An Investigation into the Oxidation of Organic Sulphides, Organic Selenides and Simple Hydrocarbons by *Mortierella isabellina* NRRL 1757

by

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Abstract

The work presented in this thesis is divided into three separate sections. Each section is involved with a different problem, however all three are involved with a microbial oxidation of a substrate.

A series of aryl substituted phenyl and benzyl methyl sulphides were oxidized to the corresponding sulphoxides by Mortierella isabellina NRRL 1757. For this enzymic oxidation, based on ^{18}O labeled experiments, the oxygen atom is derived from the atmosphere and not from water. By way of an ultraviolet analysis, the rates of oxidation, in terms of sulphoxide appearance, were obtained and correlated with the Hammett p sigma constants for the phenyl methyl sulphide series. A value of -0.67 was obtained and is interpreted in terms of a mechanism of oxidation that involves an electrophilic attack on the sulphide sulphur by an enzymic iron-oxygen activated complex and the conversion of the resulting sulphur cation to sulphoxide.

A series of alkyl phenyl selenides have been incubated with the fungi, Aspergillus niger ATCC 9142, Aspergillus foetidus NRRL 337, M. isabellina NRRL 1757 and Helminthosporium species NRRL 4671. These fungi have been reported to be capable of carrying out the efficient oxidation of sulphide to sulphoxide, but in no case was there any evidence to support the occurrence of a microbial oxidation. A more extensive investigation was carried out with M. isabellina, this fungus was capable of oxidizing the corresponding

sulphides to sulphoxides. Using a labeled substrate, [Methyl-^{14}C]-methyl phenyl selenide, the fate of this compound was investigated following an incubation with M. isabellina. Besides the ^{14}C-analysis, a quantitative selenium analysis was carried out with phenyl methyl selenide. These techniques indicate that the selenium was capable of entering the fungal cell efficiently but that some metabolic cleavage of the selenium-carbon bond may take place.

The ^{13}C NMR shifts were assigned to the synthesized alkyl phenyl sulphides and selenides.

The final section involved the incubation of ethylbenzene and p-ethyltoluene with M. isabellina NRRL 1757. Following this incubation an hydroxylated product was isolated from the medium. The ^{1}H NMR and mass spectral data identify the products as 1-phenylethanol and p-methyl-1-phenylethanol. Employing a chiral shift reagent, tris-(3-heptafluorobutyl-d-camphorato)-europium III, the enantiomeric purity of these products was investigated. An optical rotation measurement of 1-phenylethanol was in agreement with the results obtained with the chiral shift reagent. M. isabellina is capable of carrying out an hydroxylation of ethylbenzene and p-ethyltoluene at the α position.

Acknowledgements

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Introduction I

Cytochrome P-450 is an important monooxygenase enzyme responsible for the oxidation of metabolites and foreign compounds such as drugs, pesticides, carcinogens and environmental pollutants. Even though this enzyme frequently prevents lethal doses of ingested compounds from accumulating within an organism, there are instances where these oxidations lead to the production of toxic and carcinogenic metabolites.[1] The reactions *in vivo* are primarily concerned with the breakdown of aromatic and cyclic species into smaller fragments, and the transformation, breakdown and the synthesis of certain steroidal hormones.[2]

It was not until 1937 that Michel[3] discovered that the major site of the conversion of foreign compounds into polar derivatives was the liver and in 1955 it was suggested by Brodie et al.[4] that the enzymes effecting these transformations was located in the endoplasmic reticulum. The endoplasmic reticulum was later isolated as a "microsome fraction" by Claude[5] and shown to contain respiratory pigments that were not blood pigments. These same microsomal pigments were also reported by Kingenberg[6] and Garfinkel[7] in 1958 but it was not until 1962 that Omura and Sato[8] demonstrated their hemoprotein nature and showed that, once reduced by sodium dithionite, they gave an absorption at 450 nm when combined with carbon monoxide.[9,10]

This was the discovery of cytochrome P-450, its name

originating from its characteristic absorption at 450 nm, when the enzyme is chelated with carbon monoxide.

The actual mechanism of the activation and incorporation of oxygen by this monooxygenase is still not fully understood. However, due to its importance in both the mammalian system, fungi, yeast and in bacteria, a large amount of data has been compiled since its discovery over two decades ago.

This enzyme system is not restricted to any one organism but has been found in mitochondria and microsomes of mammalian tissues[11], in yeast[12] and in bacteria.[13] The enzyme system that has received most of the research is that of the mitochondria and microsomes (particularly the liver microsomes). It appears that more than one form of the enzyme, differing in molecular weight has been identified and purified,[14] these differences being based upon their origin, i.e. liver microsomes, and their mobilities when subjected to gel-electrophoresis.

In studying liver microsomes containing cytochrome P-450, antibodies were produced for $P\text{-}450_{LM2}$ (liver microsomes, number 2 position by gel-electrophoresis) and when they were subjected to $P\text{-}450_{LM4}$, the antibodies did not react with this enzyme preparation. The reverse was also tried with similar results, leading to the conclusion that the two cytochrome enzyme preparations were different.[15] Further identifiable differences between the two enzyme preparations, $P\text{-}450_{LM2}$ and $P\text{-}450_{LM4}$ are contained in Table 1.

It was also discovered that specific forms of cyto-

Table 1

Properties of two major forms of liver microsomal cytochrome P-450.

Property studied	Values obtained	
	$P\text{-}450_{LM2}$	$P\text{-}450_{LM4}$
Polypeptide molecular weight	48,700	55,300
Apparent molecular weight	200,000	500,000
Haem content, per polypeptide chain	1	1
C-Terminal amino acid residue	Arginine	Lysine
N-Terminal amino acid residue	Methionine	?
Absorption maxima		
Oxidized form	418 nm	394 nm
reduced CO complex	451 nm	448 nm

chrome P-450 are inducible by the administration of different drugs to animals, prior to the preparation of the microsomes. The most common drug injected into the various species is phenobarbitol.

The specificity of this monooxygenase was also different than that of most enzyme systems, which are usually specific for one group of substrates. This microsomal enzyme system is capable of catalyzing the oxidation of a variety of compounds. Not only is it able to react with fatty acids and alkanes[16] but steroids[17], polycyclic hydrocarbon carcinogens and a variety of drugs and other foreign compounds.[18] It appears to be one of the most versatile catalysts available, for example, hydroxylation, N- and O-demethylation, deamination, sulphoxidation and N-oxidation of a range of different substrate types are just a few of the hydroxylation reactions attributed to this system.[19] The versatility of this monooxygenase can be demonstrated by a consideration of the oxidation of chloropromazine, a tranquilizer in the treatment of schizophrenia.[20] Often a single substrate will be attacked at more than one site, as is the case with this drug. This substrate undergoes N-dealkylation, S-oxidation and hydroxylation of the aromatic ring (Figure 1).

The reason for the wide range of substrate specificity is not quite understood, partly because of the problems encountered in solublizing the membrane bound cytochrome P-450 system. One possibility could be the existence of more than one form of the hepatic microsomal system[14,21]. Studies related to the various enzyme systems (mitochondria, liver,

Figure 1. Reaction of Chloropromazine with Cytochrome P-450

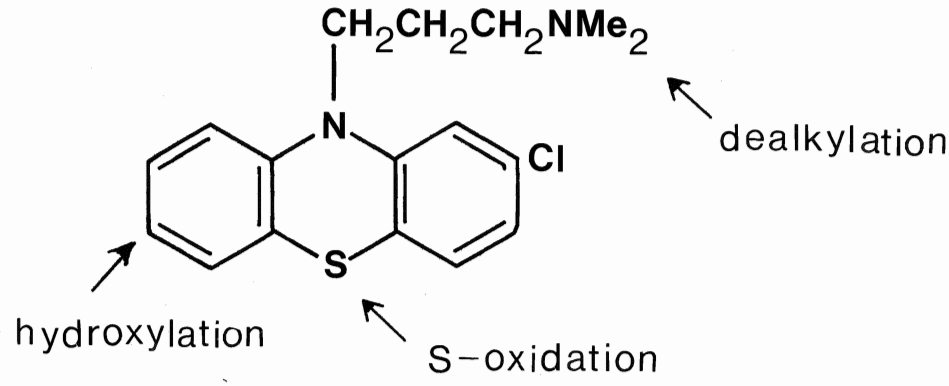
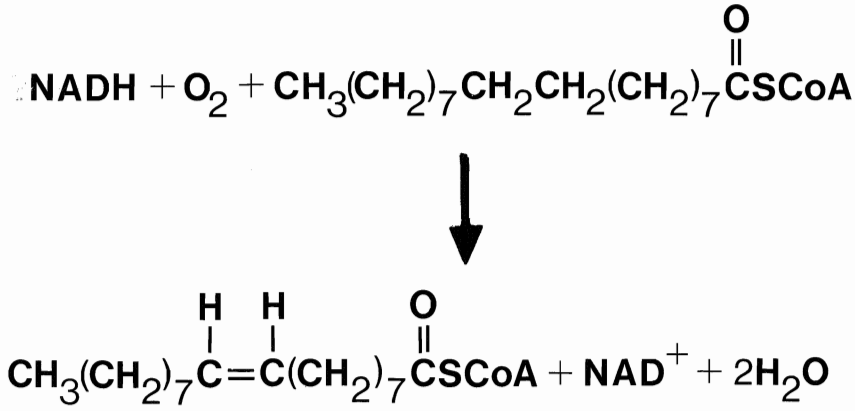


Figure 2. Desaturation of Stearoyl-CoA



yeast and bacteria), have discovered that the microsomal P-450 linked monooxygenase requires an external source of electrons such as NADH or NADPH for the insertion of oxygen into the substrate. These electrons flow from NAD(P)H, to a flavoprotein (for bacterial systems it is an iron-sulphur protein), to cytochrome P-450 which then interacts with oxygen to form the active oxygen species and then with the substrate to form products.

Isotopic studies of cytochrome P-450 have shown that this species is a true monooxygenase, obtaining oxygen for the oxidation reaction from molecular oxygen. Also, these oxidative reactions are under stereochemical control where there is retention of configuration and a low intermolecular kinetic isotope effect (KIE).[22]

Since this enzymic reaction involves the oxidation of a substrate, the oxidation of NAD(P)H to $NAD(P)^+$, and the incorporation of a single molecule of oxygen from 0_2, the group of enzymes responsible for such reactions are referred to as mixed function oxidases.[23] The cytochrome P-450 enzyme is therefore referred to as a monooxygenase or mixed function oxidase.

In addition to monooxygenases, there is another class of enzymes which are also capable of carrying out various oxidation reactions. This group can be referred to as oxygen-transferases or dioxygenases and can be distinguished from monooxygenases as they incorporate both oxygen atoms into the substrate. In discussing enzymic oxidation reactions in

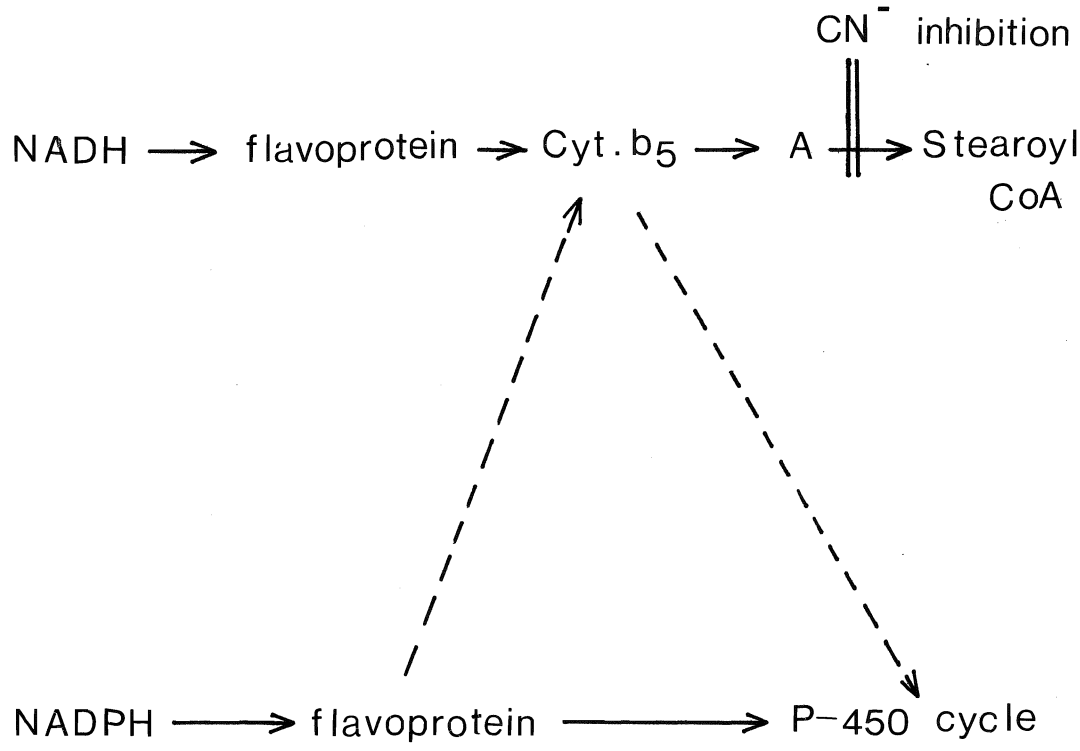
general, both the dioxygenase and monooxygenase classes can be grouped together and referred to as oxygenases. A true monooxygenase or dioxygenase can readily be identified by how they incorporate molecular oxygen into the various substrates.

Almost from the initial description of the microsomal cytochrome P-450 enzyme system, both NADH and NADPH were linked with this system.[24] It seemed that NADH alone was unable to carry out a demethylation reaction but when both nucleotides were present the system worked effectively. Further experiments were set up dealing with NADPH cytochrome c reductase and cytchrome b_5 reductase, both the fluorescent and optical changes of these microsomal flavoproteins were monitored following the addition of NADPH and NADH. The addition of NADH caused a decrease in absorption and fluorescence, inferring that the flavoprotein underwent reduction and with the addition of NADPH, there was no fluorescence or absorbance change. Conversely, similar results were obtained when NADPH was added prior to NADH, there was no reduction of the second nucleotide. The reduction of both flavoproteins left the authors perplexed as it was known that each flavoprotein had a high degree of specificity towards the reduced nucleotide. This phenomena was explained by Estabook and Cohen[25] as a result of cross reactions between the two flavoprotein species, allowing them to function with either nucleotide. Using a fatty acid desaturation system, this idea was examined. In this system long chain acyl-CoA thioesters undergo desaturation to monosaturated acylthioesters (Figure 2) (these monosaturated species are then

incorporated into phospholipids giving biological membrane their fluidity). With this system and the cytochrome P-450 system, electrons from NADH are transferred to the fatty acyl-Co substrate via cytochrome b_5 and a cyanide sensitive factor. If NADH and cytochrome b_5 is involved with the transfer of electrons to cytochrome P-450, then the diversion of electrons from NADH to the desaturation system should cause a shut down of the cytochrome P-450 system with a loss of oxidative activity. Cytochrome b_5 does not have the same reactivity with 0_2 as P-450 and does not react directly with it.[26] The addition of stearoyl-CoA did in fact cause a loss in cytochrome P-450 activity, as electrons were diverted for the desaturation of stearoyl-CoA.[27] Also, if this desaturation pathway was inhibited, then the flow of electrons should then be available for the cytochrome P-450 cycle. The addition of cyanide to these coupled systems did in fact increase the rate of drug oxidation, due to the presence of NADH. The mechanism proposed by the authors for this cross reactivity, is contained in Figure 3. The first electron for the reduction of cytochrome P-450 comes from NADPH since NADH can not support the oxidation by itself. The second electron can come from either NADH or NADPH and if it does come from NADH, then it is transferred to the cytochrome P-450 cycle via cytochrome b_5. This cross linking and the involvement of cytochrome b_5 is present only in mammalian systems.[28]

In attempting to understand how the enzyme carries out these oxidation reactions, an accurate description of its structure and how the active site works is needed. Cytochrome

Figure 3. Possible Interaction of Cytochrome b_5 with Cytochrome P-450.



