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Enzyme Catalysis

Direct Observation of an Oxepin from a Bacterial Cytochrome P450-Catalyzed Oxidation

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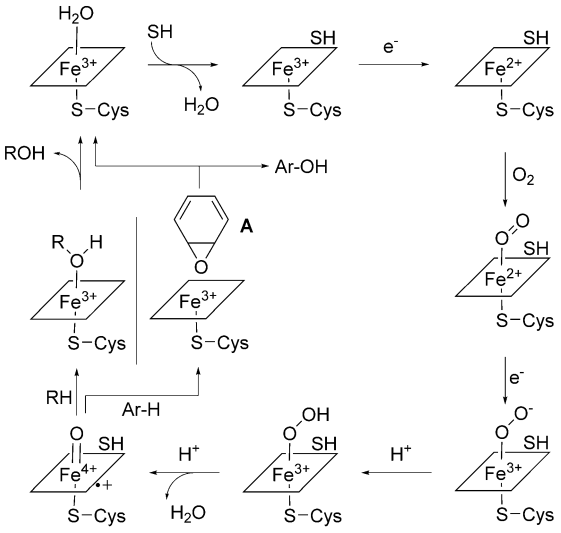
In memory of John (Kappa) Cornforth

Abstract: The cytochromes P450 are hemoproteins that catalyze a range of oxidative C–H functionalization reactions, including aliphatic and aromatic hydroxylation. These transformations are important in a range of biological contexts, including biosynthesis and xenobiotic biodegradation. Much work has been carried out on the mechanism of aliphatic hydroxylation, implicating hydrogen atom abstraction, but aromatic hydroxylation is postulated to proceed differently. One mechanism invokes as the key intermediate an arene oxide (and/or its oxepin tautomer). Conclusive isolation of this intermediate has remained elusive and, currently, direct formation of phenols from a Meisenheimer intermediate is believed to be favored. We report here the identification of a P450 [$P450_{cam}$ (CYP101A1) and $P450_{cin}$ (CYP176A1)]-generated arene oxide as a product of in vitro oxidation of *tert*-butylbenzene. Computations (CBS-QB3) predict that the arene oxide and oxepin have similar stabilities to other arene oxides/oxepins implicated (but not detected) in P450-mediated transformations, suggesting that arene oxides can be unstable terminal products of P450-catalyzed aromatic oxidation that can explain the origin of some observed metabolites.

The cytochromes P450 (P450s) comprise a superfamily of hemoproteins with over 11,000 members, which catalyze an array of oxidative transformations.[1] Found in most kingdoms of life, they play integral roles in the biosynthesis of crucial cellular components, for example, sterols, secondary metabolites, and in the biodegradation of xenobiotics for cellular protection and energy generation.[2] Archetypically, they activate molecular oxygen with two electrons derived ultimately from a nicotinamide cofactor, and insert an oxygen atom into their substrate through a mechanism that involves a complex catalytic cycle (Scheme 1).

The prototypical P450-catalyzed hydroxylation of alkanes involves initial H atom abstraction by a highly reactive iron-oxo porphyrin cation radical[3,4] followed by recombination of an intermediate carbon-centered radical with the iron-tethered hydroxyl. This gives rise to the hydroxylated product that is then released from the enzyme (Scheme 1).[3a,5] P450-mediated hydroxylation of aromatic substrates is an intriguing and important reaction in both biosynthetic and drug metabolism contexts, but is still not fully understood. Direct H atom abstraction is much less likely with an aromatic substrate than an aliphatic one, due to the greater strength of aromatic C–H bonds. Moreover, aromatic hydroxylation is accompanied by the so-called 'NIH shift', a rearrangement observed in isotopically labelled substrates in which a deuterium originally present at the site of oxidation moves to an adjacent carbon.[6] The formation of arene oxides (**A**, Scheme 1) as obligate intermediates in P450-catalyzed oxidation of an arene (e.g., **1**, Scheme 2) has been invoked to explain both the NIH shift and the extensive array of subsequent transformations that are seen coupled to aromatic oxidation (e.g., production of diols and glutathione



Scheme 1. The mechanism of P450-catalyzed oxidation of substrates (SH), which may be either aliphatic (R–H) or aromatic (Ar–H). Hydroxylation of the latter has been proposed to proceed via an intermediate arene oxide **A**.

