Mechanistic studies on the chemical oxidation of substituted tetrahydrobenzofurans
by Kirk Paul Manfredi

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry
Montana State University

Abstract:
here indicates that 15 does not undergo a NIH Shift but loses a proton to form the enol 109 which isomerizes to 81.

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Chemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

January 1985

APPROVAL

of a thesis submitted by

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INTRODUCTION

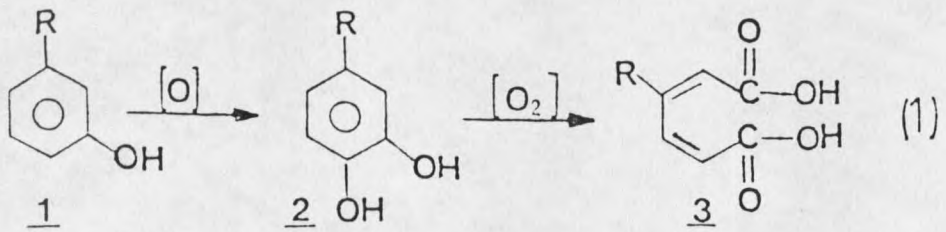
An increasing number of organic substrates that are either toxic or carcinogenic are now believed to have their effects enhanced (or even caused) by the products of their metabolic oxidation. The conversion of polyaromatic hydrocarbons to epoxides and diols and the conversion of nitrites to nitrosamines are just two examples. These oxidations are believed to occur in the liver of mammals and are catalyzed by a diverse class of hepatic enzymes known as mixed function oxidase enzymes (MFO). It is the purpose of this study to investigate a number of MFO chemical mimics and their subsequent reactions with alkyl substituted furans. It is appropriate, however, to define what is meant by the term mixed function oxidase and to examine the literature with regard to their mechanism of oxygen fixation, nature of the active oxygen species and evaluation of the various MFO mimics.

Xenobiotic substances in mammalian systems are oxidized in the liver by a diverse class of enzymes termed oxidases. In 1950, during a study on tryptophan metabolism, Hayaishi discovered a new type of oxidizing enzyme and gave it the classification of an

"oxygenase".[1,2]

Oxygenases are, therefore, a special class of oxidase. The term oxidase is given to an enzyme when the final acceptor of oxygen is a hydrogen. In an oxidase, either one, two or four electrons are transferred to a molecule of oxygen to form HO_2^-, H_2O_2, or $2H_2O$, respectively.[3]

Oxygenases which will be the focus of this introduction can be classified into two major categories. The first category is called dioxygenases. These are enzymes that catalyze reactions in which both atoms of a molecule of oxygen are transferred to a substrate. The second category is termed monooxygenase, these enzymes insert one atom of molecular oxygen into the substrate, while the other atom is made into H_2O. Since these enzymes appear to be bifunctional (i.e., hydrogen is the acceptor of an oxygen atom as in an oxidase) they are generally referred to as mixed function oxidases or MFOs. The functions of monooxygenases and dioxygenases can be coupled as shown in the hypothetical reaction scheme shown below (equation 1).



There are a number of known classes of MFOs that are based on the nature of the electron donor. Only the two classes that are known to be responsible for the oxidation of xenobiotic substances will be considered in this text. These two classes are termed flavoprotein monooxygenases and heme dependent microsomal cytochrome P-450 monooxygenase.

Flavin protein dependent monooxygenases require reduced purine as an electron donor. These enzymes are found in the liver microsomes of mammals and are known to hydroxylate aromatic substrates, epoxidize double bonds and oxidize sulfur and nitrogen containing compounds to sulfoxide and N-oxides respectively.[4,5] The second class is the cytochrome P-450 heme dependent MFOs. These are much less specific enzyme systems which hydroxylate alkanes as well as the same type of substrates as the flavin dependent MFOs. Flavoprotein monooxygenases are much more specific and better characterized than P-450 enzymes.[3a] Both of these enzyme systems appear to have different and unique methods of "fixing" atmospheric oxygen to a more reactive oxidizing agent.