A = CYANIDE sensitive species

P-450 is a heme protein, made up of a prosthetic group containing an iron atom (Fe^{3+} or Fe^{2+}) coordinated to a macrocyclic tetrapyrrole ring (Figure 4). The pyrrole nitrogens of this ring make up four ligands to the iron, in an equatorial position which leaves the two axial positions filled by other ligands. The two major oxidation states of the iron in the protein are Fe^{2+} and Fe^{3+}, both of which are stable in an aqueous environment. Iron in the 2+ oxidation state has six electrons in the 3d orbitals while Fe^{3+} has only five. Since this is a biological system containing ligands, the orbitals are no longer degenerate and ligand field-splitting occurs.[29] In this heme protein there is an octahedral ligand field where the orbitals are split into two discrete groups of three low lying and two high lying orbitals (t_{2g} and eg). The difference between the two is the ligand field splitting energy, Δ (Figure 5).

In filling these orbitals, when Δ is small, relative to the pairing energy, then all of the five 3d orbitals will be filled. This is called high-spin iron. For high-spin Fe^{3+} there are five unpaired electrons; and Fe^{2+} has four unpaired.

Low-spin iron occurs when the field splitting energy is greater than the pairing energy and the two higher orbitals are not populated. In both cases, the electrons distribute themselves in the three low lying orbitals. Iron (III) has only one unpaired electron while Fe^{2+} has none (Figure 5). Since the 3d electrons are not in the upper antibonding orbitals, the low-spin state iron is more compact than the high-spin state.

Figure 4. Protoporphyrin lX

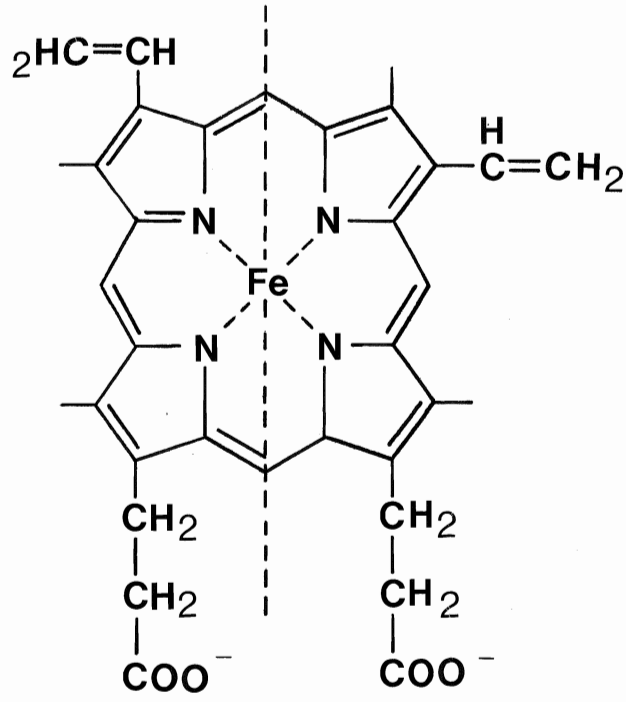
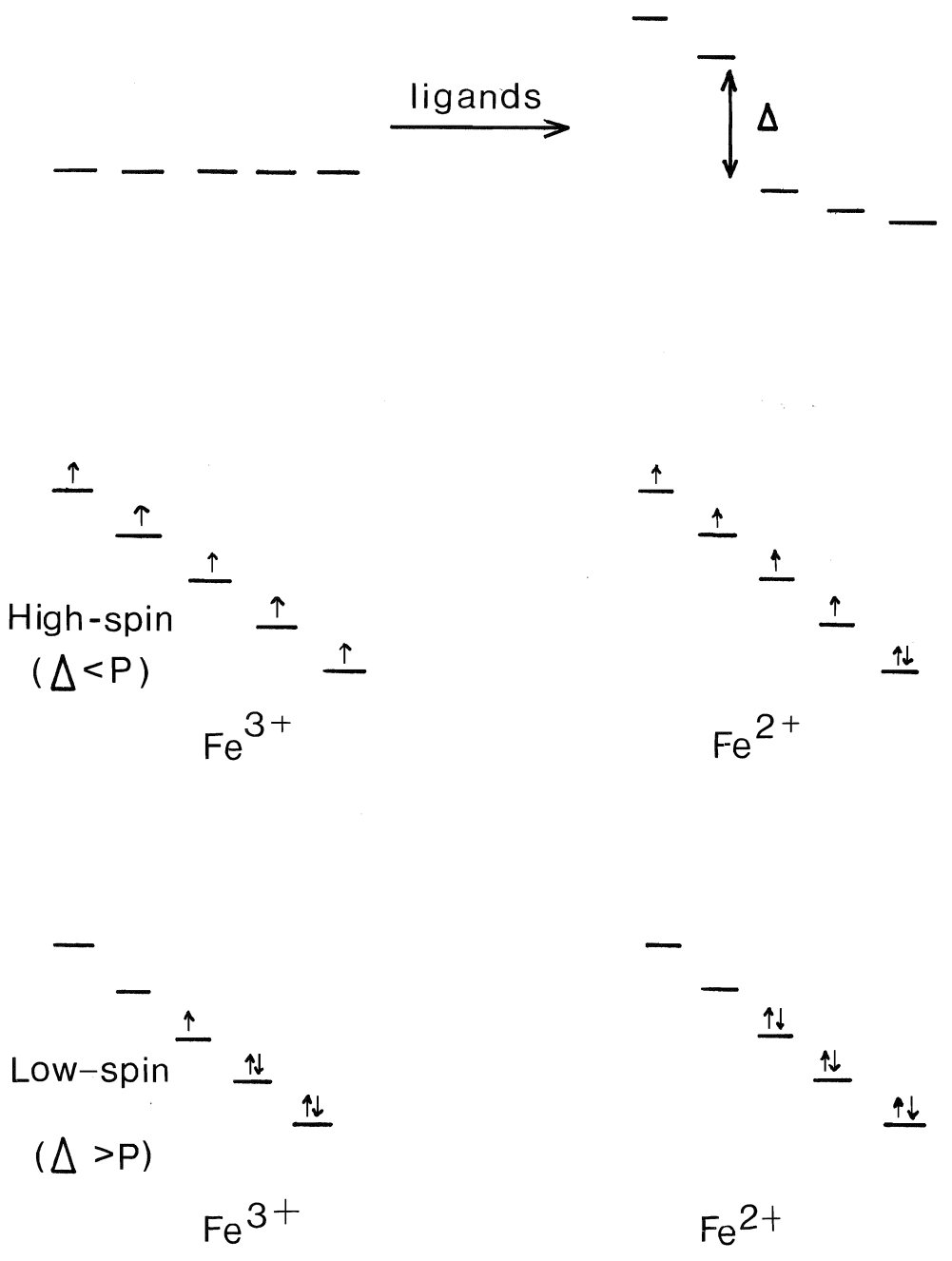


Figure 5. Spin States of Iron

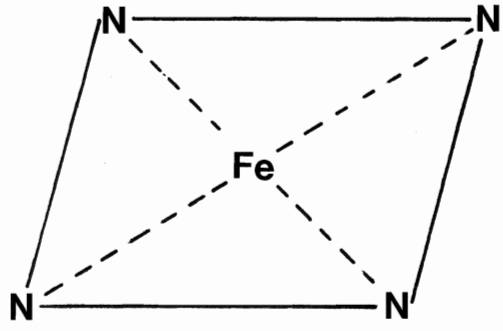


The difference in size between the two spin states of iron is an important point in heme-iron chemistry. From x-ray studies on heme proteins, the tetrapyrrole molecule is 2.02Å in diameter and when Fe^{2+} or Fe^{3+} is in a low-spin state, it is capable of resting in this cavity (1.91Å diameter and is in the plane of the molecule).[26] When the iron is in the high-spin state, the diameter changes to about 2.06Å [26] and experimental results show that it is no longer resting in the plane of the macrocycle but is above the plane[30,31] (Figure 6). The iron (III) is about 0.3Å out of the plane and iron (II) is 0.7Å. The splitting of the ligand field energy appears to be balanced somewhere between these two states and it is the axial ligands which can determine the equilibrium position. These axial ligands are of great interest as they hold the key to understanding how the enzyme functions and how the various reactions are performed.[31]

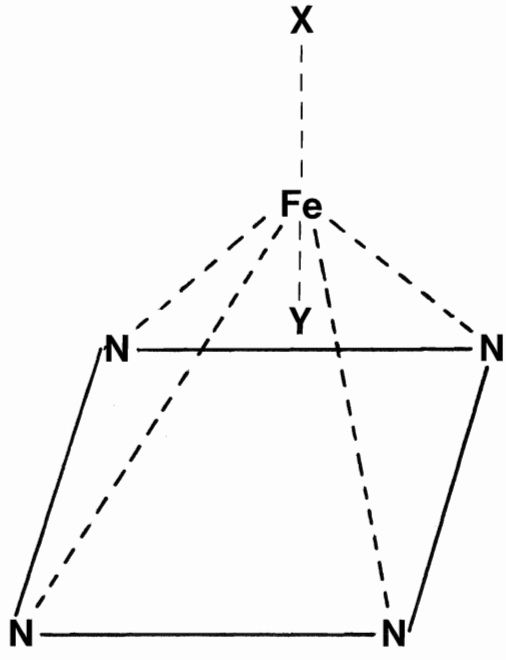
In a strong ligand field, one where an nucleophile is bonded, the donation of electrons from the ligand will force electrons into the orbitals of lowest energy (t_{2g}). This should reduce the number of unpaired electrons on the metal iron and cause a "low-spin" state to occur. In a weak ligand field, the converse is also true where the electrons associated with the complex will cause the electrons to occupy all the molecular orbitals, Fe^{2+} will have four unpaired and Fe^{3+} will have five (high-spin state).

Work on an isolated enzyme fraction of soluble cytochrome $P\text{-}450_{CAM}$ from Pseudomomas putida showed that approximately 95% of the enzyme was in the low-spin form, with sulphur as

Figure 6. Configuration of Iron in Low and High Spin States.



low-spin Fe



X ≠ Y

high-spin Fe

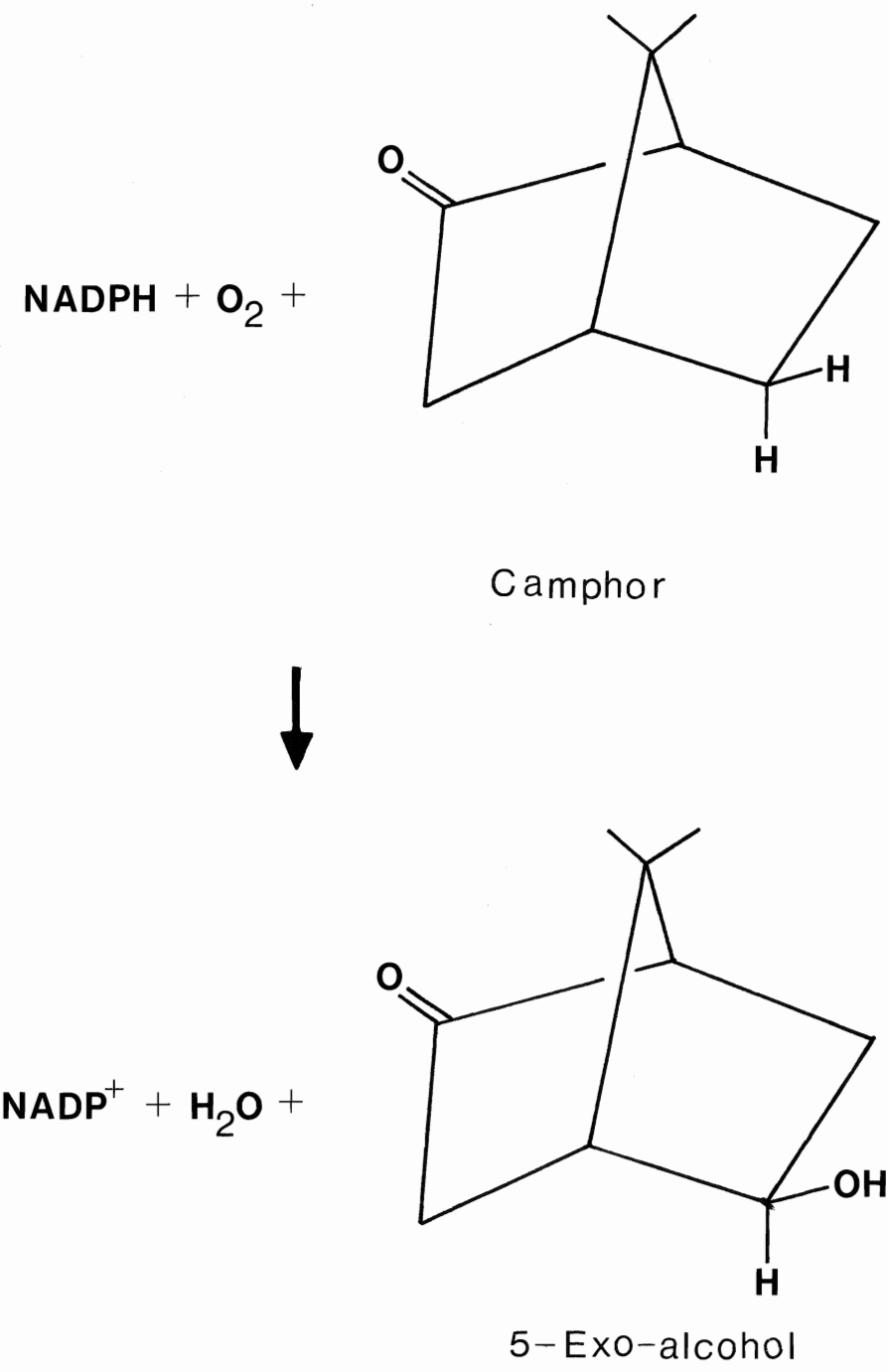
one of its axial ligands[32] and 93.8% in the high-spin state when the substrate was bound to the enzyme.[33] It is generally accepted that one of the axial ligands of cytochrome P-450 is a sulphur atom, usually that of a cysteine amino acid and that possibly the interaction of the sulphur ligand on the bottom of cytochrome P-450 affects the coordination of dioxygen to the top axial position.

The problem with working with a cytochrome P-450 system is that it is difficult to isolate and purify a soluble form of the enzyme. This is why the enzyme preparation from camphor-grown Pseudomonas putida is important; it is the most precisely defined cytochrome P-450 and has been obtained in a crystal form,[34] allowing the possibility that an x-ray structure determination can eventually be obtained.

This is a bacterial system and at this time is the easiest to work with as all the components of the multienzyme complex are soluble proteins and can be easily purified.[35] The three enzymes are a NADH oxidizing flavoprotein dehydrogenase; a 2Fe/2S cluster protein (putidaredoxin); and the P-450 hemeprotein hydroxylase. The system is responsible for the hydroxylation of camphor to 5-exo-alcohol (Figure 7). Based on studies pertaining to this system, the enzyme has a molecular weight of 45,000, a single polypeptide chain and one protohaem unit.[34] The substrate used with this preparation is D(+) camphor but the (L) isomer of camphor is also accepted.

In order to obtain a clearer picture of the mechanism of P-450, two valuable pieces of information are required: an

Figure 7. Hydroxylation of Camphor



amino acid sequence of the enzyme and an x-ray structure determination. The amino acid sequence is important as it would aid in determining the x-ray structure but until recently, problems relating to solubility and multiple forms[36] of the mammaliam monooxygenase have hampered the efforts. The first complete amino acid sequence from the camphor-grown Pseudomonas putida of cytochrome P-450 has been reported in the literature[37] and an equally impressive feat was the elucidation of the primary structure of the enzyme isolated from rat liver.[38] Both pieces of information coupled with an x-ray structure will drastically narrow the gap between our understanding of the enzyme and its actual operation.