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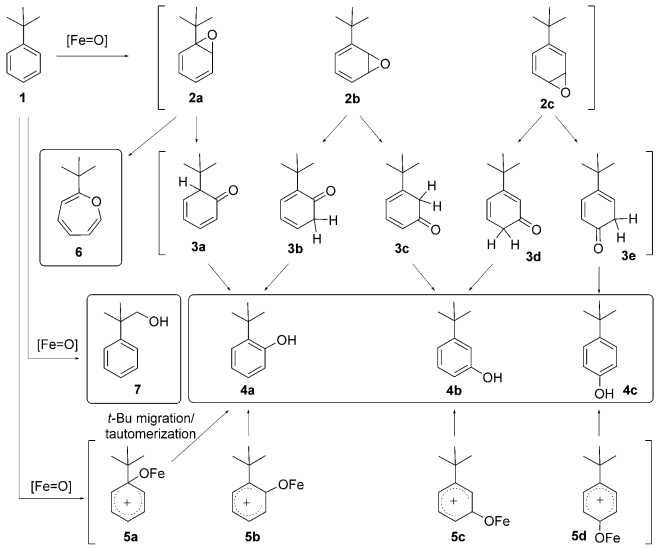


Scheme 2. Possible pathways for P450-catalyzed oxidation of *tert*-butyl benzene (**1**).

adducts (in drug metabolism)[7] and the dearomatization of substituted benzenes).[8] In addition, cyclizations in the biosynthesis of a variety of secondary metabolites have also been rationalized via the formation of arene oxides.[9] Conversion of an arene oxide to the corresponding phenol via a ketone (**2** to **4**, Scheme 2) would be subject to a significant isotope effect in the enolization step, thus explaining the deuterium retention observed in the classical NIH shift.[6,10]

Considerable effort has been expended to identify the proposed arene oxide intermediates involved in P450-catalyzed aromatic oxidations and minor amounts have been identified from oxidation of isolated aromatic systems by in vivo systems. Benzene oxide itself was detected in the blood of rats after administration of benzene. Arene oxides were also detected after catalytic oxidation by liver microsomes of benzene or chlorobenzene; the latter using radioisotope dilution assays.[11] Whilst these results supported the hypothesis that arene oxides were intermediates, kinetic isotope effects found in the hydroxylation of chlorobenzene[12] and the regiochemistry of fluorobenzene oxidation, suggested other mechanisms.[13] Recent computational studies provided evidence that P450-catalyzed aromatic oxidations do not involve arene oxide intermediates (**A**, Scheme 1),[12–14] but rather that aromatic hydroxylation occurs instead via a Meisenheimer tetrahedral intermediate (**5**, Scheme 2). This directly introduces the hydroxyl group onto the aromatic ring[14a,15] and a 1,2-hydride shift then yields the ketone **3**, which tautomerizes to the phenol. This process proceeds with the required isotope effect to yield the deuterium retention of the NIH shift. In this scenario, arene oxides—if they are formed at all—would be terminal side products and would only be converted to phenols through a non-enzymatic, acid-catalyzed reaction.[14a,c,d]

Chemically, arene oxides exist in a valence tautomeric equilibrium with the corresponding, less-reactive oxepins (e.g., **6**).[16] This valence-tautomeric equilibrium is influenced by both the substituents on the aromatic ring as well as the solvent and temperature.[6,10] The formation of arene oxides or oxepins has never been directly observed with a purified, reconstituted P450 system in vitro. In this study, we report the first direct observation of an oxepin (**6**) as a product of a P450-catalyzed oxidation and this, somewhat counter-intuitively, provides evidence that it is not an intermediate but a somewhat unstable product in aromatic oxidations.

