

# Mechanisms of Cytochrome P450 Reactions

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## Abstract

The cytochrome P450 enzymes (P450s) catalyze a number of different reactions using a basic chemical template. The course of events follows the order of substrate binding, 1-electron reduction, O<sub>2</sub> binding, a second 1-electron reduction, and then a series of less well-defined steps understood as protonation, hemolytic scission of the O–O bond to yield an active perferryl FeO species (depicted as FeO<sup>3+</sup>), reaction with the substrate, and release of the product, although the point should be made that the sequence of some of the events may vary. The chemical events are generally applicable to most of the oxidative reactions. Exactly why rates of individual P450 reactions vary is still not clear. The basic P450 reactions include C-hydroxylation, heteroatom oxygenation, heteroatom release (dealkylation), epoxide formation, and 1,2-migration. However, P450 enzymes also use variations of the basic chemistry to catalyze what appear to be a myriad of unusual reactions, including reductions, isomerizations, and oxidative coupling.

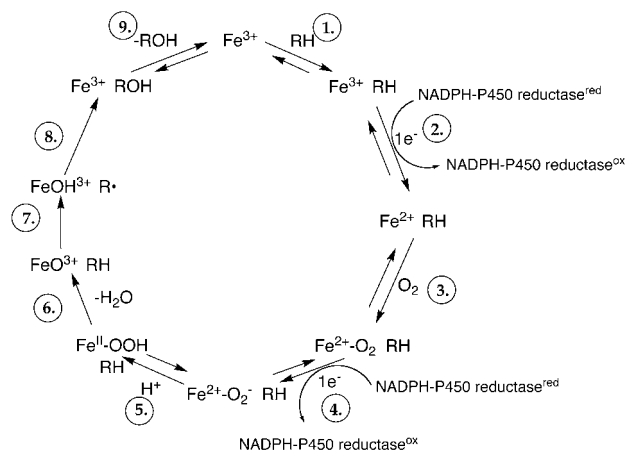
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## 1. Introduction

Cytochrome P450 (P450) reactions are primarily mixed-function oxidations. These monooxygenations are of interest in biochemistry because they are so ubiquitous in nature and because these generally chemically difficult reactions occur with so many substrates. The P450s are found from eubacteria and archebacteria to humans, with relatively few exceptions (e.g. the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* are devoid of P450 genes). The repertoire of substrates includes a wide variety of natural products ranging from terpenes in microorganisms to steroids in mammals. The P450s are involved in both anabolic and catabolic reactions. In the latter category, the degradation of drugs by humans has received special attention,<sup>1</sup> in that 75% of the enzymatic processing of drugs is due to P450s<sup>2</sup> and changes in the interaction of P450s and drugs can be the basis of major side effects.<sup>3</sup>

The chemistry of these reactions has not come easily. Early in P450 research the enzymes were difficult to purify. Research on several of the catalytic steps requires anaerobic methods, rapid kinetics, and sophisticated chemical and spectroscopic approaches. One of the most

challenging aspects is the instability of all of the complexes in Fig. 1 between steps 3 and 8. In this latter regard, much of the inference has been based on biomimetic models and theoretical calculations. In this brief review, we will consider some of the salient points at each reaction step, as well as controversies and open questions.



**Fig. 1.** General scheme for P450 catalysis. See text for discussion of individual steps.

## 2. General Features of the Catalytic Mechanism

Introducing an oxygen into an unactivated methylene or methyl group is very energetically unfavorable. Enzymes can do this using high-valent iron- or copper-oxygen complexes; some of the P450-type oxygenations can be done by flavoproteins, but often only with partially-activated substrates. The reaction of triplet  $O_2$  with organic molecules is generally spin-forbidden<sup>4</sup> and the use of metalloenzymes provides a means of circumventing the barrier in a controlled manner. Catalyzing such reactions with a stepwise series of intermediates allows for control of the oxidation process without generating dangerous reactants, in principle, although we will see that there is considerable inefficiency in many cases. This inefficiency can be considered the cost of having systems that can process so many individual substrates.

As mentioned earlier, biomimetic models can be used to achieve most of the known P450 reactions. Most of these systems use metalloporphyrins with high-valent oxidants such as peracids or iodosyl compounds. The work indicates that neither the protein, the specific metal (iron), nor the ligand matrix (porphyrin) is essential,<sup>5</sup> although in these simple systems the control of regioselectivity is usually simply thermodynamic.

Before we begin our discussion of the steps in Fig. 1, we should emphasize that these do not necessarily occur in the order discussed. Substrate binding and dissociation can occur with ferrous P450, at least in some cases.<sup>6</sup> Also, some mammalian (ferric) P450s are reduced at equally fast rates in the absence or presence of substrate.<sup>7</sup> Another consideration is that it is conceivable that some P450s might even be in a ferrous resting state in the highly reducing environment of (some) cells with low  $O_2$  tension, if in equilibrium with the NAD(P)H/NAD(P)<sup>+</sup> pool.

## 3. Individual Reaction Steps

**Substrate Binding (Step 1)** – Historically substrate binding has been considered to be a relatively rapid, univalent process. This has been shown to be the case for P450s with relatively small binding sites, such as bacterial P450 101A1 and human P450 2A6, with analysis of the kinetics of spectral perturbation yielding  $k_{on} \sim 10^6$ – $10^7$  M<sup>-1</sup> s<sup>-1</sup>.<sup>6,8,9</sup> These rates are not necessarily diffusion-limited but may be attenuated by hidden steps and/or non-productive binding modes.

One point to be made is that even in these cases, a  $K_d$  of 1  $\mu$ M gives a  $k_{off}$  of 1–10 s<sup>-1</sup>, corresponding to experimental measurements,<sup>6,8</sup> so the protein must be changing conformation rapidly to allow these on and off rates. With a P450 such as 2A6, the substrate is deeply imbedded and there must be rapid opening and closing of much of the protein.<sup>10</sup>

In the past decade the cooperative behavior of some prokaryotic and especially human P450s has been studied extensively.<sup>1,11,12</sup> Some models proposed to explain this behavior have two or more substrates in the active site simultaneously.<sup>13,14</sup> Many models<sup>15,16</sup> have been made, although a large number of these are (i) based only on steady-state kinetics and/or (ii) not detailed enough (in terms of data) to support conclusions about mechanisms derived from deconvolution. In particular, many of the data sets give very low  $n$  values (<1.5) in (Hill) plots of  $v = k_{cat} [S]^n (S_{50} + S^n)^{-1}$ <sup>17</sup> and may be suspect.

The binding of substrates to some of the P450s with large active sites (e.g. P450 3A4, 1A2) is relatively slow as judged by rates of perturbation of the heme spectra (<  $10^6$  M<sup>-1</sup> s<sup>-1</sup>).<sup>18</sup> However, with fluorescent substrates a more rapid initial step can be detected by measuring the rate of initial fluorescence quenching.<sup>18,19</sup> The kinetics for P450s 3A4 and also 1A2<sup>20</sup> are interpreted as being most consistent with a scheme in which the substrate binds first to a peripheral site on P450 (in a diffusion-limited reaction) followed by a conformational change that moves the substrate near the heme and another conformational change that further perturbs the heme interaction. In most reactions, substrate oxidation is at a rate approximating one of the latter steps, so steps 2–9 of Fig. 1 are competing with conformational changes and the kinetic complexity of these reactions is not surprising.

