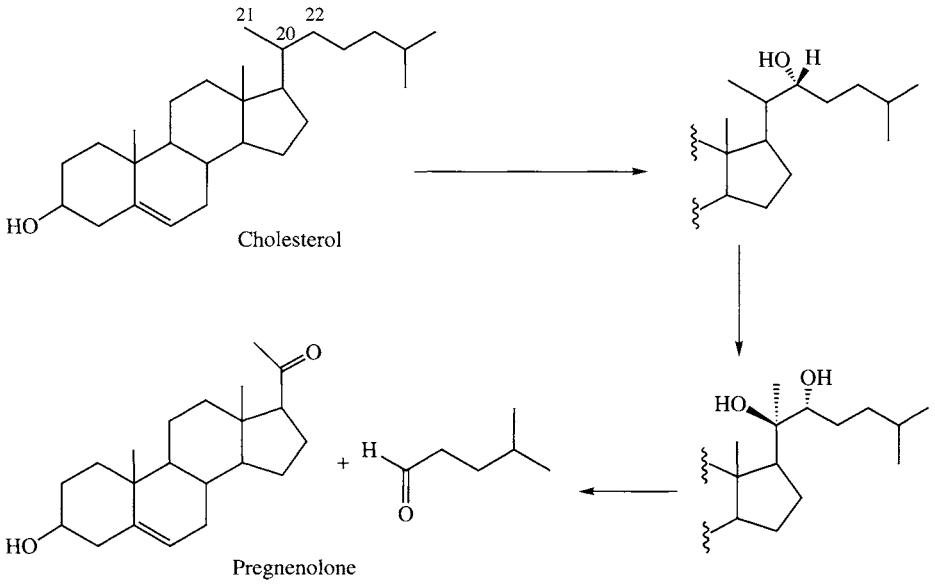


Figure 6.30. The intermediates in the $P450_{scc}$ catalyzed conversion of cholesterol to pregnenolone and 4-methylpentanal.

one molecule of dioxygen and one molecule of NADPH. The first two regio- and stereospecific hydroxylation reactions lead to, respectively, 22(*R*)-hydroxy and 20(*R*), 22(*R*) dihydroxycholesterol. These reactions are unremarkable P450-catalyzed oxidations, proceeding with retention of configuration as expected[235, 236]. The third oxidative transformation leads to cleavage of the C–C bond between the two oxygenated carbons and is of considerable mechanistic interest. The overall transformation is quite efficient as the intermediate hydroxylated cholesterol derivatives are bound up to 300 times more tightly than the parent substrate[237] and the ferrous–dioxygen complex is more stable in each successive turnover[238, 239].

Mechanisms for cleavage of the intermediate diol that involve further oxidation at C-22 are excluded by the fact that the 22(*S*) hydrogen is retained in the 4-methylpentanal produced as a result of C–C bond cleavage[235]. The most likely mechanism is thus one in which one of the hydroxyl moieties is activated in some fashion, followed by decomposition with C–C bond cleavage. The nature of this activation has led to a number of mechanistic proposals. First, a hydrogen may be abstracted from one of the alcohols by the ferryl species to form an alkoxy radical, which decomposes to release one carbonyl fragment and a carbon radical. This radical is then intercepted by the Fe(IV)OH species to yield the second product (Figure 6.31, path B). An alternative mechanism suggests that one of the hydroxyls of the diol intermediate intercepts an activated oxygen species to produce a peroxy complex. Loss of a proton from the adjacent alcohol initiates a heterolytic fragmentation reaction that leads directly to the two carbonyl products (Figure 6.31, path A). Formation of a hydroperoxide may be seen as precedented by the exchange of oxygen between hydrogen peroxide and water via a putative ferryl species in model systems[240]. The chemistry of such a hydroperoxide would also explain nicely the intriguing early observation that (20*S*)-20-(*p*-tolyl)-5-pregnen-3β-ol is cleaved to pregnenolone and presumably phenol by $P450_{scc}$ (Figure 6.32)[241]. This remarkable transformation would be analogous to the well-known formation of acetone and phenol from cumene hydroperoxide under acid catalysis.

Recently another biosynthetic enzyme, $P450_{BioI}$ (CYP107H1) has been shown to cleave an aliphatic chain via a diol intermediate. First found as a gene of unknown function in the biotin biosynthetic operon of *Bacillus subtilis*, $P450_{BioI}$ was implicated

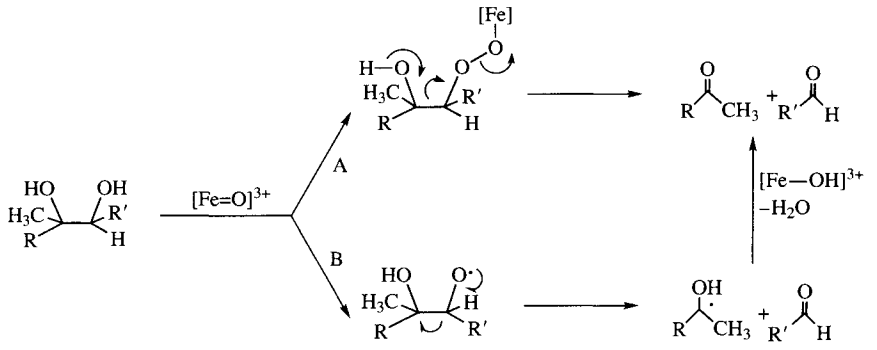


Figure 6.31. Possible mechanisms for the final step in the cholesterol side-chain cleavage reaction, where R = the sterol nucleus and R′ = $CH_2CH_2CH(CH_3)_2$.

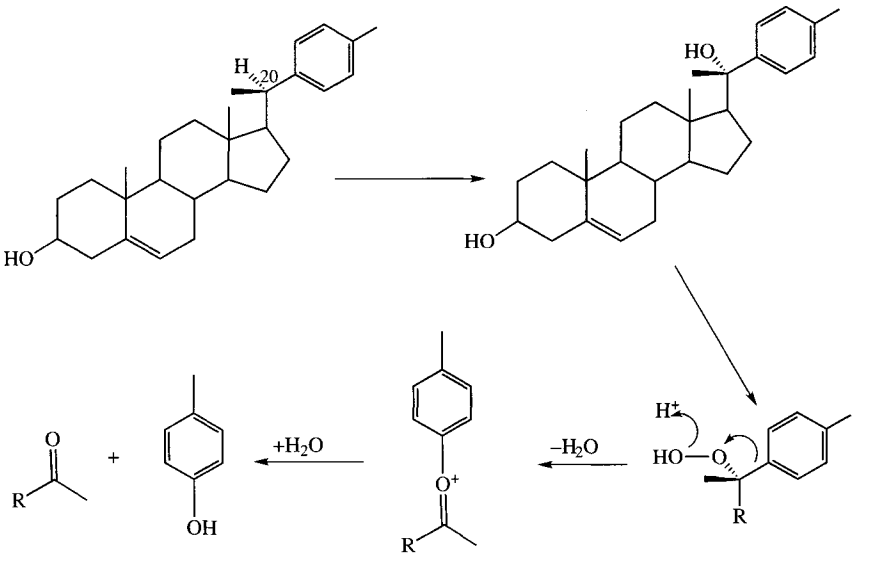


Figure 6.32. A possible mechanism for the $P450_{scc}$ catalyzed conversion of (20*S*)-20-(*p*-tolyl)-5-pregnen-3β-ol to pregnenolone and phenol (R = sterol nucleus).

through analysis of mutants in the formation of a biological equivalent of pimelic acid (heptanedioic acid)[242]. Cloning and overexpression in *E. coli* resulted in isolation of a complex between the P450 and acylated acyl carrier protein (ACP) as well as the P450 alone[243]. It was shown that the acyl moiety of the complex could be cleaved to produce a pimeloyl ACP utilizing a novel flavodoxin as the redox partner. $P450_{Biol}$ was also shown to act on free fatty acids to produce pimelic acid as well as a range of hydroxylated fatty acids[243,244]. Careful analysis of these latter products indicated that a range of hydroxy fatty acids was produced but no ω-oxidation was observed, indicating that production of a long-chain diacid as a pimeloate precursor was not the biological function of this P450[245]. Subsequently, a series of potential intermediates in the C–C bond cleavage reaction were synthesized and incubated with the enzyme[246]. It was shown that pimelic acid production increased when the substrate was changed from the C_{14} fatty acid to the 7-hydroxy derivative with the *threo*-7,8-diol as the best substrate (Figure 6.33). Other derivatives such as the 7- or

8-oxo, 8-hydroxy or *erythro*-7,8 diol gave no increase in pimelic acid formation. It was also found that, as with $P450_{scc}$, the postulated oxygenated intermediates bound much more tightly to the enzyme than the parent substrate[246]. These results are clearly in keeping with a C–C bond cleavage mechanism in which the P450 operates on one face of the extended conformation of the fatty acid chain to produce the *threo*-diol, which is then cleaved to produce two aldehyde fragments. The pimeloyl semialdehyde initially formed is somewhat unstable to aerial oxidation and both it and pimelic acid are seen in the cleavage of the *threo*-7,8-diol. Interestingly, only a small enantioselectivity was seen for the 7-(*S*) alcohol and the derived *threo* diol. This perhaps reflects the fact that the true substrate is an ACP-bound acyl group, making $P450_{BioI}$ one of a growing number of P450s found to act on carrier protein bound substrates[247].

One example of P450 mediated C–C cleavage via a presumed diol intermediate during xenobiotic metabolism has been reported. Olanexidine, an antimicrobial agent, is metabolized mainly to a range of chain shortened carboxylic acids in both rats and dogs, as well as to various other oxygenated metabolites (Figure 6.34)[248, 249]. Dog liver microsome studies indicated that vicinal diol metabolites were further transformed to the C–C bond cleavage products and specific inhibitor studies implicated enzymes of the CYP2D family in all of the oxidative transformations[248]. Interestingly, in contrast to $P450_{scc}$ and $P450_{BioI}$, no diastereoselectivity was observed in the further oxidation of the diols investigated (Figure 6.34)[248]. This may be due to the position of the diol near the terminus of the aliphatic chain such that there is little difference in the energy of binding or oxidation of the conformations accessible to the *erythro* and *threo* isomers. An alternative explanation is that C–C

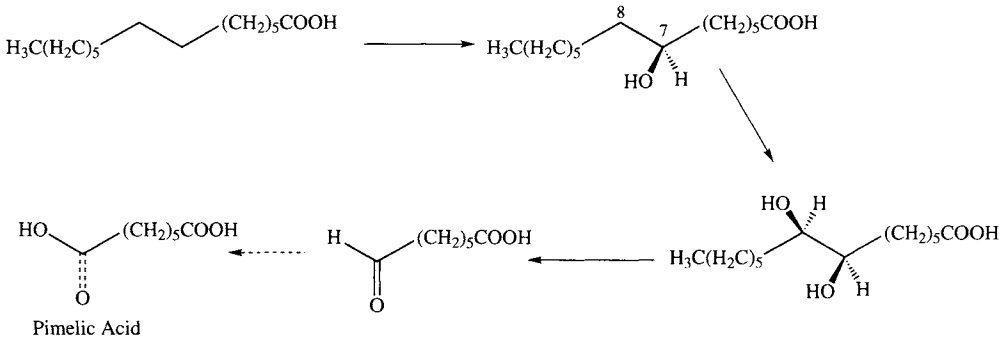


Figure 6.33. The intermediates in the C–C bond cleavage reaction catalyzed by $P450_{BioI}$ that produces pimelic acid from tetradecanoic acid.

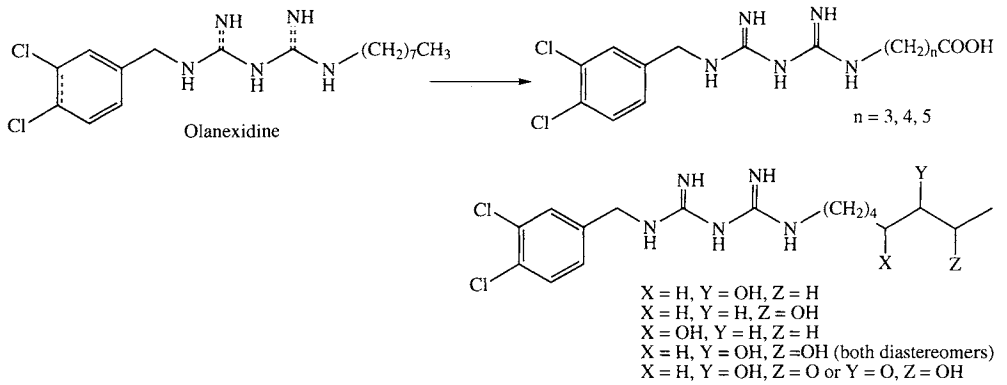


Figure 6.34. Oxygenated metabolites produced by P450-mediated oxidation of olanexidine.

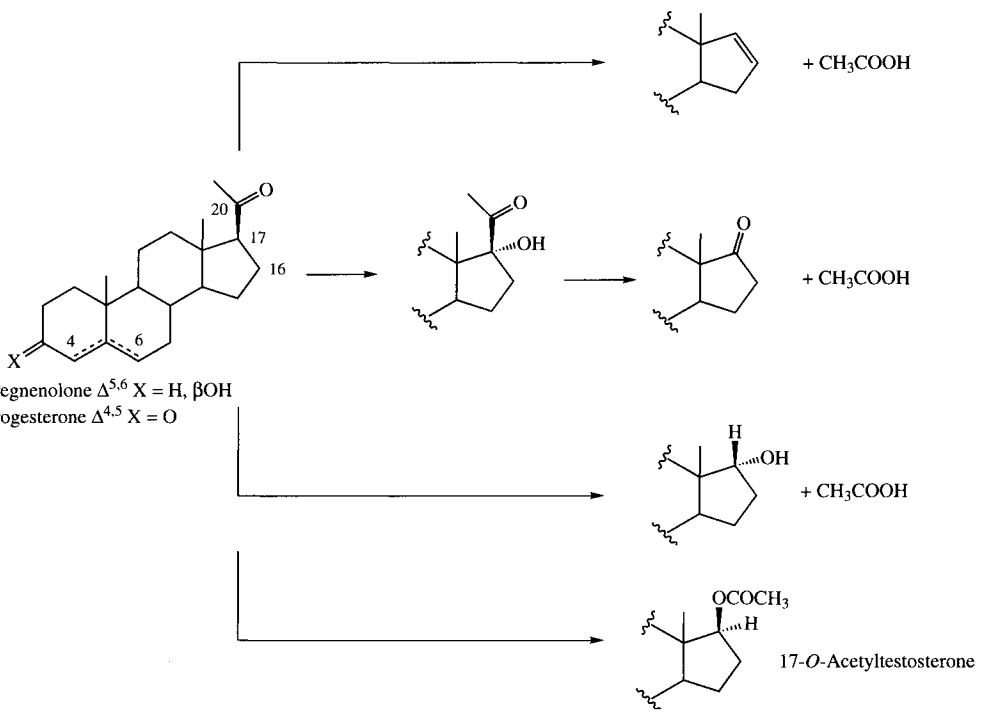


Figure 6.35. Various oxidation products reported to be formed from pregnenolone/progesterone via the action of CYP17A.

bond cleavage does not proceed directly from the diol but rather an α-hydroxy ketone which is also an observed metabolite (cf. CYP17A below). However, the different effects of specific P450 inhibitors on α-hydroxy ketone formation and C–C bond cleavage argue against a precursor product relationship[248]. Final delineation of the pathway awaits studies with purified isoforms but does suggest that C–C bond cleavage may be a significant metabolic pathway for compounds with aliphatic chains.

Keto Alcohols. CYP17A is a remarkable, multifunctional P450 that is primarily responsible for the 17α-hydroxylation of pregnenolone (or progesterone) and the subsequent lysis of the C17–C20 bond to produce dehydroepiandrosterone (or androstendione)[250, 251]. In addition, it catalyzes the cleavage of this same C17–C20 bond in mechanistically distinct ways to yield a number of minor products. These less common pathways lead to the formation of 17α-hydroxyandrost-5-en-3β-ol (note inversion of stereochemistry at C17) and the corresponding $\Delta^{16,17}$-olefin from pregnenolone[252–254] as well as 17-*O*-acetyltestosterone from progesterone (Figure 6.35)[255]. The proposed mechanism for the dominant reaction involves an unremarkable hydroxylation at C17 of the steroid nucleus (Figure 6.36)[256]. This is then followed by an attack of the ferric peroxo moiety on the carbonyl to yield a species that fragments to an alkoxy radical and a one-electron oxidized ferryl species. The alkoxy radical subsequently decomposes to produce acetic acid and a carbon radical that recombines with the ferryl species to yield a *gem*-diol, which dehydrates to the C17 carbonyl of the product. This mechanism is in accord with a wealth of labeling studies and can be modified simply to explain the origin of the other observed products.

Studies with $^{18}O_2$ have demonstrated the incorporation of one atom of ^{18}O into the acetic acid fragment produced upon C17–C20 cleavage of pregnenolone in all of the observed pathways[254, 256, 257]. Additionally, ^{18}O incorporation from $^{18}O_2$ is seen at the C17 position of the steroidal products from pregnenolone bearing an oxygen atom at this position[256]. The minor products from C17–C20 cleavage are proposed to arise from attack

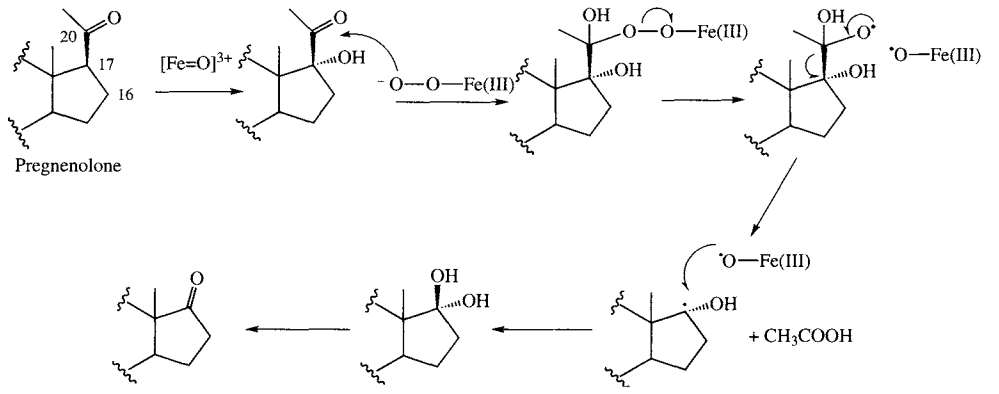


Figure 6.36. Proposed mechanism for the C17–C20 lyase reaction catalyzed by CYP17A. The key steps involve addition of a P450 ferric peroxide species to the C20 carbonyl and subsequent free radical fragmentation of the peroxyhemiacetal.

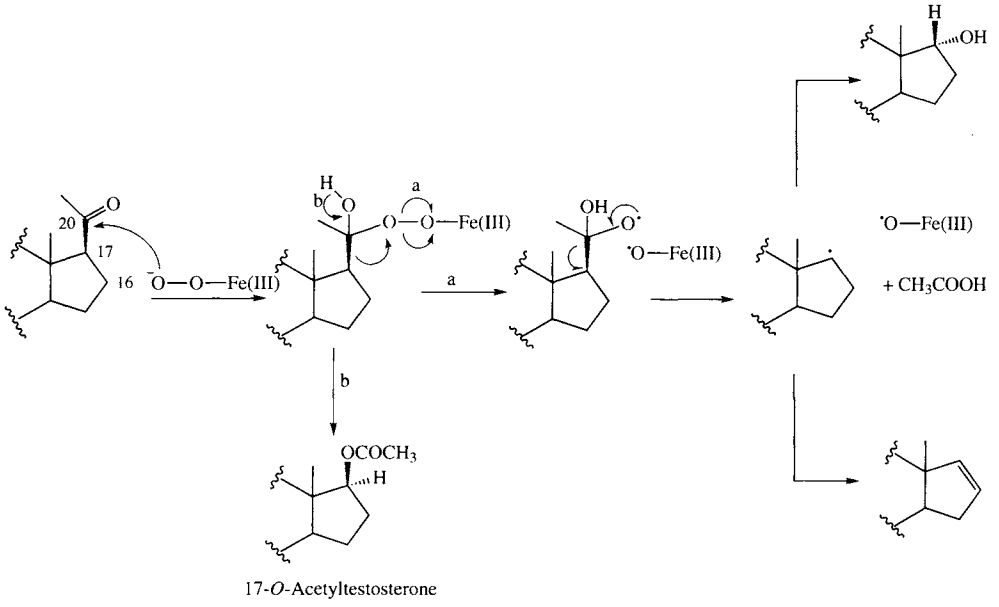


Figure 6.37. Proposed mechanistic manifold to account for the formation of other products in the CYP17A catalyzed oxidation of pregnenolone/progesterone. Ionic decomposition (pathway b) of the peroxyhemiacetal competes with free-radical fragmentation (pathway a) to yield the observed mixture of minor products.

of the ferric peroxo species on the carbonyl prior to any C17 hydroxylation (Figure 6.37). The resultant adduct then fragments to an alkoxy radical which loses acetic acid to produce a C17 radical in a fashion analogous to that proposed for the major pathway. This C17 radical then partitions between direct oxygen rebound to the 17α-hydroxy product and elimination to the $\Delta^{16,17}$-olefin. This latter product may arise via direct hydrogen abstraction or via a single-electron oxidation of the radical to the cation followed by elimination of a proton[256]. Results of CYP17A catalyzed oxidation of substrates bearing deuterium labels at the C16, C17 and the methyl group α to the ketone are in agreement with this mechanism[256]. The 17-*O*-acetyl-testosterone is probably best explained as

the result of a Baeyer–Villiger like decomposition of the peroxo adduct derived initially from an attack on the carbonyl (Figure 6.37)[255]. This indicates that the peroxo adduct may decompose by an ionic mechanism as well as by the radical pathway proposed to explain the other observed products.