Our investigations focused on the catalytic oxidation of the C_{10} hydrocarbon, *tert*-butylbenzene (**1**, Scheme 2) by two P450s [$P450_{cam}$ (CYP101 A1) and $P450_{cin}$ (CYP176 A1)]. The native substrates of these enzymes are the similarly sized C_{10} monoterpenes (1*R*)-(+)-camphor and 1,8-cineole, respectively.[2a,18] In principle, P450-catalyzed hydroxylation of **1** could yield any of the species depicted in Scheme 2. The oxidations of **1** by $P450_{cam}$ and $P450_{cin}$ were observed to give the expected four products, alcohol **7** and phenols **4a–c** in different ratios (Table 1). The 3- and 4-*tert*-butyl phenols **4b** and **4c** were found to co-elute under the coupled GC-MS conditions employed (Figure 1), but could be separated after acetylation, allowing determination of the product distribution. Broadly, the physical parameters for the interaction of **1** with $P450_{cam}$ and $P450_{cin}$ are similar. However, the differences observed (see Table 1) are consistent with varying ratios of **4** and **7** produced when these P450s catalyze the oxidation of **1**. Though the dissociation constants (K_d) of $P450_{cam}$ and $P450_{cin}$ with **1** were similar, $P450_{cin}$ showed both a greater change in spin state [low spin $Fe^{III}{\cdot}H_2O$ (417 nm) to high spin Fe^{III} (392 nm)] upon substrate binding, usually indicative of substrate binding closer to the heme iron. $P450_{cin}$ also exhibited a higher rate of NAD(P)H (nicotinamide cofactor) consumption in the presence of **1** but $P450_{cam}$ was found to be more efficient at coupling NAD(P)H consumption to substrate oxidation. The interaction of **1** with $P450_{cam}$ has been investigated previously[17] and the results reported are in good qualitative agreement with those observed here (Table 1).

Table 1. Characterization and analysis of oxidation products of *tert*-butyl benzene (**1**) by cytochromes $P450_{cam}$ and $P450_{cin}$.

	$P450_{cam}$[c]	$P450_{cin}$[d]
K_d [μM]	639 ± 71	542 ± 87
High spin [%][a]	48 ± 1	90 ± 1
Rate of NAD(P)H consumption [%][a]	28[17]	106 ± 4
[$\mu M\,min^{-1}\,\mu M^{-1}$ P450]	89[17]	258 ± 9
Coupling [%][b]	33[17]	14 ± 1
Products [%]		
Alkyl hydroxylation **7**	73 (51)[c]	2 (2)[d]
Aromatic hydroxylation -*ortho* **4a**	11 (22)[c]	47 (40)[d]
Aromatic hydroxylation -*meta* **4b**	10 (18)[c]	44 (48)[d]
Aromatic hydroxylation -*para* **4c**	5 (9)[c]	5 (6)[d]
Oxepin formation **6**	1 (–)[c]	2 (4)[d]

[a] The percentage spin state and rate of NADH ($P450_{cam}$)/NADPH ($P450_{cin}$) consumption were standardized against camphor and cineole with the respective P450, assuming $P450_{cam}$:camphor and $P450_{cin}$:cineole was 100%. [b] The ratio of the amount of NAD(P)H consumed to the amount of product formed. [c] Numbers in parentheses are previously reported results.[17] [d] Numbers in parentheses indicate percentage of product at 6 min.

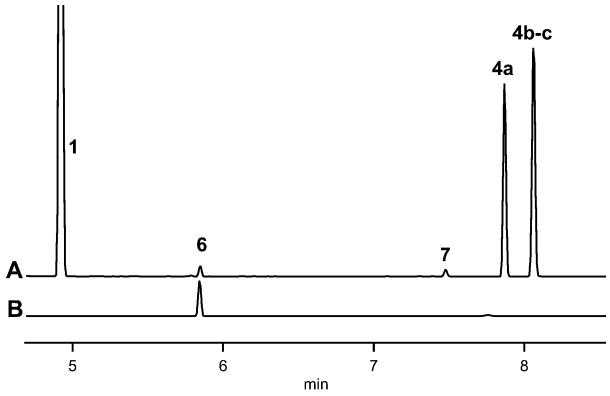


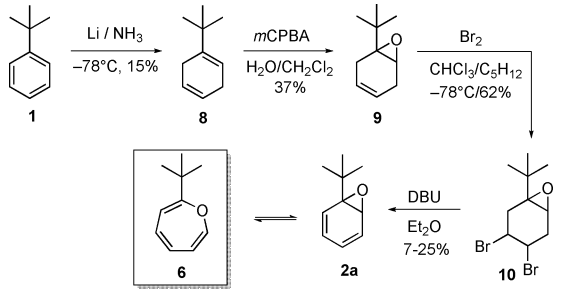
Figure 1. A) GCMS chromatogram of the products from oxidation of *tert*-butyl benzene **1** catalyzed by $P450_{cin}$; B) synthetic oxepin **6** as the standard.