Despite the plethora of literature about multiple substrate complexes, the only direct physical evidence is with one crystal structure of P450 3A4<sup>21</sup> and fluorescence spectra of dimers (excimers) of pyrene in P450 3A4<sup>22</sup> and possibly in P450 1A2.<sup>20</sup> The “initial” complex may be related to complexes of P450 3A4 with a steroid bound outside of the formal active site.<sup>23, 24</sup>

**Reduction (Step 2)** – The electron used to reduce ferric P450 comes from either a flavoprotein or iron-sulfur protein. These reductions have been studied extensively, usually in the presence of CO, which traps the ferrous iron and produces a very distinct spectral signal.

Reduction rates can vary considerably.<sup>7</sup> With the rapid rates of product formation by the bacterial P450s (at least 101A1 and 102A1), reduction is reported to be highly dependent upon the presence of substrate<sup>25</sup> and can be correlated with substrate-induced changes in spin-state and the oxidation-reduction potential.<sup>9,26</sup> With mammalian P450s, a variety of results are observed. Some P450s behave similarly to the bacterial P450s mentioned above, but with others the rate of reduction is independent of the presence of substrate and also the iron spin-state.<sup>7</sup>

One issue in microsomal preparations of mammalian tissues is that the concentration of NADPH-P450 reductase is an order of magnitude less than that of P450 and lower apparent rates of P450 reduction are observed because the reductase must diffuse to multiple P450s. This phenomenon appears to account for the multiphasic nature of the reduction kinetics.<sup>27</sup>

**O<sub>2</sub> Binding (Step 3)** – Relatively few studies of the rate of O<sub>2</sub> binding to reduced P450 have been made, because the product is rather unstable. The results for several P450s indicate that the rate is on the order of  $> 10^6 \text{ M}^{-1} \text{ s}^{-1}$ .<sup>9,25,28</sup> Under laboratory conditions, the O<sub>2</sub> concentration in buffer is  $\sim 180 \text{ } \mu\text{M}$ , so the rate is not limiting. Within cells the O<sub>2</sub> tension is not as high, but because the “on” rates for O<sub>2</sub> and organic substrates are similar, the rates of O<sub>2</sub> binding are considered to be higher than for substrates (step 1) because the cellular concentration of O<sub>2</sub> is generally higher than that for most substrates, at least drugs and most steroids.

**Reactions of the FeO<sub>2</sub><sup>2+</sup> Complex (Step 4 etc.)** – The FeO<sub>2</sub><sup>2+</sup> complex is unstable; the bacterial P450 101A1 complex was the first observed and has been most extensively described.<sup>25,29</sup> The stabilities of the bacterial and mammalian P450 FeO<sub>2</sub><sup>2+</sup> complexes vary considerably and have been compiled,<sup>28,30</sup> with the rate of decay varying from 0.01 to 140 s<sup>-1</sup> (i.e.  $t_{1/2}$  varying from 5 ms to 70 s at 25 °C).

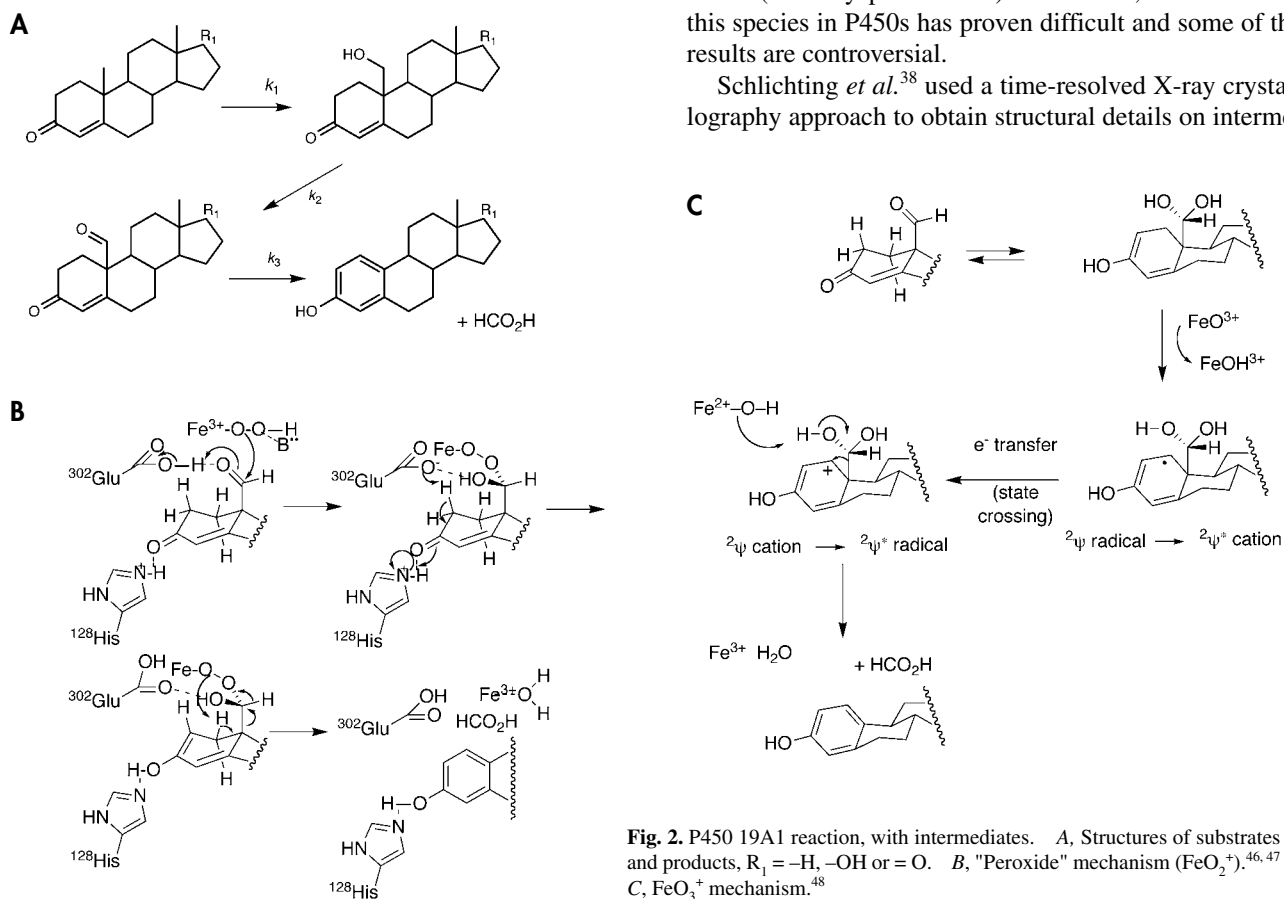
In the absence of import of electrons the complex decomposes, apparently to yield Fe<sup>3+</sup> and O<sub>2</sub><sup>-</sup>, although this analysis has only been done rigorously with bacterial P450 101A1.<sup>31</sup> However, in the presence of substrate some dismutation of a FeO<sub>2</sub><sup>2+</sup> – substrate complex can oc-

cur, with one molecule apparently transferring an electron to another to generate oxidized product (in a yield as high as 60%).<sup>6,28</sup>

The kinetics of the reaction of reduced putidaredoxin with bacterial P450 101A1 have been studied extensively, with the two proteins reacting in a second-order reaction to generate a productive complex involved in electron transfer and product formation.<sup>25,32</sup> These reactions have been more difficult to study with mammalian proteins, due to the slow interactions of P450 and NADPH-P450 reductase that occur when the two proteins are mixed, at least in vesicular systems.<sup>7,33</sup> Presumably the situation is the same with cytochrome *b*<sub>5</sub>, although some attempts have been made.<sup>34</sup> However, Waskell and her colleagues have recently used approaches with rabbit P450 2B4 and either cytochrome *b*<sub>5</sub>, NADPH-P450 reductase, or a deazaflavin-substituted NADPH-P450 reductase in which the two proteins are pre-mixed, reduced, and reacted with O<sub>2</sub>.<sup>35</sup> Of interest is the report that cytochrome *b*<sub>5</sub> is considerably more efficient than the reductase in delivering the second electron to rabbit P450 2B4, in the presence of the substrate benzphetamine.<sup>36</sup> These are technically challenging studies but should be extended to more systems.