The role of the ferric peroxo moiety in the mechanism has been supported by mutagenesis studies in which Thr306 has been replaced by an alanine[258]. This threonine is believed to be the active site residue that directs the delivery of protons required to cleave the O–O bond and form the ferryl species. As expected, its loss results in an approximately 20-fold decrease in the ferryl dependent C17 hydroxylation activity but a much smaller decrease in C17–C20 lyase activity mediated by the ferric peroxo moiety[258]. Experiments involving analysis of the solvent deuterium isotope effect as a function of pH have suggested that the protonation of the ferric peroxide intermediate governs whether the reaction proceeds via a ferryl dependent (17α hydroxylation) or a peroxy adduct (C17–C20 lyase) pathway[259].

The aldehyde corresponding to pregnenolone has also been used as a mechanistic probe with CYP17A as it is reported to undergo exclusive cleavage of the C17–C20 bond to produce formic acid and the 17α alcohol or $\Delta^{16,17}$-olefin[260]. These reactions are believed to proceed via pathways analogous to those proposed for the formation of minor cleavage products of CYP17A catalyzed oxidation of pregnenolone. The more electrophilic carbonyl of the aldehyde favors the direct bond scission pathways by more effectively trapping the ferric peroxide moiety. The aldehyde is not reported to be oxidized to the corresponding acid[260]. This suggests that an ionic cleavage of the proposed peroxy intermediate (Baeyer–Villiger pathway) does not occur to any great extent as hydrogen migration, which would lead to acid formation, is known to be favored for this type of reaction. The experiments with this aldehyde do, however, provide evidence for the bifurcation of a single pathway leading to the two minor products of pregnenolone oxidation (Figure 6.37). Deuteration of the C16α position led to an apparent enrichment in deuteration of the 17α-hydroxy product, suggesting an isotope-induced partitioning away from the $\Delta^{16,17}$-olefin that requires cleavage of the C–D bond[260]. Cleavage of the C–C bond α to an aldehyde is discussed further in Section 8.3.

8.2. Cleavage Alpha to Oxygenated Carbon

Ketones. The CYP17A-mediated cleavage of the C17–C20 bond of pregnenolone (or progesterone) without prior C17 hydroxylation provides the only clearly documented example of cleavage of a C–C bond α to a ketone (Figure 6.37, Section 8.1). The manifold of products formed, however, nicely indicates the variety of mechanistic pathways that might be envisioned. A peroxo adduct from the carbonyl and the ferric peroxide intermediate forms and subsequently decomposes by one of two pathways. A radical mechanism leads to an alkoxy radical that eventually gives C–C bond cleavage to form an alcohol or olefinic product. An ionic mechanism (Baeyer–Villiger) leads to an ester product in which insertion of oxygen has occurred with retention of configuration. It will be of interest to determine whether such pathways might provide the dominant activity of some P450s.

Aldehydes. Cleavage of a C–C bond α to an aldehyde has already been discussed in the context of the CYP17A-catalyzed oxidation of an aldehyde analogue of pregnenolone (Section 8.1). However, such reactions are believed to play a central role in the activities of several other P450s including the important steroid biosynthetic enzymes aromatase (CYP19) and 14α-demethylase (CYP51). It is worth emphasizing that P450-catalyzed aldehyde oxidation does not necessarily result in C–C bond cleavage and that in fact often oxidation to the corresponding carboxylic acid occurs[261]. The factors that govern partitioning between these different modes of oxidation are unknown at present[261].

Aromatase (CYP19), like $P450_{scc}$, plays an essential role in the biosynthesis of steroid hormones. It catalyzes the aromatization of the C_{19} androgen, androstendione to the C_{18} estrogen estrone (Figure 6.38), as well as similar aromatizations of testosterone and 16α-hyroxyandrostendione[262, 263]. These conversions involve three sequential oxidations at the angular C19 methyl group that result in its eventual loss and aromatization of the A-ring of the substrate. Each oxidation requires a molecule of NADPH and of

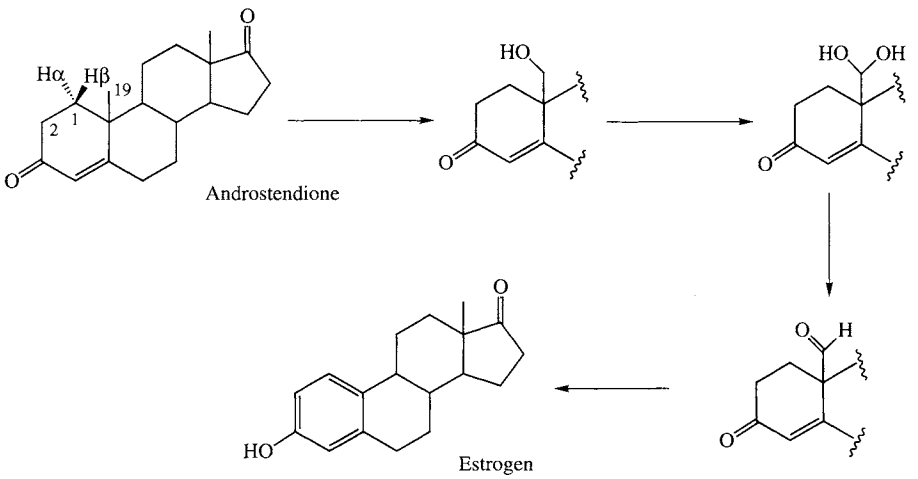


Figure 6.38. Intermediates in the catalytic turnover of aromatase (CYP19).

oxygen[264]. The first two steps appear to be unexceptional P450-catalyzed hydroxylation steps. The initial reaction produces the C19 primary alcohol and proceeds, as expected, with retention of configuration[265, 266], while the second oxidation abstracts the 19-*pro-R* hydrogen to yield a *gem*-diol intermediate[267, 268]. This latter compound is believed to dehydrate to yield the more stable, observed C19 aldehyde. There is an observable tritium isotope effect on the first hydroxylation step[269], but not on the subsequent one with [19-^{3}H] androst-4-ene-3,17-dione or analogues[270, 271]. This is understandable as the first step can discriminate between the hydrogen and tritium atoms on a given methyl group. An isotope effect on the second step, however, which stereospecifically removes the *pro-R* hydrogen, would require the kind of *inter*-molecular effect commonly suppressed in P450 reactions. It is the mechanism of the third oxidative transformation that involves C–C bond cleavage and aromatization that has attracted the most attention. In this reaction, the 1β and 2β hydrogens are lost[272–277] into water and the C19 carbon as formate which contains an oxygen atom from the first and third oxidation steps[278, 279]. A large number of different mechanisms have been proposed to account for this transformation involving the intermediacy of a steroid containing a C19 formyl group and, variously, a 4,5-epoxide[280], a 1β-hydroxyl[276], a 2β-hydroxyl [281, 282], or a C19 peroxide [278, 283] as well as a possible enzymic Schiff base formed from the 3-keto moiety[284]. Several of these intermediates are known to be converted spontaneously[281] or by aromatase[285] into estrone but none of them are currently accepted as lying upon the major pathway for aromatization. This is primarily due to the ^{18}O labeling studies indicating that the third oxygen atom is incorporated into formate[278, 280]. The difficulties in establishing the mechanism are illustrated nicely with the postulated 2β-hydroxy intermediate. This was synthesized and shown to aromatize rapidly in the absence of enzyme[281] and it was also detected in enzymic incubations at low pH (which slows the aromatization reaction)[282]. However, the facts that the 2β-hydroxyl was not incorporated into the released formate[279], and the stereochemistry of loss of hydrogen from C-2 appears to be substrate dependent ruled this compound out as an obligatory intermediate[286, 287]. The currently accepted mechanism[256, 288–290] explains all experimental observations and is supported by model studies[291–293] and analogy with the mechanisms of other P450s such as CYP17A and CYP2B4 (*vide infra*) (Figure 6.39). Thus, the ferric peroxide intermediate is believed to add to the electrophilic aldehyde carbonyl to yield a peroxyhemiacetal. This can fragment to give an alkoxy radical that loses formic acid to produce a C10 radical. Loss of the 1β hydrogen and enolization of the carbonyl is required to produce the aromatized A ring. Recent model studies by Valentine and coworkers provide

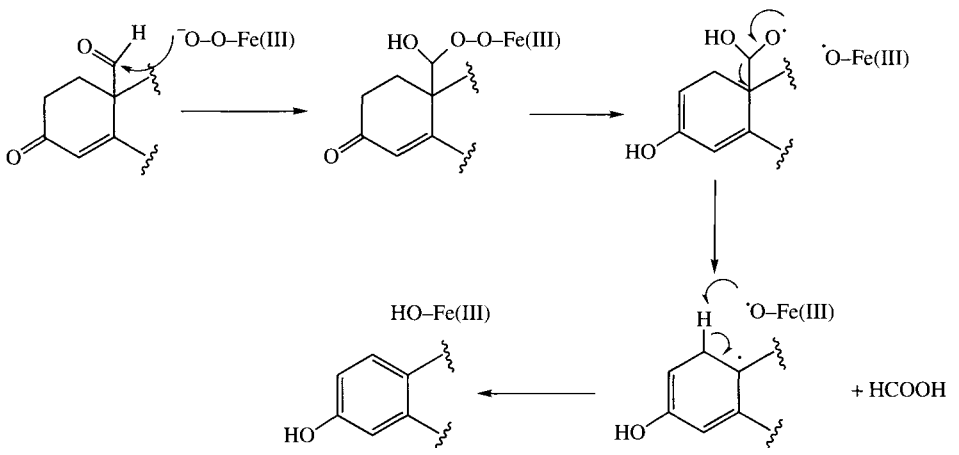
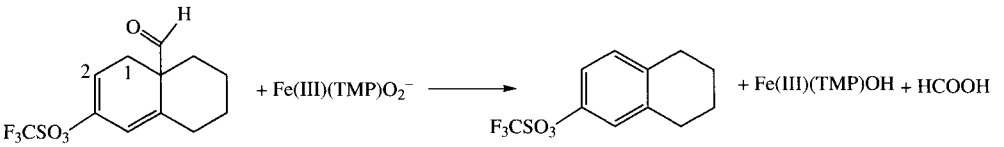


Figure 6.39. The currently accepted mechanism for the final step in the aromatase catalyzed reaction. The timing of enolization of the carbonyl with respect to the addition of the ferric peroxide to the aldehyde and to C–C and O–O bond fission is still uncertain.



TMP = Tetramesitylporphyrin

Figure 6.40. Conversion of enolized analogue of the natural aromatase substrate to the corresponding aromatized compound is catalyzed by a model peroxo ferric porphyrin complex.

strong support for the involvement of the ferric peroxo in the mechanism[291]. They demonstrated that a model peroxo ferric porphyrin complex will quantitatively convert an enolized model of androstendione to the corresponding aromatic compound and formate (Figure 6.40). Reaction of the ferric peroxo model with androstendione itself results in the chemically reasonable epoxidation of the electron deficient C4–C5 double bond. This follows suggestions in the literature that enolization of the C3 carbonyl occurs prior to C19–C10 bond cleavage and additionally activates the 1β hydrogen toward loss[293, 294]. However, whether the chemoselectivity required (C–C bond cleavage vs epoxidation) is achieved enzymatically via prior enolization or by selective positioning of the substrate within the active site remains to be established.

Enzymes of the CYP51 family (sterol 14α-demethylases) catalyze the removal of the 14α-methyl group from a variety of steroidal nuclei with concomitant introduction of a carbon–carbon double bond (Figure 6.41). The archetypal reaction is the loss of the C14 angular methyl group (C32) from lanosterol with formation of a C14–C15 double bond during cholesterol biosynthesis[295, 296]. Once again, this conversion is believed to involve three sequential oxidation steps and proceed initially via an alcohol that is subsequently converted into an aldehyde[297–307]. These steps parallel the first two steps catalyzed by aromatase and are believed to be unexceptional hydroxylation reactions[308–310]. The stereochemical course of the second hydroxylation is unknown, but studies with mechanism-based inhibitors have shown that steroidal 32-*S*-vinylalcohols are transformed to covalent inhibitors, presumably with a C32 carbonyl via a C32 *gem*-diol while the 32-*R* isomers are not oxidized[311]. These results do demonstrate stereospecificity in the oxidation of

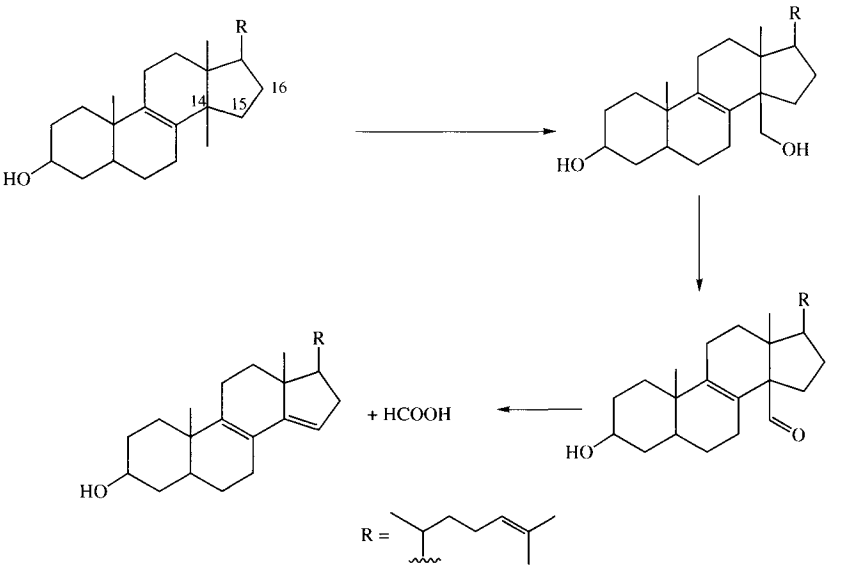


Figure 6.41. The stable intermediates in the 14α-demethylation of lanosterol.

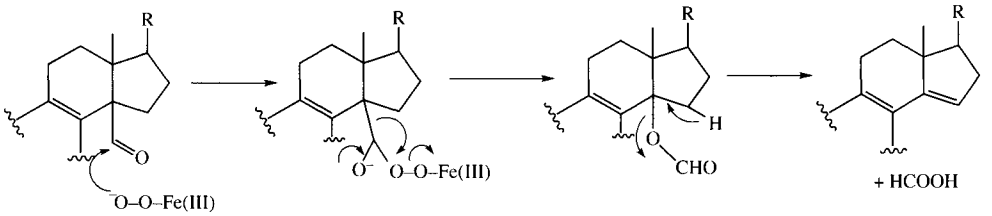


Figure 6.42. A possible mechanism for the final step in the 14α-demethylation of lanosterol that employs the isolated Baeyer–Villiger rearrangement product. Radical decomposition of the peroxyhemiacetal intermediate may also lead to the observed demethylated product.

a C32 alcohol but the deduction that the *proS* hydrogen is removed is more questionable. The 32-*S*-vinyl alcohols that are oxidized have a hydrogen that is stereochemically equivalent to the *proR* hydrogen of the hydroxymethyl intermediate in the demethylation reaction. This would suggest that it is this *proR* hydrogen that is abstracted in the second hydroxylation step catalyzed by CYP51.

The facts on the oxidation of the C32 aldehyde are remarkably similar to those seen for the aromatase catalyzed reaction. The formate moiety that is expelled contains one dioxygen-derived oxygen atom and incorporates one oxygen and one hydrogen atom from the aldehyde precursor[312]. There is also a stereospecific loss of the *syn* 15α hydrogen in the formation of the $\Delta^{14,15}$ double bond[298, 313, 314]. By analogy with the aromatase reaction, the oxidation is proposed to be initiated by addition of the ferric peroxo intermediate to the electrophilic aldehyde to produce a peroxyhemiacetal (Figure 6.42). However, the decomposition of this intermediate is proposed to occur via an ionic Baeyer–Villiger-like mechanism rather than a radical one. The primary reason for this is the isolation and spectroscopic identification of the 14α-formyloxy compound required as an intermediate in the ionic mechanism[315]. It was also demonstrated that the 14α-formyloxy intermediate could be converted into the final product by the enzyme[315]. The mechanism of this elimination has

not been studied in detail but loss of the tertiary allylic formate would be expected to be a facile reaction, subject to standard acid–base catalysis. However, the proposed mechanism invokes an ionic decomposition of a peroxyhemiacetal while those suggested for CYP17 and CYP19 invoke radical pathways. The possibility remains therefore that, as is believed to be the case with CYP17, the Baeyer–Villiger product is formed as a result of a minor pathway while the major route proceeds via simultaneous elimination of C32 and the 15α hydrogen. A unified view of the mechanism of the three P450 families would suggest that this occurs via radical decomposition of the peroxyhemiacetal (cf. Figure 6.39). It is perhaps also possible that the isolated formyloxy compound arises from formate trapping of a C14 cation, the major fate of which would be to undergo elimination with loss of the C15α hydrogen. The cation would derive from a single-electron oxidation of a C14 radical, the intermediate in the radical decomposition of the peroxyhemiacetal. The availability of cloned and overexpressed CYP51 from animals[316, 317], plants[318], fungi[312], and bacteria[319–321] with differing substrate specificity[322] and also of a crystal structure of one of the bacterial enzymes[18] should facilitate complete elucidation of the mechanism of this interesting family of P450s.

Some xenobiotic metabolizing enzymes, particularly CYP2B4, are also reported to be capable of catalyzing the conversion of some aldehydes into the one-carbon diminished alkene and formate[323, 324]. It appears that there is a structural requirement for α or β branching of the aldehyde for the reaction, with alkene formation occurring with compounds such as isobutyraldehyde and 2-methylbutyraldehyde but not with the straight-chain propionaldehyde or valeraldehyde[323]. Although most work has been carried out with CYP2B4, other isoforms such as CYP1A2, 2E1, 2C3, and 3A6 are all reported to catalyze this type of transformation[323]. The extent to which this occurs relative to oxidation of the aldehyde to the corresponding carboxylic acid appears small with a ratio of 50:1 favoring acid formation in the CYP2B4 catalyzed oxidation of 2-phenylpropionaldehyde[325]. However, the reaction has proved very valuable as a model for understanding the mechanism of the C–C bond cleavage reactions of CYP17, 19, and 51 and has played a significant role in the formulation of their mechanisms above. It was shown that the deformylation reaction was supported by P450 reductase/NADPH or H_2O_2 but not cumene hydroperoxide or iodosyl benzene[324]. This concurs with the hypothesis that it is the ferric peroxo species that adds to the aldehyde rather than the ferryl species. Fragmentation of the hydroperoxyhemiacetal then occurs to produce formate and the alkene (Figure 6.43). Significantly, formation of the one-carbon reduced alcohol has also been

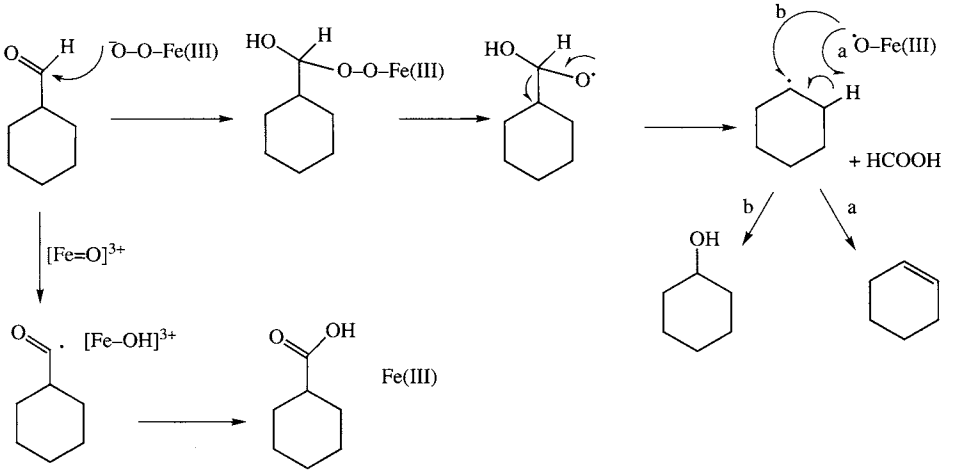


Figure 6.43. The oxidation of cyclohexanecarboxaldehyde by CYP2B4 is believed to proceed via the ferryl species to yield the carboxylic acid and via the ferric peroxo species to yield the deformylated products, cyclohexene and cyclohexanol.

reported, although only in passing![325] This product is analogous to the 17α-hydroxy C_{19} products reported from CYP17A oxidation of pregnenolone and its analogues. It should be noted that while deformylation is thought to involve the ferric peroxo species, oxidation to the acid is believed to proceed via the ferryl species[325].

The relevance of the CYP2B4 catalyzed deformylation reaction as a model for CYP51 is clearly demonstrated by the aromatization of the androstendione analogue 3-oxodecalin-4-ene-10-carboxaldehyde to the corresponding tetrahydronaphthalene (cf. Figure 6.40) with concomitant formate production[326, 327]. Deuterium isotope studies showed that the formyl hydrogen was retained in the formate, that the 1β hydrogen was specifically lost, and that loss of the C2 hydrogen was not stereoselective. These results faithfully reproduce the characteristics of the aromatase catalyzed reaction.