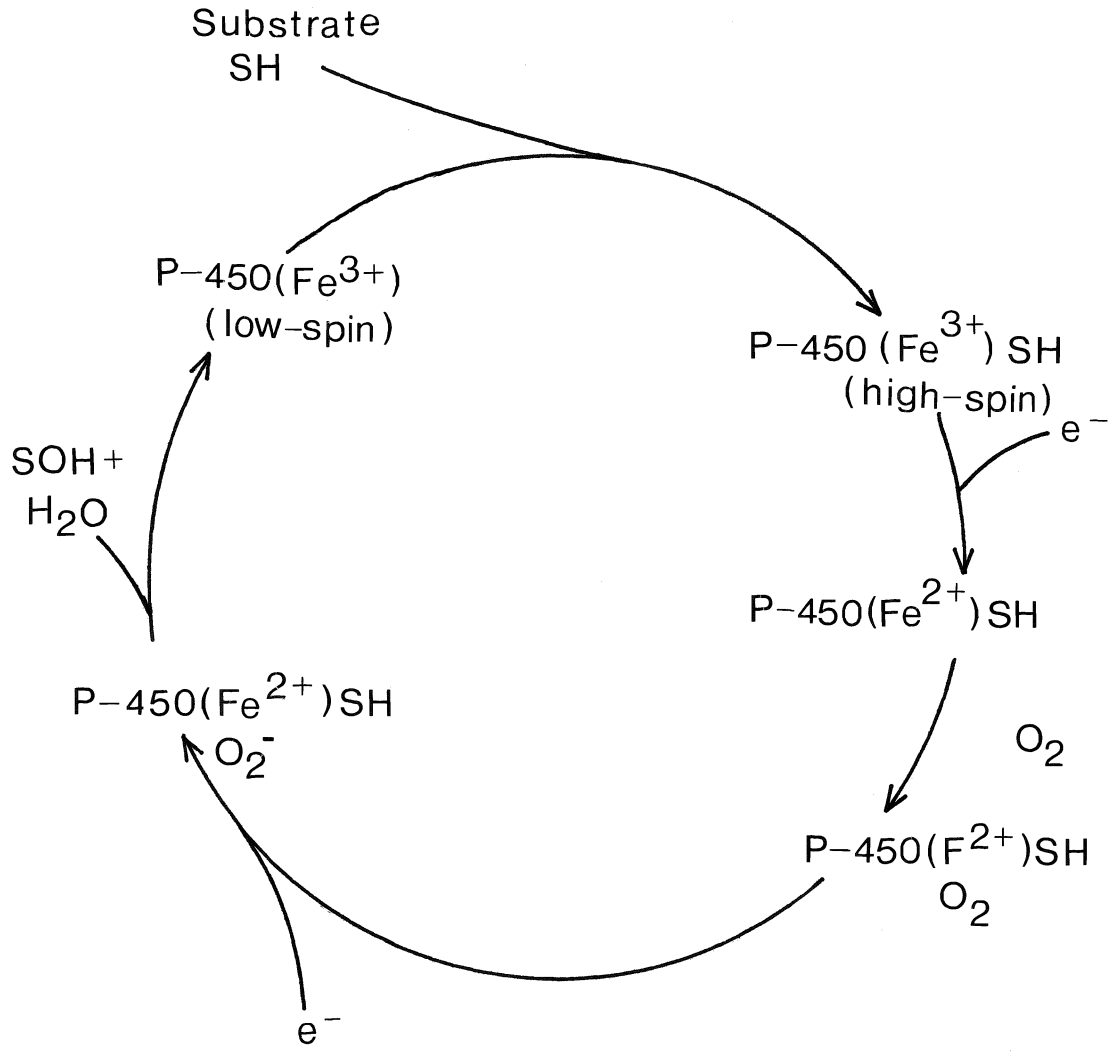
Continued work on the structure of the hemeprotein[39] has reinforced the proposal that a sulphur atom is present as the ligand trans to oxygen. It appears that sulphur is present in both spin states of the ferric compound as well as the high-spin and carbon-monoxide bound ferrous states of $P\text{-}450_{CAM}$. This work represents the first direct observation of sulphur ligation in cytochrome P-450 and various measurements of the bond lengths were recorded. The iron sulphur distances are consistent with that of thiolate binding, presumably from cysteinate, in all known conformational states of the enzyme.[40] Thiol and disulphide coordination however, can not be dismissed as a possible route for binding and this is where present research is being channeled.

Even though the bacterial cytochrome P-450 system is NADH rather than NADPH linked, it is believed that in spite of these differences, any mechanistic information derived

from this species will also be relevant to the membrane P-450 system.

Research on the camphor system of cytochrome P-450 has yielded some information regarding the kinetic catalysis[26]. The Fe^{3+} - P-450 resting state is in low spin Fe^{3+} (based on optical and EPR spectra), which binds the substrate molecule in the first step and then the first redox step is the passage of one electron into the active site of the enzyme. This generates a Fe^{2+} substrate complex where the iron is now in the high spin state (from ESR and optical spectra analysis). This therefore results in the movement of the iron out of the plane of the heme group and could cause other conformational changes in the protein. At this point dioxygen is now capable of binding (reversibily), as the top axial ligand and the spin state of this species is not as of yet known. One possibility is that the dioxygen is capable of inducing a low spin condition. The system is now capable of receiving a second electron from the iron-sulphur protein. Following the addition of a second electron, the enzyme complex now becomes the active species which dissociates readily to product, water and the regeneration of iron in the free state. After the addition of the second electron the mechanism becomes unclear because of this rapid dissociation; the proposed cycle is contained in Figure 8. There are at the present time, no suitable techniques for capturing and observing the active oxygen species in cytochrome P-450. Most of the information obtained regarding this species has resulted from studying the oxidation products of the organic substrates and

Figure 8. Proposed Mechanism for Cytochrome P-450 involving a Superoxide Ion.



chemical models for this enzyme.

Mechanistic studies on this enzyme have revealed that the active species during the oxidation is somewhat specific, as tertiary carbon hydrogen bonds are often selectively oxidized over secondary bonds and that benzylic and allylic carbon atoms are especially reactive,[41] as are positions α to heteroatoms. Also, regions of high π bond density are selectively epoxidized over regions of lower π bond density in polycyclic aromatic hydrocarbons. The hydroxylation reactions of cytochrome P-450 are known to proceed with retention of configuration,[22] suggesting that there is an insertion of the active oxygen molecule rather than a back-side displacement which causes an inversion of stereochemistry or an S_N2 mechanism which racemizes the center.[42]

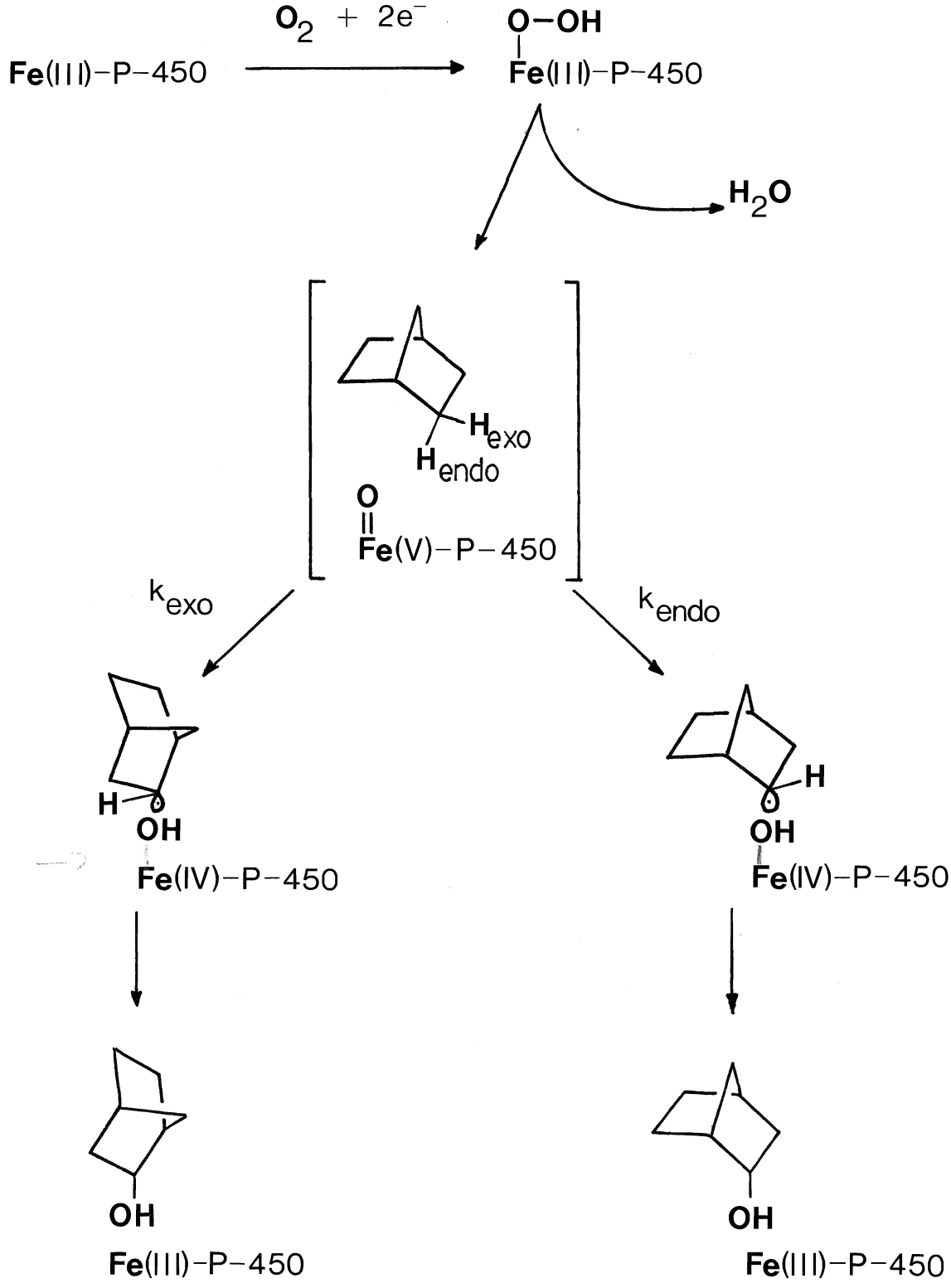
Since the monooxygenase reactions of cytochrome P-450, are enzymic, once the substrate reacts with the enzyme it is presumably held in place by the configuration of the active site. If substrate bonds are broken at this stage, then any rotation or configuration change may be hampered, and thus retention of its stereochemistry may occur. In the oxidation of sulphides, for example, this reaction proceeds through a definite steric course from which attack is mainly from one side.[43]

In spite of all the present information regarding mammaliam cytochrome P-450, the precise form of the active species is unknown. However, studies pertaining to the hepatic and bacterial systems have helped in narrowing the search for these answers. In both the bacterial cytochrome

$P\text{-}450_{CAM}$[44] and hepatic systems[45] there is some evidence which suggests that the superoxide ion (0_2^-) is the active species responsible for the oxidations. This superoxide ion could possibly be an iron ferrous peroxide complex containing the sulphur atom of cysteine as a ligand $S\text{-}Fe^{2+}\text{-}0_2^-$.[46] Copper-tyrosine chelates are known to have superoxide dismutase activity and are small enough to reach the active site of P-450,were capable of inhibiting drug metabolism but did not affect the rate of P-450 reduction by NADPH.[47] This indicates that the point of inhibition was the active site and not the prevention of electron transfer from NADPH to the cytochrome P-450 cycle. In a similar experiment, the addition of superoxide dismutase to an enzyme preparation did in fact inhibit product formation. The regeneration of the superoxide anions resulted in the retransformation to product.[48]

Another possibility is the existence of a free radical mechanism, which has recently received renewed support.[49-51] Research by Groves and McClusky[52] using deuterium labelling as a probe on carbon hydroxylations has provided some evidence for the existence of a nonconcerted (free radical) mechanism. These workers reported a fairly large kinetic isotope effect KH/KD=11.5 (usually the KIE values are less than 2[22]), and found that the reaction was not completely stereospecific. The loss of stereochemistry was postulated to occur as a result of an inital hydrogen abstraction to form a carbon radical followed by some epimerization which would account for the loss of stereochemistry (Figure 9). If it is a radical species, then the addition of radical

Figure 9. Proposed Mechanism for Cytochrome P-450, Based on Isotope Studies.[52]



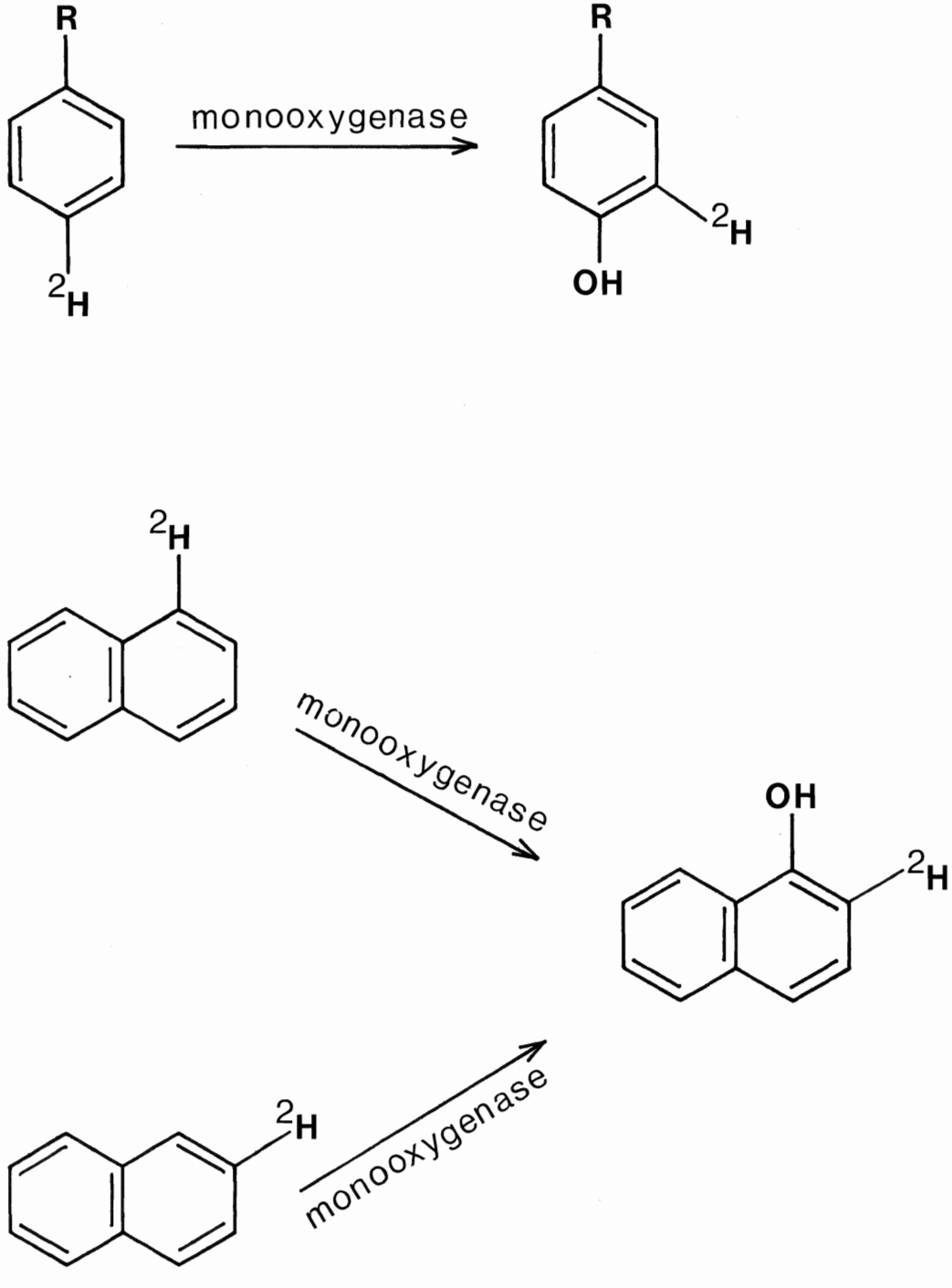
scavengers may prevent the oxidation process from occurring, if these scavengers are able to reach the active site. The addition of an hydroxyl radical scavenger caused a shutdown of the system, suggesting the involvement of an -OH radical.[8]

The similarity between the cytochrome P-450 active species and carbenes has been suggested, since both species undergo the insertion into carbon hydrogen bonds with retention of configuration. This similarity has led to the supposition that the active species in P-450 enzyme systems was in fact an oxene or neutral atomic oxygen, by analogy to carbenes.[54]

In addition to looking at the products from the various enzyme oxidations to discover the mechanism of cytochrome P-450, chemical reactions have also been investigated. Various chemical reagents have been known to produce oxidation products similar to those of the monooxygenase. The peracids fit this description, as does Fenton's reagent, chromylchloride and various tin-oxygen complexes.[55]

In attempting to find a suitable chemical species to act as a model for cytochrome P-450, the chemical reagent must fulfill certain requirements. One requirement is the formation of the "NIH shift."[56] In looking at the hydroxylation of an aromatic ring, with P-450, the hydrogen at the point of hydroxylation undergoes an intramolecular shift and turns up at an adjacent position in the product (Figure 10). This result was obtained by monitoring the tritium label in various aromatic substrates. This NIH shift seems

Figure 10. NlH Shift



to be characteristic of monooxygenase and those chemical systems which do show the same reactivity are considered suitable candidates for further study. They then become useful models with similar mechanistic features, for comparison with the enzyme. Fenton's reagent however, did not show any appreciable shift[57] but other reagents such as peroxytrifluoroacetic acid[58] and hypofluorous acid[59] were acceptable. Peracids are the best characterized system which are capable of delivering an electrophilic oxygen to a nucleophile and elicit the NIH shift in the oxidation of aromatic compounds.