**Experimental Studies on the P450 FeO<sup>3+</sup> Complex (Steps 5, 6, and 7)** – The FeO<sup>3+</sup> complex corresponds electronically to peroxidase Compound I, which is relatively stable (in many peroxidases).<sup>37</sup> However, characterizing this species in P450s has proven difficult and some of the results are controversial.

Schlichting *et al.*<sup>38</sup> used a time-resolved X-ray crystallography approach to obtain structural details on interme-



**Fig. 2.** P450 19A1 reaction, with intermediates. **A**, Structures of substrates and products, R<sub>1</sub> = -H, -OH or = O. **B**, “Peroxide” mechanism (FeO<sub>2</sub><sup>+</sup>).<sup>46,47</sup> **C**, FeO<sub>3</sub><sup>+</sup> mechanism.<sup>48</sup>

diates in the P450 catalytic reaction cycle. Irradiation of the  $\text{FeO}_2^{2+}$  complex with the appropriate energy led to the production of a complex which was attributed to be  $\text{FeO}^{3+}$ , although the results are not unambiguous. Ferric P450 was recovered, but product yield was not quantified.<sup>38</sup> The assignment of the product as 5-*exo*-hydroxycamphor is based on ENDOR studies.<sup>39</sup>

Largely on the basis of a number of studies with peroxidases and biomimetic models, the  $\text{FeO}^{3+}$  complex is considered to exist in an  $\text{Fe}^{\text{IV}} = \text{O}$ /porphyrin radical electronic configuration, as opposed to  $\text{Fe}^{\text{V}} = \text{O}$ .<sup>5,37</sup>

In attempts to circumvent the complexities of reactions proceeding from the  $\text{FeO}_2^{2+}$  species, several investigators have reported the use of the alternate strategy of adding peracids to ferric P450, a method used with biomimetic model metalloporphyrins.<sup>5</sup> However, the results are not in agreement, even for a set of experiments all done with P450 101A1. Spectra have been described with  $t_{1/2}$  in the ms range with differing UV-visible spectra,<sup>40,41</sup> with controversy about the pH used and the significance of oxidation of a tyrosine near the active site.<sup>42</sup> However, de Vries' group has used microsecond freeze-quench methods and reported that the complex assigned as  $\text{FeO}^{3+}$  has a  $t_{1/2}$  of only 16  $\mu\text{s}$ ,<sup>43</sup> based on UV-visible and ESR spectrometry of the complex (S. de Vries, personal communication). One problem with all of these studies is that none have involved attempts to measure the kinetics of product formation (hydroxycamphor), which should track with the decomposition of a valid  $\text{FeO}^{3+}$  species.

**Viability of Alternative Species as Oxidants instead of  $\text{FeO}^{3+}$  ( $\text{FeOOH}^{2+}$ ,  $\text{FeO}_2^+$ ) (Step 5)**—Most of the attention has been given to  $\text{FeO}^{3+}$  ("Compound I") as the oxidant but the possibility exists that other species could be involved. Attention has been given to  $\text{FeO}_2^+$  and  $\text{FeOOH}^{2+}$ , two intermediates shown earlier in the catalytic cycle (Fig. 1). In principle, these should be considered in light of the known reactions of alkyl hydroperoxides and flavin hydroperoxides with olefins and heteroatoms.<sup>44,45</sup> The peroxide pathway was proposed primarily to explain the third step in the aromatization of estrogens, a reaction catalyzed by P450 19A1<sup>46,47</sup> (Fig. 2). Compelling evidence indicates that some P450 reactions can involve such intermediates.<sup>49</sup> A large fraction of the evidence comes from work in which a conserved threonine, considered to be involved in proton donation to  $\text{FeO}_2^+$ ,<sup>50,51</sup> has been mutated to alanine. However, alternate effects of the mutations are possible and cannot be ruled out.<sup>52</sup> Even in the aromatase reaction an alternate pathway involving a  $\text{FeO}^{3+}$  step with hydrogen atom abstraction at C-1 has been proposed, and density functional theory calculations have been interpreted in favor of this.<sup>48</sup>

Discerning the roles of the potential oxygenated intermediates is not trivial, in that these can be formed consecutively (Fig. 1). Some quantum mechanical calculations have been done and interpreted as evidence for  $\text{FeOO}(\text{H})$  involvement,<sup>53</sup> but others have also concluded from differ-

ent calculations that  $\text{FeOO}(\text{H})$  species cannot perform many of the most common P450 reactions.<sup>54</sup> The proposition has been made that P450s can catalyze some oxidations using  $\text{FeOO}(\text{H})$  and some with  $\text{FeO}^{3+}$ .<sup>55</sup> Alternatively, Shaik has developed a model in which all reactions proceed from  $\text{FeO}^{3+}$  but from either of two spin states, high or low<sup>56,57</sup> (these should not be directly equated with the ferric spin states mentioned earlier in the steps involving substrate binding (Fig. 1, step 1) and reduction (Fig. 1, step 2)).

Another issue is the nature of intermediates (i.e. caged carbon radicals) in the oxidation reaction, or whether they even exist. The lack of complete scrambling of stereochemistry and rearrangement in oxidation reactions has been interpreted as evidence for concerted reactions, or even in terms of carbocationic intermediates<sup>58</sup> or possibly the contribution of the two spin states of  $\text{FeO}^{3+}$ .<sup>57</sup> One of the problems with interpretation of studies with "radical clocks" is that the rates of rearrangement in solution (or gas phase) do not necessarily be interpreted as conclusive (but positive evidence can). Studies with norcaradiene and spiro[2.5]octane yield rearrangement products only consistent with radical intermediates, with 16 to 52 ps lifetimes for several bacterial and mammalian P450s ( $k = 5 \times 10^7$  to  $2 \times 10^8 \text{ s}^{-1}$  for rebound) and are not consistent with either concerted or cationic mechanisms.<sup>60</sup>

**Electron Transfer and Hydrogen Atom Transfer (Step 7)**—The high valent complex  $\text{FeO}^{3+}$  should have the potential to remove electrons from substrates, just as a peroxidase Compound I species does, in that the electronic state is the same. The oxidation potentials ( $E_{1/2}$ ) of horseradish peroxidase Compounds I and II are both  $\sim 1.0 \text{ V}$ , and P450  $\text{FeO}^{3+}$  should have a similar potential. Marcus Theory considerations

$$\Delta G^\ddagger = \lambda/4 (1 + \Delta G^\circ/\lambda)^2 \quad (1)$$

which relate rates of enzyme catalysis and  $E_{1/2(\text{substrate})}$  differences among substrates, argue that the effective  $E_{1/2}$  should be a function of the distance of  $\text{FeO}^{3+}$  and the reductant site (and increasing in a low dielectric constant), so the effective  $E_{1/2}$  may be even higher.<sup>61</sup>

$$E_{1/2(\text{app})} = E_{1/2(\text{int})} + 14.4/r_{1,2}D \quad (2)$$

where  $E_{1/2(\text{app})}$  is the apparent potential,  $E_{1/2(\text{int})}$  is the intrinsic potential,  $r_{1,2}$  is the internuclear distance between the interacting spheres in the transition state, and  $D$  is the static dielectric constant of the active site.