Experiments with $^{18}O_2$ have shown that the oxygen incorporation in organic substrates during biological oxidations comes from atmospheric oxygen and not H_2O.[3] This may seem surprising since atmospheric oxygen is

relatively inert to organic substrates. Hamilton has proposed that the relative inertness of molecular oxygen is do to the fact that oxygen's ground state is a triplet.[6] A reaction between a triplet reagent and singlet substrate to give a singlet product is a spin forbidden process and requires a high activation energy. This is not to imply that free radical chemistry does not occur, just that the free radical chemistry of molecular oxygen is endothermic. What is then one of the intriguing properties of MFOs, is how they activate molecular oxygen.

It had been suggested that MFOs activate molecular oxygen by transforming it to a singlet state.[7,8] This, however, is now thought to be unlikely for a number of reasons.[6] For example, substrates which normally react with MFOs, such as olefins and aromatic compounds, give different products when reacted with singlet oxygen. Olefins give epoxides when reacted with MFOs but give allylic hydroperoxides when reacted with singlet oxygen. Secondly, the energy required to convert triplet oxygen to its lowest singlet excited state is 22 Kcal/mol. This energy barrier is close to the upper limit for many enzyme catalyzed reations. Finally, one must consider the incredible reactivity of singlet oxygen with some substrates such as alkenes which react extremely fast.

Alkanes, on the other hand, are inert to singlet oxygen but are substrates for MFO oxidations. Based on the above observations, singlet oxygen is probably not the reactive species in biological oxidations nor is it a good chemical mimic.

There appear to be two other pathways in which biological systems can activate molecular oxygen. The first pathway entails the reaction(s) between molecular oxygen and a transition metal. When oxygen interacts with a transition metal, the oxygen orbitals interact with the metal orbitals, in such a way that one can not speak of the number of unpaired electrons on either the oxygen or the metal, but only the number of unpaired electrons on the complex. Hamilton has suggested that when oxygen is in this environment it is neither a triplet or a singlet state and can react ionically with substrates to give singlet products.[6] When oxygen is complexed in this way, it is proposed to have low activation barriers.

The other proposed mechanism to activate oxygen can be achieved by having the initial reaction occur as a free radical process. A triplet molecule and a singlet molecule can react with each other to give two free radicals. These two radicals can then recombine to form an active singlet species. Oxygen does not normally

react with organic compounds at low temperature because free radical reactions with O_2 are normally very endothermic. There are, however, certain biological molecules that react with oxygen at low temperature. In particular they are the reduced flavins that are the prosthetic group of the flavin monooxygenases. The initial flavin free radicals that are formed are highly resonance stabilized and therefore decrease the activation energy required for the initial free radical reaction. The oxygen radical and flavin radical can then recombine to form an active oxygen donor.

It can be argued that the relative inertness of molecular oxygen is not related directly to the triplet ground state as Hamilton has suggested, just simply because of the energy of the oxygen-oxygen bond (e.g., the energy required to form a carbon-oxygen bond is greater than that required to break a O_2 bond). Regardless of how enzymes fix oxygen to oxidize organic substrates, it is the nature of the oxygen that is important when considering chemical mimics for MFOs.

When considering the chemical nature of the active oxygen species in MFOs, it was noticed that they catalyze reactions similar to carbene and nitrene reactions.[9] Carbenes will insert a methylene group into carbon-hydrogen bonds in alkanes, while MFOs will insert

oxygen to form alcohols. Carbenes will react with olefins to form cyclopropanes while MFOs give epoxides. MFOs will also react with aromatic substrates to give epoxides and diols while carbenes give alkyl benzenes and norocaradienes. These similarities have led researchers to postulate that the active oxygen donating species in MFOs donate an oxygen atom. This is now known as the oxenoid mechanism.[9]

The strongest evidence for the oxenoid mechanism comes from the experimental results obtained by the NIH group led by D.M. Jerina on what is now termed the NIH Shift[10-16] (Figure 1).