Recently, support for the role of ferric peroxo species in CYP2B4 catalyzed deformylation, and by analogy for the mechanisms of CYP17, -19, and -51, has come from mutagenesis studies[327]. Vaz and Coon reported the effect of replacing Thr302, the residue thought to facilitate O–O bond cleavage in CYP2B4, with alanine. It was expected that this would favor the peroxo pathway and decrease the availability of the ferryl species. In line with these expectations, normal P450-catalyzed reactions, including aldehyde to carboxylic acid oxidation, were suppressed but deformylation to the alkene and alcohol products was significantly enhanced[327]. Evidence for the radical nature of the decomposition of the peroxyhemiacetal has come from examination of the mechanism-based inactivation of P450s that occurs concurrently with aldehyde oxidation[325, 328]. For saturated aldehydes, it was shown that inactivation of CYP2B4 paralleled their ability to undergo a deformylation reaction, suggesting that both of these processes flowed from a common intermediate, the peroxyhemiacetal (Figure 6.43)[325]. It was shown that inactivation of the P450 was due to addition of the carbon radical, formed in a homolytic process, to the γ-*meso* position of the prosthetic heme[328]. Interestingly, although $P450_{BM3}$ is reported to oxidize a variety of aldehydes without detectable deformylation[261], it was demonstrated that a mutant is deactivated by aldehydes when the co-oxidant is H_2O_2[329]. This presumably again occurs through an alkyl radical formed by homolytic decomposition of the peroxyhemiacetal intermediate. An intermediate was detected in this work that was spectroscopically consistent with an isoporphyrin which would be formed upon addition of a carbon radical to the heme cofactor[329]. Finally, it is of note that the ferryl catalyzed oxidation of aldehydes to acids can also cause enzyme inactivation by heme adduct formation, but in this case as predicted for an H abstraction mechanism, an acylated heme is formed[328].

Cytochrome P450s can also interact with aldehydes in a different way to generate the corresponding hydrocarbon and CO_2,[330]. Hydrocarbons are abundant components of cuticular lipids in most insects and can also play a role in their chemical communication. It has been demonstrated that in microsomes derived from the house fly, *Musca domestica*, hydrocarbons are formed from the corresponding aldehyde with concomitant generation of CO_2 and with all the characteristics expected of a P450-mediated reaction:

$$CH_3(CH_2)_8CH{=}CH(CH_2)_{12}CD_2CDO + NADPH + O_2 + H^+ \rightarrow CH_3(CH_2)_8CH{=}CH(CH_2)_{12}CD_3 + NADP^+ + CO_2 + H_2O$$

There is a requirement for NADPH and oxygen and the reaction is inhibited by both CO and an antibody to house fly P450 reductase[330]. Labeling studies showed that deuterium atoms at the C-1, C-2, and C-3 positions were all retained[331]. In addition, active oxygen donors such as hydrogen peroxide, cumene hydroperoxide, and iodosylbenzene all support hydrocarbon production to some extent. The ability of the latter species to support oxidation clearly indicates that the ferric peroxide species is not the active oxidant in this case. On the basis of these results, an unusual mechanism has been proposed[331] and a slightly more conventional version is presented here (Figure 6.44). The first step is the oxidation of the aldehyde to a dioxirane or its resonance form, a carbonyl oxide. Dioxiranes are known to decompose with release of CO_2 and formation of two radicals that can recombine as shown to form a hydrocarbon[332]. Presumably, this recombination would be favored by retention of the fragments within the active site. Complete elucidation of the reaction mechanism awaits identification and purification of the P450 but recent studies have shown this to be a

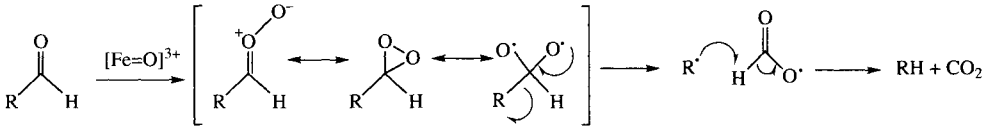


Figure 6.44. Possible mechanism for the P450-catalyzed conversion of an aldehyde into the corresponding hydrocarbon and CO_2 seen in the biosynthesis of insect-derived hydrocarbons.

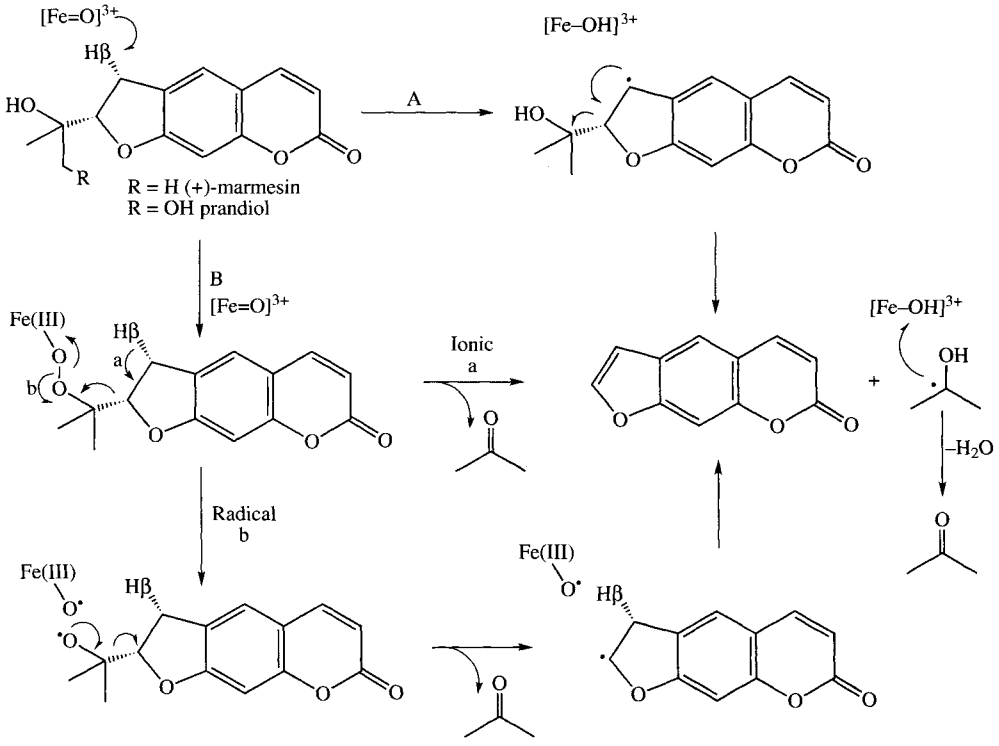


Figure 6.45. Mechanistic possibilities for the P450-catalyzed conversion of (+)-marmesin into psoralen and acetone. Prandiol is known not to be an intermediate in this process.

widespread reaction in insects for the formation of hydrocarbons[333].

Alcohols. Boland and coworkers demonstrated conclusively that a P450 can catalyze the direct fragmentation of an alcohol into an olefin and a carbonyl-containing fragment[69]. They studied the conversion of marmesin to psoralen in microsomes derived from cell cultures of the plant *Ammi majus* (Figure 6.45)[69]. Deuterium labeled precursors allowed them to demonstrate that marmesin was converted into an equimolar mixture of acetone and psoralen, excluding the possibility of other oxygenated intermediates such as the known prandiol (Figure 6.45). The stereochemistry of the elimination was exclusively *syn* and a small isotope effect ($k_H/k_D = 4$) was observed when the abstracted hydrogen was replaced with deuterium. The mechanism proposed (Figure 6.45, pathway A) consists of β-hydrogen atom abstraction, decomposition of the radical intermediate to produce the olefin and an isopropoxyl radical, the latter of which is intercepted by the Fe(IV)OH species[69]. A possible mechanistic alternative (Figure 6.45, pathway B) invokes intermediates analogous to those proposed for the diol cleavage reactions above. In these, it would be the alcohol moiety that is initially

attacked and initiates fragmentation to the observed products.

This type of C–C cleavage reaction, however, appears to be a general and important biosynthetic one in plants and a number of other analogous oxidative C–C bond cleavage reactions seen in bacteria and plants have now been postulated to be P450 catalyzed[334, 335]. In particular, secologanin synthase from *Catharanthus roseus* (CYP72A1) is believed to catalyze the C–C bond cleavage that transforms loganin into secologanin, the final common non-nitrogenous precursor of many plant indole alkaloids (Figure 6.46)[335, 336]. In this case, the reaction involves cleavage of a carbocyclic ring rather than fragmentation of the substrate. This activity was demonstrated *in vitro* with CYP72A1 heterologously expressed in *E. coli* as a fusion with its homologous P450 reductase[336].

A reaction that involves cleavage of the C–C bond α to a phenol occurs in aflatoxin biosynthesis[337]. Aflatoxins are mycotoxins produced by strains in the fungal genus *Aspergillus* and are notable for the complexity of their biogenesis. Genetic evidence suggested that a single P450 was responsible for the transformation of *O*-methylsterigmatocystin to aflatoxin B_1 (Figure 6.47)[338]. A P450 from *Aspergillus parasiticus* was subsequently cloned, heterologously expressed in yeast, and was demonstrated to be capable of catalyzing this remarkable conversion *in vivo*[337]. The first formed 11-hydroxy-*O*-methylsterigmatocystin (Figure 6.47) was also synthesized and shown to be converted to aflatoxin B_1. These experiments interlocked with a wealth of previous results from *in vivo* isotope labeling studies and led to the mechanistic hypothesis shown[337]. After C–C bond cleavage and formation of the proposed hydrolytically unstable lactone, the ensuing decarboxylation, dehydration, rearrangement, and *O*-demethylation reactions are presumed to proceed spontaneously. Two plausible mechanisms for the

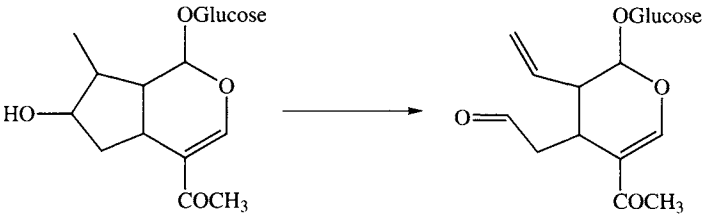
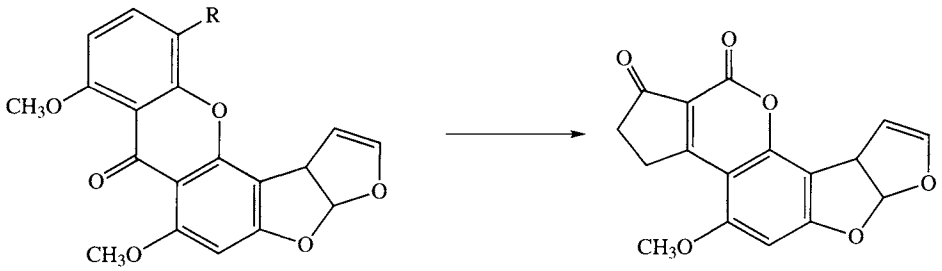


Figure 6.46. Loganin is converted into secologanin via a P450-catalyzed C–C bond cleavage reaction analogous to that seen in psoralen biosynthesis.



R = H *O*-Methylsterigmatocystin
R = OH 11-hydroxy *O*-Methylsterigmatocystin

Figure 6.47. A single cytochrome P450 is responsible for the conversion of *O*-methylsterigmatocystin to aflatoxin B_1 via 11-hydroxy *O*-methylsterigmatocystin.

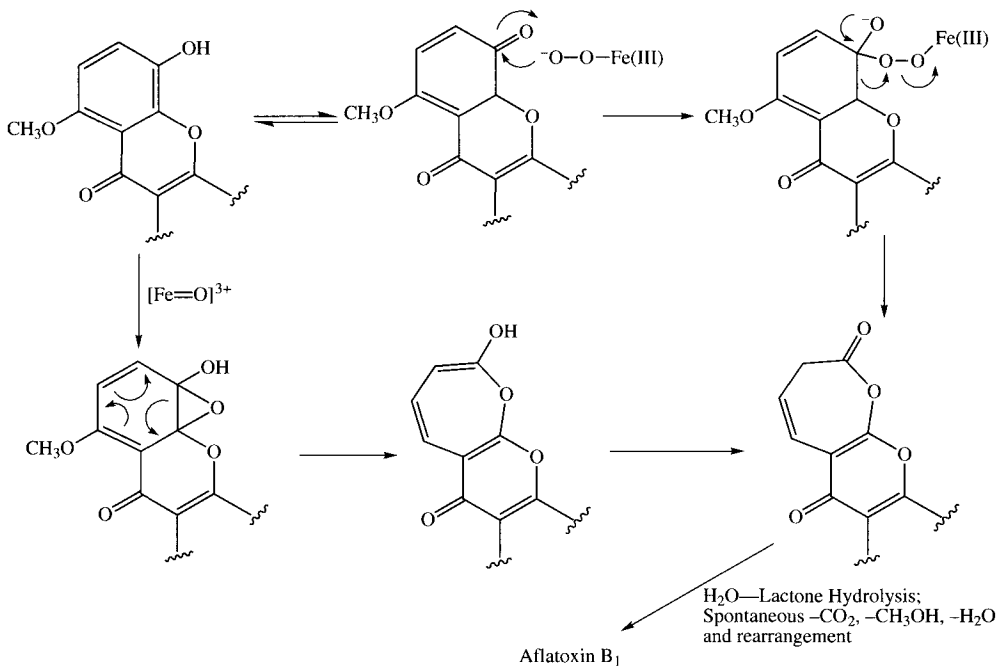


Figure 6.48. Mechanistic proposals for the P450-catalyzed C–C bond cleavage during the biosynthesis of aflatoxin B_1. One possibility involves a Baeyer–Villiger-like reaction of the ferric peroxo species with the keto tautomer of the phenolic substrate while the other proceeds via the epoxide intermediate typical of ferryl catalyzed aromatic oxidations.

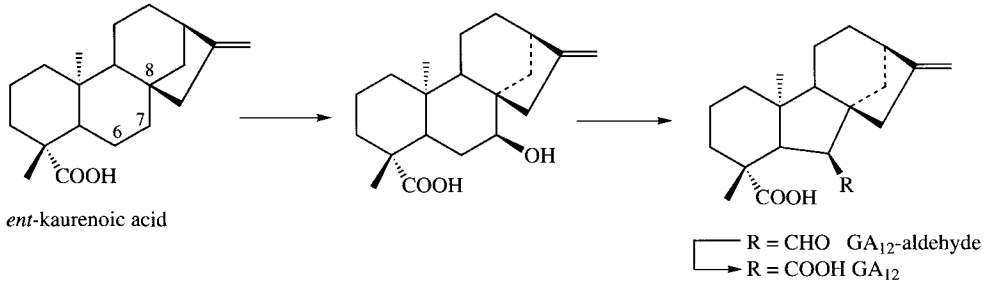


Figure 6.49. CYP88A catalyzes the three oxidative transformations required to convert *ent*-kaurenoic acid into GA_{12}.

C–C bond cleavage process have been proposed (Figure 6.48)[337]. The first involves a Baeyer–Villiger-like oxidation of the keto tautomer of the phenol and the second a rearrangement of the epoxide intermediate in aromatic oxidation. Delineation of the mechanism will require experimentation with purified enzyme, mutants, and substrate analogues.

The gibberellins (GAs) are important plant hormones with remarkably complex structures. Several similarly remarkable multifunctional P450s have been implicated in their biosynthesis in both plants and fungi[339, 340]. CYP88A from *Arabidopsis thaliana* and barley has been shown to catalyze the three oxidative steps required to convert *ent*-kaurenoic acid to GA_{12} (Figure 6.49)[341]. The experiments involved the expression of CYP88A in yeast strains containing *A. thaliana* P450 reductase and monitoring *in vivo* oxidation of potential substrates. The key step in the proposed

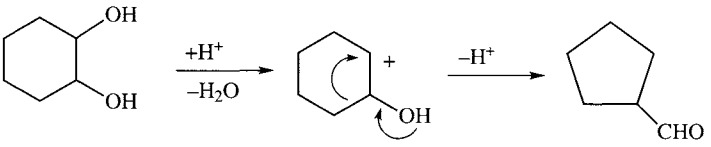


Figure 6.50. Mechanism of a pinacol rearrangement of a diol.

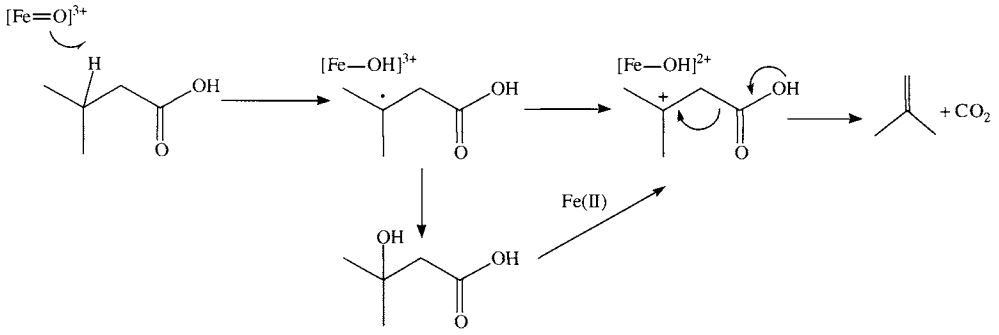


Figure 6.51. Likely mechanisms for the P450-catalyzed conversion of valerate into isobutene and CO_2. A pathway involving direct hydride abstraction has also been proposed but appears less probable.

reaction involves cleavage of a C–C bond α to an alcohol in an oxidative ring contraction to yield an aldehyde (GA_{12}-aldehyde, Figure 6.49)[341]. The mechanism has not been investigated but the process follows the pathway predicted for an α-hydroxy carbocation, such as the intermediate proposed for a pinacol rearrangement of a diol (Figure 6.50). Such a cation could arise from a diol formed by CYP88A catalyzed C6 hydroxylation under the influence of the Lewis acidic heme iron or directly via a SET process from the hydroxylation radical intermediate. Subsequently, a P450 from the fungus *Gibberella fujikuroi* was also shown to catalyze the same pinacol-like transformation, once again by expression and *in vivo* monitoring of putative substrate transformation[342]. In this case, a 6,7-diol was also isolated but was not further transformed via ring contraction, suggesting that such a compound is not an intermediate in this pathway. This single fungal P450 was also proposed to be capable of catalyzing at least *seven* other biosynthetically significant oxidative transformations as well as the three assigned to CYP88A, explaining the various GA metabolites found in *G. fujikuroi*. One of these other transformations is a proposed oxidative cleavage of the vicinal 6,7-diol. Clearly, the results of *in vitro* characterization of the catalytic capabilities of this enzyme will be of great interest.

Acids. Two isolated examples of P450-catalyzed oxidative decarboxylation have appeared in the literature. The first concerns the formation of isobutene from isovalerate by the yeast *Rhodotorula minuta*[343]. A P450 and a homologous reductase were purified and a reconstituted system that produced isobutene from isovalerate was constructed[343]. A large isotope effect upon isobutene formation was found when the β-hydrogen was substituted with deuterium (k_H/k_D = 14), clearly implicating cleavage of this bond in the rate-determining step. It was also found that β-branching appeared to be necessary for alkene formation. A mechanism involving direct hydride abstraction and decarboxylation of the resultant cation was proposed[343]. However, more conventional pathways are also possible in which either (a) hydrogen atom abstraction to give a carbon radical is followed by electron transfer to generate the corresponding carbocation, or (b) the tertiary alcohol is formed but ionizes to the carbocation under the influence of the Lewis acidic heme iron (Figure 6.51). One caveat with this system is that the P450 was subsequently shown to hydroxylate benzoate to 4-hydroxybenzoate as part of phenylalanine catabolism and this latter reaction

appears to be its primary metabolic function[344, 345]. It is also unclear whether other nonvolatile products of isovalerate oxidation are formed in the incubations which were monitored by headspace gas chromatography[343]. Thus, the exact nature of this decarboxylation reaction remains to be established.

Hirobe has reported the P450-catalyzed decarboxylation of carboxylic acids to a one-carbon shorter alcohol[346]. The acid must have an α-carbon bearing either a phenyl group or three substituents. This transformation was originally observed in iron–porphyrin model systems but was subsequently reproduced *in vivo* in rats and in rat liver microsomes for two therapeutic carboxylic acids (Figure 6.52)[346]. Once again, the mechanism has not been investigated but the

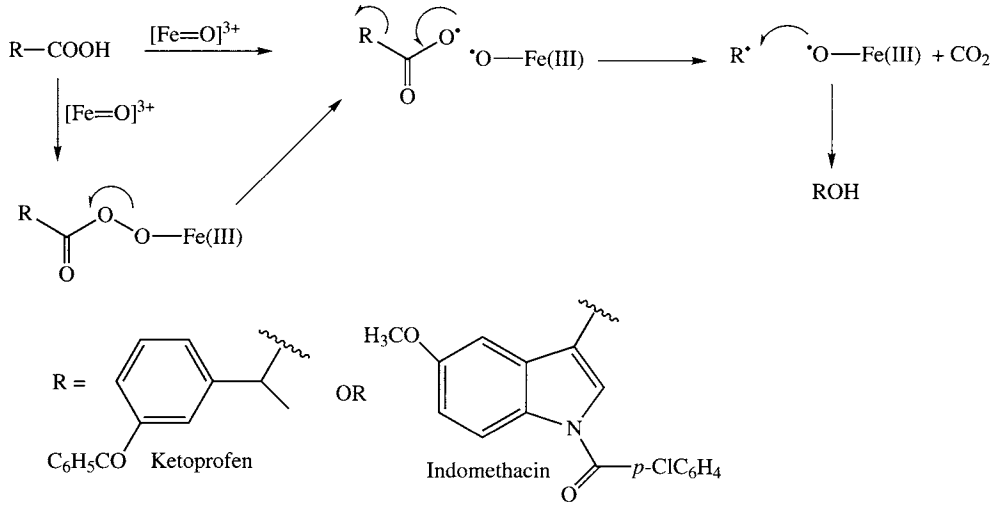


Figure 6.52. Possible pathways for the oxidative decarboxylation of some therapeutic carboxylic acids catalyzed by both P450s and some iron–porphyrin model systems.