Evidence for the involvement of 1-electron oxidation reactions (with substrates having electrons that can be removed with potentials of  $<1.5 \text{ V}$ ) is based on the following pieces of information: (i) Cyclopropyl and cyclobutyl heteroatoms (mainly amines) undergo rearrangements characteristic of aminium radicals or the equivalent.<sup>62,63</sup> (ii) Dealkylation reactions show lower kinetic hydrogen

isotope effects (H/D, H/T) with amines but not amides.<sup>64,65</sup> (iii) 1,4-Dihydropyridines, which are vinylogous amines, undergo ring oxidation to pyridines with extrusion of 4-alkyl substituents as radicals, consistent only with initial 1-electron oxidation. (iv) Amines and sulfides can undergo facile heteroatom oxygenation in cases where  $\alpha$ -hydrogens cannot be removed.<sup>66,67</sup> (v) Rates of *N*-demethylation follow a Hammett relationship with *para*-substituted *N,N*-dimethylanilines, yielding a negative  $\rho$  value<sup>68</sup> or parameters consistent with a Marcus relationship (*vide supra*).<sup>61</sup> (vi) Several reactions with low  $E_{1/2}$  compounds other than amines yield either products that can only be rationalized on the basis of 1-electron oxidation (e.g. quadricyclane)<sup>69</sup> or yield spectrally observable stable cation radicals (1,2,4,5-tetramethoxybenzene).<sup>70</sup> (vii) Finally, perhaps the simplest and most powerful piece of evidence is that the dealkylation of *N*-methyl, *N*-ethylaniline by P450 2B1 is 20:1 in favor of *N*-deethylation,<sup>71,72</sup> which is consistent with the known stability of an *N*-methyl aminium radical (relative to *N*-ethyl)<sup>73</sup> but not the relative stabilities of methyl vs. ethyl radicals.<sup>74</sup>

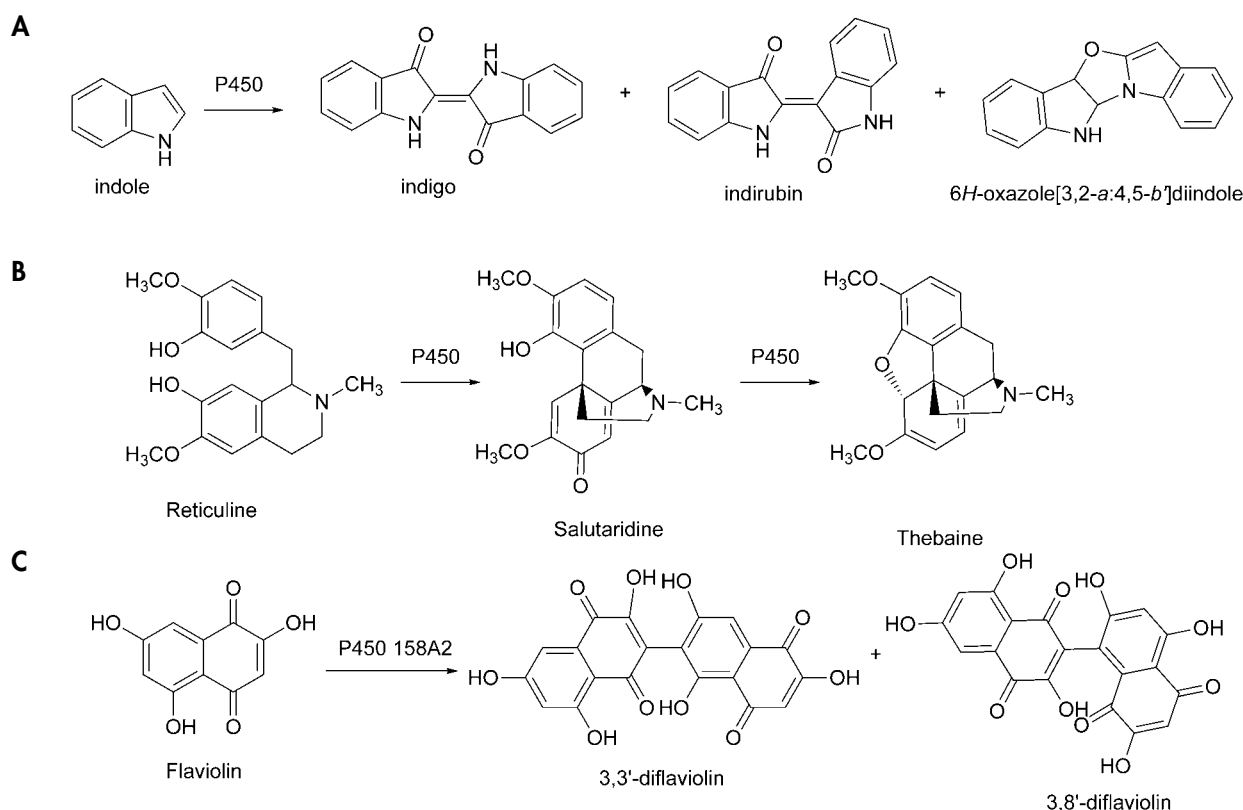
Several arguments against 1-electron transfer can be discounted, including the lack of rearrangement of some radical clock probes (*vide supra*) and some of the kinetic isotope work,<sup>75</sup> which was not reproduced.<sup>76</sup> The conclu-

sions about 1-electron transfer do not, however, rule out the possibility of hydrogen atom transfer or other oxidation modes when the reaction geometry and electronics are appropriate with heteroatoms etc. in the active site.<sup>76</sup>

**Events Occurring after Product Formation (Step 9)** – In general the products of P450 reactions are slightly more polar than the substrates, and their behavior is similar to that of substrates. Dissociation constants and on and off rates are similar to those measured for substrates.<sup>6</sup> In many cases, the products are substrates in that multiple oxidations occur.

When a step following product formation is rate-limiting, kinetic analysis is very characteristic with a first-order “burst” of product formation occurring for the first cycle of the reaction, followed by a slower, linear, steady-state reaction.<sup>77</sup> This behavior has been observed for the oxidation of ethanol by human P450 2E1.<sup>78,79</sup> In this kinetic situation,  $K_m \ll K_d$ .<sup>80–82</sup> Presumably the slow step is a conformational change (following product formation) and not product release *per se*, in that the product acetaldehyde (and acetic acid) has low affinity for P450 2E1 and therefore a high off rate.<sup>79</sup>

Some steroid-oxidizing P450s normally catalyze three sequential oxidations (mammalian P450s 11A1, 19A1, and 51A1) (Fig. 2). The intermediate products can be seen



**Fig. 3.** Coupling reactions catalyzed by P450s. **A**, Intermolecular coupling products from P450 2A6-catalyzed oxidation of indole. **B**, Intramolecular coupling reactions by P450s observed in mammalian morphine biosynthesis. **C**, Dimerization of flaviolin by P450 158A2, most likely taking place in the active site of the enzyme.