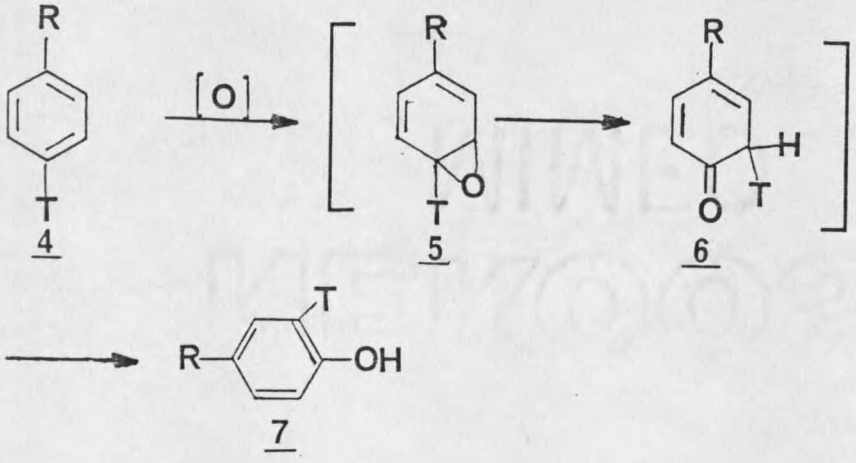


Figure 1. An example of an NIH Shift reaction.

During the enzyme catalyzed hydroxylation of an aromatic substrate, the intermediate arene oxide rearranges to form the ketone. During this transformation , a proton

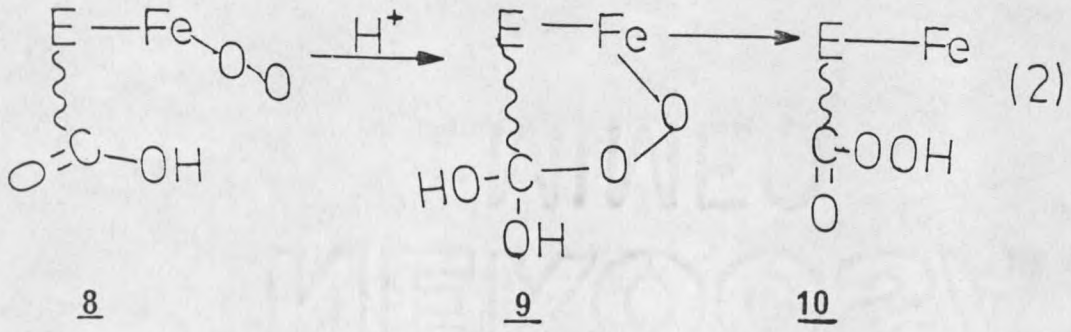
(T) isomerizes to the incipient carbonium ion and is referred to as the NIH Shift. A large portion of the label changes position relative to the R group at this time. This rearrangement causes more of the label to stay on the ring because of the primary isotope effect. The NIH Shift is observed with both liver microsomal suspension and isolated enzymes.[10,16] The importance of the NIH Shift to the oxenoid mechanism is that the intermediate arene oxide would be the product that one would expect from an oxene (carbene) type reaction. Sources of oxene such as m-chloroperbenzoic acid (mCPBA), peroxyacetic acid and trifluoroperacetic acid all give NIH Shift products when reacted with various aromatic substrates.[17]

Jerina has supported a loose set of criteria to determine the relevancy of chemical model systems to actual enzymes.[18] Chemical mimics for P-450 systems should display a broad spectrum of chemical reactivity. A chemical mimic should possess the ability to hydroxylate both aromatic and aliphatic compounds, epoxidize double bonds and oxidize nitrogen and sulfur containing compounds to N-oxides and sulfoxides, respectively. The most important criteria is that the reagent should give the NIH Shift product when oxidizing an aromatic substrate.