The simplest mechanism for the peroxy acid oxidation[60] would involve either the generation or a transfer of ^{+}OH to form the cationoid intermediate by an electrophilic substitution reaction. From here the loss of the label or migration could occur. The driving force for the migration would be the formation of a positively charged species. This intermediate could then lose either a hydrogen or label to produce the aromatic compound (Figure 11).

The oxidation of the peroxy acid could also lead to an arene oxide being generated in the normal fashion of an epoxidation of a double bond, as seen in Figure 12. The arene oxide would then undergo cleavage to give the more stable carbonium iron which could form the common keto intermediate and then form the hydroxylated product.[61] This mechanism accounts for the NIH shift as deuterated arene oxides are known to rearrange to phenols with migration and retention of deuterium.[62] Rahimtula et al.[63] using an enzyme preparation of cumenehydroperoxide, NADPH, 0_2, and a flavoprotein NADPH

Figure 11. Mechanism for Oxidation by Peroxy Acids

(Peroxytrifluoroacetic acid)

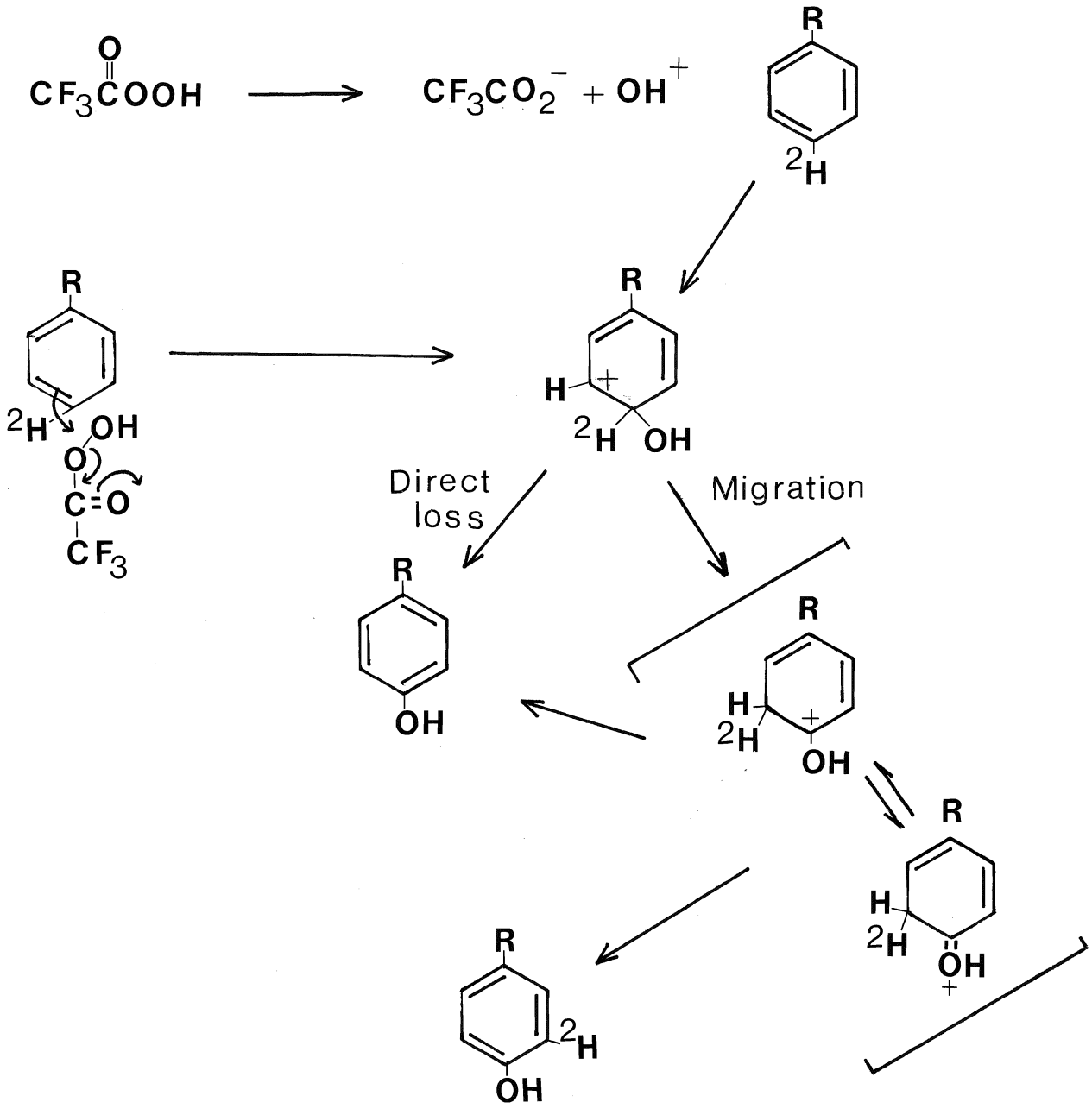
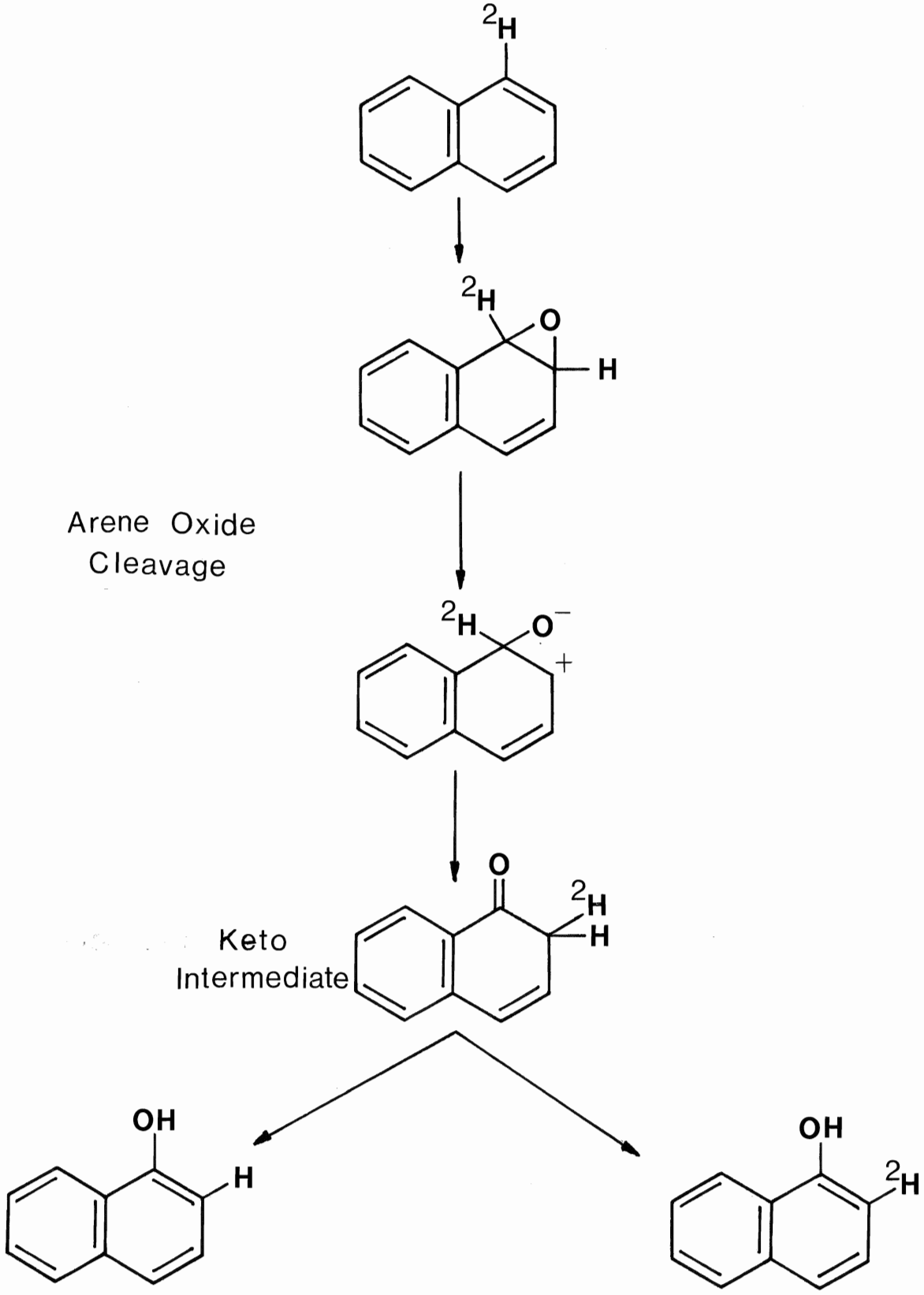


Figure 12. Arene Oxide Mechanism for Hydroxylation of 1-Deuterated naphthalene.



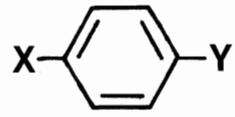
system has demonstrated that the same amount of tritium retention does occur, suggesting an arene oxide mechanism.

If the active oxygen species can not be isolated due to its rapid dissociation, then one alternate approach would be to investigate the electron density requirements of the enzyme towards the substrate. The work presented in this thesis (in part), involves the use of a micro-organism Mortierella isabellina in the oxidation of substituted sulphur compounds. The monooxygenases present in this species are capable of carrying out an effective oxidation of certain phenyl and benzyl sulphides.[64] The enzymic oxidation of sulphides to sulphides and to sulphones is a relatively common phenomena when dealing with enzymes from mammalian[65-67] or microbial sources.[68-70]

Early in this field of research, the prime concern was towards the stereospecificity of these reactions[68-70] and it has only been recently that endeavors have shifted towards a mechanistic investigation of the various oxidation reactions.[66,67,71,72]

It was Hammett[73] who proposed that the electronic influence of a substituent X might be assessed by studying reactions in a side chain Y of a benzene derivative and even though X and Y are separated physically, the electronic influence of X can be transmitted to the reaction site Y (Figure 13). The original work of Hammett[73,74] was based on the dissociation of substituted benzoic acids and introduced the Hammett equation based on his results (Equation 1).

Figure 13. Electronic Influence of X on a Side Chain Y.



$$\text{Log}\frac{K}{K_0} = \sigma\rho \qquad (1)$$

Sigma (σ) is the substituent constant and depends on the nature and position of the substituent, K represents the rate or equilibrium constant for the substituted reaction and Ko applies to the unsubstituted reaction. The value of rho(ρ) is the important variable as it indicates the effect that X induces on the reaction.

A normal polar displacement involves bond rupturing and bond formation at the transition state. Depending on which of the two is in control, the formation of a positive charge will be less than or greater than the ground state. Supposing the positive charge is greater at the transition state, then the stabilization of this species will be aided by an electron supplying substituent and the value of rho will be negative. Conversely, if the positive charge is less at the transition state, then an electron withdrawing substituent will stabilize the species and rho will be positive. There is also the possibility of a concerted mechanism and if this occurs the

electron density surrounding the reacting atom should not effect the reaction or its rate. There would be no charged intermediate and as such there would be no requirement for charge stabilization, therefore rho should be zero.

The procedure for obtaining the reaction constant (rho), consists of obtaining the values of K and Ko and ploting the logarithmicvalue of K/Ko versus sigma. The values for sigma, depending on the substituent and its position on the aromatic ring have already been calculated by Hammett.[73] This plot should produce a straight line with a slope of rho.

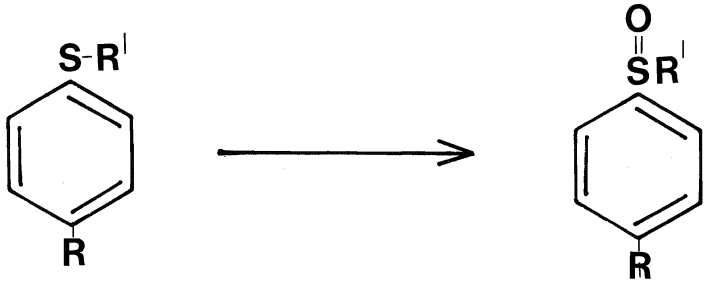
The benefits from such an equation would be enormous provided it produced results consistent with known reaction mechanisms, and that a linear relationship was obtained from the graph. All of this would only be possible if what is being monitored is the effect of the electron density surrounding the site at which the reaction is taking place and not a variation due to other factors such as steric size.

In the study of chemical oxidation reactions, Howe and Hiatt[75] investigated substituent effects on the oxidation of aniline to nitrobenzene employing metal catalysts and tert-butyl hydroperoxide. The authors found a good correlation of their data using the Hammett equation. The values of rho were - 1.42 and -1.97. The first number was obtained using standard sigma values and the second was from sigma (+) values. Similar results were obtained by Furia and Modena[76] with metal catalysts, peroxide and substituted compounds of thioanisole. Their value of rho (-.5) however was lower than the previous example. These same researchers then looked

into an acid catalyzed reaction of arylmethyl sulphide with hydrogen peroxide[77] and obtained a rho value of -1.13.

Bortolini et al.[78] undertook an investigation into the effect of metal catalysts with peroxides on various para substituted phenyl methyl sulphides. The authors reported that the substituent effect on the oxidation states of the sulphides followed a Hammett plot with rho equal to -0.7. These findings supported an electrophilic mechanism for the oxidation, as did the previous examples.

Other studies employing the Hammett equation have been directed towards enzymic systems and some of the rho values associated with these studies are contained in Table 2. May et al.[71] discovered that a copper containing monooxygenase dopamine β-hydroxylase, was capable of completing the sulphoxidation of substituted aryl sulphides (Equation 2). By varying the R group and measuring the oxidation rates, they concluded



(2)

Table 2

Hammett p values obtained for oxidation at sulphur and hydroxylation at carbon.

Oxidation at	Oxidizing species	p	Reference
S	Dopamine B-hydroxylase	-3.6	71
S	Rabbit liver microsomes	-0.16*	66
S	*M. isabellina*	-0.67	
S	Rabbit liver microsomes	-0.2 *	85
C	Dopamine B-hydroxylase	-0.4	71
C	*Pseudomonas putida*	-1.14	154
C	Rat liver microsomes/ Cumenehydroperoxide	-1.6	87

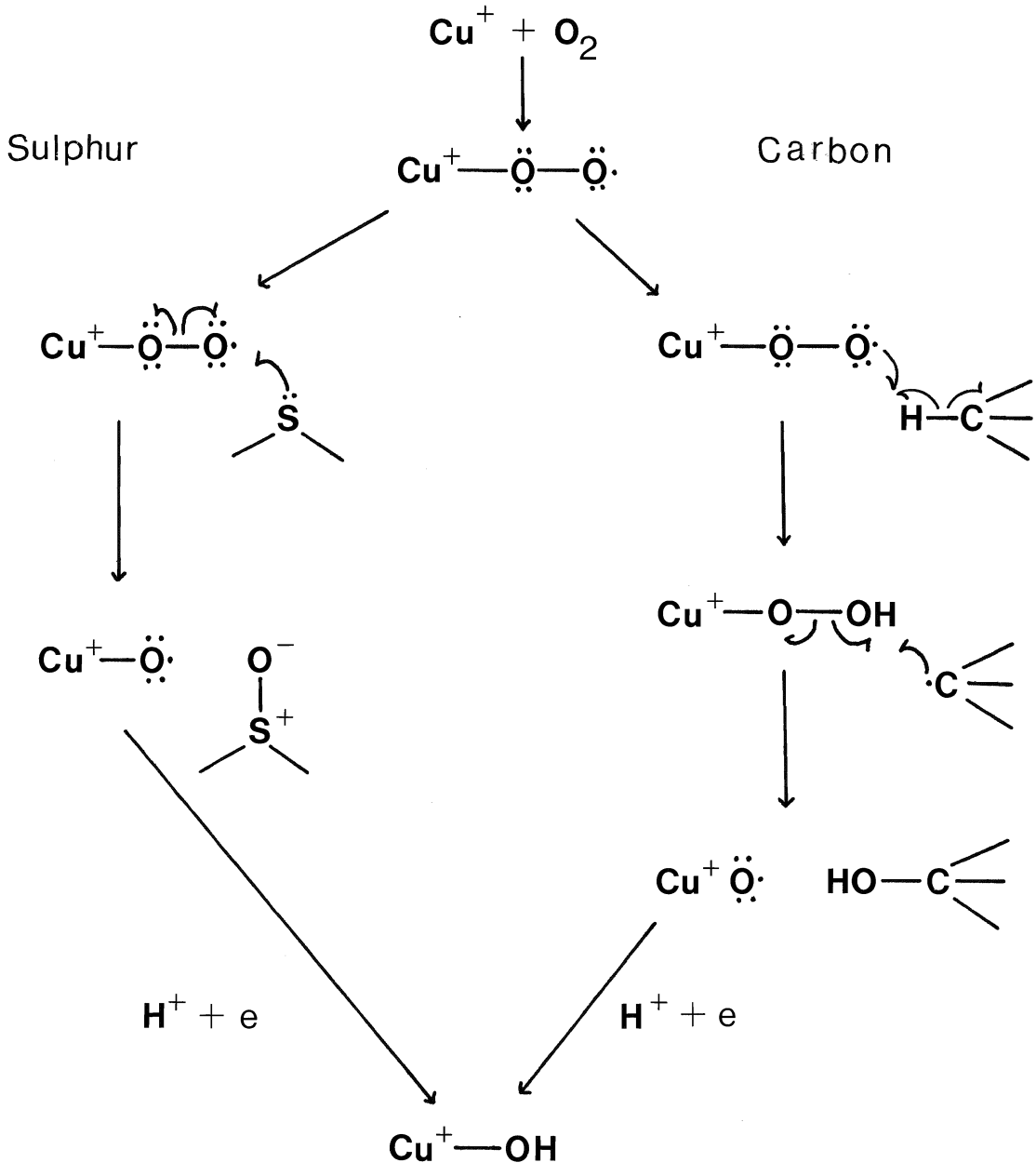
* P^+ value

that with this enzyme, the reaction proceeded at rates which produced a rho value of -3.6. Due to the low range of the sigma values used (the electron donating and withdrawing substituents were not varied to any great extent), this figure of -3.6 should be regarded as an approximate value. These same researchers looked into the hydroxylation of various dopamine derivatives and reported a rho value of -0.4.[71] Both these values were interpreted by the author in terms of a mechanism of enzymic oxidation similar to that of a hydrogen peroxide oxidation of sulphides. They failed however to comment on the difference in the value of the enzymic sulphoxidation versus that of the chemical reaction (-1.13)[77] but did propose a mechanism based on their two results which is contained in Figure 14.