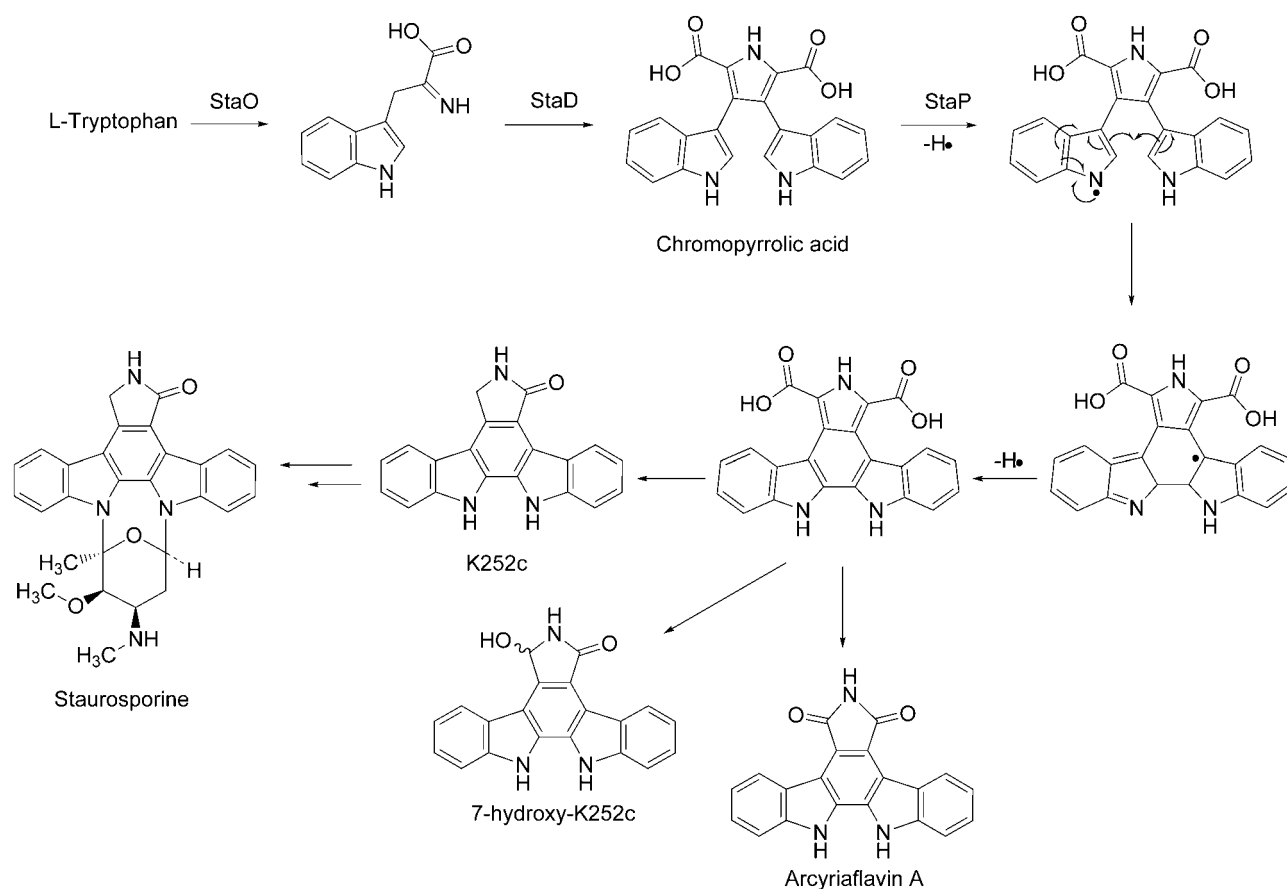


Fig. 4. Inter- and intra-molecular coupling reactions in staurosporine biosynthesis.

in some *in vitro* situations but exactly how fast these products diffuse from the enzymes has not been studied in detail. In the P450 2E1-catalyzed oxidation of ethanol, only trace acetaldehyde appears to leave the enzyme and most proceeds directly to acetic acid.<sup>79</sup>

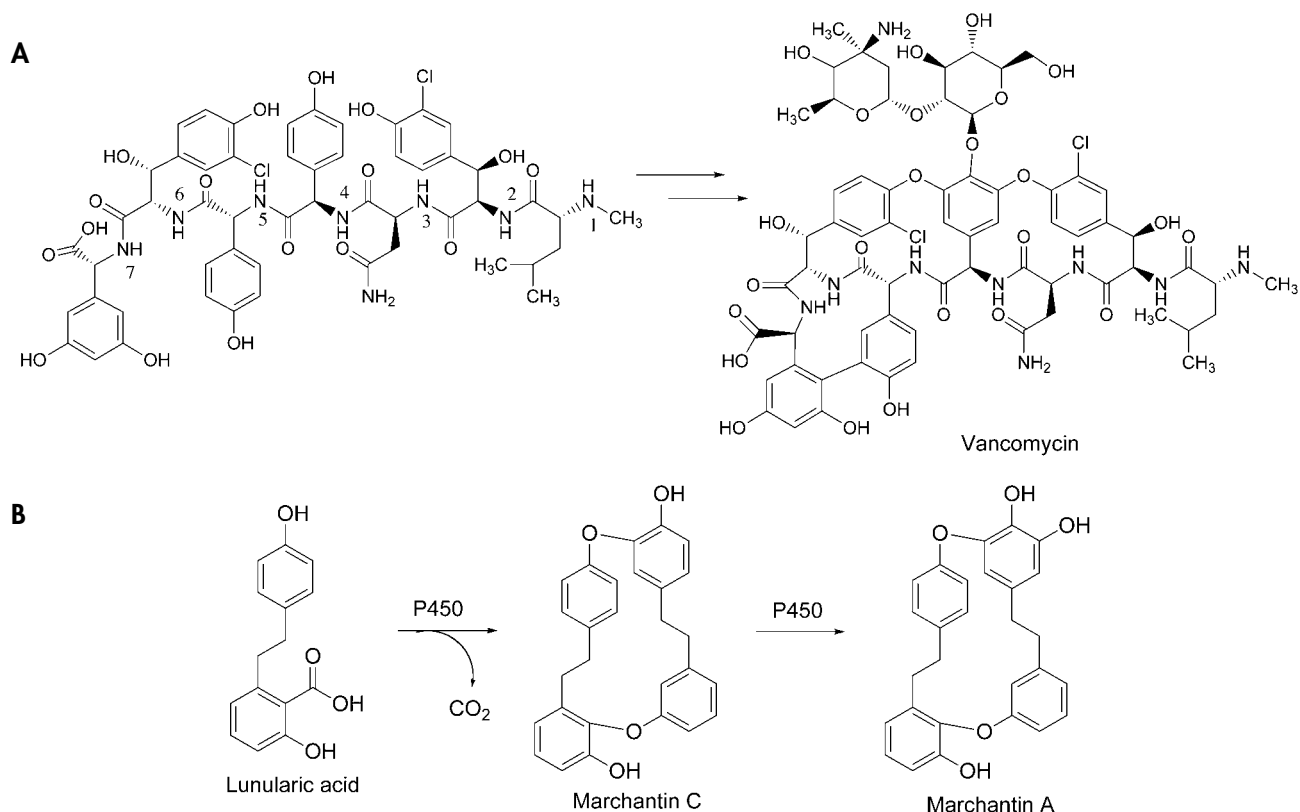
#### 4. Variations on the Theme: Unusual P450 Reactions

Despite a common mechanism of catalysis, some P450-mediated reactions may lead to unexpected products. Such “unusual” reactions have been documented in the literature for both mammalian and plant P450s. In some cases, unexpected products are believed to form in the active site of the P450 enzyme involved in the transformation, whereas in others the spontaneous chemical reactions taking outside of the enzyme (following catalysis) are responsible for the formation of such products. Uncommon P450 reactions have been reviewed in detail elsewhere<sup>83,84</sup> and only a few representative examples will be summarized below under the headings of coupling reactions, reductions, and rearrangements.