GCMS analysis of the products of $P450_{cin}$ catalyzed oxidation of **1** revealed the presence of a fifth, unexpected product, comprising up to approximately 4% of the total metabolites formed. This compound eluted much earlier during GCMS analysis than either alcohol **7** or phenols **4a–c** (R_t 5.8 min, Figure 1A). Interestingly, however, the observed molecular ion (*m/z* 150) was consistent with incorporation of a single oxygen atom into the substrate (c.f. **1**, $M^{+\cdot}$ *m/z* 134), strongly supporting the catalytic production of oxepin **6**/benzene oxide **2a** during oxidation of **1**.

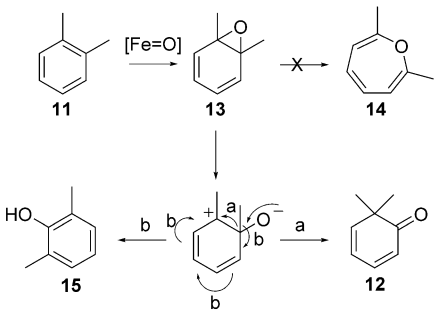
Thus, an authentic sample of oxepin **6** was prepared (Scheme 3) using the generalized synthetic procedure reported by Vogel.[16a] Birch reduction of *tert*-butyl benzene (**1**) gave 1-*tert*-butyl-1,4-cyclohexadiene (**8**) as reported.[19] Subsequent oxidation with *meta*-chloroperbenzoic acid (*m*CPBA) at the more reactive double bond afforded the regioisomerically pure epoxide **9**, which was reacted with bromine to give **10** as a mixture of two chromatographically inseparable diastereomers. Finally, treatment of dibromide **10** with 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) afforded the volatile target oxepin **6** with expected spectroscopic properties. The ^{1}H NMR spectrum of **6** in $CDCl_3$ revealed that the equilibrium between **6** and **2a**, which is expected to be fast on the NMR timescale, lies predominantly towards the oxepin. The diagnostic signal is that for the proton α to the oxygen, which would be expected to occur at approximately 4 ppm in arene oxide **2a** and at 6 ppm in oxepin **6**; the experimental value (reflecting a concentration-weighted average of the two tautomers) is 5.70 ppm.

Co-injection of synthetic oxepin **6** with the products from the $P450_{cin}$-catalyzed oxidation of **1** confirmed that the additional product was indeed **6**. To our knowledge, this constitutes the first reported observation of significant quantities of an oxepin arising from a P450-catalyzed oxidation of a simple aromatic compound in vitro. A time course experiment (Supporting Information, Supplementary Figure 1) revealed that all products, including **6**, increased linearly for the first six minutes of the reaction after which time the reaction is complete. Products **4a–c** and **7** then remained unchanged whilst **6** slowly decomposed ($t_{1/2}$ approximately 13 min). This behavior is consistent with **6** being an (unstable) product formed concomitantly with **4a–c** and **7** and inconsistent with it being an intermediate. However, despite its apparent stability under assay conditions, oxepin **6** was not previously reported among the products of oxidation of **1** catalyzed by $P450_{cam}$.[17] Upon careful examination of the $P450_{cam}$ catalyzed oxidation of **1**, we found that a small amount of oxepin **6** was indeed formed, constituting approximately 1% of the total oxidized product after 30 min. Given that neither of the P450s employed are specific for **1**, this suggests that production of **6** is a function of the substrate structure and not a specific enzyme effect. Thus, we sought examples of other aromatic oxidations in which a benzene oxide has been proposed as a key intermediate.

In work on the oxidation, and particularly the dearomatization of *ortho*-xylene (**11**) by $P450_{BM-3}$ (Scheme 4), Wong and co-workers reported the isolation of 6,6-dimethylcyclohexa-2,4-dienone (**12**) and 2,6-dimethylphenol (**15**) which were suggested to arise from rearrangement of benzene oxide intermediate **13**. It is known from experimental studies that the tautomeric equilibrium between *ortho*-xylene 1,2-oxide (**13**) and 2,7-dimethyloxepin (**14**) lies overwhelmingly toward the oxepin.[16a] However, no oxepin formation was detected in the $P450_{BM-3}$-catalyzed reaction. Wong et al. proposed that it did not form because the ring opening of **13** to give a zwitterionic intermediate was faster than valence tautomerization to give **14**.[8] In order to determine if this might be due to fundamental chemical differences between the **2a**/**6** and **13**/**14** pairs, we repeated the $P450_{BM-3}$ catalyzed oxidation of **11** with sampling times and analysis methods identical to that used for $P450_{cin}$ catalyzed oxidation of **1**. The product distributions were identi-



Scheme 3. The synthetic route utilized to access 2-*tert*-butyloxepin (**6**). *m*CPBA = *meta*-chloroperbenzoic acid; DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene.