The sulphoxidation reaction involves a nucleophilic attack of sulphur on an electron deficient oxygen intermediate and the hydroxylation reaction proceeds via a neutral radical intermediate. This neutral species would therefore cause very little charge separation at the transition state, which is consistent with their results.

The oxidation of thioanisole derivatives by an isoalloxazine hydroxylase was later attempted by Miller.[79] The Hammett plot produced a value of -1.67 which was consistent with similar experiments working with hydrogen peroxide.[77] This value suggests that the hydroperoxide mechanism involves[80] a nucleophilic attack of sulphur with little or no electron transfer. The researchers also suggested that this same

Figure 14. Proposed Mechanism for Dopamine B-hydroxylase.



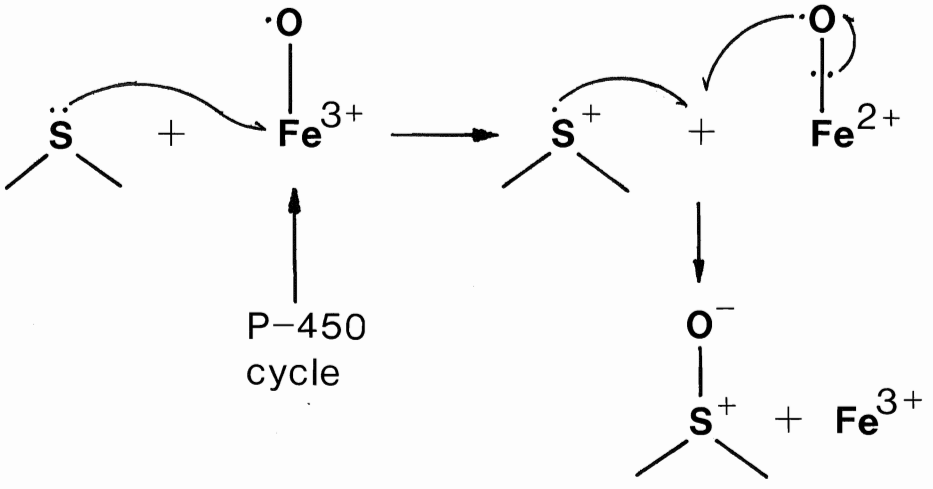
mechanism is present in FAD containing monooxygenases.

About the same time, Oae and co-workers were studying the sulphoxidation of substituted phenylmethyl sulphides using a cytochromime P-450 dependent enzyme preparation from rat liver microsomes.[66,67,72] Their kinetic results produced a rho value of -0.16, which is lower than the value obtained from the dopamine B-hydroxylase system. The authors proposed a mechanism based on their low value of rho, model reactions involving hydroxyl radical generating systems[81-85] and a study of one electron oxidation potentials of various sulphides. The rate determining step based upon studies involves the existence of an activated iron oxygen species (Figure 15).

Their work was later extended to the oxidation of substituted sulphoxides to sulphones,[85] employing the same enzyme preparation by the same research group. The authors state that with this system, the general progression from sulphoxide to sulphone is a generally accepted event but do not mention this in their previous work[66,67,72] with sulphides. Their results showed that when an electron donating species was present on the aromatic ring, the rate of oxidation increased producing a rho(+) values of -0.2. This work therefore reinforced their previous study dealing with an initial one electron transfer from sulphur to the iron-oxygen intermediate, to produce the activated oxenoid species which would then react with the substrate to produce the oxygenated species.

A chemical analogy can be drawn from the oxidation of

Figure 15. Proposed Mechanism for Sulphoxidation by Rat Liver Microsomes.



substituted diphenylsulphoxides by m-chloroperoxybenzoic acid.[86] The rho value which Cerniani and Modena [86] obtained was -0.54 and was explained as a typical electrophilic oxidation reaction.

One final piece of information regarding cytochrome P-450, pertaining to its role in substrate hydroxylation by peroxides[87] is a study of the hydroxylation of various substituted tolerene dirivatives. Instead of the usual oxygen donar, cumenehydroperoxide was replaced for dioxygen (Equation 3).

$$Rx + XOOH \longrightarrow ROH + XOH \qquad (3)$$

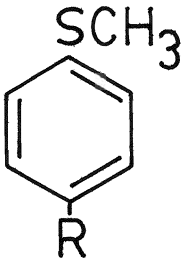
A conventional Hammett plot of the various hydroxylation rates resulted in a rho value of -1.16 which reflects the possibility of an abstraction of a hydrogen atom from toluene by an electrophilic active oxygen species. The existence of a hydrogen abstraction has previously been proposed due to various studies with isotope labels.[52] These results reflect the possibility of a similar mechanism in the NAD(P)H/0_2/ cytochrome P-450 system. The hydrogen peroxide could be produced at the active site by a 2 electron reduction of molecular oxygen. Another possibility could be the existence of a protein bound peracid or peramide. If peroxide is being produced in the cytochrome P-450 system, the addition of catalase should prevent the oxidation from occurring.

Since the oxidation of certain benzyl sulphides is possible with microorganisms,[68-70] what requirements in terms of electron density on the sulpher atom are required for the oxidation to occur? Is the electron density a critical factor

in these enzyme reactions and if so, what effect would increasing or decreasing the density occur to oxidation rate? The electron density surrounding a sulphur can be changed by using different substituents in the para position of an aromatic ring. The electron donating and electron withdrawing capacities of the various groups should be sufficient to change the electron cloud, through the polarizable π - electron system. These changes should therefore cause the rate of sulphoxide production to deviate from that species which is unsubstituted and should be of a significant magnitude in order to be monitored. By measuring the rate of sulphoxide production and using the Hammett equation, some mechanistic information regarding the monooxygenase present in Mortierella isabellina should be obtainable.

This study was undertaken to investigate the S-oxidation of a series of substituted methyl phenyl (1) and benzyl methyl sulphides (3) by the fungus Mortierella isabellina NRRL 1757. The fungus was chosen due to its ability to oxidize sulphides (1,3) to the corresponding sulphoxides (2,4) without any contamination of sulphoxide by sulphone.[70] This is a novel reaction as most microbial systems that perform this oxidation, also produce some sulphone.[88]

The importance of such a property by a fungus does not become apparent until one attempts to obtain the various rates of sulphoxidation from the sulphides. The presence of sulphone could interfere producing inconsistent results.



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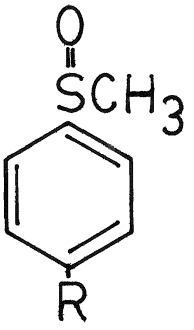

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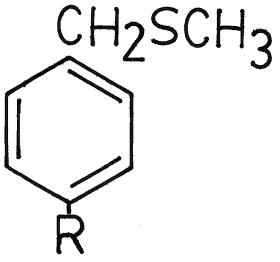

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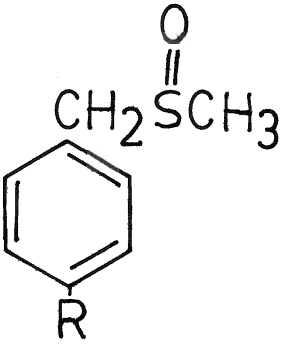
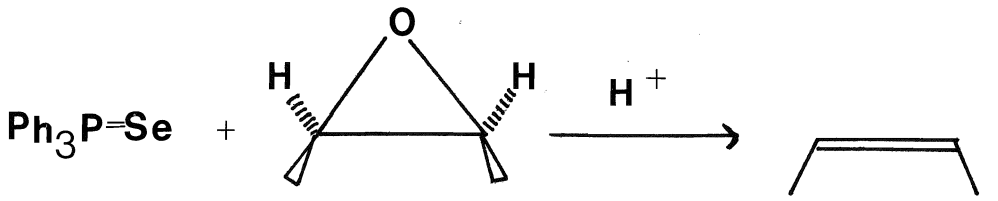


4

An Investigation of the Biotransformation of Organic Selenides by Fungi

Introduction II

The application of selenium in organic synthesis has until recently been of minor importance. The beginning of research into its practical uses did not appear until 1973 with the discovery of several new reagents, where selenium was attached to the organic structure. One of the first modern synthetic reactions was the conversion of epoxides into olefins as reported by Clive and Denyer[89] in 1973. It seems that in the presence of triphenylphosphine selenide and acid, various epoxides can be converted to the corresponding olefins (equation 4).



(4)

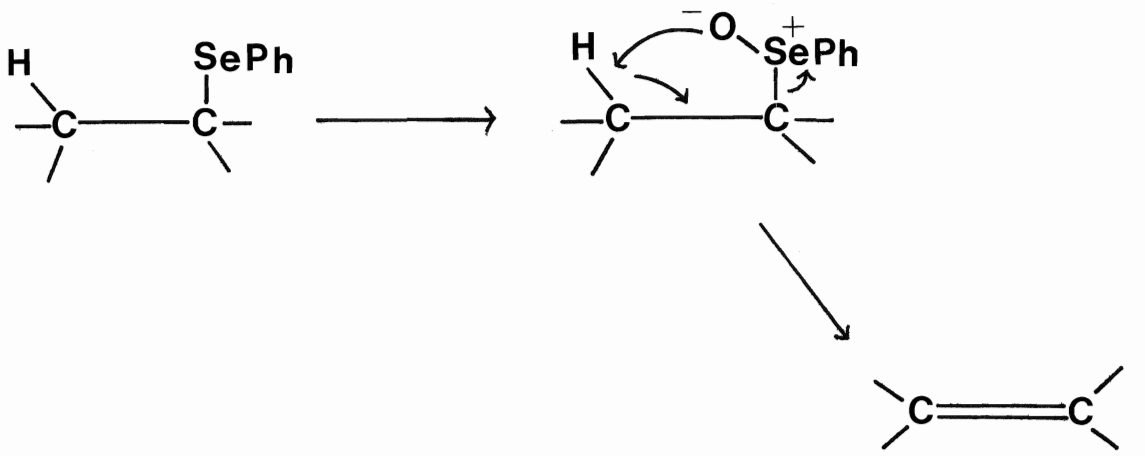
The usefulness of selenium (and also sulphur) is derived from the ease at which the organoselenium compounds can be prepared and the ease at which these compounds can effect functional group interconversions. The only drawback to these

reactions is cost of the reagents and the toxicity associated in working with them.[90]

It seems that selenium forms weaker σ bonds than sulphur and because of this, many reactions which do involve C-Se, or O-Se cleavage are more rapid than the analogous sulphur cleavage. In this respect, the syn elimination occurs about 1000 times as fast as the analogous sulphur reactions[91] and the [2,3]-sigmatropic rearrangements occur at lower temperatures.[92]

This relatively new topic of organoselenium chemistry encompasses a large volume of material. This project is primarily concerned with the oxidation of selenides and the fragmentation patterns that occur due to the addition of an oxygen atom. Therefore only those reactions that involve these processes will be discussed in the following sections.

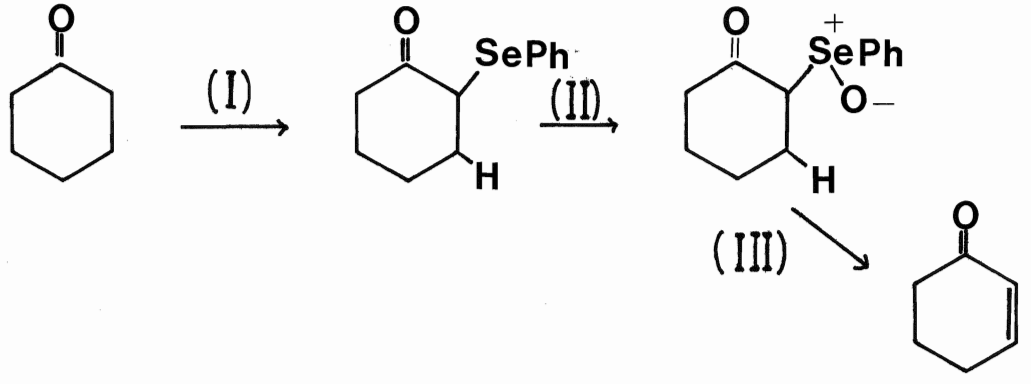
One of the major applications of organoselenium chemistry came about by three independent groups.[93-95] This reaction was however first reported by Huget et al.[96] and Mundy and Whitehouse.[91] They discovered that the introduction of an oxygen molecule into the selenium compound (selenoxide formation) resulted in a fragmentation of the species by a syn elimination,[94] this fragmentation occurring rapidly and efficiently at room temperature. The utility of this reaction (equation 5) is the ability to introduce unsaturation into a carbon sigma bond system using a mild procedure. More specifically, a standard method for making α,β-unsaturated carbonyl compounds (equation 6).



(5)

The reaction has two advantages over the analogous sulphoxide elimination, in that it occurs at a lower temperature (~ 100°C lower) and also that the oxidation is normally carried out with inexpensive oxidizing reagents. In trying to synthesize the corresponding sulphoxide, $NaIO_4$ is often used as H_2O_2 and other standard oxidizing agents often result in the production of sulphone.

The procedure for making the α,β-unsaturated carbonyl compounds can be completed in three steps:

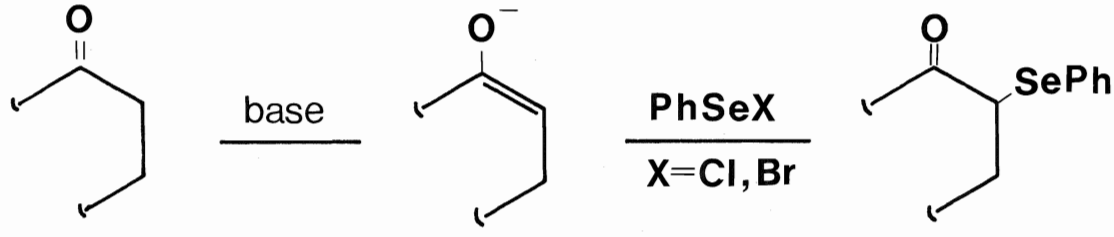


(6)

(I) The introduction of the benzeneseleno-group (PhSe), to the carbonyl usually by way of a lithiumenolate at low temperature and then reacted with PhSe-SePh, PhSeCl or PhSeBr (equation 7).