**Coupling Reactions** – Inter- as well as intra-molecular

coupling of the substrate molecules may lead to unexpected products. One of the earlier examples of a coupling reaction catalyzed by a P450 comes from this laboratory, with P450 2A6-catalyzed oxidation of indole leading to dimerized products of oxidized indole including the dyes indigo (blue), indirubin (red), and 6*H*-oxazolo[3,2-*a*:4,5-*b'*]diindole (Fig. 3A).<sup>85,86</sup> A proposed mechanism for this transformation (starting from tryptophan) was given in detail previously.<sup>83,85</sup> Another example is the oxidative intramolecular coupling of (*R*)-reticuline to form salutaridin, followed by a further cyclization to form thebaine (Fig. 3B),<sup>87</sup> as key steps in morphine biosynthesis by mammals, although the P450s involved in this transformation have not been identified.<sup>88,89</sup> A more recent example is flaviolin dimer formation catalyzed by P450 158A2 from *Streptomyces coelicolor* (Fig. 3C), most likely taking place in the active site of the enzyme, which was shown by x-ray crystal structural studies to accommodate two flaviolin molecules.<sup>90</sup>

P450 enzymes are reported to be involved in the biosynthesis of the indolocarbazole core of the antibiotic staurosporine, isolated from *Streptomyces* sp. TP-A0274,<sup>91,92</sup> which displays broad spectrum antitumor activity via the inhibition of protein kinases.<sup>93</sup> Following the forma-



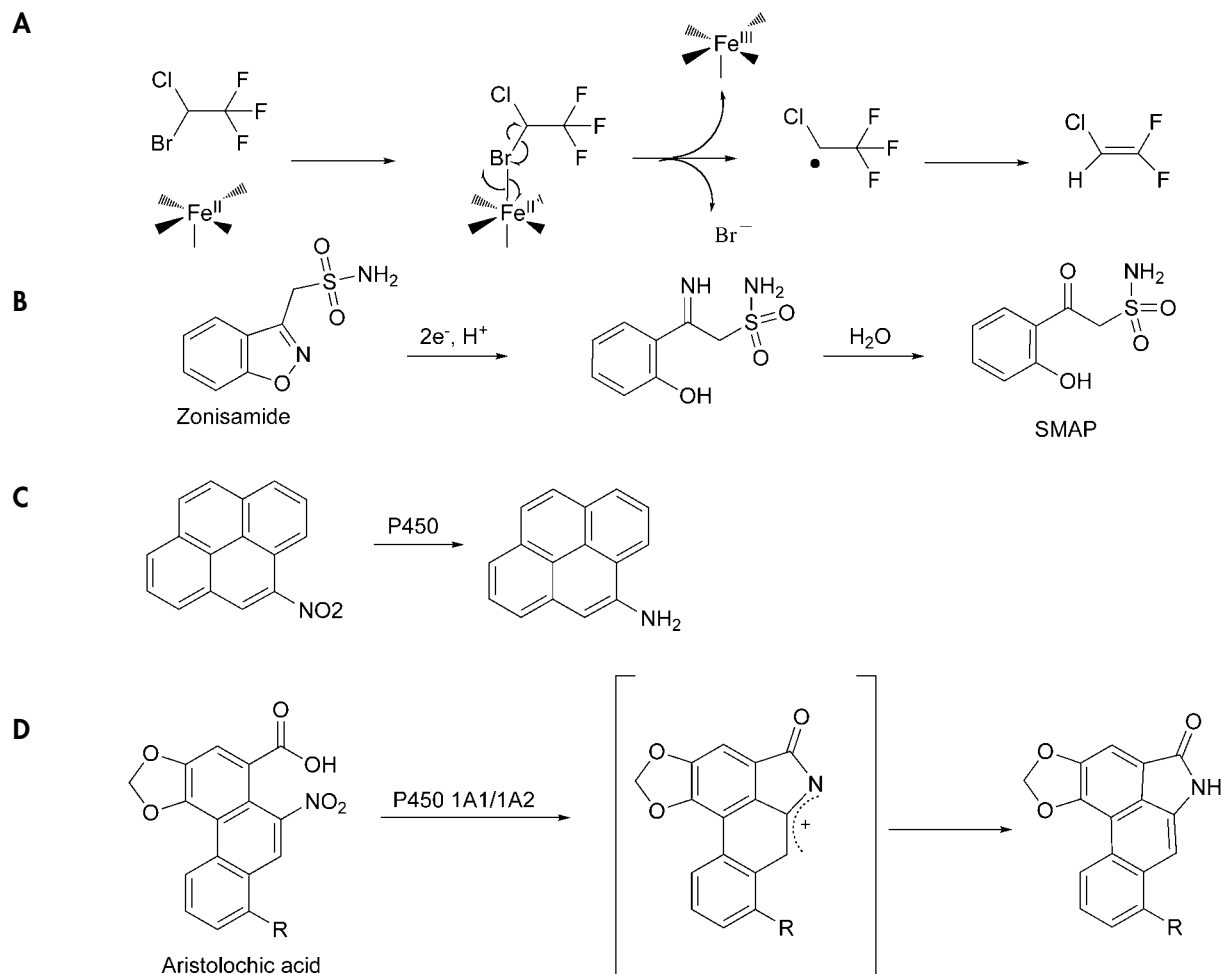
**Fig. 5.** Oxidative phenol coupling reactions catalyzed by plant P450s. **A**, Three sequential intramolecular oxidative phenol coupling reactions catalyzed by P450s 165B1, 165A1, and 165C1 result in the formation of vancomycin. **B**, Intermolecular phenol coupling of lunularic acid to give marchantin C, which is further oxidized to marchantin A.

tion of chromopyrrolic acid from indole-3-pyruvic acid imine, StaP (P450 245A1) catalyzes the aryl-aryl coupling reaction leading to three different indolocarbazole compounds: K252c, arcyliaflavin A, and 7-hydroxy-K252c (Fig. 4).<sup>94</sup> Because P450 245A1 does not catalyze monooxygenation reactions<sup>94</sup> and on the basis of a recent crystal structure,<sup>95</sup> it was proposed that the Compound I complex of P450 245A1 removes an electron from the substrate to form an indole radical cation and a ferryl-oxo heme species<sup>95</sup> in a similar fashion to cytochrome *c* peroxidase.<sup>96</sup> Deprotonation of the indole radical cation results in the indole radical shown in Fig. 4.