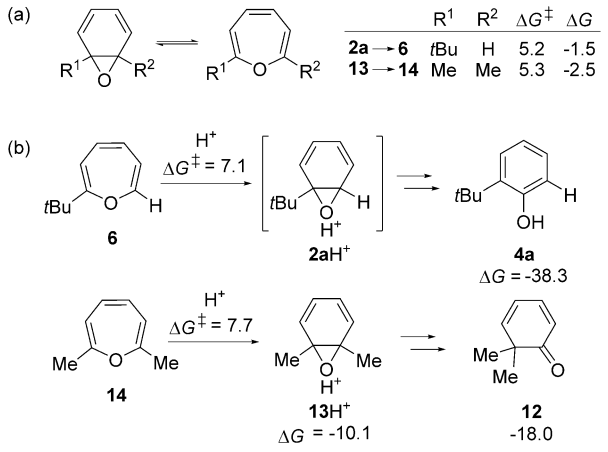
Scheme 4. Mechanism proposed by Wong et al. for the $P450_{BM-3}$ catalyzed oxidation of *ortho*-xylene (**11**) to 6,6-dimethylcyclohexa-2,4-dienone (**12**).[8]

cal to those previously reported,[8] within experimental error, and showed no trace of **14**.

One of the primary difficulties in identifying arene oxides as intermediates is their rapid rearrangement to more stable compounds (e.g., phenols) under physiological conditions.[6] If arene oxides are produced in the oxidations of both **1** and **11**, then the failure to detect oxepin **14** could in principle be due to either a much slower valence bond isomerisation of arene oxide **13** to **14**, as proposed by Wong et al.,[8] or to a lower stability of oxepin **14** under the experimental conditions. We thus investigated the differences between oxepins **6** and **14** experimentally and computationally. An authentic, synthetic sample of **14** was prepared using Vogel's reported route.[16a,20,21]

High-accuracy CBS-QB3 calculations (see the Supporting Information) were performed to evaluate the stabilities of oxepins **6** and **14** (Scheme 5).[22] The isomerization of *tert*-butyl benzene oxide **2a** to oxepin **6**, and of dimethyl benzene oxide **13** to oxepin **14** (Scheme 5a), are both computed to have small activation barriers ($\Delta G^{\ddagger} \approx 5$ kcal mol^{-1}), indicating that equilibrium will be rapidly attained for both pairs. The values of ΔG (-1.5 and -2.5 kcal mol^{-1}, respectively) predict that in both cases the oxepin will be the dominant species at equilibrium and that, in fact, the arene oxide/oxepin equilibrium for **13**/**14** lies more strongly towards the oxepin than that of **2a**/**6** (although the equilibrium position in polar solvents may contain somewhat more benzene oxide).[16a] These computational data agree well with our experimental observations, where NMR and GCMS analysis suggested that both **6** and **14** were stable to rearrangement at room temperature in organic solvents and at high temperature in the gas phase and that the equilibrium between **6** and **2a** lay largely toward **6**. Vogel had previously reported that the **13**/**14** equilibrium similarly strongly favored **14** even in polar solvents.[16a]

Furthermore, calculations predict that, under acidic conditions, the conversion of both oxepin **6** to phenol **4a**, and of oxepin **14** to cyclohexadienone **12** (Scheme 5b), are strongly exergonic reactions, with low barriers (7–8 kcal mol^{-1}). Computations suggest that, in the absence of acid, the ring opening



Scheme 5. a) Computed activation barriers and energetics (kcal mol^{-1}) of a ring opening of benzene oxides to oxepins, and b) conversion of oxepin **6** to phenol **4a** and of oxepin **14** to cyclohexadienone **12** under acidic conditions, calculated with CBS-QB3.