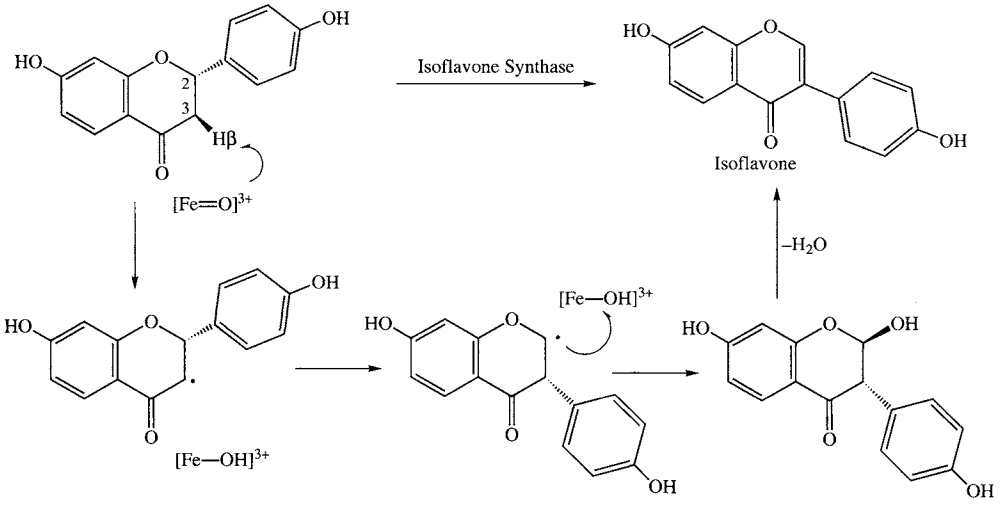


Figure 6.53. Isoflavone synthase (CYP93C) catalyzes the formation of isoflavone from 2*S*-flavone via an oxidative aryl migration. A possible mechanism involving an anchimerically stabilized radical is shown.

proposed decomposition of a carboxyl radical is attractive, especially as this process is known to be quite sensitive to α-substitution. The radical might be produced either directly from the carboxylate by the ferryl species or by decomposition of a peroxyacid initially formed by reaction of the acid and an ironoxo species (Figure 6.52). This latter mechanism would then be analogous to the reported CYP2B4 catalyzed conversion of 2-phenylperacetic acid to CO_2 and benzyl alcohol by homolysis of the O–O bond[347].

Ethers. Isoflavone synthase (CYP93C) catalyzes the formation of isoflavone from 2*S*-flavone via an unusual oxidative aryl migration (Figure 6.53)[348–350]. (This C–C bond cleavage occurs α to an ether and is classified as such here, but it is unclear whether this is a mechanistically significant feature.) Little is known about the reaction, but it is postulated to proceed via 3β HAT to give a carbon radical anchimerically stabilized by the adjacent phenol[348, 351]. Oxygen rebound can then occur at the C2 position to give the unstable 2-hydroxyisoflavone that dehydrates to isoflavone (Figure 6.53). Support for this mechanism is provided by the isolation of the 3β-hydroxyisoflavone as a side product of the reaction[351]. The availability of heterologously overexpressed wild-type protein and site-directed mutants should facilitate investigation of this unusual transformation[351].

8.3. Cleavage Alpha to Carbon Bearing a Nitrogen Atom

Amines. Recently, an example of C–C bond cleavage α to an amine has been reported (Figure 6.54)[352]. Interestingly, this is also a rearrangement reaction and one of the few examples of C–C bond scission catalyzed by non-biosynthetic enzymes. It was found that a variety of tetramethylpiperidine containing compounds were transformed into the corresponding dimethylpyrrolidine derivatives (Figure 6.54). By incubations with recombinant human liver P450s and immuno-inhibition studies, this reaction was shown to be catalyzed by a variety of P450s, with CYP3A4 the major isoform responsible for this transformation. The authors suggest that this is a general metabolic pathway for compounds containing a tetramethylpiperidine moiety as they have also observed similar metabolism in other mammals[352]. The mechanism of the reaction has not been investigated in detail but clearly appears to be a transformation of a secondary amine, formed via *N*-dealkylation if necessary, given the structures of the pyrrolidines produced. The intermediacy of hydroxylamines or the corresponding nitroxyl radical in this reaction has been suggested. One possibility (Figure 6.54) is that the heme iron may promote ionization of a hydroxylamine to an incipient nitrogen cation that rearranges, a pathway comparable to the P450-catalyzed dehydration of oximes to nitriles[353]. Alternatively, it may simply be a rearrangement of the intermediate nitrogen cation radical formed during amine oxidation. This can no longer be stabilized by α-hydrogen elimination and the steric congestion of the surrounding methyl groups may slow the oxygen rebound, allowing rearrangement (Figure 6.54). The piperidine to pyrrolidine rearrangement has precedent in the chemistry of *N*-fluoroamines that undergo the same ring contraction in the presence of a Lewis acid[354]. This latter reaction, however, presumably involves the equivalent of a nitrogen cation, rather than a cation radical species favoring the former pathway. More detailed investigations are required to determine the mechanism of this interesting transformation.

Finally, another of the remarkable multifunctional P450s involved in GA formation in fungi has recently been demonstrated to catalyze the demethylation of an angular carbon along the biosynthetic pathway (Figure 6.55)[355]. The apparently concomitant formation of the lactone with demethylation suggests that a different pathway is followed from that seen in aromatase and 14α-demethylase. It is tempting to speculate that this represents a biosynthetically novel oxidative decarbonylation or decarboxylation reaction in which an alcohol or the corresponding cation is the initial product. This could then be intercepted by the adjacent carboxylate to form the observed lactone (Figure 6.55). Clearly, however, the cytochrome P450s are capable of catalyzing C–C cleavage by a variety of mechanisms and much work remains to understand all the possible permutations of these interesting reactions.

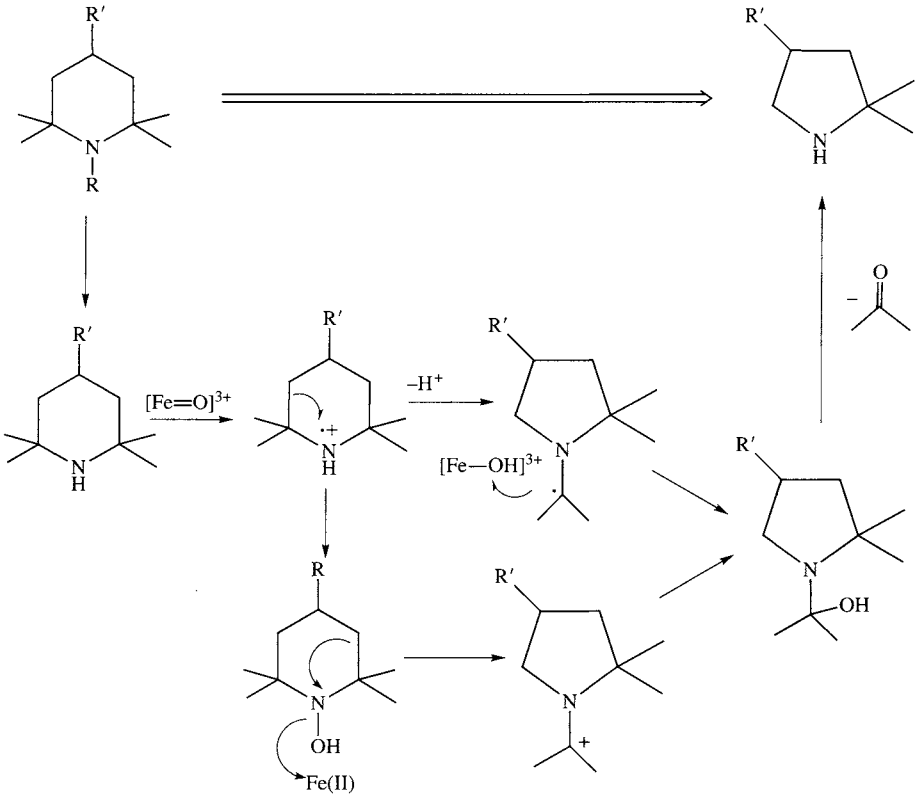


Figure 6.54. A variety of tetramethylpiperidine compounds are converted into the corresponding dimethylpyrrolidine derivatives by a number of xenobiotic metabolizing P450s, particularly CYP3A4. Two mechanistic possibilities for this process are shown. (R = H, R′ = *p*-nitrophenyl-NH- or R = CH_3, R′ = $(C_6H_5)_2HCO$-)

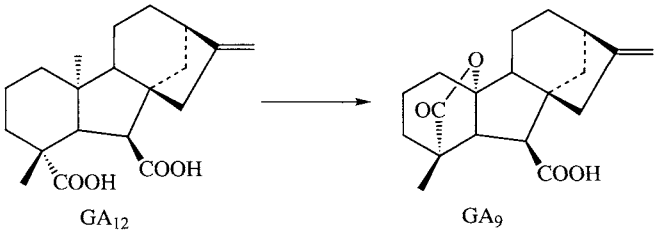


Figure 6.55. Gibberellin 20-oxidase from the fungus *Gibberella fujikuroi* is a multifunctional P450 that catalyzes the angular demethylation of GA_{12} to produce the lactone GA_9.

9. Conclusions

Cytochrome P450 mechanisms continue to surprise and delight, although the field is growing to maturity and the completely unexpected is less frequently encountered. Experimentally, the past few years have seen major progress in characterizing the intermediates that are formed as molecular oxygen is activated to the final oxidizing species. All the intermediates, with the exception of the

critical ferryl species, have now been directly observed by various spectroscopic and crystallographic methods. The ferric peroxo anion has been found to act as the oxidizing agent with a growing range of highly electrophilic substrates. In contrast, the proposed role for the ferric hydroperoxo complex as an electrophilic oxidizing agent remains a matter of debate, as the evidence advanced in support of the proposal is circumstantial and contradictory. Although the ferryl species remains elusive, it is increasingly clear that it plays the predominant role as the oxidizing agent in the P450 catalytic cycle. A second area that has recently received considerable attention is the mechanism of hydrocarbon hydroxylation, the key question being whether the radical rebound mechanism that has held sway for three decades is in fact valid. The contradictory results obtained with radical and cation probes, which have provided most of the new evidence, must be resolved by further experimentation in order for this question to be settled. The development of a two-state model for the catalytic action of P450 enzymes may be one of the most important recent advances in the field, as it provides a ready explanation for a variety of otherwise contradictory data, some of which argues for concerted and some for nonconcerted oxidation mechanisms. No doubt, the next few years will uncover novel aspects of P450 function and will lead to deeper and more sophisticated understanding of the catalytic mechanisms of the amazing family of P450 enzymes.

Acknowledgments

The work from the authors' laboratories and the preparation of this review were supported by National Institutes of Health Grant GM25515 (PROM) and Australian Research Council Grant DP0210635 (JJDV).

References

1. Dawson, J.H. and M. Sono (1987). Cytochrome P-450 and chloroperoxidase: Thiolate-ligated heme enzymes. Spectroscopic determination of their active-site structures and mechanistic implications of thiolate ligation. *Chem. Rev.* **87**, 1255–1276.
2. Shimizu, T., K. Hirano, M. Takahashi, M. Hatano, and Y. Fujii-Kuriyama (1988). Site-directed mutagenesis of cytochrome P-450d: Axial ligand and heme incorporation. *Biochemistry* **27**, 4138–4141.
3. Unger, B. (1988). Ph.D. Thesis. University of Illinois Urbana, Champaign, IL.
4. Auclair, K., P. Moënne-Loccoz, and P.R. Ortiz de Montellano (2001). Roles of the proximal heme thiolate ligand in cytochrome P450cam. *J. Am. Chem. Soc.* **123**, 4877–4885.
5. Yoshioka, S., S. Takahashi, H. Hori, K. Ishimori, and I. Morishima (2001). Proximal cysteine residue is essential for the enzymatic activities of cytochrome $P450_{cam}$. *Eur. J. Biochem.* **268**, 252–259.
6. Tani, F., M. Matsu-ura, S. Nakayama, M. Ichimura, N. Nakamura, and Y. Naruta (2001). Synthesis and characterization of alkanethiolate-coordinated iron porphyrins and their dioxygen adducts as models for the active center of cytochrome P450: Direct evidence for hydrogen bonding to bound dioxygen. *J. Am. Chem. Soc.* **123**, 1133–1142.
7. Woggon, W.-D., H.-A. Wagennecht, and C. Claude (2001). Synthetic active site analogues of heme-thiolate proteins. Characterization and identification of intermediates of the catalytic cycles of cytochrome P450cam and chloroperoxidase. *J. Inorg. Biochem.* **83**, 289–300.
8. Ogliaro, F., S. Cohen, M. Filatov, N. Harris, and S. Shaik (2000). The high-valent compound of cytochrome PA50: The nature of the Fe-S bond and the role of the thiolate ligand as an internal electron donor. *Angew. Chem. Int. Ed.* **39**, 3851–3855.
9. Green, M.T. (1999). Evidence for sulfur-based radicals in thiolate compound I intermediates. *J. Am. Chem. Soc.* **121**, 7939–7940.
10. Poulos, T.L., B.C. Finzel, and A.J. Howard (1987). High-resolution crystal structure of cytochrome P450cam. *J. Mol. Biol.* **192**, 687–700.
11. Ravichandran, K.G., S.S. Boddupalli, C.A. Hasemann, J.A. Peterson, and J. Deisenhofer (1993). Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science* **261**, 731–736.
12. Li, H. and T.L. Poulos (1997). The structure of the cytochrome p450BM-3 haem domain complexed with the fatty acid substrate, palmitoleic acid. *Nat. Struct. Biol.* **4**, 140–146.
13. Hasemann, C.A., K.G. Ravichandran, J.A. Peterson, and J. Deisenhofer (1994). Crystal structure and refinement of cytochrome P450terp at 2.3 A resolution. *J. Mol. Biol.* **236**, 1169–1185.
14. Cupp-Vickery, J. and T.L. Poulos (1995). Structure of cytochrome P450eryF involved in erythromycin biosynthesis. *Nat. Struct. Biol.* **2**, 144–153.
15. Shimizu, H., S.-Y. Park, Y. Gomi, H. Arakawa, H. Nakamura, S.-I. Adachi *et al.* (2000). Proton delivery in NO reduction by fungal nitric-oxide

reductase. Cryogenic crystallography, spectroscopy, and kinetics of ferric-NO complexes of wild-type and mutant enzymes. *J. Biol. Chem.* **275**, 4816–4826.

16. Yano, J.K., L.S. Koo, D.J. Schuller, H. Li, P.R. Ortiz de Montellano, and T.L. Poulos (2000). Crystal structure of a thermophilic cytochrome P450 from the archaeon *Sulfolobus solfataricus*. *J. Biol. Chem.* **275**, 31086–31092.
17. Podust, L.M., Y. Kim, M. Arase, B.A. Neely, B.J. Beck, H. Bach *et al.* (2003). The 1.92-A structure of *Streptomyces coelicolor* A3(2) CYP154C1. A new monooxygenase that functionalizes macrolide ring systems, *J. Biol. Chem.* **278**, 12214–12221.
18. Podust, L.M., T.L. Poulos, and M.R. Waterman (2001). Crystal structure of cytochrome P450 14alpha-sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. *Proc. Natl. Acad. Sci. USA* **98**, 3068–3073.
19. Nagano, S., H. Li, H. Shimizu, C. Nishida, H. Ogura, P.R. Ortiz de Montellano *et al.* (2003). Crystal structure of epothilone-D bound, epothilone-B bound, and susbstrate-free forms of cytochrome P450epoK. *J. Biol. Chem.* **278**, 44886–44893.
20. Williams, P.A., J. Cosme, V. Sridhar, E.F. Johnson, and D.E. McRee (2000). Mammalian microsomal cytochrome P450 monooxygenase: Structural adaptations for membrane binding and functional diversity. *Mol. Cell.* **5**, 121–131.
21. Guengerich, F.P. and W.W. Johnson (1997). Kinetics of ferric cytochrome P450 reduction by NADPH-cytochrome P450 reductase: Rapid reduction in the absence of substrate and variations among cytochrome P450 systems. *Biochemistry* **36**, 14741–14750.
22. Imai, M., H. Shimada, Y. Watanabe, Y. Matsushima-Hibiya, R. Makino, H. Koga *et al.* (1989). Uncoupling of the cytochrome P-450cam monooxygenase reaction by a single mutation, threonine-252 to alanine or valine: Possible role of the hydroxy amino acid in oxygen activation. *Proc. Natl. Acad. Sci. USA* **86**, 7823–7827.
23. Martinis, S.A., W.M. Atkins, P.S. Stayton, and S.G. Sligar (1989). A conserved residue of cytochrome P-450 is involved in heme-oxygen stability and activation. *J. Am. Chem. Soc.* **111**, 9252–9253.
24. Kimata, Y., H. Shimada, T. Hirose, and Y. Ishimura (1995). Role of Thr-252 in cytochrome P450cam: A study with unnatural amino acid mutagenesis. *Biochem. Biophys. Res. Commun.* **208**, 96–102.
25. Ortiz de Montellano, P.R. (1995). Oxygen activation and reactivity. In P.R. Ortiz de Montellano (ed.), *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd edn. Plenum, New York, pp. 245–304.
26. Estabrook, R.W., C. Martin-Wixtrom, Y. Saeki, R. Renneberg, A. Hildebrandt, and J. Werringloer (1984). The peroxidatic function of liver microsomal cytochrome P450: Comparison of hydrogen peroxide and NADPH-catalyzed N-demethylation reactions. *Xenobiotica* **14**, 87–104.
27. Renneberg, R., J. Capdevila, N. Chacos, R.W. Estabrook, and R.A. Prough (1981). Hydrogen peroxide-supported oxidation of benzo[a]pyrene by rat liver microsomal fractions. *Biochem. Pharmacol.* **30**, 843–848.
28. Fasco, M.J., L.J. Piper, and L.S. Kaminsky (1979). Cumene hydroperoxide-supported microsomal hydroxylations of warfarin—A probe of cytochrome P450 multiplicity and specificity. *Biochem. Pharmacol.* **28**, 97–103.
29. Kelly, W.G. and A.H. Stolee (1978). Stabilization of placental aromatase by dithiothreitol in the presence of oxidizing agents. *Steroids* **31**, 533–539.
30. Kupfer, R., S.Y. Liu, A.J. Allentoff, and J.A. Thompson (2001). Comparisons of hydroperoxide isomerase and monooxygenase activities of cytochrome P450 for conversions of allylic hydroperoxides and alcohols to epoxyalcohols and diols: Probing substrate reorientation in the active site. *Biochemistry* **40**, 11490–11501.
31. He, K., L.M. Bornheim, A.M. Falick, D. Maltby, H. Yin, and M.A. Correia (1998). Identification of the heme-modified peptides from cumene hydroperoxide-inactivated cytochrome P450 3A4. *Biochemistry* **37**, 17448–17457.
32. Mueller, E.J., P.J. Loida, and S.G. Sligar (1995). Twenty-five years of P450cam research. In P.R. Ortiz de Montellano (ed.), *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd edn. Plenum, New York, pp. 83–124.
33. Loida, P.J. and S.G. Sligar (1993). Engineering cytochrome P-450$_{cam}$ to increase the stereospecificity and coupling of aliphatic hydroxylation. *Protein Eng.* **6**, 207–212.
34. Atkins, W.M. and S.G. Sligar (1987). Metabolic switching in cyctochrome P-450$_{cam}$: Deuterium isotope effects on regiospecificity and the monooxygenase/oxidase ratio. *J. Am. Chem. Soc.* **109**, 3754–3760.
35. Fruetel, J.A., J.R. Collins, D.L. Camper, G.H. Loew, and P.R. Ortiz de Montellano (1992). Calculated and experimental absolute stereochemistry of the styrene and beta-methylstyrene epoxides formed by cytochrome P 450cam. *J. Am. Chem. Soc.* **114**, 6987–6993.
36. Perret, A. and D. Pompon (1998). Electron shuttle between membrane-bound cytochrome P450 3A4 and b_5 rules uncoupling mechanism. *Biochemistry* **37**, 11412–11424.
37. Reed, J.R. and P.F. Hollenberg (2003). Comparison of substrate metabolism by cytochromes P450 2B1, 2B4, and 2B6: Relationship of heme spin state, catalysis, and the effects of cytochrome b_5. *J. Inorg. Biochem.* **93**, 152–160.