Another oxidative coupling catalyzed by P450s was reported for the biosynthesis of vancomycin, a glycopeptide antibiotic (Fig. 5A).<sup>97</sup> Based on the gene knock-out experiments carried out for balhimycin biosynthesis,<sup>98</sup> the coupling steps were shown to take place in a sequence catalyzed in order by OxyB (P450 165B1),<sup>99</sup> OxyA (P450 165A1), and OxyC (P450 165C1),<sup>100</sup> with first coupling being between the phenol rings of residues 4 and 6, followed by the coupling between the phenol rings of residues 2 and 4, and finally between the residues 5 and 7.<sup>101</sup> It was demonstrated that a P450 enzyme (marchantin C synthase) is involved in the formation of marchantin

C,<sup>102</sup> a cyclic bi(bibenzyl) isolated from the liverwort *Marchantia polymorpha* L.<sup>103</sup> The intermolecular phenol coupling of lunularic acid is catalyzed by P450 to form marchantin C (Fig. 5B).<sup>102</sup>

**Reductions** – P450s have been shown to catalyze reductions of certain substrates<sup>104,105</sup> in addition to their main function of catalyzing oxidative biotransformation reactions. Reductions of alkyl halides by P450s are proposed to take place as shown in Fig. 6A, where homolytic cleavage of carbon-halogen bonds takes place following the binding of substrate to the reduced heme iron in a “Type II” fashion. This proposal is supported by the documented formation of halides upon reduction of aryl halides.<sup>106</sup> The resulting alkyl radicals may react with cellular macromolecules as well as other radicals, including O<sub>2</sub>. The reductive dehalogenation of alkyl halides also lead to the formation of olefins and reduced haloalkanes. P450-catalyzed reduction of the anticonvulsant drug zonisamide results in the formation of the ring-opened metabolite 2-sulfamoylacetyl phenol via the hydrolysis of the ketimine intermediate (Fig. 6B).<sup>107,108</sup> Further studies suggested that P450 3A4 is the (mammalian) enzyme involved in the reductive ring-cleavage of zonisamide.<sup>109,110</sup> Reduction of a nitro group by P450s



**Fig. 6.** Examples of reduction reactions catalyzed by P450s. *A*, Reductive dehalogenation of haloethane via "Type II" binding to reduced heme iron. *B*, Reductive ring cleavage of the anticonvulsant drug zonisamide by P450. *C*, Reduction of 4-nitropyrene to 4-aminopyrene. *D*, Reductive cyclization of two aristolochic acid components via the formation of a nitrenium intermediate.

has been well documented, with reduction of 4-nitropyrene to 4-aminopyrene being a classic example (Fig. 6C).<sup>111</sup> P450s 1A1 and 1A2 were reported to be involved in the reductive cyclization of the two major components of the aristolochic acid (Fig. 6D, R = H or CH<sub>3</sub>). The intermediacy of the cyclic nitrenium ion has been demonstrated via the isolation of corresponding DNA adducts.<sup>112</sup>

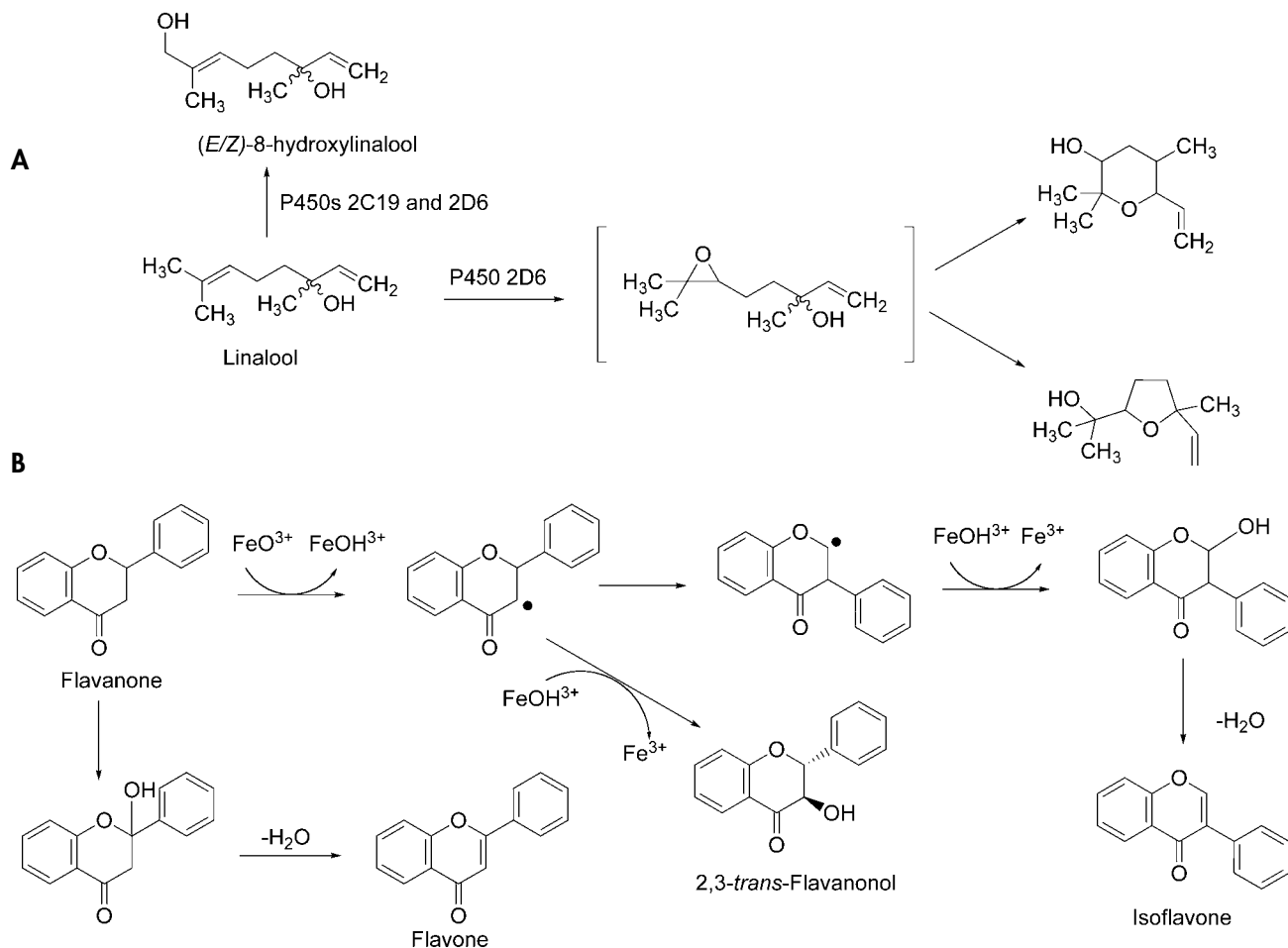
**Rearrangements** – Following the initial oxidation of a substrate molecule by P450, a rearrangement reaction may take place resulting in the formation of an unexpected product. In some cases, a rearrangement reaction is proposed to take place following formation of a free radical from the substrate via hydrogen atom abstraction by P450. In other cases, the product of the P450 oxidation is a reactive product and undergoes a spontaneous chemical rearrangement reaction.