(II) The oxidation of the selenide to the corresponding selenoxide using a standard oxidizing reagent and

(III) The fragmentation of the selenoxide to produce the olefin.

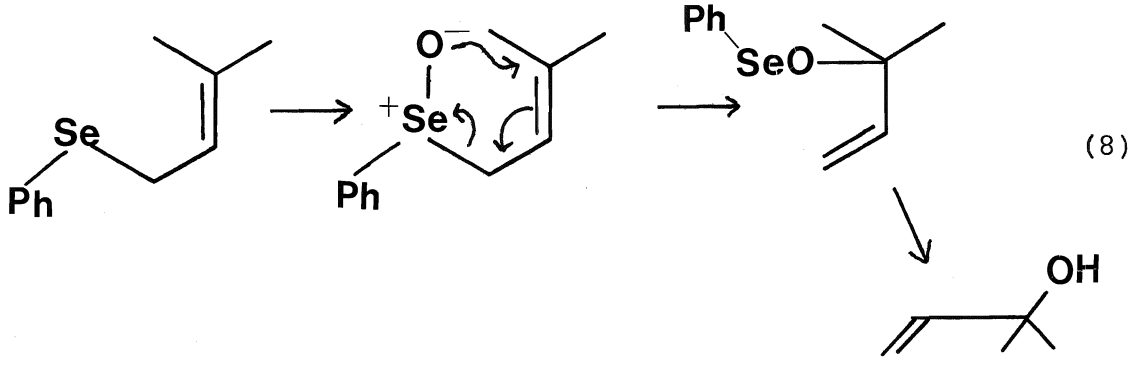


(7)

The fragmentation normally occurs only when there is a β-hydrogen which can be abstracted by the selenoxide. This method has been tested with some success on ketones,[97] esters,[98] lactones,[99] nitriles,[100] aldehydes[101] and lactams.[102] With the realization of such a successful procedure further research was then devoted towards the attachment of the phenylseleno-group (PhSe) to the organic molecule in order to carry out the selenoxide fragmentation. An extensive summary of such work is contained in a review article by Clive.[103]

Continuing research towards useful synthetic reactions

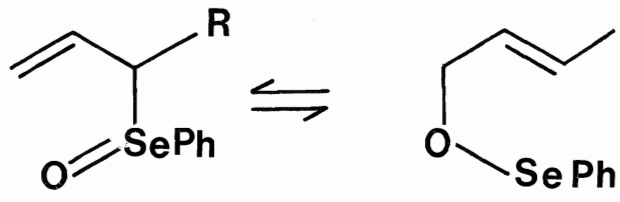
containing selenium resulted in the discovery of the decomposition of allyl and related selenoxides to allylic alcohols[104] as seen in equation 8.



(8)

The formation of alcohol is a result of two consecutive and spontaneous processes: a [2, 3]-sigmatropic rearrangement of the selenoxide and the subsequent hydroxylsis of the selenic ester. In comparison, for allylic sulphoxides, the corresponding sigmatropic rearrangement is not a spontaneous reaction.[105] Thus the use of selenium in the reaction facilitates transformation to the alcohol under mild conditions.

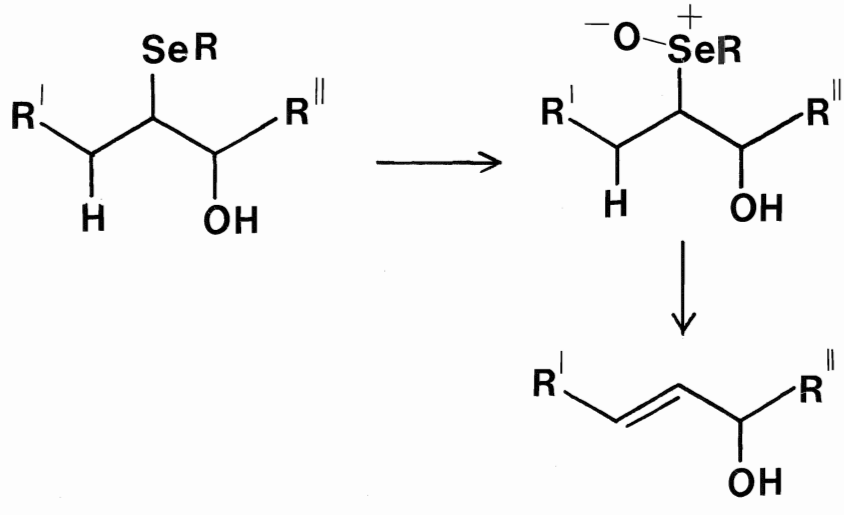
The equilibrium condition of this species is an important aspect when dealing with this type of selenoxide compound. The equilibrium lies principally to the right of equation 9, in contrast to that of the sulphoxides.[106] Therefore the use of selenium in this case, results in the formation of the alcohol.



(9)

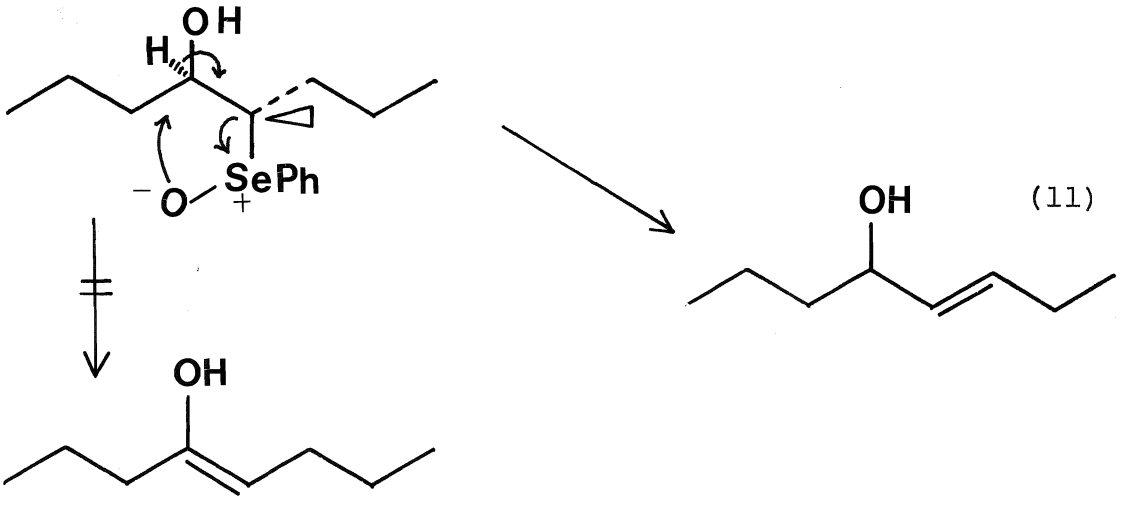
Evidently, this [2, 3]-sigmatropic rearrangement is faster than the corresponding selenoxide fragmentation[107] and as such the end result is an allylic alcohol rather than an olefin.

One final reaction which is of some importance involving the usual selenoxide fragmentation, is the formation of an allylic alcohol, similar to the previous example. In this case the starting compound is a β-hydroxylalkyl selenide and the oxidation of this selenide produces the alcohol (equation 10) after fragmentation.[108]



(10)

From the experiments present in the literature it seems that three generalities can be drawn regarding olefin formation. Firstly, if there is conformational freedom present, then any disubstituted olefins forming do so with trans-stereochemistry. The exception is when dealing with α , β unsaturated nitriles which give both cis and trans isomers. Secondly, if there are adjacent oxygen substituents present then the syn elimination is strictly away from the oxygen bearing carbon to produce the allylic alcohol (equation 11). Finally, when attempting to produce a terminal olefin using this oxidation-fragmentation procedure, the yield is often quite low unless specially substituted aryl selenides are used or the selenoxide is made to collapse by heating in a basic solution.[103]



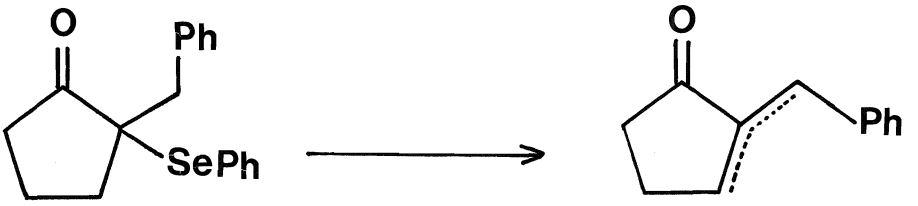
(11)

As with any synthetic route, side reactions are always a possibility. The same is true for the syn elimination, where the formation of PhSeOH and its disproportionation

products react with olefins under acidic conditions to produce β-hydroxyselenides.[109] It seems that even the solvent employed can also alter the reaction slightly as protic solvents reduce the rate of syn elimination. Also in reactions that produce methyl alkyl selenoxides, this species catalytically decomposes H_2O_2 thereby preventing olefin formation. However most of these problems with PhSeOH can be prevented if the elimination is carried out by a brief thermolysis in the presence of alkylamine. These syn elimination reactions do not appear to be reversible as do the sulphoxide reactions.[110]

Early studies dealing with the oxidation of selenides[111] and the decomposition of these species did not reveal their true potential in the synthesis for a number of years. An important aspect of this reaction became apparent when Jones, Mundy and Whitehouse[91] were studying the oxidation of 6-β-phenylselenide-5-∝-cholestane with ozone. The researchers discovered that this oxidation led to a mixture of the R and S selenoxide, which was separated, and found to decompose rapidly in hexane to give only 5-∝-cholest-6 ene. Further studies on the oxidation of selenides[112] eventually led to the discovery that selenoxides could be employed to produce olefins. They were actually synthetically equivalent to olefins and further studies were needed to pinpoint their practical importance in organic synthesis. Although in non-cyclic systems the fragmentation of the various selenoxides almost always produces the olefin of (E) geometry, problems do however arise when trisubstituted species are involved and

also when there is more than one position that can lose a proton[113] during the fragmentation process (equation 12).

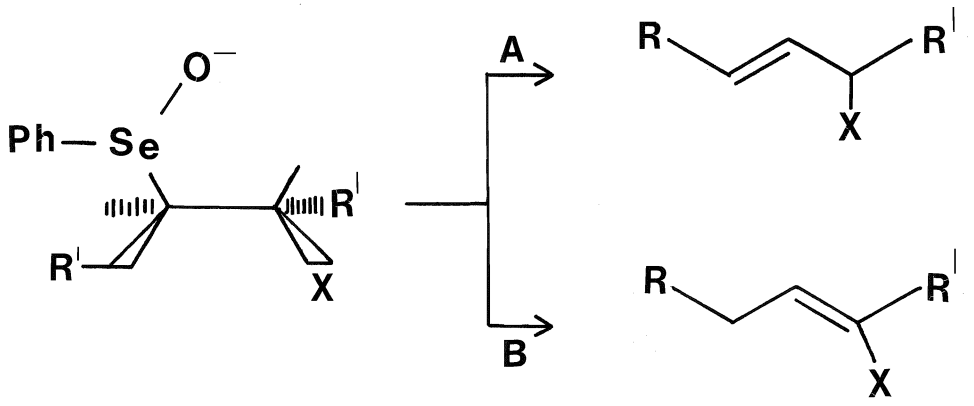


(12)

Also, if the syn conformation is not possible or if the resulting strain of double bonds produced by the fragmentation is high, then the reaction does not occur.

In most of the cyclic systems, the geometry of the systems ensures that a cis-olefin is produced. However it appears that in most of the reactions, the actual bond geometry is determined by the conformational effects of the ring.[103]

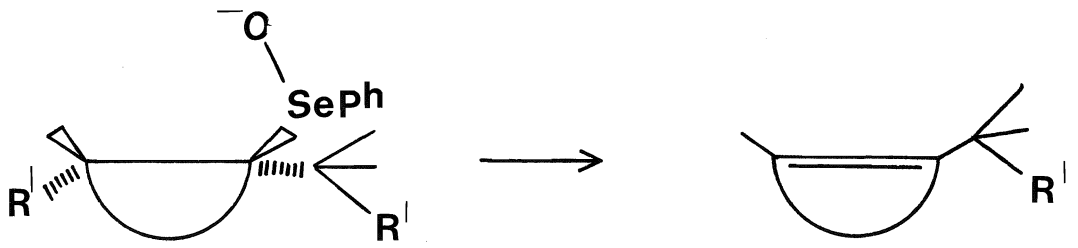
The regiochemistry of this fragmentation pattern can be explained in situations similar to that of equation 13. In most cases the route to product A is followed even when the double bond of the alternate product is in conjugation with a phenyl group.[103]



(13)

In the case where X is a chlorine either product is possible.

In cyclic compounds where there is a possibility of the removal of a proton from the ring or the exocyclic chain, in general the loss of the proton occurs from the ring (equation 14).



(14)

This is possibly due to its fixed geometry thereby lowering the entropy factors involved in obtaining a <u>syn</u> transition state.

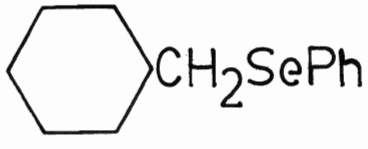
In all of these reactions the ability to introduce a

selenide group (benzeneselenol) into the starting material and oxidize the compound is of prime importance. The oxidation of the various selenides is made possible through the use of four types of oxidants: NaIO4 (expensive), peracids (moderate cost), O_3 (low temperature reactions) and H_2O_2 which is relatively easy to obtain. With most of these oxidants, the unstable selenoxides are produced at low temperature and the solution is then allowed to warm to room temperature. While the solution is equilibrating to room temperature, the selenoxide usually decomposes in situ to produce the desired products.

The occurrance of diastereomeric selenoxides, of a stable configuration is noted, in the literature, in a small number of instances.[91,114,115] The synthesis of chiral selenoxides however is not normally possible through the usual methods of asymmetric synthesis e.g. for the oxidation with a chiral acid. If chiral selenoxides were readily available, then the potential application of these species in equations 6, 8, and 10 becomes apparent. In both cases it may be possible to transfer this chirality from selenium to the carbon, resulting in the production of olefins of defined geometry. The problem then becomes how to introduce chirality into the substrates to produce optically active selenoxides. The possibility of producing chiral selenoxides was attempted by the microbial oxidation of selenides in cases where the carbon beta to selenium was a methylene group.

Previous work pertaining to the microbial oxidation of

sulphides using actively growing or resting cultures of fungi has provided a convenient and relatively high yield method for producing sulphoxides of high enantromeric purity.[68-70] It is therefore the aim of this study to attempt the production of chiral selenoxides employing four fungi which are capable of performing the asymmetric oxidation of alkyl aryl-sulfides. For this study, Aspergillus niger, Aspergillus foetidus, Helminthosporium sp., and Mortierella isabellina were employed to attempt this oxidation. With Mortierella isabellina, a more extensive study was carried out due to its previous involvement with the (efficient asymmetric) oxidation of various sulphides.[64] For this study, the following compounds were required for the incubation with the various fungi: cyclohexylmethyl phenyl selenide 5, cyclohexylmethyl phenyl sulphide 6, crotyl phenyl selenide 7, crotyl phenyl sulphide 8, phenylmethyl selenide 9.



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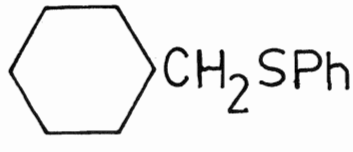

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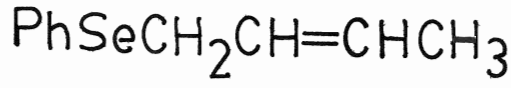

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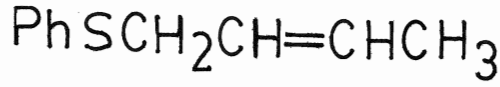

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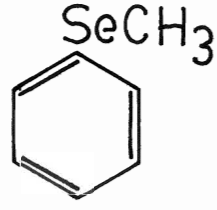


9

An Investigation into the Hydroxylation of Ethylbenzene and p-Ethyltoluene by *Mortierella isabellina* NRRL 1757 and their Enantiomeric Purity

Introduction III

Apart from the oxidation of various sulphur and selenium derivatives, the ability of microorganisms and mammals to oxidize nonpolar aromatic hydrocarbons has been recognized and studied extensively. The substrates for this type of oxidation varies from the simple hydrocarbons benzene to water insoluble polycyclic hydrocarbons.[116] Some of these reactions can involve a simple hydroxylation of the aromatic ring,[117] whereas in some instances this hydroxylation can occur at both sidechain and aromatic ring.[118] Kaubisch, Daly and Jerina[117] investigated the hydroxylation of alkyl benzenes by rat liver microsomes and attempted to explain their findings in terms of an arene oxide intermediate. Various deuteriated substrates were employed and in some cases a "NIH" shift occurred (Figure 10). Similar results were reported by other researchers.[119]

Apart from the oxidation of the arcmatic regions, transformations of the alkyl sidechain are also possible. Webley et al.[120] looked into the oxidation of 1-phenyl-dodecane by Nocardia opaca strain T_{16}, and strain P_2. The T_{16} strain hydroxylated the aromatic ring and also transformed the sidecahin to phenylacetic acid. The P_2 strain however, simply oxidized the substrate to 1-phenylacetic acid. Another strain of Norcardia oxidized ethylbenzene and butylbenzene to phenylacetic acid.[121] Most of these transformations occur via the β-oxidation of the alkyl substituent to produce the

various substituted acetic acids which were resistant to further metabolism.[121,88]

In some instances, the initial reaction of these oxidation processes often involves the formation of 1,2-dihydrodiols[122] (equation 15a) where the elements of H_2O_2 have been added across one of the aromatic double bonds.