38. Schlichting, I., J. Berendzen, K. Chu, A.M. Stock, S.A. Maves, D.E. Benson *et al.* (2000). The catalytic pathway of cytochrome $P450_{cam}$ at atomic resolution. *Science* **287**, 1615–1622.
39. Davydov, R., T.M. Makris, V. Kofman, D.E. Werst, S.G. Sligar, and B.M. Hoffman (2001). Hydroxylation of camphor by reduced oxy-cytochrome P450cam: Mechanistic implications of EPR and ENDOR studies of catalytic intermediates in native and mutant enzymes. *J. Am. Chem. Soc.* **123**, 1403–1415.
40. Denisov, I.G., T.M. Makris, and S.G. Sligar (2001). Cryotrapped reaction intermediates of cytochrome P450 studied by radiolytic reduction with phosphorus-32. *J. Biol. Chem.* **276**, 11648–11652.
41. Rahimtula, A.D., P.J. O'Brien, E.G. Hrycay, J.A. Peterson, and R.W. Estabrook (1974). Possible higher valence states of cytochrome P-450 during oxidative reactions. *Biochem. Biophys. Res. Commun.* **60**, 695–702.
42. Blake, R.C. and M.J. Coon (1981). On the mechanism of action of cytochrome P-450. Role of peroxy spectral intermediates in substrate hydroxylation. *J. Biol. Chem.* **256**, 5755–5763.
43. Wagner, G.C., M.M. Palcic, and H.B. Dunford (1983). Absorption spectra of cytochrome P450CAM in the reaction with peroxy acids. *FEBS Lett.* **156**, 244–248.
44. Egawa, T., H. Shimada, and Y. Ishimura (1994). Evidence for compound I formation in the reaction of cytochrome P450cam with m-chloroperbenzoic acid. *Biochem. Biophys. Res. Commun.* **201**, 1464–1469.
45. Schünemann, V., C. Jung, A.X. Trautwein, D. Mandon, and R. Weiss (2000). Intermediates in the reaction of substrate-free cytochrome P450cam with peroxy acetic acid. *FEBS Lett.* **179**, 149–154.
46. Schünemann, V., C. Jung, J. Terner, A.X. Trautwein, and R. Weiss (2002). Spectroscopic studies of peroxyacetic acid reaction intermediates of cytochrome $P450_{cam}$ and chloroperoxidase. *J. Inorg. Biochem.* **91**, 586–596.
47. Kellner, D.G., S.-C. Nung, K.E. Weiss, and S.G. Sligar (2002). Kinetic characterization of compound I formation in the thermostable cytochrome P450 CYP119. *J. Biol. Chem.* **277**, 9641–9644.
48. Vaz, A.D.N., D.F. McGinnity, and M.J. Coon (1998). Epoxidation of olefins by cytochrome P450: Evidence from site-specific mutagenesis for hydroperoxo-iron as an electrophilic oxidant. *Proc. Natl. Acad. Sci. USA* **95**, 3555–3560.
49. Jin, S., T.M. Makris, T.A. Bryson, S.G. Sligar, and J.H. Dawson (2003). Epoxidation of olefins by hydroperoxo-ferric cytochrome P450. *J. Am. Chem. Soc.* **125**, 3406–3407.
50. Ogliaro, F., S.P. de Visser, S. Cohen, P.K. Sharma, and S. Shaik (2002). Searching for the second oxidant in the catalytic cycle of cytochrome P450: A theoretical investigation of the iron(III)-hydroperoxo species and its epoxidation pathways. *J. Am. Chem. Soc.* **124**, 2806–2817.
51. Guengerich, F.P., A.D.N. Vaz, G.N. Raner, S.J. Pernecky, and M.J. Coon (1997). Evidence for a role of a perferryl-oxygen complex, FeO^{3+}, in the N-oxygenation of amines by cytochrome P450 enzymes. *Mol. Pharmacol.* **51**, 147–151.
52. Vatsis, K.P. and M.J. Coon (2002). Ipso-substitution by cytochrome P450 with conversion of *p*-hydroxybenzene derivatives to hydroquinone: Evidence for hydroperoxo-iron as the active oxygen species. *Arch. Biochem. Biophys.* **397**, 119–129.
53. Toy, P.H., B. Dhanabalasingam, M. Newcomb, I.H. Hanna, and P.F. Hollenberg (1997). A substituted hypersensitive radical probe for enzyme-catalyzed hydroxylations: Synthesis of racemic and enantiomerically enriched forms and application in a cytochrome P450-catalyzed oxidation. *J. Org. Chem.* **62**, 9114–9122.
54. Toy, P.H., M. Newcomb, and P.F. Hollenberg (1998). Hypersensitive mechanistic probe studies of cytochrome P450-catalyzed hydroxylation reactions. Implications for the cationic pathway. *J. Am. Chem. Soc.* **120**, 7719–7729.
55. Toy, P.H., M. Newcomb, M.J. Coon, and A.D.N. Vaz (1998). Two distinct electrophilic oxidants effect hydroxylation in cytochrome P-450-catalyzed reactions. *J. Am. Chem. Soc.* **120**, 9718–9719.
56. Schöneboom, J.C., H. Lin, N. Reuter, W. Thiel, S. Cohen, F. Ogliaro *et al.* (2002). The elusive oxidant species of cytochrome P450 enzymes: Characterization by combined quantum mechanical/molecular mechanical (QM/MM) calculations. *J. Am. Chem. Soc.* **124**, 8142–8151.
57. Ogliaro, F., S.P. de Visser, S. Cohen, P.K. Sharma, and S. Shaik (2002). Searching for the second oxidant in the catalytic cycle of cytochrome P450: A theoretical investigation of the iron(III)-hydroperoxo species and its epoxidation pathways. *J. Am. Chem. Soc.* **124**, 2806–2817.
58. Kamachi, T., Y. Shiota, T. Ohta, and K. Yoshizawa (2003). Does the hydroperoxo species of cytochrome P450 participate in olefin epoxidation with the main oxidant, Compound I: Criticism from density functional theory calculations. *Bull. Chem. Soc. Jpn.* **76**, 721–732.
59. Groves, J.T., G.A. McClusky, R.E. White, and M.J. Coon (1978). Aliphatic hydroxylation by highly purified liver microsomal cytochrome P-450. Evidence for a carbon radical intermediate. *Biochem. Biophys. Res. Commun.* **81**, 154–160.
60. Ogliaro, F., S.P. de Visser, S. Cohen, J. Kaneti, and S. Shaik (2001). The experimentally elusive oxidant of cytochrome P450: A theoretical "trapping" defining more closely the "real" species. *ChemBiochem.* **11**, 848–851.

61. Ogliaro, F., N. Harris, S. Cohen, M. Filatov, S.P. de Visser, and S. Shaik (2000). A model "rebound" mechanism of hydroxylation by cytochrome P450: Stepwise and effectively concerted pathways and their reactivity patterns. *J. Am. Chem. Soc.* **122**, 8977–8989.
62. Foster, A.B. (1985). Deuterium isotope effects in the metabolism of drugs and xenobiotics: Implications for drug design. *Adv. Drug Res.* **14**, 2–40.
63. Hjelmeland, L.M., L. Aronow, and J.R. Trudell (1977). Intramolecular determination of primary kinetic isotope effects in hydroxylations catalyzed by cytochrome P-450. *Biochem. Biophys. Res. Commun.* **76**, 541–549.
64. White, R.E., J.P. Miller, L.V. Favreau, and A. Bhattacharyaa (1986). Stereochemical dynamics of aliphatic hydroxylation by cytochrome P-450. *J. Am. Chem. Soc.* **108**, 6024–6031.
65. Gelb, M.H., D.C. Heimbrook, P. Malkonen, and S.G. Sligar (1982). Stereochemistry and deuterium isotope effects in camphor hydroxylation by the cytochrome $P450_{cam}$ monoxygenase system. *Biochemistry* **21**, 370–377.
66. Groves, J.T. and D.V. Subramanian (1984). Hydroxylation by cytochrome P-450 and metalloporphyrin models. Evidence for allylic rearrangement. *J. Am. Chem. Soc.* **106**, 2177–2181.
67. Oliw, E.H., I.D. Brodowsky, L. Hörnsten, and M. Hamberg (1993). Bis-allylic hydroxylation of polyunsaturated fatty acids by hepatic monooxygenases and its relation to the enzymatic and nonenzymatic formation of conjugated hydroxy fatty acids. *Arch. Biochem. Biophys.* **300**, 434–439.
68. Tanaka, K., N. Kurihara, and M. Nakajima (1979). Oxidative metabolism of tetrachlorocyclohexenes, pentachlorocyclohexenes, and hexachlorocyclohexenes with microsomes from rat liver and house fly abdomen. *Pestic. Biochem. Physiol.* **10**, 79–95.
69. Stanjek, V., M. Miksch, P. Lueer, U. Matern, and W. Boland (1999). Biosynthesis of psoralen: Mechanism of a cytochrome P450 catalyzed oxidative bond cleavage. *Angew. Chem. Int. Ed.* **38**, 400–402.
70. Ortiz de Montellano, P.R. and R.A. Stearns (1987). Timing of the radical recombination step in cytochrome P-450 catalysis with ring-strained probes. *J. Am. Chem. Soc.* **109**, 3415–3420.
71. White, R.E., J.T. Groves, and G.A. McClusky (1979). Electronic and steric factors in regioselective hydroxylation catalyzed by purified cytochrome P-450. *Acta Biol. Med. Ger.* **38**, 475–482.
72. Sligar. S.G., M.H. Gelb, and D.C. Heimbrook (1984). Bio-organic chemistry and cytochrome P-450-dependent catalysis. *Xenobiotica* **14**, 63–86.
73. Houghton, J.D., S.E. Beddows, K.E. Suckling, L. Brown, and C.J. Suckling (1986). 5α,6α-Methanocholestan-3β-ol as a probe of the mechanism of action of cholesterol 7α-hydroxylase. *Tetrahedron Lett.* **27**, 4655–4658.
74. Bowry, V.W. and K.U. Ingold (1991). A radical clock investigation of microsomal cytochrome P-450 hydroxylation of hydrocarbons. Rate of oxygen rebound. *J. Am. Chem. Soc.* **113**, 5699–5707.
75. Newcomb, M. and P.H. Toy (2000). Hypersensitive radical probes and the mechanisms of cytochrome P450-catalyzed hydroxylation reactions. *Acc. Chem. Res.* **33**, 449–455.
76. Atkinson, J.K. and K.U. Ingold (1993). Cytochrome P450 hydroxylation of hydrocarbons: Variation in the rate of oxygen rebound using cyclopropyl radical clocks including two new ultrafast probes. *Biochemistry* **32**, 9209–9214.
77. Atkinson, J.K., P.F. Hollenberg, K.U. Ingold, C.C. Johnson, M.-H. Le Tadic, M. Newcomb *et al.* (1994). Cytochrome P450-catalyzed hydroxylation of hydrocarbons: Kinetic deuterium isotope effects for the hydroxylation of an ultrafast radical clock. *Biochemistry* **33**, 10630–10637.
78. Newcomb, M., M.-H. Le Tadic, D.A. Putt, and P.F. Hollenberg (1995). An incredibly fast apparent oxygen rebound rate constant for hydrocarbon hydroxylation by cytochrome P-450 enzymes. *J. Am. Chem. Soc.* **117**, 3312–3313.
79. Newcomb, M., M.-H. Le Tadic-Biadatti, D.L. Chestney, E.S. Roberts, and P.F. Hollenberg (1995). A nonsynchronous concerted mechanism for cytochrome P450 catalyzed hydroxylation. *J. Am. Chem. Soc.* **117**, 12085–12091.
80. Auclair, K., Z. Hu, D.M. Little, P.R. Ortiz de Montellano, and J.T. Groves (2002). Revisiting the mechanism of P450 enzymes using the radical clocks norcarane and spiro[2,5]bicyclooctane. *J. Am. Chem Soc.* **124**, 6020–6027.
81. Newcomb, M., R. Shen, Y. Lu, M.J. Coon, P.F. Hollenberg, D.A. Kopp *et al.* (2002). Evaluation of norcarane as a probe for radicals in cytochrome P450- and soluble methane monooxygenase-catalyzed hydroxylation reactions. *J. Am. Chem. Soc.* **124**, 6879–6886.
82. Hino, F. and D. Dolphin (1999). The biomimetic oxidation of dieldrin using polyhalogenated metalloporphyrins. *J. Chem. Soc. Chem. Commun.* 629–630.
83. Shaik, S., M. Filatov, D. Schröder, and H. Schwarz (1998). Electronic structure makes a difference: Cytochrome P450 mediated hydroxylations of hydrocarbons as a two-state reactivity paradigm. *Chem. Eur.* **4**, 193–199.
84. Harris, N., S. Cohen, M. Filatov, and F. Ogliaro (2000). Two-state reactivity in the rebound step of

alkane hydroxylation by cytochrome P-450: Origins of free radicals with finite lifetimes. *Angew. Chem. Int. Ed.* **39**, 2003–2007.

85. Schröder, D., S. Shaik, and H. Schwarz (2000). Two-state reactivity as a new concept in organometallic chemistry. *Acc. Chem. Res.* **33**, 139–145.
86. Ogliaro, F., S.P. de Visser, J.T. Groves, and S. Shaik (2001). Chameleon states: High-valent metal-oxo species of cytochrome P450 and its ruthenium analogue. *Angew. Chem. Int. Ed.* **40**, 2874–2878.
87. Manchester, J.I., J.P. Dinnocenzo, L. Higgins, and J.P. Jones (1997). A new mechanistic probe for cytochrome P450: An application of isotope effect profiles. *J. Am. Chem. Soc.* **119**, 5069–5070.
88. Choi, S.-Y., P.E. Eaton, P.F. Hollenberg, K.E. Liiu, S.J. Lippard, M. Newcomb *et al.* (1996). Regiochemical variations in reactions of methylcubane with *tert*-butoxyl radical, cytochrome P-450 enzymes and a methane monooxygenase system. *J. Am. Chem. Soc.* **118**, 6547–6555.
89. Newcomb, M., R. Shen, S.-Y. Choi, P.H. Toy, P.F. Hollenberg, A.D.N. Vaz *et al.* (2000). Cytochrome P450-catalyzed hydroxylation of mechanistic probes that distinguish between radicals and cations. Evidence for cationic but not for radical intermediates. *J. Am. Chem. Soc.* **122**, 2677–2686.
90. Stearns, R.A. and P.R. Ortiz de Montellano (1985). Cytochrome P-450 catalyzed oxidation of quadricyclane. Evidence for a radical cation intermediate. *J. Am. Chem. Soc.* **107**, 4081–4082.
91. Ortiz de Montellano, P.R., H.S. Beilan, and K.L. Kunze (1981). N-Alkylprotoporphyrin IX formation in 3,5-dicarbethoxy-1,4-dihydrocollidine-treated rats. Transfer of the alkyl group from the substrate to the porphyrin. *J. Biol. Chem.* **256**, 6708–6713.
92. Augusto, O., H.S. Beilan, and P.R. Ortiz de Montellano (1982). The catalytic mechanism of cytochrome P-450. Spin-trapping evidence for one-electron substrate oxidation. *J. Biol. Chem.* **257**, 11288–11295.
93. Kennedy, C.H. and R.P. Mason (1990). A reexamination of the cytochrome P-450-catalyzed free radical production from a dihydropyridine. Evidence of trace transition metal catalysis. *J. Biol. Chem.* **265**, 11425–11428.
94. Guengerich, F.P., C.-H. Yun, and T.L. Macdonald (1996). Evidence for a 1-electron oxidation mechanism in N-dealkylation of N,N-dialkylanilines by cytochrome P450 2B1. Kinetic hydrogen isotope effects, linear free energy relationships, comparisons with horseradish peroxidase and studies with oxygen surrogates. *J. Biol. Chem.* **271**, 27321–27329.
95. Miwa, G.T., J.S. Walsh, and A.Y.H. Lu (1984). Kinetic isotope effects on cytochrome P-450-catalyzed oxidation reactions. The oxidative O-dealkylation of 7-ethoxycoumarin. *J. Biol. Chem.* **259**, 3000–3004.
96. Baciocchi, E., O. Lanzalunga, A. Lapi, and L. Manduchi (1998). Kinetic deuterium isotope effect profiles and substituent effects in the oxidative N-demethylation of N,N-dimethylanilines catalyzed by tetrakis(pentafluorophenyl)porphyrin iron(III) chloride. *J. Am. Chem. Soc.* **120**, 5783–5787.
97. Galliani, G., M. Nali, B. Rindone, S. Tollari, M. Rocchetti, and M. Salmona (1986). The rate of N-demethylation of N,N-dimethylanilines and N-methylanilines by rat-liver microsomes is related to their first ionization potential, their lipophilicity and to a steric bulk factor. *Xenobiotica* **16**, 511–517.
98. Macdonald, T.L., W.G. Gutheim, R.B. Martin, and F.P. Guengerich (1989). Oxidation of substituted N,N-dimethylanilines by cytochrome P-450: Estimation of the effective oxidation-reduction potential of cytochrome P-450. *Biochemistry* **28**, 2071–2077.
99. Goto, Y., Y. Watanabe, S. Fukuzumi, J.P. Jones, and J.P. Dinnocenzo (1998). Mechanisms of N-demethylations catalyzed by high-valent species of heme enzymes: Novel use of isotope effects and direct observation of intermediates. *J. Am. Chem. Soc.* **120**, 10762–10763.
100. Miwa, G.T., J.S. Walsh, G.L. Kedderis, and P.F. Hollenberg (1983). The use of intramolecular isotope effects to distinguish between deprotonation and hydrogen atom abstraction mechanisms in cytochrome P-450- and peroxidase-catalyzed N-demethylation reactions. *J. Biol. Chem.* **258**, 14445–14449.
101. Hall, L.R. and R.P. Hanzlik (1991). N-dealkylation of tertiary amides by cytochrome P-450. *Xenobiotica* **21**, 1127–1138.
102. Okazaki, O. and F.P. Guengerich (1993). Evidence for specific base catalysis in N-dealkylation reactions catalyzed by cytochrome P450 and chloroperoxidase. Differences in rates of deprotonation of aminium radicals as an explanation for high kinetic hydrogen isotope effects observed with peroxidases. *J. Biol. Chem.* **268**, 1546–1552.
103. Nelsen, S.F. and J.T. Ippoliti (1986). The deprotonation of trialkylamine cation radicals by amines. *J. Am. Chem. Soc.* **108**, 4879–4881.
104. Karki, S.B., J.P. Dinnocenzo, J.P. Jones, and K.R. Korzekwa (1995). Mechanism of oxidative amine dealkylation of substituted N,N-dimethylanilines by cytochrome P-450: Application of isotope effect profiles. *J. Am. Chem. Soc.* **117**, 3657–3664.
105. Tanko, J.M., R. Friedline, N.K. Suleman, and N. Castagnoli (2001). *tert*-Butoxyl as a model for

radicals in biological systems: *Caveat emptor. J. Am. Chem. Soc.* **123**, 5808–5809.
106. Macdonald, T.L., K. Zirvi, L.T. Burka, P. Peyman, and F.P. Guengerich (1982). Mechanism of cytochrome P-450 inhibition by cyclopropylamines. *J. Am. Chem. Soc.* **104**, 2050–2052.
107. Hanzlik, R.P. and R.H. Tullman (1982). Suicidal inactivation of cytochrome P-450 by cyclopropylamines. Evidence for cation-radical intermediates. *J. Am. Chem. Soc.* **104**, 2048–2050.
108. Guengerich, F.P., R.J. Willard, J.P. Shea, L.E. Richards, and T.L. Macdonald (1984). Mechanism-based inactivation of cytochrome P-450 by heteroatom-substituted cyclopropanes and formation of ring-opened products. *J. Am. Chem. Soc.* **106**, 6446–6447.
109. Shaffer, C.L., M.D. Morton, and R.P. Hanzlik (2001). *N*-Dealkylation of an *N*-cyclopropylamine by horseradish peroxidase. Fate of the cyclopropyl group. *J. Am. Chem. Soc.* **123**, 8502–8508.
110. Shaffer, C.L., S. Harriman, Y.M. Koen, and R.P. Hanzlik (2002). Formation of cyclopropanone during cytochrome P450-catalyzed N-dealkylation of a cyclopropylamine. *J. Am. Chem. Soc.* **124**, 8268–8274.
111. Kuttab, S., J. Shang, and N. Castagnoli (2001). Rat liver microsomal enzyme catalyzed oxidation of 4-phenyl-trans-1-(2-phenylcyclopropyl)-1,2,3,6-tetrahydropyridine. *Bioorg. Med. Chem.* **9**, 1685–1689.
112. Hall, L.R. and R.P. Hanzlik (1989). Kinetic deuterium isotope effects on the N-demethylation of tertiary amides by cytochrome P-450. *J. Biol. Chem.* **264**, 12349–12355.
113. Constantino, L., E. Rosa, and J. Iley (1992). The microsomal demethylation of N,N-dimethylbenzamides. Substituent and kinetic deuterium isotope effects. *Biochem. Pharmacol.* **44**, 651–658.
114. Iley, J., L. Constantino, F. Norberto, and E. Rosa (1990). Oxidation of the methyl groups of N,N-dimethylbenzamides by a cytochrome P450 mono-oxygenase model system. *Tetrahedron Lett.* **31**, 4921–4922.
115. Hall, L.R., R.T. Iwamoto, and R.P. Hanzlik (1989). Electrochemical models for cytochrome P-450. N-Demethylation of tertiary amides by anodic oxidation. *J. Org. Chem.* **54**, 2446–2451.
116. Hlavica, P. and M. Lehnerer (1995). Some aspects of the role of cytochrome P-450 isozymes in the N-oxidative transformation of secondary and tertiary amine compounds. *J. Biochem. Toxicol.* **10**, 275–285.
117. Seto, Y. and F.P. Guengerich (1993). Partitioning between N-dealkylation and N-oxygenation in the oxidation of N,N-dialkylarylamines catalyzed by cytochrome P450 2B1. *J. Biol. Chem.* **268**, 9986–9997.
118. Burstyn, J.N., M. Iskandar, J.F. Brady, J.M. Fukuto, and A.K. Cho (1991). Comparative studies of N-hydroxylation and N-demethylation by microsomal cytochrome P-450. *Chem. Res. Toxicol.* **4**, 70–76.
119. Hlavica, P. and U. Künzel-Mulas (1993). Metabolic N-oxide formation by rabbit-liver microsomal cytochrome P-4502B4: Involvement of superoxide in the NADPH-dependent N-oxygenation of N,N-dimethylaniline. *Biochim. Biophys. Acta* **1158**, 83–90.
120. Watanabe, Y., T. Numata, T. Iyanagi, and S. Oae (1981). Enzymatic oxidation of alkyl sulfides by cytochrome P450 and hydroxyl radical. *Bull. Chem. Soc. Jpn.* **54**, 1163–1170.
121. Watanabe, Y., T. Iyanagi, and S. Oae (1980). Kinetic study on enzymatic S-oxygenation promoted by a reconstituted system with purified cytochrome P450. *Tetrahedron Lett.* **21**, 3685–3688.
122. Watanabe, Y., T. Iyanagi, and S. Oae (1982). One electron transfer mechanism in the enzymatic oxygenation of sulfoxide to sulfone promoted by a reconstituted system with purified cytochrome P450. *Tetrahedron Lett.* **23**, 533–536.
123. Alvarez, J.C. and P.R. Ortiz de Montellano (1992). Thianthrene 5-oxide as a probe of the electrophilicity of hemoprotein oxidizing species. *Biochemistry* **31**, 8315–8322.
124. Bacciochi, E., O. Lanzalunga, and B. Pirozzi (1997). Oxidations of benzyl and phenethyl phenyl sulfides. Implications for the mechanism of the microsomal and biomimetic oxidation of sulfides. *Tetrahedron* **53**, 12287–12298.
125. Baciocchi, E., O. Lanzalunga, S. Malandrucco, M. Iolel, and S. Steenken (1996). Oxidation of sulfides by peroxidases. Involvement of radical cations and the rate of the oxygen rebound step. *J. Am. Chem. Soc.* **118**, 8973–8974.
126. Holland, H.L., M.J. Chernishenko, M. Conn, A. Munoz, T.S. Manoharan, and M.A. Zawadski (1990). Enzymic hydroxylation and sulfoxidation of cyclopropyl compounds by fungal biotransformation. *Can. J. Chem.* **68**, 696–700.
127. Watabe, T. and K. Akamatsu (1974). Microsomal epoxidation of *cis*-stilbene: Decrease in epoxidase activity related to lipid peroxidation. *Biochem. Pharmacol.* **23**, 1079–1085.
128. Watabe, T., Y. Ueno, and J. Imazumi (1971). Conversion of oleic acid into *threo*-dihydroxystearic acid by rat liver microsomes. *Biochem. Pharmacol.* **20**, 912–913.
129. Ortiz de Montellano, P.R., B.L.K. Mangold, C. Wheeler, K.L. Kunze, and N.O. Reich (1983). Stereochemistry of cytochrome P-450-catalyzed epoxidation and prosthetic heme alkylation. *J. Biol. Chem.* **258**, 4208–4213.