of benzene oxide **13** by C–O bond cleavage is very unfavorable. A pathway for C–O bond cleavage to give the zwitterionic intermediate proposed by Wong et al. (Scheme 4) could not be located on the potential energy surface, and only became feasible when the oxygen was protonated. Thus we believe the mechanism of decomposition of both oxepins involves conversion to the protonated benzene oxide, followed by C–O bond cleavage (and further 1,2 alkyl shifts in the case of **14**). (Supporting Information, Scheme S1). Surprisingly, however, experiments with **6** and synthetic **14**, revealed significant differences in the stabilities of the two oxepins towards decomposition. Whereas *tert*-butyloxepin **6** decomposed with a $t_{1/2}$ of 13 min in aqueous buffer (pH 7.4 50 mM Tris), the decomposition of dimethyloxepin **14** under similar conditions (or even in unbuffered water) was complete in <1 min by GCMS analysis, giving a mixture of cyclohexadienone **12** and 2,6-dimethylphenol (**15**). The ratio of these two products was essentially the same as observed in the enzyme-mediated dearomatization of **11**. In contrast, **14** did not decompose noticeably in a less-polar solution (ethyl acetate, methanol) at room temperature over a period of hours, or even in ethyl acetate/acetic acid solution. The reason for the more rapid decomposition of **14** (compared to **6**) in aqueous solution is not immediately obvious from the energetics shown in Scheme 5. We speculate that **13**/**14** is more sensitive to protonation than **2a**/**6**; the more readily formed cations **13**H$^+$/**14**H$^+$ rearrange with almost negligible barrier to give **12**H$^+$ and **15**H$^+$.

Considered together, the relatively slow decomposition of **14** in non-polar solvents (which may approximate the polarity of a P450 active site), compared with its rapid decomposition in aqueous solution, and the identical product ratios observed from the enzyme catalyzed oxidation of **11** and the rearrangement of **14** in aqueous solution together make it likely that **13**/**14** are indeed direct products of the P450$_{BM-3}$ mediated oxidation of **11**. The experimentally and theoretically determined properties of **14** are consistent with the idea that arene oxide **13** would have been formed in the P450$_{BM-3}$-catalyzed oxidation of *ortho*-xylene, as proposed by Wong et al.[8] However, in the relatively nonpolar enzyme active site, as seen with **2a**/**6**, **13** would have converted rapidly to give predominantly oxepin **14.** This (**14**), upon release from the active site into aqueous solution, would then rapidly rearrange to the observed **12** and **15**, thus eluding direct detection.

Despite a sustained interest in P450-catalyzed oxidation of aromatic compounds, our detection of **6** among the P450-catalyzed oxidation products of **1** is the first report of a discrete oxepin product from a reconstituted in vitro system, and indeed from a bacterial P450. The observation of oxepin **6** as a product from P450$_{cin}$- and P450$_{cam}$-mediated oxidation of **1** but not of **14** from P450$_{BM-3}$ catalyzed oxidation of **11** appears significant. Quantum chemical calculations reveal that the rates of equilibration and relative stabilities of the **2a**/**6** and **13**/**14** pairs are comparable. As none of the enzyme/substrate pairs (P450$_{cin}$/**1**, P450$_{cam}$/**1**, P450$_{BM-3}$/**11**) are natural pairings, it is unlikely that active site residues specifically inhibit the valence bond tautomerisation of an intermediate benzene oxide in one, but not another P450, or that the steric con-

straints of an active site significantly change the relative stability of the benzene oxide/oxepin pair, as has been proposed for some substrate specific non-heme iron oxygenase systems.[23] Therefore, the simplest explanation for these observations is that benzene oxides/oxepins are specific, if somewhat unstable, products of P450-catalyzed arene oxidation and not general intermediates. That is, aromatic oxidation would generally proceed through rearrangement of the Meisenheimer tetrahedral intermediate as previously proposed on the basis of computational studies.[14e] Benzene oxide/oxepin formation would then be governed by the structure of the arene oxidized and perhaps its interaction with the P450. The fate of the oxepin produced is governed by its stability in aqueous solution: it may be stable enough to allow observation as with **6**; it may spontaneously rearrange as with **14**; or it may react, probably via its tautomeric arene oxide, intra- or intermolecularly with nucleophiles as previously proposed to give a range of metabolites.[7,9] Although oxepins and their derivatives are known as natural products,[23,24] the oxidative enzymes associated with their biogenesis have not to date been P450s. This raises questions as to whether biosynthetic P450s are capable of catalyzing specific formation of an oxepin, and if so how they promote oxepin formation over hydroxylation and what structural features are important in stabilizing the oxepin product in aqueous solution.

Experimental Section

The Supporting Information contains details of the synthesis of **6** and **14** as well as their spectroscopic characterization including NMR spectra, biochemical methods for the P450-catalyzed oxidations as well as the analysis of these reactions, and computational methods, computed geometries, and energies.

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Keywords: arene oxide · benzene oxide · cytochrome p450 · oxepin · *tert*-butyl benzene

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