Over the years a number of investigators have attempted to develop chemical systems to mimic the P-450 microsomal MFO. The first of these systems was developed by Udenfriend and co-workers in 1954.[19] Udenfriend's reagent consists of O_2, Fe(II), EDTA, and ascorbic acid at neutral pH. This system is capable of converting aliphatic compounds to alcohols and carbonyl compounds, olefins to epoxides and aromatic substrates to phenols. The mechanism of this system has been studied extensively and it is unlikely that the reagent acts through an oxenoid pathway.[20] Further, the reagent does not give NIH Shift products. Finally, oxidation products in general are formed in very low yield.

A second system known as the Hamilton system consists of Fe(III) and catechol and uses H_2O_2 as its oxygen source.[21] This system hydroxylates aromatic substrates, but in much higher yield than Udenfriend's reagent. Although it gives low yields of NIH Shift products, it is still considered to be a model for the heme dependent P-450 systems. Recently Groves has developed a similar type system using Fe(II)-tetraphenyl-porphine chloride (FeTPPCl) and iodosylbenzene as an oxygen source.[22] This system undergoes many of the reactions that P-450 catalyzes. No work as yet has been done to determine if it gives NIH Shift products.

The above systems all use metals to activate some oxygen source. There are also chemical mimics that already have an active oxygen source. The photolysis of pyridine-N-oxide in the presence of aromatic substrates leads to high yields of NIH Shift phenolic compounds.[23] Peracetic acid, trifluoroperacetic acid and m-chloroperbenzoic acid all give NIH Shift products with various aromatic substrates.[17] In fact, Hamilton has proposed that the active oxygen in heme dependent P-450 systems is either a peracid or peramide.[6] He has proposed that the Fe(II) in P-450 first forms a complex with O_2. This complex then oxidizes an adjacent amide carbonyl or acid carbonyl to peramide or peracid with the concomitant loss of H_2O. The peracid or peramide is then the oxidizing agent (equation 2). More recent work, however, has



(2)

8 9 10

pointed to a sulfur-iron-oxene complex as the reactive intermediate.[24,25]

The uncertainty of the exact nature of the active

oxygen donor in P-450 enzymes has led to considerable difficulty in designing adequate chemical mimics to study oxidative metabolism. It has been possible to overcome this problem by using liver microsome preparations. These microsomal preparations have had considerable success in determining oxidation products of various organic substrates.

Liver microsomal preparations from rat and rabbit livers are good sources of MFOs. It is also known that by treating rabbits and rats with non lethal doses of Aroclor 1254, 3-methylcholanthrene or phenobarbital prior to microsome preparation, the P-450 activity can be greatly enhanced in in vitro studies. The induced P-450 enzymes show essentially no substrate specificity and oxidize a wide variety of substrates. Coon has isolated a number of P-450 enzymes from liver microsomes that are electrophoretically pure and show a wide range of substrate activity.[26,27]

Metabolism studies using induced liver microsomes as a source of P-450 MFO, have been examined extensively with polyaromatic hydrocarbons as substrates.[28] The oxidative metabolites of polyaromatic hydrocarbons (PAH) are known to be potent mutagens that function by binding to DNA.[29,30] Sims was the first to identify the oxidative metabolites of 3-methylcholanthrene.[31] In

this study, he isolated the microsomes from rat livers and incubated them with 3-methylcholanthrene. He then synthesized a number of potential metabolites and compared their thin layer chromatography R_f values and UV spectra to those of the 13 metabolites that he isolated from the microsomal reaction (Figure 2). Subsequent studies have identified the metabolites from benzanthracene and benzo [a] pyrene.[32] The metabolism of these compounds proved to be a variety of alcohols, phenols, diols, epoxides, and ketones. Extensive studies have also been done on the metabolism of aryl amines. Aryl amines are first acetylated, N-hydroxylated to give the hydroxamic acid derivatives which are known to be carcinogenic.[33,34]

PAHs and aromatic amines have received considerable attention because of their abundance in the environment. PAHs are produced from combustion of fossil fuels and wood products. Aryl amines are found in dyes and proteinaceous foods. There are probably a number of other toxic organic compounds whose metabolism increase their toxicity but they are less studied, perhaps because they are not as abundant in the environment as PAHs and aryl amines.