130. Hanzlik, R.P. and G.O. Shearer (1978). Secondary deuterium isotope effects on olefin epoxidation by cytochrome P450. *Biochem. Pharmacol.* **27**, 1441–1444.
131. Hanzlik, R.P. and G.O. Shearer (1975). Transition state structure for peracid epoxidation: Secondary deuterium isotope effects. *J. Am. Chem. Soc.* **97**, 5231–5233.
132. Ortiz de Montellano, P.R. and K.L. Kunze (1981). Shift of the acetylenic hydrogen during chemical and enzymatic oxidation of the biphenylacetylene triple bond. *Arch. Biochem. Biophys.* **209**, 710–712.
133. McMahon, R.E., J.C. Turner, G.W. Whitaker, and H.R. Sullivan (1981). Deuterium isotope effect in the biotransformation of 4-ethynylbiphenyls to 4-biphenylacetic acids by rat hepatic microsomes. *Biochem. Biophys. Res. Commun.* **99**, 662–667.
134. Ortiz de Montellano, P.R. and E.A. Komives (1985). Branchpoint for heme alkylation and metabolite formation in the oxidation of aryl acetylenes by cytochrome P450. *J. Biol. Chem.* **260**, 3330–3336.
135. Foroozesh, M., G. Primrose, Z. Guo, L.C. Bell, W.L. Alworth, and F.P. Guengerich (1997). Aryl acetylenes as mechanism-based inhibitors of cytochrome P450-dependent monooxygenase enzymes. *Chem. Res. Toxicol.* **10**, 91–102.
136. Ortiz de Montellano, P.R. and M.A. Correia (1995). Inhibition of cytochrome P450 enzymes. In P.R. Ortiz de Montellano (ed.), *Cytochrome P450: Structure, Mechanism and Biochemistry*, 2nd edn. Plenum, New York, pp. 305–364.
137. Ortiz de Montellano, P.R. and B.A. Mico (1980). Destruction of cytochrome P-450 by ethylene and other olefins. *Mol. Pharmacol.* **18**, 128–135.
138. Kunze, K.L., B.L.K. Mangold, C. Wheeler, H.S. Beilan, and P.R. Ortiz de Montellano (1983). The cytochrome P-450 active site. Regiospecificity of the prosthetic heme alkylation by olefins and acetylenes. *J. Biol. Chem.* **258**, 4202–4207.
139. Ortiz de Montellano, P.R., R.A. Stearns, and K.C. Langry (1984). The allylisopropylacetamide and novonal prosthetic heme adducts. *Mol. Pharmacol.* **25**, 310–317.
140. Ortiz de Montellano, P.R. and K.L. Kunze (1981). Cytochrome P-450 inactivation: Structure of the prosthetic heme adduct with propyne. *Biochemistry* **20**, 7266–7271.
141. Gan, L.-S., A.L. Acebo, and W.L. Alworth (1984). 1-Ethynylpyrene, a suicide inhibitor of cytochrome P-450 dependent benzo[a]pyrene hydroxylase activity in liver microsomes. *Biochemistry* **23**, 3827–3836.
142. CaJacob, C.A., W. Chan, E. Shephard, and P.R. Ortiz de Montellano (1988). The catalytic site of rat hepatic lauric acid omega-hydroxylase. Protein versus prosthetic heme alkylation in the omega-hydroxylation of acetylenic fatty acids. *J. Biol. Chem.* **263**, 18640–18649.
143. Henschler, D., W.R. Hoos, H. Fetz, E. Dallmeier, and M. Metzler (1979). Reactions of trichloroethylene epoxide in aqueous systems. *Biochem. Pharmacol.* **28**, 543–548.
144. Miller, R.E. and F.P. Guengerich (1982). Oxidation of trichloroethylene by liver microsomal cytochrome P-450: Evidence for chlorine migration in a transition state not involving trichloroethylene oxide. *Biochemistry* **21**, 1090–1097.
145. Liebler, D.C. and F.P. Guengerich (1983). Olefin oxidation by cytochrome P-450: Evidence for group migration in catalytic intermediates formed with vinylidene chloride and *trans*-1-phenyl-1-butene. *Biochemistry* **22**, 5482–5489.
146. Mansuy, D., J. Leclaire, M. Fontecave, and M. Momenteau (1984). Oxidation of monosubstituted olefins by cytochromes P450 and heme models: Evidence for the formation of aldehydes in addition to epoxides and allylic alcohols. *Biochem. Biophys. Res. Commun.* **119**, 319–325.
147. Miller, V.P., J.A. Fruetel, and P.R. Ortiz de Montellano (1992). Cytochrome P450*cam*-catalyzed oxidation of a hypersensitive radical probe. *Arch. Biochem. Biophys.* **298**, 697–702.
148. de Visser, S.P., F. Ogliaro, N. Harris, and S. Shaik (2001). Multi-state epoxidation of ethene by cytochrome P450: A quantum chemical study. *J. Am. Chem. Soc.* **123**, 3037–3047.
149. de Visser, S.P., F. Ogliaro, and S. Shaik (2001). Stereospecific oxidation by compound I of cytochrome P450 does not proceed in a concerted synchronous manner. *J. Chem. Soc. Chem. Commun.* 2322–2323.
150. de Visser, S.P., F. Ogliaro, and S. Shaik (2001). How does ethene inactivate cytochrome P450 en route to its epoxidation? A density functional study. *Angew. Chem. Int. Ed.* **40**, 2871–2874.
151. Lovern, M.R., M.J. Turner, M. Meyer, G.L. Kedderis, W.E. Bechtold, and P.M. Schlosser (1997). Identification of benzene oxide as a product of benzene metabolism by mouse, rat, and human liver microsomes. *Carcinogenesis* **18**, 1695–1700.
152. Jerina, D.M. and J.W. Daly (1974). Arene oxides: A new aspect of drug metabolism. *Science* **185**, 573–582.
153. Koerts, J., A.E.M.F. Soffers, J. Vervoort, A. De Jager, and I.M.C.M. Rietjens (1998). Occurrence of the NIH shift upon the cytochrome P450-catalyzed *in vivo* and *in vitro* aromatic ring hydroxylation of fluorobenzenes. *Chem. Res. Toxicol.* **11**, 503–512.

154. Hanzlik, R.P. and K.-H.J. Ling (1993). Active site dynamics of xylene hydroxylation by cytochrome P-450 as revealed by kinetic deuterium isotope effects. *J. Am. Chem. Soc.* **115**, 9363–9370.
155. Riley, P. and R.P. Hanzlik (1994). Electron transfer in P450 mechanisms. Microsomal metabolism of cyclopropylbenzene and *p*-cyclopropylanisole. *Xenobiotica* **24**, 1–16.
156. Tomaszewski, J.E., D.M. Jerina, and J.W. Daly (1975). Deuterium isotope effects during formation of phenols by hepatic monooxygenases: Evidence for an alternative to the arene oxide pathway. *Biochemistry* **14**, 2024–2030.
157. Preston, B.D., J.A. Miller, and E.C. Miller (1983). Non-arene oxide aromatic ring hydroxylation of 2,2',5,5'-tetrachlorobiphenyl as the major metabolic pathway catalyzed by phenobarbital-induced rat liver microsomes. *J. Biol. Chem.* **258**, 8304–8311.
158. Hanzlik, R.P., K. Hogberg, and C.M. Judson (1984). Microsomal hydroxylation of specifically deuterated monosubstituted benzenes: Evidence for direct aromatic hydroxylation. *Biochemistry* **23**, 3048–3055.
159. Korzekwa, K.R., D.C. Swinney, and W.F. Trager (1989). Isotopically labeled chlorobenzenes as probes for the mechanism of cytochrome P-450 catalyzed aromatic hydroxylation. *Biochemistry* **28**, 9019–9027.
160. Rietjens, I.M.C. and J. Vervoort (1992). A new hypothesis for the mechanism for cytochrome P-450 dependent aerobic conversion of hexahalogenated benzenes to pentahalogenated phenols. *Chem. Res. Toxicol.* **5**, 10–19.
161. Rietjens, I.M.C.M., A.E.M.F. Soffers, C. Veeger, and J. Vervoort (1993). Regioselectivity of cytochrome P-450 catalyzed hydroxylation of fluorobenzenes predicted by calculated frontier orbital substrate characteristics. *Biochemistry* **32**, 4801–4812.
162. Zakharieva, O., M. Grodzicki, A.X. Trautwein, C. Veeger, and I.M.C.M. Rietjens (1996). Molecular orbital study of the hydroxylation of benzene and monofluorobenzene catalysed by iron-oxo porphyrin π-cation radical complexes. *J. Biol. Inorg. Chem.* **1**, 192–204.
163. Koerts, J., M.M.C. Velraeds, A.E.M.F. Sofferse, J. Vervoort, and I.M.C.M. Rietjens (1997). Influence of substituents in fluorobenzene derivatives on the cytochrome P450-catalyzed hydroxylation at the adjacent ortho aromatic carbon center. *Chem. Res. Toxicol.* **10**, 279–288.
164. Ohe, T., T. Mashino, and M. Hirobe (1994). Novel metabolic pathway of arylethers by cytochrome P450: Cleavage of the oxygen-aromatic ring bond accompanying *ipso*-substitution by the oxygen atom of the active species in cytochrome P450 models and cytochrome P450. *Arch. Biochem. Biophys.* **310**, 402–409.
165. Ohe, T., T. Mashino, and M. Hirobe (1997). Substituent elimination from *p*-substituted phenols by cytochrome P450. *Ipso*-substitution by the oxygen atom of the active species. *Drug Metab. Dispos.* **25**, 116–122.
166. Rizk, P.N. and R.P. Hanzlik (1995). Oxidative and non-oxidative metabolism of 4-iodoanisole by rat liver microsomes. *Xenobiotica* **25**, 143–150.
167. Hinson, J.A., S.D. Nelson, and J.R. Mitchell (1977). Studies on the microsomal formation of arylating metabolites of acetaminophen and phenacetin. *Mol. Pharmacol.* **13**, 625–633.
168. Hinson, J.A., S.D. Nelson, and J.R. Gillette (1979). Metabolism of [p-^{18}O]-phenacetin: The mechanism of activation of phenacetin to reactive metabolites in hamsters. *Mol. Pharmacol.* **15**, 419–427.
169. Koymans, L., J.H.V. Lenthe, G.M.D. Den Kelder, and N.P.E. Vermeulen (1990). Mechanisms of activation of phenacetin to reactive metabolites by cytochrome P-450: A theoretical study involving radical intermediates. *Mol. Pharmacol.* **37**, 452–460.
170. Veronese, M.E., S. McLean, C.A. D'Souze, and N.W. Davies (1985). Formation of reactive metabolites of phenacetin in humans and rats. *Xenobiotica* **15**, 929–940.
171. Rietjens, I.M.C.M., B. Tyrakowska, C. Veeger, and J. Vervoort (1990). Reaction pathways for biodehalogenation of fluorinated anilines. *Eur. J. Biochem.* **194**, 945–954.
172. Stresser, D.M. and D. Kupfer (1997). Catalytic characteristics of CYP3A4: Requirement for a phenolic function in *ortho*-hydroxylation of estradiol and mono-*O*-demethylated methoxychlor. *Biochemistry* **36**, 2203–2210.
173. Jellinck, P.H., E.F. Hahn, and J. Fishman (1986). Absence of reactive intermediates in the formation of catechol estrogens by rat liver microsomes. *J. Biol. Chem.* **261**, 7729–7732.
174. Sarabia, S.F., B.T. Zhu, T. Kurosawa, M. Tohma, and J.G. Liehr (1997). Mechanism of cytochrome P450-catalyzed aromatic hydroxylation of estrogens. *Chem. Res. Toxicol.* **10**, 767–771.
175. Stadler, R. and M.H. Zenk (1993). The purification and characterization of a unique cytochrome P-450 enzyme from *Berberis solonifera* plant cell cultures. *J. Biol. Chem.* **268**, 823–831.
176. Kraus, P.F.X. and T.M. Kutchan (1995). Molecular cloning and heterologous expression of a cDNA encoding berbamunine synthase, a C-O phenol-coupling cytochrome P450 from the higher plant *Berberis stolonifera*. *Proc. Natl. Acad. Sci. USA.* **92**, 2071–2075.
177. Amann, T. and M.H. Zenk (1991). Formation of the morphine precursor salutaridine is catalyzed

by a cytochrome P-450 enzyme in mammalian liver. *Tetrahedron Lett.* **32**, 3675–3678.
178. Gerardy, R. and M.H. Zenk (1993). Formation of salutaridine from (*R*)-reticuline by a membrane-bound cytochrome P450 enzymes from *Papaver somniferum*. *Phytochemistry* **32**, 79–86.
179. Nasreen, A., M. Rueffer, and M.H. Zenk (1996). Cytochrome P-450-dependent formation of isoandrocymbine from autumnaline in colchicine biosynthesis. *Tetrahedron Lett.* **37**, 8161–8164.
180. Zerbe, K., O. Pylypenko, F. Vitali, W. Zhang, S. Rouset, M. Heck *et al.* (2002). Crystal structure of OxyB, a cytochrome P450 implicated in an oxidative phenol coupling reaction during vancomycin biosynthesis. *J. Biol. Chem.* **27**, 47476–47485.
181. Bischoff, D., S. Pelzer, B. Bister, G.J. Nicholson, S. Stockert, M. Schirle *et al.* (2001). The biosynthesis of vancomycin-type glycopeptide antibiotics—The order of the cyclization steps. *Angew. Chem. Int. Ed.* **40**, 4688–4691.
182. van Wageningen, A.M., P.N. Kirkpatrick, D.H. Williams, B.R. Harris, J.K. Kershaw, N.J. Lennard *et al.* (1998). Sequencing and analysis of genes involved in the biosynthesis of a vancomycin group antibiotic. *Chem. Biol.* **5**, 155–162.
183. Pelzer, S., R. Sussmuth, D. Heckmann, J. Recktenwald, P. Huber, G. Jung *et al.* (1999). Identification and analysis of the balhimycin biosynthetic gene cluster and its use for manipulating glycopeptide biosynthesis in *Amycolatopsis mediterranei* DSM5908. *Antimicrob. Agents Chemother.* **43**, 1565–1573.
184. Chiu, H.-T., B.K. Hubbard, A.N. Shah, J. Eide, R.A. Fredenburg, C.T. Walsh *et al.* (2001). Molecular cloning and sequence analysis of the complestatin biosynthetic gene cluster. *Proc. Natl. Acad. Sci. USA.* **98**, 8548–8553.
185. Sato, H. and F.P. Guengerich (2000). Oxidation of 1,2,4,5-tetramethoxybenzene to a cation radical by cytochrome P450. *J. Am. Chem. Soc.* **122**, 8099–8100.
186. Anzenbacher, P., T. Niwa, L.M. Tolbert, S.R. Sirimanne, and F.P. Guengerich (1996). Oxidation of 9-alkylanthracenes by cytochrome P450 2B1, horseradish peroxidase, and iron tetraphenylporphine/iodosylbenzene systems: Anaerobic and aerobic mechanisms. *Biochemistry* **35**, 2512–2520.
187. Ts'o, P.O., Caspary, W.J. and R.J. Lorentzen (1977). The involvement of free radicals in chemical carcinogenesis. In W.A. Pryor (ed.), *Free Radicals in Biology* vol. III. Academic Press, New York, pp. 251–303.
188. Cavalieri, E.L. and E.G. Rogan (1992). The approach to understanding aromatic hydrocarbon carcinogenesis. The central role of radical cations in metabolic activation. *Pharmacol. Ther.* **55**, 183–199.
189. Cavalieri, E. and E. Rogan (1995). Central role of radical cations in metabolic activation of polycyclic aromatic hydrocarbons. *Xenobiotica* **25**, 677–688.
190. Cavalieri, E., E. Rogan, R.W. Roth, R.K. Saugier, and A. Hakam (1983). The relationship between ionization potential and horseradish peroxidase/hydrogen peroxide-catalyzed binding of aromatic hydrocarbons to DNA. *Chem. Biol. Interact.* **47**, 87–109.
191. Devanesan, P., E. Rogan, and E. Cavalieri (1987). The relationship between ionization potential and prostaglandin H synthase-catalyzed binding of aromatic hydrocarbons to DNA. *Chem. Biol. Interact.* **61**, 89–95.
192. Cavalieri, E.L., E.G. Rogan, P.D. Devanesan, P. Cremonesi, R.L. Cerny, M.L. Gross *et al.* (1990). Binding of benzo[a]pyrene to DNA by cytochrome P450-catalyzed one-electron oxidation in rat liver microsomes and nuclei. *Biochemistry* **29**, 4820–4827.
193. Rogan, E.G., P.D. Devanesan, N.V.S. RamaKrishna, S. Higginbotham, N.S. Padmavathi, K. Chapman *et al.* (1993). Identification and quantitation of benzo[a]pyrene-DNA adducts formed in mouse skin. *Chem. Res. Toxicol.* **6**, 356–363.
194. Khandwala, A.S. and C.B. Kasper (1973). Preferential induction of aryl hydroxylase activity in rat liver nuclear envelope by 3-methylcholanthrene. *Biochem. Biophys. Res. Commun.* **54**, 1241–1246.
195. Cavalieri, E.L., E.G. Rogan, P. Cremonesi, and P.D. Devanesan (1988). Radical cations as precursors in the metabolic formation of quinones from benzo[a]pyrene and 6-fluorobenzo[a]pyrene. Fluoro substitution as a probe for one-electron oxidation in aromatic substrates. *Biochem. Pharmacol.* **37**, 2173–2182.
196. Rettie, A.E., A.W. Rettenmeier, W.N. Howald, and T.A. Baillie (1987). Cytochrome P-450-catalyzed formation of Δ^4-VPA, a toxic metabolite of valproic acid. *Science* **235**, 890–893.
197. Rettie, A.E., M. Boberg, A.W. Rettenmeier, and T.A. Baillie (1988). Cytochrome P-450-catalyzed desaturation of valproic acid *in vitro*. Species differences, induction effects, and mechanistic studies. *J. Biol. Chem.* **263**, 13733–13738.
198. Porubeck, D.J., H. Barnes, G.P. Meier, L.J. Theodore, and T.A. Baillie (1989). Enantiotopic differentiation during the biotransformation of valproic acid to the hepatotoxic olefin 2-*n*-propyl-4-pentenoic acid. *Chem. Res. Toxicol.* **2**, 35–40.
199. Kassahun, K. and T.A. Baillie (1993). Cytochrome P-450-mediated dehydrogenation of 2-n-propyl-2(*E*)-pentenoic acid, a pharmacologically-active metabolite of valproic acid, in rat liver

microsomal preparations. *Drug Metab. Dispos.* **21**, 242–248.