Davey and Gibson[118] looked into the bacterial metabolism of para and meta xylene and discovered that various strains of Pseudomonas were capable of carrying out successive single step oxidations of one methyl group to form para and metatoluic acid (Figure 16) and implied that 4-methyl and 3-methylcatechol were intermediates in the further degradation of these xylenes. The authors go on to propose that from these studies a dihydrodiol intermediate could be involved with the formation of these methylcatechols.

The occurrence of 1,2-dihydrodiols as intermediates in the metabolism of aromatic compounds has been known as early as 1936.[123] In studying the mammalian oxidation of naphthalene, for example, the hydrocarbon is believed to be converted to an arene oxide by the cytochrome P-450 enzyme system[124] and from here further metabolism includes conversion to naphthols (nonenzymic reaction), reactions with nucleophiles and the formation to trans 1,2-dihydroxy-1,2-dihydronaphthalene (equation 15). The discovery of trans diols in the metabolism of hydrocarbons suggested that possibly bacteria and fungi could also oxidize hydrocarbons via the same route. It seems that the bacteria Pseudomonas putida[125] is capable of oxidizing naphthalene to a diol but in this case it is the cis-1,2-

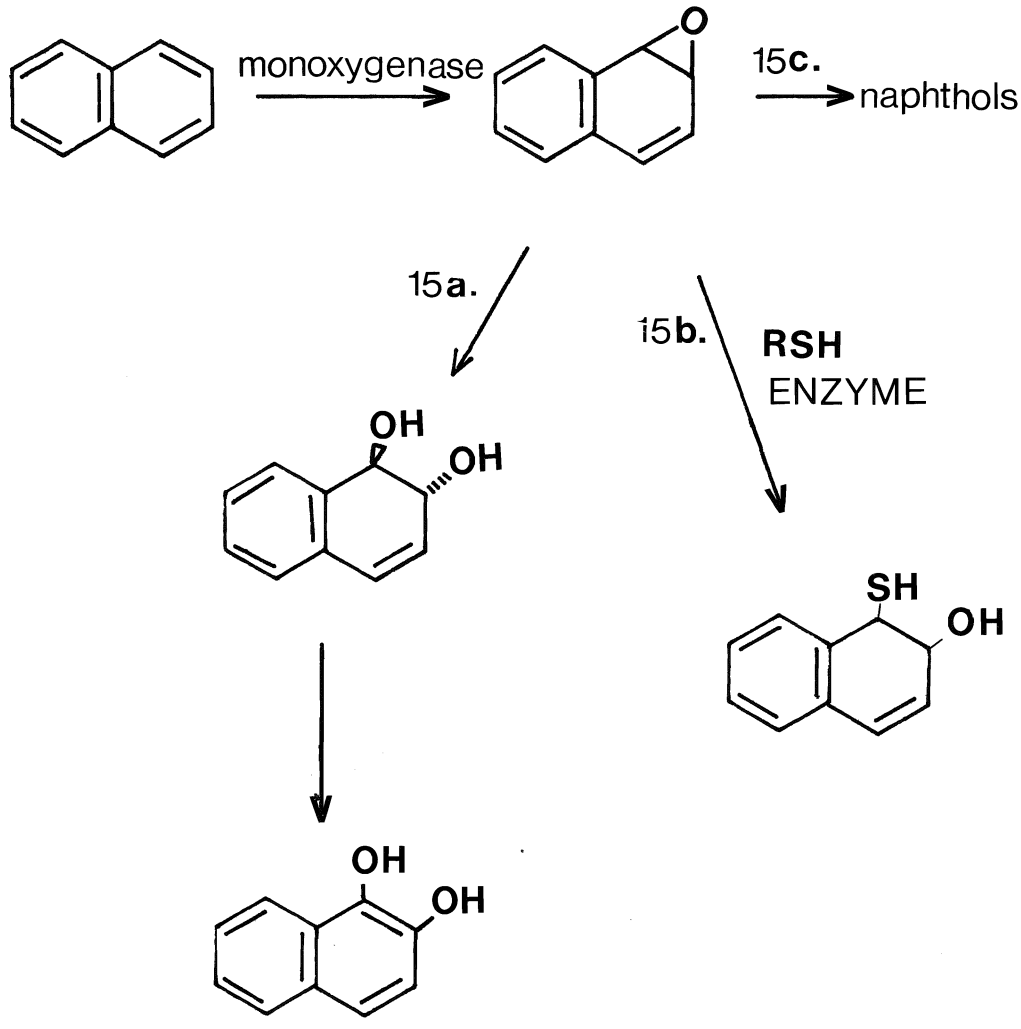
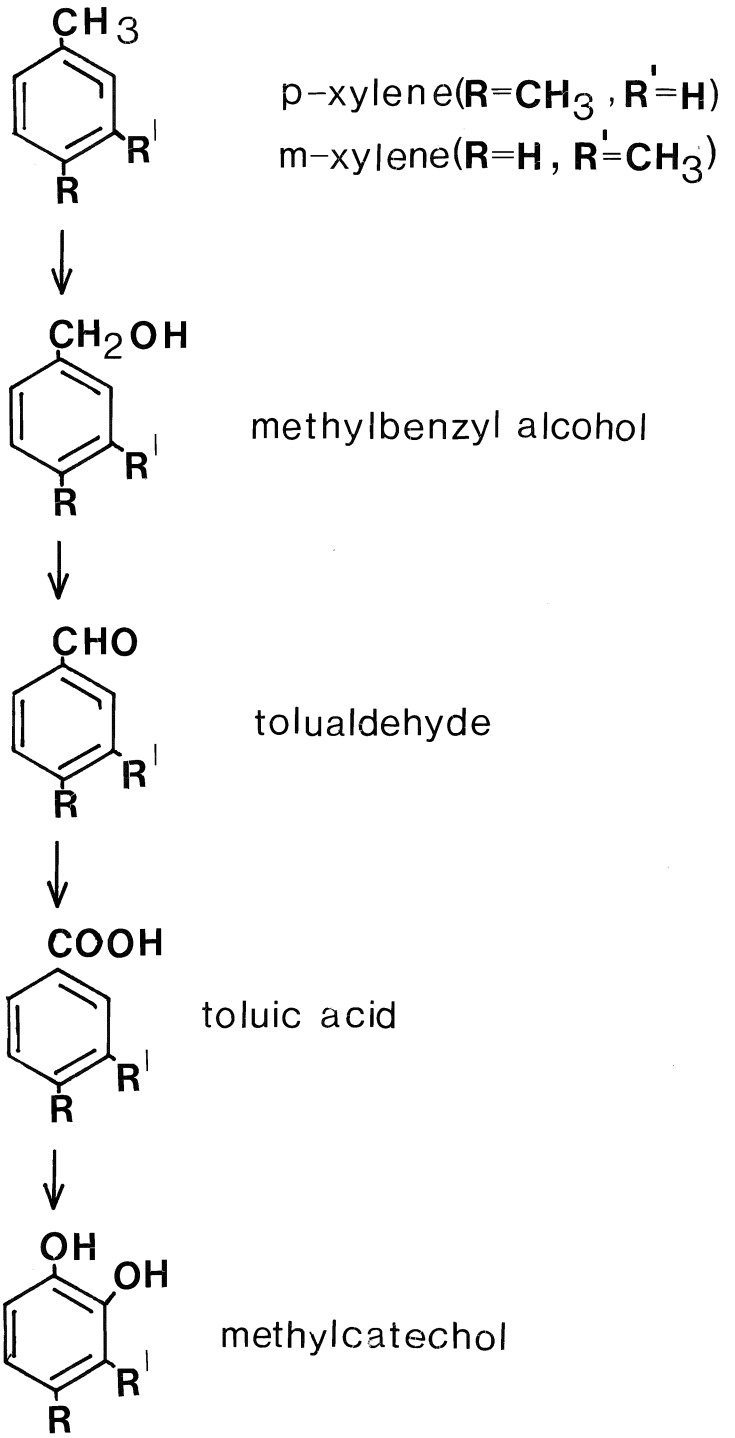

monoxygenase
O
15c.
naphthols
15a.
15b.
RSH
ENZYME
OH
OH
SH
OH
OH
OH

Figure 16. Bacterial Metabolism of para-and meta-Xylene.



dihydroxy-1,2-dihydronaphthalene. This cis isomer then reacts with NAD^+ to form the 1,2-dihydroxynaphthalene. From here the hydroxylated hydrocarbon is degraded further, to the extent that these bacteria are capable of utilizing naphthalene as its sole carbon source. This bacterial species has been studied extensively in an attempt to discover the active oxygen species in the cytochrome P-450 monooxygenase enzyme system. Possibly by studying the oxidation reactions of the various hydrocarbons further information can be obtained for the mechanism.

Recent studies have been involved with looking at the spectral changes associated with the addition of hydrocarbon substances to oxidized cytochrome P-450.[126] It seems that in monitoring these differences there are various types of ligand-induced different spectra of which type I, and modified type II are examples.

The type I change is associated with those substrates of the mixed function oxidase system. The spectral changes result in the production of a peak at 385 nm and a trough at 420 nm. By increasing the amount of substrate, this produces a proportional increase in the magnitude of the spectrum. These compounds which bring about the type I spectrum are thought to do so by binding to the active site of the enzyme.

The modified type II spectral change is produced by alcohols, ketones and certain drugs and is characterized by a peak at 420 nm and a trough at 385 nm. The modified type II spectrum is the mirror image of the type I.

The addition of ethylbenzene to the phenobarbital treated rats caused the type I spectral change[127] and also the

same spectra were obtained when guinea pig adrenal microsomes were used.[128] Research by Toftgard et al.[129] concluded that the addition of this substrate caused an increase in the concentration of cytochrome P-450 and NADPH reductase in rats.

All of these spectral differences infer that the hydrocarbon binds to the active site of cytochrome P-450. Both the in vivo and in vitro oxidation of this substrate by mammals has received some attention in the literature. An injection of this hydrocarbon into rabbits resulted in the urinary excretion of both isomers of 1-phenylethanol.[130] Similar experiments were conducted with rats in which 90.3% R(+)-1-phenylethanol and 9.7% of the S(-) isomer was isolated.[131] Further research with rat liver microsomes produced 1-phenylethanol of the S configuration (80-85%)[132] and another study concluded similarly that S(-) 1-phenylethanol was the product.[133] Working with microsomal preparations (rat liver), the oxidation of ethylbenzene proceeded with the incorporation of atmospheric oxygen[134] and the reaction occurred with the direct replacement of one of the ∝ hydrogens by oxygen. The oxidation occurred with retention of stereochemistry and the pretreatment with phenobarbitol lowered the stereospecificity of the reaction.[131,134]

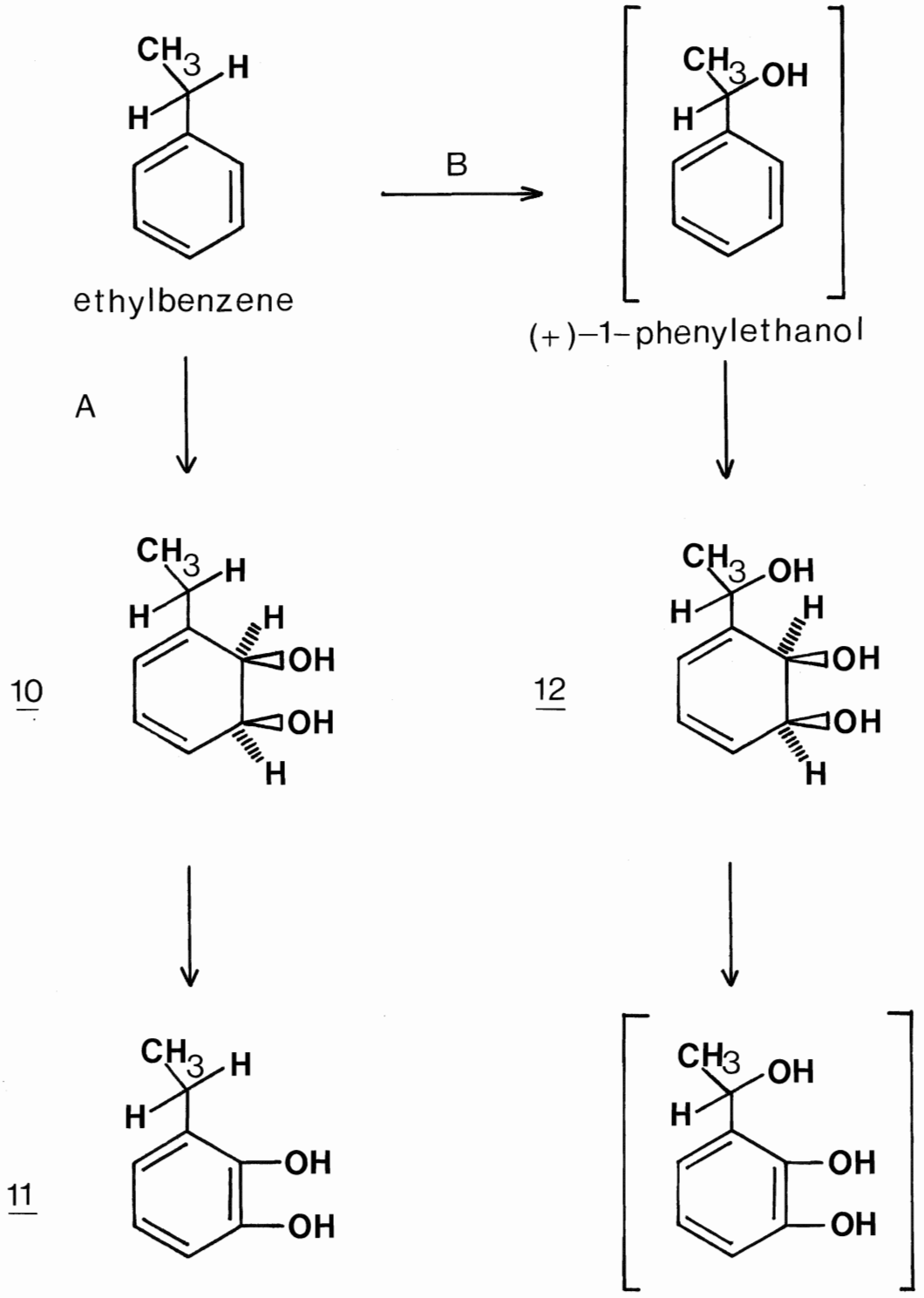
Apart from the formation of 1-phenylethanol, the oxidation of ethylbenzene by rat liver microsomes also produced 4-hydroxyethylbenzene and 2-hydroxyethylbenzene.[117] It appears, from the literature, that both rat liver microsomes and guinea pig microsomes are capable of oxidizing ethylbenzene to any one of three different substances. The reactions with micro-

organisms do not, however, look as promising. There are very few examples where a microorganism is capable of metabolizing ethylbenzene as its sole carbon source. The bacteria Pseudomonas putida[135] can however, metabolize ethylbenzene via pathway A or B (Figure 17). Pathway B, however, is believed to be of minor importance. Compounds 10, 11 and 12 were isolated from the reaction medium and both of the compounds in brackets (phenylethanol and catechol) were believed to exist but were not isolated from the medium. The authors therefore proposed their existence based on the isolation of the other three compounds.