In a recent study it was shown that the oxidation of linalool, a commonly used fragrance ingredient causing

skin sensitization,<sup>113</sup> is catalyzed by the P450s 2D6 and 2C19, both known to be expressed in the skin (Fig. 7A).<sup>114,115</sup> In addition to 8-hydroxylation, P450 2D6 is proposed to catalyze the 6,7'-epoxidation, and rearrangement yields the pyranoid and furanoid derivatives shown in Fig. 7A.<sup>116</sup>

Rearrangement of flavanone, involving a 1,2-aryl migration, is an important step in isoflavonoid biosynthesis.<sup>117</sup> Incorporation studies using <sup>18</sup>O<sub>2</sub> have shown this rearrangement to be catalyzed by P450 93C (from *Pueraria lobata*).<sup>118</sup> A similar biotransformation of flavanone was demonstrated to be catalyzed by mammalian P450s 1A1 and 2B6.<sup>119</sup> On the basis of results of kinetic isotope effect experiments, the authors proposed a pathway for the formation of isoflavone from flavanone similar to that observed in the case of plant P450s (Fig. 7B).<sup>119</sup> Flavone and 2,3-*trans*-flavanonol were reported to be the other two products resulting from P450-catalyzed oxidation of flavanone. Rearrangement of littorine in the biosynthesis of





**Fig. 7.** Rearrangement reactions initiated by P450s. **A**, Epoxidation of linalool by P450 2D6 followed by spontaneous rearrangement to give cyclic ethers. **B**, Rearrangement of flavanone to isoflavone following hydrogen atom abstraction by P450.

the tropane alkaloid hyoscyamine<sup>120</sup> is also proposed to involve a free radical intermediate (Fig. 8). Hydrogen abstraction by P450 followed by the rearrangement of the resulting radical and rebound step yields the geminal diol, dehydration of which gives hyoscyamine aldehyde (Fig. 8). Functional genomic approaches revealed P450 80F1 (from *Hyoscyamus niger*) to be the enzyme responsible for this transformation<sup>121</sup> in agreement with earlier reports, suggesting the involvement of a P450 in this rearrangement reaction.<sup>122</sup>

## 5. Conclusions

The P450s catalyze a number of different reactions using a basic chemical template. The events in Fig. 1 are generally applicable to most of the oxidative reactions, although the point should be made again that the events do not necessarily have to proceed in a linear order.<sup>6</sup>

Exactly why rates of individual P450 reactions vary so much is still not clear. At first examination, the fit of the substrate in the active site of P450 2A6 is relatively tight<sup>10</sup> and not that dissimilar from bacterial P450 101A1,<sup>123</sup> although the rates vary ~ 100-fold. In many cases high intermolecular non-competitive kinetic hydrogen isotope effects (H/D, H/T) are expressed<sup>6,28</sup> but not others.<sup>124</sup> These and other studies indicate that the rate-limiting steps in P450 reactions vary.<sup>125</sup> Some of the rate-limiting steps identified to date, in various systems, include (Fig. 1) steps 2,<sup>7,126</sup> 4<sup>36,127</sup> (based on the cytochrome *b<sub>5</sub>* effects), 7<sup>28,128</sup> (based on the kinetic hydrogen isotope effects, i.e. H/D, H/T), and 9<sup>78,79</sup> (see discussion regarding P450 2E1 oxidation of ethanol, *vide supra*). One issue is probably the extent of non-productive modes of substrate binding, which may not be obvious in all P450-ligand crystal structures. In summary, although much is now known about the chemical mechanisms of P450 catalysis, more needs to be learned about several of the major steps and the reasons why rates vary.

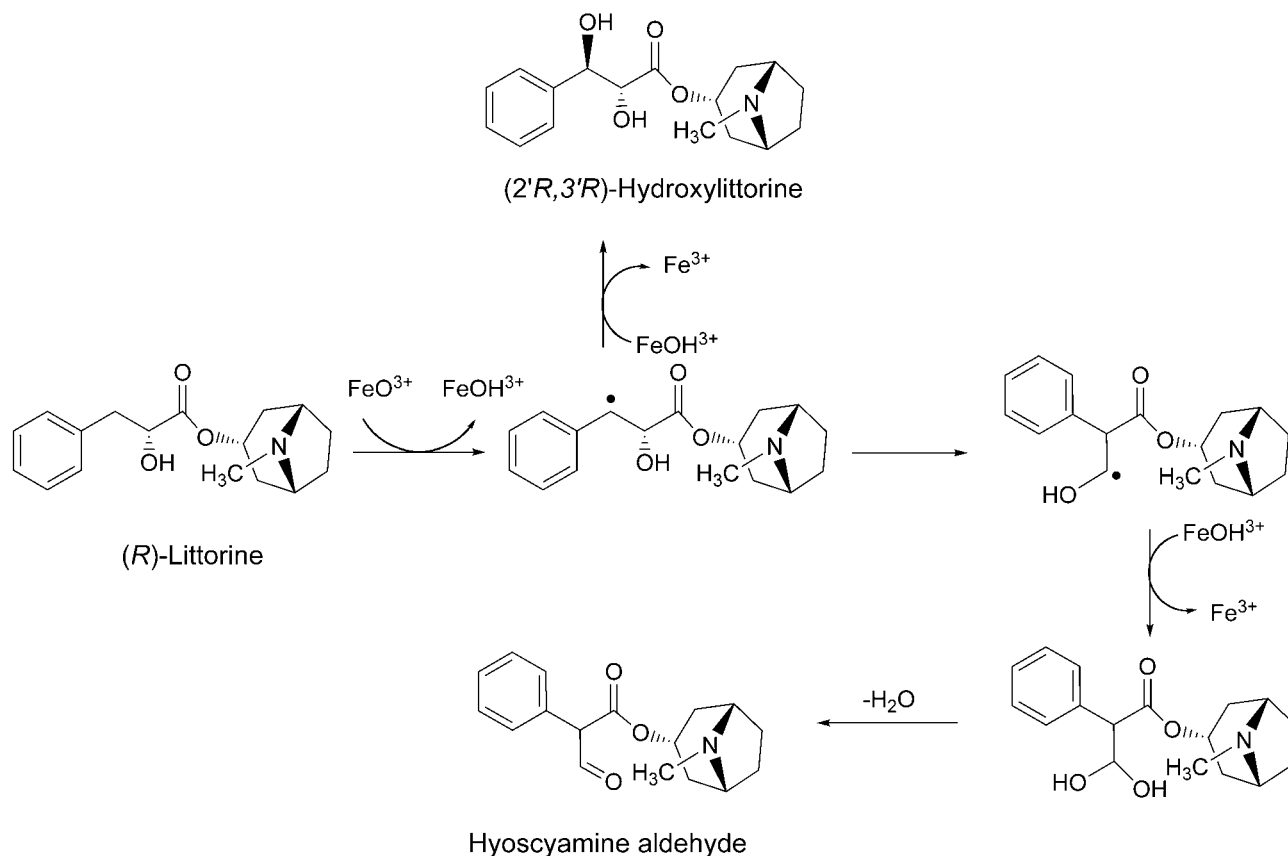


Fig. 8. Rearrangement of littorine via a free radical intermediate to give hyoscyamine aldehyde as the final product.

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## Povzetek

Encimi naddružine citokromov P450 katalizirajo veliko različnih reakcij, ki pa imajo enako kemijsko osnovo. Najprej pride do vezave substrata, sledi prva elektronska redukcija, vezava O<sub>2</sub>, druga elektronska redukcija, nato pa serija slabše definiranih stopenj, kot so protonacija, hemolitična cepitev vezi O–O, ki vodi do nastanka aktivnega perferilnega intermediate FeO (označenega kot FeO<sup>3+</sup>), reakcija s substratom in sprostitvev produkta. Poudariti velja, da se zaporedje nekaterih stopenj lahko spreminja. Kemijski dogodki so takšni, kot v večini oksidacijskih reakcij, kljub vsemu pa ni popolnoma jasno, zakaj so hitrosti posameznih P450 reakcij različne. Osnovne P450 reakcije vključujejo hidroksilacijo C atoma, oksigenacijo heteroatoma, sprostitvev heteroatoma (dealkilacija), tvorbo epoksida in 1,2-premestitev. Citokromi P450 pri katalizi uporabljajo tudi variacije osnovne kemije, kar privede do celega spektra nenavadnih reakcij, kot so redukcije, izomerizacije in oksidativne sklopitve.