200. Sadeque, A.J.M., M.B. Fisher, K.R. Korzekwa, F.J. Gonzalez, and A.E. Rettie (1997). Human CYP2C9 and CYP2A6 mediate formation of the hepatotoxin 4-ene-valproic acid. *J. Pharmacol. Exp. Ther.* **283**, 698–703.
201. Fisher, M.B., S.J. Thompson, V. Ribeiro, M.C. Lechner, and A.E. Rettie (1998). P450-catalyzed in-chain desaturation of valproic acid: Isoform selectivity and mechanism of formation of Δ^3-valproic acid generated by baculovirus-expressed CYP3A1. *Arch. Biochem. Biophys.* **356**, 63–70.
202. Rettie, A.E., P.R. Sheffels, K.R. Korzekwa, F.J. Gonzalez, R.M. Philpot, and T.A. Baillie (1995). CYP4 isozyme specificity and the relationship between ω-hydroxylation and terminal desaturation of valproic acid. *Biochemistry* **34**, 7889–7895.
203. Rettenmeier, A.W., W.P. Gordon, K.S. Prickett, R.H. Levy, J.S. Lockard, K.E. Thummel *et al.* (1986). Metabolic fate of valproic acid in the rhesus monkey. Formation of a toxic metabolite, 2-n-propyl-4-pentenoic acid. *Drug Metab. Dispos.* **14**, 443–453.
204. Pennanen, S., A. Kojo, M. Pasanen, J. Liesivuori, R.O. Juvonen, and H. Kumulainen (1996). CYP enzymes catalyze the formation of a terminal olefin from 2-ethylhexanoic acid in rat and human liver. *Hum. Exp. Toxicol.* **15**, 435–442.
205. Behrouzian, B. and P.H. Buist (2002). Fatty acid desaturation: Variations on an oxidative theme. *Curr. Opin. Chem. Biol.* **6**, 577–582.
206. Obach, R.S. (2001). Mechanism of cytochrome P4503A4- and 2D6-catalyzed dehydrogenation of ezlopitant as probed with isotope effects using five deuterated analogs. *Drug Metab. Dispos.* **29**, 1599–1607.
207. Höld, K.M., N.S. Sirisoma, and J.E. Casida (2001). Detoxification of α- and β-thujones (the active ingredients of absinthe): Site specificity and species differences in cytochrome P450 oxidation in vitro and in vivo. *Chem. Res. Toxicol.* **14**, 589–595.
208. Hata, S., T. Nishino, M. Komori, and H. Katsuki (1981). Involvement of cytochrome P-450 in Δ^{22}-desaturation in ergosterol biosynthesis in yeast. *Biochem. Biophys. Res. Commun.* **103**, 272–277.
209. Kelly, S.L., D.C. Lamb, B.C. Baldwin, A.J. Corran, and D.E. Kelly (1997). Characterization of *Saccharomyces cerevisiae* CYP61, sterol Δ22-desaturated, and inhibition by azole antifungal agents. *J. Biol. Chem.* **272**, 9986–9988.
210. Skaggs, B.A., J.F. Alexander, C.A. Pierson, K.S. Schweitzer, K.T. Chun, C. Koegel *et al.* (1996). Cloning and characterization of the *Saccharomyces cerevisiae* C-22 sterol desaturase gene, encoding a second cytochrome P-450 involved in ergosterol biosynthesis. *Gene* **169**, 105–109.
211. Lamb, D.C., S. Maspahy, D.E. Kelly, N.J. Manning, A. Geber, J.E. Bennett *et al.* (1999). Purification, reconstitution, and inhibition of cytochrome P-450 sterol Δ^{22}-desaturase from the pathogenic fungus *Candida glabrata*. *Antimicrob. Agents Chemother.* **43**, 1725–1728.
212. Rodrigues, C.M.P., B.T. Kren, C.J. Steer, and K.D.R. Setchell (1996). Formation of D22-bile acids in rats is not gender specific and occurs in the peroxisome. *J. Lipid Res.* **37**, 540–550.
213. Nagata, K., D.J. Liberato, J.R. Gillette, and H.A. Sasame (1986). An unusual metabolite of testosterone: 17β-hydroxy-4,6-androstadiene-3-one. *Drug Metab. Dispos.* **14**, 559–565.
214. Aoyama, T., K. Korzekwa, K. Nagata, J. Gillette, H.V. Gelboin, and F.J. Gonzalez (1989). cDNA-directed expression of rat testosterone 7α-hydroxylase using the modified vaccinia virus, T7-RNA-polymerase system and evidence for 6□-hydroxylation and Δ^6-testosterone formation. *Eur. J. Biochem.* **181**, 331–336.
215. Korzekwa, K.R., W.F. Trager, K. Nagata, A. Parkinson, and J.R. Gillette (1990). Isotope effect studies on the mechanism of the cytochrome P-450IIA1-catalyzed formation of Δ^6-testosterone from testosterone. *Drug Metab. Dispos.* **18**, 974–979.
216. Chadwick, R.W., L.T. Chuang, and K. Williams (1975). Dehydrogenation: A previously unreported pathway of lindane metabolism in mammals. *Pestic. Biochem. Physiol.* **5**, 575–586.
217. Mochizuki, H., K. Suhara, and M. Katagiri (1992). Steroid 6β-hydroxylase and 6-desaturase reactions catalyzed by adrenocortical mitochondrial P-450. *J. Steroid Biochem. Mol. Biol.* **42**, 95–101.
218. Boyd, D.R., N.D. Sharma, R. Agarwal, R.A.S. McMordie, J.G.M. Bessems, B. van Ommen *et al.* (1993). Biotransformation of 1,2-dihydronaphthalene and 1,2-dihydroanthracene by rat liver microsomes and purified cytochromes P-450. Formation of arene hydrates of naphthalene and anthracene. *Chem. Res. Toxicol.* **6**, 808–812.
219. Adams, J.D., H. Yagi, W. Levin, and D.M. Jerina (1995). Stereo-selectivity and regio-selectivity in the metabolism of 7,8-dihydrobenzo[a]pyrene by cytochrome P450, epoxide hydrolase and hepatic microsomes from 3-methylcholanthrene-treated rats. *Chem. Biol. Interact.* **95**, 57–77.
220. Kaminsky, L.S., M.J. Fasco, and F.P. Guengerich (1980). Comparison of different forms of purified cytochrome P-450 from rat liver by immunological inhibition of regio- and stereoselective metabolism of warfarin. *J. Biol. Chem.* **255**, 85–91.
221. Vyas, K.P., P.H. Kari, S.R. Prakash, and D.E. Duggan (1990). Biotransformation of lovastatin. II. *In vitro* metabolism by rat and mouse liver

microsomes and involvement of cytochrome P-450 in dehydrogenation of lovastatin. *Drug Metab. Dispos.* **18**, 218–222.

222. Wang, R.W., P.H. Kari, A.Y.H. Lu, P.E. Thomas, F.P. Guengerich, and K.P. Vyas (1991). Biotransformation of lovostatin. IV. Identification of cytochrome P450 3A proteins as the major enzymes responsible for the oxidative metabolism of lovostatin in rat and human liver microsomes. *Arch. Biochem. Biophys.* **290**, 355–361.
223. Vickers, S. and C.A. Duncan (1991). Studies on the metabolic inversion of the 6' chiral center of simvastatin. *Biochem. Biophys. Res. Commun.* **181**, 1508–1515.
224. Guan, X., M.B. Fisher, D.H. Lang, Y.-M. Zhen, D.R. Koop, and A.E. Rettie (1998). Cytochrome P450-dependent desaturation of lauric acid: Isoform selectivity and mechanism of formation of 11-dodecenoic acid. *Chem. Biol. Interact.* **110**, 103–131.
225. Kelkar, H.S., T.W. Skloss, J.F. Haw, N.P. Keller, and T.H. Adams (1997). *Aspergillus nidulans stcL* encodes a putative cytochrome P-450 monooxygenase required for bisfuran desaturation during aflatoxin and sterigmatocystin biosynthesis. *J. Biol. Chem.* **272**, 1589–1594.
226. Akashi, T., M. Fukuchi-Mizutani, T. Aoki, Y. Ueyama, K. Yonekura-Sakakibara, Y. Tanaka *et al.* (1999). Molecular cloning and biochemical characterization of a novel cytochrome P450, flavone synthase II, that catalyzes direct conversion of flavanones to flavones. *Plant Cell Physiol.* **40**, 1182–1186.
227. Guengerich, F.P. and D.H. Kim (1991). Enzymatic formation of ethyl carbamate to vinyl carbamate and its role as an intermediate in the formation of 1,N^6-ethenoadenosine. *Chem. Res. Toxicol.* **4**, 413–421.
228. Wood, A.W., D.C. Swinney, P.E. Thomas, D.E. Ryan, P.F. Hall, W. Levin *et al.* (1988). Mechanism of androstenedione formation from testosterone and epitestosterone catalyzed by purified cytochrome P-450b. *J. Biol. Chem.* **263**, 17322–17332.
229. Swinney, D.C., D.E. Ryan, P.E. Thomas, and W. Levin (1988). Evidence for concerted kinetic oxidation of progesterone by purified rat hepatic cytochrome P-450g. *Biochemistry* **27**, 5461–5470.
230. Nelson, S.D., A.J. Forte, and D.C. Dhalin (1980). Lack of evidence for N-hydroxyacetaminophen as a reactive metabolite of acetaminophen *in vitro*. *Biochem. Pharmacol.* **29**, 1617–1620.
231. Lee, J.S., N.E. Jacobsen, and P.R. Ortiz de Montellano (1988). 4-Alkyl radical extrusion in the cytochrome P-450-catalyzed oxidation of 4-alkyl-1,4-dihydropyridines. *Biochemistry* **27**, 7703–7710.
232. Guengerich, F.P., W.R. Brian, M. Iwasaki, M.-A. Sari, C. Bäärnhielm, and P. Berntsson (1991). Oxidation of dihydropyridine calcium channel blockers and analogues by human liver cytochrome P-450 IIIA4. *J. Med. Chem.* **34**, 1838–1844.
233. Skiles, G.L. and G.S. Yost (1996). Mechanistic studies on the cytochrome P450-catalyzed dehydrogenation of 3-methylindole. *Chem. Res. Toxicol.* **9**, 291–297.
234. Zuleski, F.R., K.M. Kirkland, M.D. Melgar and J.O. Malbica (1985). Tracazolate metabolites in rat tissue. *Drug Metab. Dispos.* **13**, 129–147.
235. Byon, C.-Y. and M. Gut (1980). Steric considerations regarding the biodegradation of cholesterol to pregnenolone. Exclusion of (22*S*)-22-hydroxycholesterol and 22-ketocholesterol as intermediates. *Biochem. Biophys. Res. Commun.* **94**, 549–552.
236. Burstein, S., B.S. Middleditch, and M. Gut (1975). Mass spectrometric study of the enzymic conversion of cholesterol to (22*R*)-22-hydroxycholesterol, (20*R*,22*R*)-20,22-dihydroxycholesterol, and pregnenolone, and of (22*R*)-22- hydroxycholesterol to the glycol and pregnenolone in bovine adrenocortical preparations. Mode of oxygen incorporation. *J. Biol. Chem.* **250**, 9028–9037.
237. Lambeth, J.D., S.E. Kitchen, A.A. Farooqui, R. Tuckey, and H. Kamin (1982). Cytochrome P-450scc-substrate interactions. Studies of binding and catalytic activity using hydroxycholesterols. *J. Biol. Chem.* **257**, 1876–1884.
238. Tuckey, R.C. and H. Kamin (1983). Kinetics of oxygen and carbon monoxide binding to adrenal cytochrome P-450scc. Effect of cholesterol, intermediates, and phosphatidylcholine vesicles. *J. Biol. Chem.* **258**, 4232–4237.
239. Tuckey, R.C. and H. Kamin (1982). The oxyferro complex of adrenal cytochrome P-450scc. Effect of cholesterol and intermediates on its stability and optical characteristics. *J. Biol. Chem.* **257**, 9309–9314.
240. Primus, J.-L., K. Teunis, D. Mandon, C. Veeger, and I.M.C.M. Rietjens (2000). A Mechanism for oxygen exchange between ligated oxometalloporphinates and bulk water. *Biochem. Biophys. Res. Commun.* **272**, 551–556.
241. Hochberg, R.B., P.D. McDonald, M. Feldman, and S. Lieberman (1974). Biosynthetic conversion of cholesterol into pregnenolone. Side chain cleavage of some 20-*p*-tolyl analogs of cholesterol and 20α-hydroxycholesterol. *J. Biol. Chem.* **249**, 1274–1285.
242. Bower, S., J.B. Perkins, R.R. Yocum, C.L. Howitt, P. Rahaim, and J. Pero (1996). Cloning, sequencing, and characterization of the *Bacillus subtilis* biotin biosynthetic operon. *J. Bacteriol.* **178**, 4122–4130.

243. Stok, J.E. and J.J. De Voss (2000). Expression, purification, and characterization of BioI: A carbon–carbon bond cleaving cytochrome P450 involved in biotin biosynthesis in *Bacillus subtilis*. *Arch. Biochem. Biophys.* **384**, 351–360.
244. Green, A.J., S.L. Rivers, M. Cheesman, G.A. Reid, L.G. Quaroni, I.D.G. Macdonald *et al.* (2001). Expression, purification and characterization of cytochrome P450 BioI: A novel P450 involved in biotin synthesis in *Bacillus subtilis*. *J. Biol. Inorg. Chem.* **6**, 523–533.
245. Cryle, M.J., N.J. Matovic, and J.J. De Voss (2003). Products of cytochrome P450BioI (CYP107H1)-catalysed oxidation of fatty acids. *Org. Lett.* **5**, 3341–3344.
246. Cryle, M.J. and J.J. De Voss (2004). Carbon carbon bond cleavage by cytochrome P450BioI (CYP107H1). *Chem. Commun.* 86–87.
247. Cryle, M.J., J.E. Stok, and J.J. De Voss (2003). Reactions catalyzed by bacterial cytochromes P450. *Aust. J. Chem.* **56**, 749–762.
248. Umehara, K., S. Kudo, Y. Hirao, S. Morita, T. Ohtani, M. Uchida *et al.* (2000). *In vitro* characterization of the oxidative cleavage of the octyl side chain of olanexidine, a novel antimicrobial agent, in dog liver microsomes. *Drug Metab. Dispos.* **28**, 1417–1424.
249. Umehara, K., S. Kudo, Y. Hirao, S. Morita, M. Uchida, M. Odomi *et al.* (2000). Oxidative cleavage of the octyl side chain of 1-(3,4-dichlorobenzyl)-5-octylbiguanide (OPB-2045) in rat and dog liver preparations. *Drug Metab. Dispos.* **28**, 887–894.
250. Barnes, H.J., M.P. Arlotto, and M.R. Waterman (1991). Expression and enzymatic activity of recombinant cytochrome P450 17 α-hydroxylase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **88**, 5597–5601.
251. Zuber, M.X., E.R. Simpson, and M.R. Waterman (1986). Expression of bovine 17 α-hydroxylase cytochrome P-450 cDNA in nonsteroidogenic (COS 1) cells. *Science* **234**, 1258–1261.
252. Nakajin, S., M. Takahashi, M. Shinoda, and P.F. Hall (1985). Cytochrome b5 promotes the synthesis of Δ^{16}-C19 steroids by homogeneous cytochrome P-450 C21 side-chain cleavage from pig testis. *Biochem. Biophys. Res. Commun.* **132**, 708–713.
253. Shimizu, K. (1978). Formation of 5-[17β-^{2}H] androstene-3β,17α-diol from 3β-hydroxy-5-[17,21,21,21-^{2}H]pregnen-20-one by the microsomal fraction of boar testis. *J. Biol. Chem.* **253**, 4237–4241.
254. Corina, D.L., S.L. Miller, J.N. Wright, and M. Akhtar (1991). The mechanism of cytochrome P-450 dependent carbon–carbon bond cleavage: Studies on 17α-hydroxylase-17,20-lyase. *Chem. Commun.* 782–783.
255. Mak, A.Y. and D.C. Swinney (1992). 17-*O*-Acetyltestosterone formation from progesterone in microsomes from pig testes: Evidence for the Baeyer-Villiger rearrangement in androgen formation catalyzed by CYP17. *J. Am. Chem. Soc.* **114**, 8309–8310.
256. Akhtar, M., D. Corina, S. Miller, A.Z. Shyadehi, and J.N. Wright (1994). Mechanism of the acyl-carbon cleavage and related reactions catalyzed by multifunctional P-450s: Studies on cytochrome P-45017α. *Biochemistry* **33**, 4410–4418.
257. Akhtar, M., D.L. Corina, S.L. Miller, A.Z. Shyadehi, and J.N. Wright (1994). Incorporation of label from $^{18}O_2$ into acetate during side-chain cleavage catalyzed by cytochrome P-45017α (17α-hydroxylase-17,20-lyase). *J. Chem. Soc., Perkin Trans. I*, 263–267.
258. Lee-Robichaud, P., M.E. Akhtar, and M. Akhtar (1998). An analysis of the role of active site protic residues of cytochrome P-450s: Mechanistic and mutational studies on 17α-hydroxylase-17,20-lyase (P-45017α also CYP17). *Biochem. J.* **330**, 967–974.
259. Swinney, D.C. and A.Y. Mak (1994). Androgen formation by cytochrome P450 CYP17. Solvent isotope effect and pL studies suggest a role for protons in the regulation of oxene versus peroxide chemistry. *Biochemistry* **33**, 2185–2190.
260. Lee-Robichaud, P., A.Z. Shyadehi, J.N. Wright, M. Akhtar, and M. Akhtar (1995). Mechanistic kinship between hydroxylation and desaturation reactions: Acyl-carbon bond cleavage promoted by pig and human CYP17 (P-45017α; 17α-hydroxylase-17,20-lyase). *Biochemistry* **34**, 14104–14113.
261. Davis, S.C., Z. Sui, J.A. Peterson, and P.R. Ortiz de Montellano (1996). Oxidation of w-oxo fatty acids by cytochrome P450BM-3 (CYP102). *Arch. Biochem. Biophys.* **328**, 35–42.
262. Thompson, E.A., Jr. and P.K. Siiteri (1974). The involvement of human placental microsomal cytochrome P-450 in aromatization. *J. Biol. Chem.* **249**, 5373–5378.
263. Kellis, J.T., Jr. and L.E. Vickery (1987). Purification and characterization of human placental aromatase cytochrome P-450. *J. Biol. Chem.* **262**,4413–4420.
264. Thompson, E.A., Jr. and P.K. Siiteri (1974). Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. *J. Biol. Chem.* **249**, 5364–5372.
265. Caspi, E., T. Arunachalam, and P.A. Nelson (1983). Biosynthesis of estrogens: The steric mode of the initial C-19 hydroxylation of androgens by human placental aromatase. *J. Am. Chem. Soc.* **105**, 6987–6989.

266. Caspi, E., T. Arunachalam, and P.A. Nelson (1986). Biosynthesis of estrogens: Aromatization of (19*R*)-, (19*S*)-, and (19*RS*)-[19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-ones by human placental aromatase. *J. Am. Chem. Soc.* **108**, 1847–1852.
267. Osawa, Y., K. Shibata, D. Rohrer, C. Weeks, and W.L. Duax (1975). Reassignment of the absolute configuration of 19-substituted 19-hydroxysteroids and stereomechanism of estrogen biosynthesis. *J. Am. Chem. Soc.* **97**, 4400–4402.
268. Arigoni, D., R. Battaglia, M. Akhtar, and T. Smith (1975). Stereospecificity of oxidation at C-19 in estrogen biosynthesis. *Chem. Comm.* 185–186.
269. Miyairi, S. and J. Fishman (1983). Novel method of evaluating biological 19-hydroxylation and aromatization of androgens. *Biochem. Biophys. Res. Commun.* **117**, 392–398.
270. Miyairi, S. and J. Fishman (1985). Radiometric analysis of oxidative reactions in aromatization by placental microsomes. Presence of differential isotope effects. *J. Biol. Chem.* **260**, 320–325.
271. Numazawa, M., K. Midzuhashi, and M. Nagaoka (1994). Metabolic aspects of the 1β-proton and the 19-methyl group of androst-4-ene-3,6,17-trione during aromatization by placental microsomes and inactivation of aromatase. *Biochem. Pharmacol.* **47**, 717–726.
272. Brodie, H.J., K.J. Kripalani, and G. Possanza (1969). Mechanism of estrogen biosynthesis. VI. The stereochemistry of hydrogen elimination at C-2 during aromatization. *J. Am. Chem. Soc.* **91**, 1241–1242.
273. Fishman, J. and H. Guzik, (1969). Stereochemistry of estrogen biosynthesis. *J. Am. Chem. Soc.* **91**, 2805–2806.
274. Fishman, J., H. Guzik, and D. Dixon (1969). Stereochemistry of estrogen biosynthesis. *Biochemistry* **8**, 4304–4309.
275. Fishman, J. and M.S. Raju (1981). Mechanism of estrogen biosynthesis. Stereochemistry of C-1 hydrogen elimination in the aromatization of 2β-hydroxy-19-oxoandrostenedione. *J. Biol. Chem.* **256**, 4472–4477.
276. Townsley, J.D. and H.J. Brodie (1968). Mechanism of estrogen biosynthesis. III. Stereochemistry of aromatization of C19 and C18 steroids. *Biochemistry* **7**, 33–40.
277. Osawa, Y., N. Yoshida, M. Fronckowiak, and J. Kitawaki (1987). Immunoaffinity purification of aromatase cytochrome P-450 from human placental microsomes, metabolic switching from aromatization at 1β and 2β-monohydroxylation, and recognition of aromatase isozymes. *Steroids* **50**, 11–28.
278. Akhtar, M., M.R. Calder, D.L. Corina, and J.N. Wright (1982). Mechanistic studies on C-19 demethylation in estrogen biosynthesis. *Biochem. J.* **201**, 569–580.
279. Caspi, E., J. Wicha, T. Arunachalam, P. Nelson, and G. Spiteller (1984). Estrogen biosynthesis: Concerning the obligatory intermediacy of 2β-hydroxy-10β-formyl androst-4-ene-3,17-dione. *J. Am. Chem. Soc.* **106**, 7282–7283.
280. Morand, P., D.G. Williamson, D.S. Layne, L. Lompa-Krzymien, and J. Salvador (1975). Conversion of an androgen epoxide into 17β-estradiol by human placental microsomes. *Biochemistry* **14**, 635–638.
281. Hosoda, H. and J. Fishman (1974). Unusually facile aromatization of 2b-hydroxy-19-oxo-4-androstene-3,17-dione to estrone. Implications in estrogen biosynthesis. *J. Am. Chem. Soc.* **96**, 7325–7329.
282. Goto, J. and J. Fishman (1977). Participation of a nonenzymic transformation in the biosynthesis of estrogens from androgens. *Science* **195**, 80–81.
283. Covey, D.F. and W.F. Hood (1982). A new hypothesis based on suicide substrate inhibitor studies for the mechanism of action of aromatase. *Cancer Res.* **42**, 3327–3333.
284. Beusen, D.D. and D.F. Covey (1984). Study of the role of Schiff base formation in the aromatization of 3-[^{18}O]testosterone and 3,17-di-[^{18}O] androstenedione by human placental aromatase. *J. Steroid Biochem.* **20**, 931–934.
285. Numazawa, M., A. Yoshimura, M. Tachibana, M. Shelangouski, and M. Ishikawa (2002). Time-dependent aromatase inactivation by 4β,5β-epoxides of the natural substrate androstenedione and its 19-oxygenated analogs. *Steroids* **67**, 185–193.
286. Cole, P.A. and C.H. Robinson (1990). Conversion of 19-oxo[2b-2H]androgens into estrogens by human placental aromatase. An unexpected stereochemical outcome. *Biochem. J.* **268**, 553–561.
287. Swinney, D.C., D.M. Watson, and O.Y. So (1993). Accumulation of intermediates and isotopically sensitive enolization distinguish between aromatase (cytochrome P450 CYP19) from rat ovary and human placenta. *Arch. Biochem. Biophys.* **305**, 61–67.
288. Akhtar, M., D. Corina, J. Pratt, and T. Smith (1976). Studies on the removal of C-19 in estrogen biosynthesis using oxygen-18. *Chem. Commun.* 854–856.
289. Stevenson, D.E., J.N. Wright, and M. Akhtar (1988). Mechanistic consideration of P-450 dependent enzymic reactions: Studies on oestriol biosynthesis. *J. Chem. Soc., Perkin Trans. I* 2043–2052.
290. Akhtar, M., V.C.O. Njar, and J.N. Wright (1993). Mechanistic studies on aromatase and related carbon–carbon bond cleaving P-450 enzymes. *J. Steroid. Biochem. Mol. Biol.* **44**, 375–387.

291. Wertz, D.L., M.F. Sisemore, M. Selke, J. Driscoll, and J.S. Valentine (1998). Mimicking cytochrome P-450 2B4 and aromatase: Aromatization of a substrate analog by a peroxo Fe(III) porphyrin complex. *J. Am. Chem. Soc.* **120**, 5331–5332.
292. Goto, Y., S. Wada, I. Morishima, and Y. Watanabe (1998). Reactivity of peroxoiron(III) porphyrin complexes: Models for deformylation reactions catalyzed by cytochrome P-450. *J. Inorg. Biochem.* **69**, 241–247.
293. Cole, P.A. and C.H. Robinson (1988). Peroxide model reaction for placental aromatase. *J. Am. Chem. Soc.* **110**, 1284–1285.
294. Graham-Lorence, S., B. Amarneh, R.E. White, J.A. Peterson, and E.R. Simpson (1995). A three-dimensional model of aromatase cytochrome P450. *Protein. Sci.* **4**, 1065–1080.
295. Alexander, K., M. Akhtar, R.B. Boar, J.F. McGhie, and D.H.R. Barton (1972). Removal of the 32-carbon atom as formic acid in cholesterol biosynthesis. *Chem. Commun.* 383–385.
296. Mitropoulos, K.A., G.F. Gibbons, and B.E.A. Reeves (1976). Lanosterol 14α-demethylase. Similarity of the enzyme system from yeast and rat liver. *Steroids* **27**, 821–829.
297. Canonica, L., A. Fiecchi, K.M. Galli, A.A. Scala, G. Galli, E. Grossi-Paoletti *et al.* (1968). Evidence for the biological conversion of $\Delta^{8,14}$ sterol dienes into cholesterol. *J. Am. Chem. Soc.* **90**, 6532–6534.
298. Gibbons, G.F., L.J. Goad, and T.W. Goodwin (1968). Stereochemistry of hydrogen elimination from C-15 during cholesterol biosynthesis. *J. Chem. Soc. Chem. Commun.* 1458–1460.
299. Watkinson, I.A., D.C. Wilton, K.A. Munday, and M. Akhtar (1971). Formation and reduction of the 14,15-double bond in cholesterol biosynthesis. *Biochem. J.* **121**, 131–137.
300. Shafiee, A., J.M. Trzaskos, Y.K. Paik, and J.L. Gaylor (1986). Oxidative demethylation of lanosterol in cholesterol biosynthesis: Accumulation of sterol intermediates. *J. Lipid Res.* **27**, 1–10.
301. Trzaskos, J.M., R.T. Fischer, and M.F. Favata (1986). Mechanistic studies of lanosterol C-32 demethylation. Conditions which promote oxysterol intermediate accumulation during the demethylation process. *J. Biol. Chem.* **261**, 16936–16937.
302. Saucier, S.E., A.A. Kandutsch, S. Phirwa, and T.A. Spencer (1987). Accumulation of regulatory oxysterols, 32-oxolanosterol and 32-hydroxylanosterol in mevalonate-treated cell cultures. *J. Biol. Chem.* **262**, 14056–14062.
303. Aoyama, Y., Y. Yoshida, Y. Sonoda, and Y. Sato (1987). Metabolism of 32-hydroxy-24,25-dihydrolanosterol by purified cytochrome P 45014DM from yeast. Evidence for contribution of the cytochrome to whole process of lanosterol 14α-demethylation. *J. Biol. Chem.* **262**, 1239–1243.
304. Aoyama, Y., Y. Yoshida, Y. Sonoda, and Y. Sato (1989). Deformylation of 32-oxo-24,25-dihydrolanosterol by the purified cytochrome P-45014DM (lanosterol 14α-demethylase) from yeast evidence confirming the intermediate step of lanosterol 14α-demethylation. *J. Biol. Chem.* **264**, 18502–18505.
305. Trzaskos, J., S. Kawata, and J.L. Gaylor (1986). Microsomal enzymes of cholesterol biosynthesis. Purification of lanosterol 14α-methyl demethylase cytochrome P-450 from hepatic microsomes. *J. Biol. Chem.* **261**, 14651–14657.
306. Sono, H., Y. Sonoda, and Y. Sato (1991). Purification and characterization of cytochrome P-45014DM (lanosterol 14α-demethylase) from pig liver microsomes. *Biochim. Biophys. Acta* **1078**, 388–394.
307. Sonoda, Y., M. Endo, K. Ishida, Y. Sato, N. Fukusen, and M. Fukuhara (1993). Purification of a human cytochrome P-450 isoenzyme catalyzing lanosterol 14α-demethylation. *Biochim. Biophys. Acta* **1170**, 92–97.
308. Alexander, K.T.W., M. Akhtar, R.B. Boar, J.F. McGhie, and D.H.R. Barton (1971). Pathway for the removal of C-32 in cholesterol biosynthesis. *J. Chem. Soc., Chem. Commun.* 1479–1481.
309. Akhtar, M., C.W. Freeman, D.C. Wilton, R.B. Boar, and D.B. Copsey (1977). The pathway for the removal of the 15α-methyl group of lanosterol. The role of lanost-8-ene-3β,32-diol in cholesterol biosynthesis. *Bioorg. Chem.* **6**, 473–481.
310. Akhtar, M., K. Alexander, R.B. Boar, J.F. McGhie, and D.H.R. Barton (1978). Chemical and enzymic studies on the characterization of intermediates during the removal of the 14α-methyl group in cholesterol biosynthesis. The use of 32-functionalized lanostane derivatives. *Biochem. J.* **169**, 449–463.
311. Trzaskos, J.M., R.T. Fischer, S.S. Ko, R.L. Magolda, S. Stam, P. Johnson *et al.* (1995). Substrate-based inhibitors of lanosterol 14α-methyl demethylase: II. Time-dependent enzyme inactivation by selected oxylanosterol analogs. *Biochemistry* **34**, 9677–9681.
312. Shyadehi, A.Z., D.C. Lamb, S.L. Kelly, D.E. Kelly, W.-H. Schunck, J.N. Wright *et al.* (1996). The mechanism of the acyl-carbon bond cleavage reaction catalyzed by recombinant sterol 14α-demethylase of *Candida albicans* (other names are: lanosterol 14α-demethylase, P-45014DM, and CYP51). *J. Biol. Chem.* **271**, 12445–12450.
313. Ramm, P.J. and E. Caspi (1969). Stereochemistry of tritium at carbon atoms 1, 7, and 15 in cholesterol derived from mevalonic-(3*R*,2*R*)-2-^{3}H acid. *J. Biol. Chem.* **244**, 6064–6073.

314. Akhtar, M., A.D. Rahimtula, I.A. Watkinson, D.C. Wilton, and K.A. Munday (1969). Status of C-6, C-7, C-15, and C-16 hydrogen atoms in cholesterol biosynthesis. *Eur. J. Biochem.* **9**, 107–111.
315. R.T. Fischer, J.M. Trzaskos, R.L. Magolda, S.S. Ko, C.S. Brosz, and B. Larsen (1991). Lanosterol 14α-methyl demethylase. Isolation and characterization of the third metabolically generated oxidative demethylation intermediate. *J. Biol. Chem.* **266**, 6124–6132.
316. Lamb, D.C., D.E. Kelly, M.R. Waterman, M. Stromstedt, D. Rozman, and S.L. Kelly (1999). Characteristics of the heterologously expressed human lanosterol 14α-demethylase (other names: P45014DM, CYP51, P45051) and inhibition of the purified human and *Candida albicans* CYP51 with azole antifungal agents. *Yeast* **15**, 755–763.
317. Sloane, D.L., O.-Y. So, R. Leung, L.E. Scarafia, N. Saldou, K. Jarnagin *et al.* (1995). Cloning and functional expression of the cDNA encoding rat lanosterol 14α-demethylase. *Gene* **161**, 243–248.
318. Cabello-Hurtado, F., M. Taton, N. Forthoffer, R. Kahn, S. Bak, A. Rahier *et al.* (1999). Optimized expression and catalytic properties of a wheat obtusifoliol 14α-demethylase (CYP51) expressed in yeast. Complementation of erg11D yeast mutants by plant CYP51. *Eur. J. Biochem.* **262**, 435–446.
319. Bellamine, A., A.T. Mangla, W.D. Nes, and M.R. Waterman (1999). Characterization and catalytic properties of the sterol 14α-demethylase from *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. USA* **96**, 8937–8942.
320. Lamb, D.C., K. Fowler, T. Kieser, N. Manning, L.M. Podust, M.R. Waterman *et al.* (2002). Sterol 14α-demethylase activity in *Streptomyces coelicolor* A3(2) is associated with an unusual member of the CYP51 gene family. *Biochem. J.* **364**, 555–562.
321. Jackson, C.J., D.C. Lamb, T.H. Marczylo, A.G.S. Warrilow, N.J. Manning, D.J. Lowe *et al.* (2002). A novel sterol 14α-Demethylase/Ferredoxin fusion protein (MCCYP51FX) from *Methylococcus capsulatus* represents a new class of the cytochrome P450 superfamily. *J. Biol. Chem.* **277**, 46959–46965.
322. Lamb, D.C., D.E. Kelly, and S.L. Kelly (1998). Molecular diversity of sterol 14α-demethylase substrates in plants, fungi and humans. *FEBS Lett.* **425**, 263–265.
323. Roberts, E.S., A.D.N. Vaz, and M.J. Coon (1991). Catalysis by cytochrome P-450 of an oxidative reaction in xenobiotic aldehyde metabolism: Deformylation with olefin formation. *Proc. Natl. Acad. Sci. USA* **88**, 8963–8966.
324. Vaz, A.D.N., E.A. Roberts, and M.J. Coon (1991). Olefin formation in the oxidative deformylation of aldehydes by cytochrome P-450. Mechanistic implications for catalysis by oxygen-derived peroxide. *J. Am. Chem. Soc.* **113**, 5886–5887.
325. Raner, G.M., E.W. Chiang, A.D.N. Vaz, and M.J. Coon (1997). Mechanism-based inactivation of cytochrome P450 2B4 by aldehydes: Relationship to aldehyde deformylation via a peroxyhemiacetal intermediate. *Biochemistry* **36**, 4895–4902.
326. Vaz, A.D.N., K.J. Kessell, and M.J. Coon (1994). Aromatization of a bicyclic steroid analog, 3-oxodecalin-4-ene-10-carboxaldehyde, by liver microsomal cytochrome P450 2B4. *Biochemistry* **33**, 13651–13661.
327. Vaz, A.D., S.J. Pernecky, G.M. Raner, and M.J. Coon (1996). Peroxo-iron and oxenoid-iron species as alternative oxygenating agents in cytochrome P450-catalyzed reactions: Switching by threonine-302 to alanine mutagenesis of cytochrome P450 2B4. *Proc. Natl. Acad. Sci. USA* **93**, 4644–4648.
328. Kuo, C.L., G.M. Raner, A.D. Vaz, and M.J. Coon (1999). Discrete species of activated oxygen yield different cytochrome P450 heme adducts from aldehydes. *Biochemistry* **38**, 10511–10518.
329. Raner, G.M., J.A. Hatchell, M.U. Dixon, T.L. Joy, A.E. Haddy, and E.R. Johnston (2002). Regioselective peroxo-dependent heme alkylation in P450BM3-F87G by aromatic aldehydes: Effects of alkylation on catalysis. *Biochemistry* **41**, 9601–9610.
330. Reed, J.R., D. Vanderwel, S. Choi, G. Pomonis, R.C. Reitz, and G.J. Blomquist (1994). Unusual mechanism of hydrocarbon formation in the housefly: Cytochrome P450 converts aldehyde to the sex pheromone component (*Z*)-9-tricosene and CO_2. *Proc. Natl. Acad. Sci. USA* **91**, 10000–10004.
331. Reed, J.R., D.R. Quilici, G.J. Blomquist, and R.C. Reitz (1995). Proposed mechanism for the cytochrome P450-catalyzed conversion of aldehydes to hydrocarbons in the house fly, *Musca domestica*. *Biochemistry* **34**, 16221–16227.
332. Adam, W., R. Curci, M.E. Gonzalez Nunez, and R. Mello (1991). Thermally and photochemically initiated radical chain decomposition of ketone-free methyl(trifluoromethyl)dioxirane. *J. Am. Chem. Soc.* **113**, 7654–7658.
333. Mpuru, S., J.R. Reed, R.C. Reitz, and G.J. Blomquist (1996). Mechanism of hydrocarbon biosynthesis from aldehyde in selected insect species: Requirement for O_2 and NADPH and carbonyl group released as CO_2. *Insect Biochem. Mol. Biol.* **26**, 203–208.
334. Spiteller, D., A. Jux, J. Piel, and W. Boland (2002). Feeding of [5,5-$^2H_2$]-1-desoxy-D-xylulose and [4,4,6,6,6-$^2H_5$]-mevalolactone to a geosmin-producing *Streptomyces sp.* and *Fossombronia pusilla*. *Phytochemistry* **61**, 827–834.

335. Yamamoto, H., N. Katano, A. Ooi, and K. Inoue (2000). Secologanin synthase which catalyzes the oxidative cleavage of loganin into secologanin is a cytochrome P450. *Phytochemistry* **53**, 7–12.
336. Irmler, S., G. Schroder, B. St-Pierre, N.P. Crouch, M. Hotze, J. Schmidt *et al.* (2000). Indole alkaloid biosynthesis in *Catharanthus roseus*: New enzyme activities and identification of cytochrome P450 CYP72A1 as secologanin synthase. *Plant J.* **24**, 797–804.
337. Udwary, D.W., L.K. Casillas, and C.A. Townsend (2002). Synthesis of 11-hydroxyl O-methylsterigmatocystin and the role of a cytochrome P-450 in the final step of aflatoxin biosynthesis. *J. Am. Chem. Soc.* **124**, 5294–5303.
338. Prieto, R. and C.P. Woloshuk (1997). *ord1*, an oxidoreductase gene responsible for conversion of O-methylsterigmatocystin to aflatoxin in *Aspergillus flavus*. *Appl. Environ. Microbiol.* **63**, 1661–1666.
339. Coolbaugh, R.C. (1997). Cytochrome P450-dependent steps in gibberellin biosynthesis. *Proc. Plant Growth Regulator Soc. Am.* **24**, 10–14.
340. Helliwell, C.A., W.J. Peacock, and E.S. Dennis (2002). Isolation and functional characterization of cytochrome P450s in gibberellin biosynthesis pathway. *Meth. Enzymol.* **357**, 381–388.
341. Helliwell, C.A., P.M. Chandler, A. Poole, E.S. Dennis, and W.J. Peacock (2001). The CYP88A cytochrome P450, *ent*-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. *Proc. Natl. Acad. Sci. USA* **98**, 2065–2070.
342. Rojas, M.C., P. Hedden, P. Gaskin, and B. Tudzynki (2001). The P450-1 gene of *Gibberella fujikuroi* encodes a multifunctional enzyme in gibberellin biosynthesis. *Proc. Natl. Acad. Sci. USA* **98**, 5838–5843.
343. Fukuda, H., T. Fujii, E. Sukita, M. Tazaki, S. Nagahama, and T. Ogawa (1994). Reconstitution of the isobutene-forming reaction catalyzed by cytochrome P450 and P450 reductase from *Rhodotorula minuta*: Decarboxylation with the formation of isobutene. *Biochem. Biophys. Res. Commun.* **201**, 516–522.
344. Shimaya, C. and T. Fujii (2000). Cytochrome P450rm of *Rhodotorula* functions in the β-ketoadipate pathway for dissimilation of L-phenylalanine. *J. Biosci. Bioeng.* **90**, 465–467.
345. Fukuda, H., K. Nakamura, E. Sukita, T. Ogawa, and T. Fujii (1996). Cytochrome P450rm from *Rhodotorula minuta* catalyzes 4-hydroxylation of benzoate. *J. Biochem.* **119**, 314–318.
346. Komuro, M., T. Higuchi, and M. Hirobe (1995). Application of chemical cytochrome P-450 model systems to studies on drug metabolism-VIII. Novel metabolism of carboxylic acids via oxidative decarboxylation. *Bioorg. Med. Chem.* **3**, 55–65.
347. White, R.E., S.G. Sligar, and M.J. Coon (1980). Evidence for a homolytic mechanism of peroxide oxygen–oxygen bond cleavage during substrate hydroxylation by cytochrome P-450. *J. Biol. Chem.* **255**, 11108–11111.
348. Hashim, M.F., T. Hakamatsuka, Y. Ebizuka, and U. Sankawa (1990). Reaction mechanism of oxidative rearrangement of flavanone in isoflavone biosynthesis. *FEBS Lett.* **271**, 219–222.
349. Akashi, T., T. Aoki, and S.-I. Ayabe (1999). Cloning and functional expression of a cytochrome P450 cDNA encoding 2-hydroxyisoflavanone synthase involved in biosynthesis of the isoflavonoid skeleton in licorice. *Plant Physiol.* **121**, 821–828.
350. Hakamatsuka, T., M.F. Hashim, Y. Ebizuka, and U. Sankawa (1991). P-450-dependent oxidative rearrangement in isoflavone biosynthesis: Reconstitution of P-450 and NADPH:P-450 reductase. *Tetrahedron* **47**, 5969–5978.
351. Sawada, Y., K. Kinoshita, T. Akashi, T. Aoki, and S.-I. Ayabe (2002). Key amino acid residues required for aryl migration catalysed by the cytochrome P450 2-hydroxyisoflavanone synthase. *Plant J.* **31**, 555–564.
352. Yin, W., G.A. Doss, R.A. Stearns, A.G. Chaudhary, C.E. Hop, R.B.Franklin *et al.* (2003). A novel P450-catalyzed transformation of the 2,2,6,6-tetramethyl piperidine moiety to a 2,2-dimethyl pyrrolidine in human liver microsomes: Characterization by high resolution quadrupole-time-of-flight mass spectrometry and ^{1}H-NMR. *Drug Metab. Dispos.* **31**, 215–223.
353. Boucher, J.-L., M. Delaforge, and D. Mansuy (1994). Dehydration of alkyl- and arylaldoximes as a new cytochrome P450-catalyzed reaction: Mechanism and stereochemical characteristics. *Biochemistry* **33**, 7811–7818.
354. Gupta, O.D., R.L. Kirchmeier, and J.M. Shreeve (1990). Reactions of trifluoroamine oxide: A route to acyclic and cyclic fluoroamines and N-nitrosoamines. *J. Am. Chem. Soc.* **112**, 2383–2386.
355. Tudzynski, B., M.C. Rojas, P. Gaskin, and P. Hedden (2002). The gibberellin 20-oxidase of *Gibberella fujikuroi* is a multifunctional monooxygenase. *J. Biol. Chem.* **277**, 21246–21253.