Previous work with M isabellina has shown that it is capable of carrying out the conversion of thioethers to sulfoxide[64,70] but this by itself is a common occurrance.[65-70] However, the monooxygenase system in M. isabellina responsible for this oxidation produces the optically active R(+) isomer (100% in a 60% yield).[70] The other isomer S(-) can be obtained from Helminthosporium sp.[70] Since Mortierella isabellina is capable of producing an optically active S-oxide, if the same monooxygenase system is carrying out the hydroxylation reaction, then it may be proposed that an optical active alcohol is produced. Once the hydroxylated product is isolated from the medium, the enantiomeric purity of the product can be assayed with the use of a chiral shift reagent.

Since the discovery of NMR lanthanide shift reagents,[136,137] these have become an important tool to the organic and natural product chemist in a short period of time. Due to its

Figure 17. Oxidation of Ethylbenzene (Pseudomonas putida.)



importance, a large variety of these shift reagents have been synthesized and are now available for practical purposes. With these reagents, it has been shown that signals of enantiotopic groups on all of the chiral molecules containing the correct functionality can be distinguished under certain conditions.[138] These chiral shift reagents are therefore capable of shifting the resonances of many enantiomeric organic substances to varying extents. This technique is therefore simpler than previous methods for determining the enantomeric purity of an organic compound.[139] This usually involved the derivatization of a chiral compound with an optically pure reagent and then determining the diastereomeric composition of the derivative by NMR analysis. The problem with this procedure is the small shift differences in diastereotopic atoms.

The most commonly used shift reagents are the lanthanide β-diketonates[140] and in most cases the lanthanide is the Europium III (Figure 18). These reagents function by acting as a lewis acid, forming a complex with the substrate in question, which acts as a nucleophile. The shifts which are induced are due to pseudo-contact or a dipolar interaction between the shift reagent and substrate. The result of this interaction, "the shifted spectra " are the averaged environments of the nuclei in the complexed and uncomplexed nucleophiles. The principle criteria for these shift reagents is; in order for an organic substrate to be influenced by a shift reagent, it must contain one or more atoms which are capable of coordination to the lanthanide.

The important practical application of these chiral NMR shift reagents is in determining the enantiomeric purity of a compound, they are also useful in following the resolution of racemic mixtures or the racemization of enantiomers. Since this method does not require the possession of both optically pure species, or even one in the pure form, the determination of the enantiomeric purity can be obtained by a single NMR scan of the chelated species, without the need of performing the tedious separation and identification of each of the isomers. The whole process takes, on the average, half an hour.

There are, however, certain problems with this technique, for instance the actual choice of the shift reagent can be one, as each reagent causes a different shift of the signal ($\Delta\Delta\delta$). In some cases one chiral reagent would produce a cleaner separation of the signal in question.[141] Other problems are peak broadening[142] (which is a concentration related problem), temperature factors,[142] the NMR solvent[141] and also steric problems. Usually with the proper shift reagent and the correct conditions for the NMR study, the shift ($\Delta\Delta\delta$) between at least one set of enantrotopic protons is sufficiently large, so that their enantiomeric compositions can be determined from their relative peak areas.

In determining the enantiomeric purity of phenols, Goering et al[143] reported that the derivatization of the alcohol group to an acetate group increases the $\Delta\Delta\delta$ value. Another possibility of increasing the shift magnitudes would

be to substitute a deuterium for a hydrogen vicinal to the OH group.[141] The deuterium would possibly undergo greater hydrogen bonding and increase the complexing to the shift reagent.

The procedure for using these chiral shift reagents consists of the addition of the shift reagent to the organic compound dissolved in the appropriate NMR solvent in a NMR tube. The most frequently used solvents are chloroform and carbontetrachloride.[141] Since NMR is used to monitor the chelation, only 25 mg or less of the substrate is required. If the scanned spectrum does not show any appreciable change then more of the shift reagent may be added to the NMR tube. There is, however, a point at which the continued addition of the chiral shift reagent does not cause a greater separation of the signals. This is probably due to line broadening and the saturation of the organic substrate with the shift reagent.

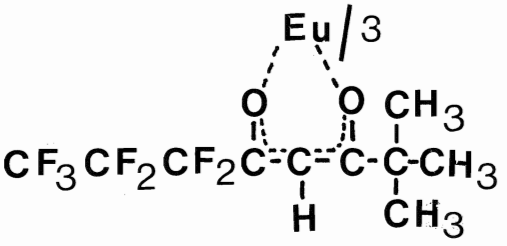
Once the correct concentration is obtained, (molar ratio), then this should produce a spectrum which contains two signals from which the enantiomeric purity can be obtained. This can be obtained by a simple integration of the peaks.

The choice of the correct shift reagent for an organic substrate can only be obtained through process of trial and error. It seems, however, that the superior reagents are those that contain fluorine, as it increases their lewis acidity. Often it is the fluorinated species which provide suitable results that could not have been obtained otherwise.[141] The most widely used shift reagent and the one which is

normally recommended to try first is $Eu(fod)_3$ (Figure 18).

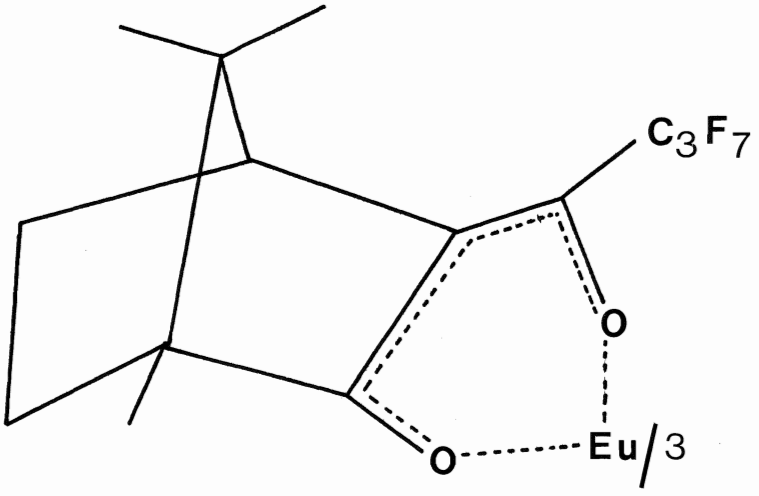
For this study the monooxygenases present in Mortierella isabellina will be used to oxidize both ethylbenzene and para-ethyltoluene. Using a chiral shift reagent, the enantomeric purity of both of these compounds will be determined. The chiral shift reagent which will be used in this study is $Eu(hfbc)_3$ (Figure 19), tris-(3-heptafluorobutyl-d-camphorato)-europium (lll).

Figure 18. The Structure of $Eu(fod)_3$.



Eu(fod)$_3$ = tris(6,6,7,7,8,8,8–heptafluoro–2,2–dimethyl–3,5–octanedione)europium(III)

Figure 19. The Structure of $Eu(hfbc)_3$, the Chiral Shift Reagent Employed in this study.



Eu(hfbc)$_3$ = tris(3–heptafluorobutyl–d–camphorato)–europium(III)

The Mechanism of Sulphide Oxidation by *Mortierella isabellina* NRRL 1757

Experimental I

Apparatus, Materials and Methods.

Ultraviolet spectra were obtained on a Hitachi Perkin-Elmer 124 spectrophotometer with HPLC methanol as solvent and reference. Proton NMR were obtained at 60 MHz with a Varian A-60 or Bruker WP-60 or At 80 MHz on a Bruker WP-80 CW using $CDCl_3$ as solvent and TMS as internal standard, and ^{13}C NMR at 15.18 MHz with a Bruker WP-60 using $CDCl_3$ as solvent and TMS as internal standard. For the chiral shift reagent experiments, spectroscopic grade benzene was employed as an internal standard. Mass spectra were obtained with an AEI MS 30 double beam spectrometer interfaced to a Kratos DS 55 data system: the isotope composition of the various samples were obtained by repeated data accumulation over the appropriate ion regions and suitable corrections were applied for the presence of natural isotope abundancies; these values were accurate to $\pm$0.5%. The fast atom bombardment (FAB) spectra were obtained from a KRATOS FAB source (Ion Tech. Ltd. atom gun), operating at an energy of 6 KeV and using a glycerol matrix for the sample. Column chromatography was performed on silica gel (60-200 mesh), and thin layer chromatography on Merck silica gel 60 F-254 (0.2 mm). Radioactivity was assayed by liquid scintillation counting using a Searle Deta 300 counter. Samples were dissolved in methanol and dispersed in Aquasol.

In order to obtain accurate measurements, duplicate samples were run under similar conditions of quenching. The selenium analysis was acquired using a Beckman Spectraspan V DC plasma emission spectrometer on samples which were diluted using distilled water. A Texas Instruments Programmable 58C with an Applied Statistics Module was responsible for all the kinetic data calculations. In all of the kinetic runs, the rate of sulphoxide appearance was obtained through a linear regression analysis. The optical rotation measurements were obtained at McMaster University using a Perkin-Elmer 241 MC Polarimeter at R.T. with a 10 cm cell and methanol as solvent. The incubation flasks were kept on a 1 in. New Brunswick Scientific rotary shaker (25^{o}, 150 rpm).

Mortierella isabellina NRRL 1757, Aspergillus niger ATCC 337 were maintained on malt agar slopes. Helminthosporium species NRRL 4671 was maintained on V-8 juice agar slopes.

The syntheses of the substrates and products isolated from the oxidation reaction were obtained through summer research and are therefore not discussed here. Both the calculations of the extinction coefficients and the preliminary data regarding the various sulphide and sulphoxides will also be omitted as this work was completed as a summer project. These values are however contained in Table 3 and an explanation as to their methods of synthesis is contained in the publication of Holland and Carter.[64]

Incubations with Mortierella isabellina.

The fungus was grown in a medium composed of glucose

(40 g), soya grits (5 g), yeast extract (5 g), sodium chloride (5 g) and potassium phosphate (dibasic) (5 g), per Litre of distilled water. One hundred and fifty milliLitres of the above medium were placed in 1-L Erlenmeyer flasks, fitted with sponge plugs, sterilized, allowed to cool and inoculated with Mortierella isabellina from growing slopes. The flasks were then placed on a 1-in rotary shaker (25°c, 150 rpm). After three days of growth, the flasks were removed from the shaker, filtered, mycelia washed twice with 50 ml of distilled water and then resuspended in distilled water. For this replacement culture technique, two batches of the fungus, from the original growth medium were combined into a 1-L flask with 130-150 mL of distilled water.

For the kinetic studies with the various sulphides, 100 mg in 1.15 mL methanol was added to each flask at time zero and the flasks were returned to the rotary shaker. At the appropriate time periods, 1-mL samples were removed from the flask by filtration and diluted to 5, 10 or 20 mL depending on the ultraviolet spectral properties of the substrate with spectroscopic grade methanol. This solution was then utilized for the untraviolet spectral analysis. The relative proportion of sulphoxide in the solution was then determined by the standard technique of a two component analysis, using the wavelengths and molar absorption coefficients listed in Table 3. Using this technique, the increase of sulphoxide concentration gave the rate constant which was then used in a Hammett plot.

Table 3. Ultraviolet spectral data for sulphides 1 and 3 and sulphoxides 2 and 4, and rate constants K for the production of sulphoxides 2 and 4.

Compound	λ max (nm)	log ε (nm)	K (mole sec^{-1})	log k/k
1a	254	4.0(254), 3.57(237)		
1b	255	4.05(255), 3.65(236)		
1c	257	4.04(257), 3.69(235)		
1d	249	3.86(249), 3.51(230)		
1e	260	4.16(260), 3.71(242)		
1f	262	4.21(262), 3.79(243)		
1g	340	4.10(340), 3.61(248)		
1h	257	3.92(257), 3.78(243)		
3a	247	2.89(247), 2.61(260)		
3b	220	3.24(220), 2.79(232)		
3c	267	3.92(267), 3.79(278)		
2a	237	3.66(237), 3.35(254)	$1.37\pm0.05 \times 10^{-8}$	0
2b	236	3.58(236), 3.30(255)	$6.1\pm0.1 \times 10^{-9}$	-0.35
2c	235	3.78(235), 3.27(257)	$6.5\pm0.1 \times 10^{-9}$	-0.32
2d	230	3.53(230), 3.27(249)	$1.20\pm0.05 \times 10^{-8}$	-0.06
2e	242	3.72(242), 3.27(260)	$1.0\pm0.05 \times 10^{-8}$	-0.14
2f	243	4.0(243), 3.50(262)	$3.2\pm0.1 \times 10^{-9}$	-0.63
2g	248	4.03(248), 2.71(340)	$3.9\pm0.1 \times 10^{-9}$	-0.56
2h	243	4.02(243), 3.59(257)	$1.9\pm0.1 \times 10^{-8}$	+0.14
4a	260 (sh)	2.40(260), 2.50(247)	$2.2\pm0.2 \times 10^{-8}$	
4b	232	3.83(232), 3.83(220)	$2.1\pm0.2 \times 10^{-8}$	
4c	278	3.94(278), 3.94(267)	$2.1\pm0.2 \times 10^{-8}$	

Calculation of the Sulphoxide Concentration

a = sulphide, λ max x s = sulphide

b = sulphoxide, λ max y so = sulphoxide

$ODx = (ODso)x + (ODs)x$

$ODy = (ODso)y + (ODs)y$

OD = E.C.I (I = 1), Beer-Lambert absorption law

(1) $ODx = (Eso)x \cdot Cso + (Es)x \cdot Cs$

(2) $ODy = (Eso)y \cdot Cso + (Es)y \cdot Cs$

From (1) $Cs = \left[\frac{OD\ x - (Eso)x \cdot Cso}{(Es)x}\right]$

Sub. into (2)

$$ODy = (Eso)y \cdot Cso + (Es)y \cdot \left[\frac{ODx - (Eso)x \cdot Cso}{(Es)x}\right]$$

$$ODy.(Es)x = (Eso)y \cdot Cso \cdot (Es)x + (Es)y \cdot ODx - (Eso)x \cdot (Es)y \cdot Cso$$

$$ODy \cdot (Es)x - (Es)y \cdot ODx = Cso\ [(Eso)y \cdot (Es)x - (Eso)x \cdot (Es)y]$$

$$Cso = \left[\frac{ODy \cdot (Es)x - (Es)y \cdot ODx}{(Eso)y \cdot (Es)x - (Eso)x \cdot (Es)y}\right]$$

Kinetic Analysis

The following tables contain all of the preliminary data from which the rate of sulphoxide appearance was obtained. In order to remove any apparent inconsistencies in the data, graphs of sulphoxide concentration versus time were originally prepared. Those few values which were far removed from a fit to an imaginary straight line were discarded.

The values contained at the bottom of each of the tables

(4 to 14), represent the rate of sulphoxide production. In most cases, the two values (from each table) were averaged to come up with the sulphoxide rate and these values were then used in a standard Hammett plot (Figure 20). The linear relationship of the aryl alkyl sulphides produced a rho value of -0.67.

Table 4. Calculation of the Rate of Sulphoxide Production

(Phenyl methyl sulphide, la)

(x = 254, y = 237)

(Dilution 1:25)

(A)

Time (hr)	OD_{237}(y)	OD_{254}(x)	Cso
3.37	0.125	0.192	3.57×10^{-4}
4.42	0.102	0.138	3.38×10^{-4}
5.33	0.118	0.153	4.08×10^{-4}
9.28	0.142	0.171	5.23×10^{-4}
10.28	0.180	0.215	6.68×10^{-4}
22.83	0.308	0.260	14.10×10^{-4}
24.33	0.340	0.284	15.64×10^{-4}
26.42	0.358	0.299	16.47×10^{-4}
27.83	0.328	0.265	15.31×10^{-4}
30.58	0.341	0.265	16.18×10^{-4}
32.83	0.354	0.260	17.17×10^{-4}
34/58	0.371	0.26	18.08×10^{-4}

Slope = 1.40×10^{-8} M s^{-1}

r = 0.996

Table 4. Calculation of the Rate of Sulphoxide Production

(Phenyl methyl sulphide, la)

(x = 254, y = 237)

(Dilution 1:25)

(B)

Time (hr)	OD_{237}(y)	OD_{254}(x)	Cso
0.5	0.099	0.153	2.80×10^{-4}
1.0	0.11	0.171	3.09×10^{-4}
2.0	0.118	0.165	3.77×10^{-4}
3.5	0.124	0.177	3.88×10^{-4}
4.5	0.125	0.179	3.89×10^{-4}
5.0	0.126	0.179	3.96×10^{-4}
7.0	0.161	0.193	5.95×10^{-4}
11.0	0.185	0.190	7.63×10^{-4}
12.0	0.198	0.190	8.50×10^{-4}

$$\text{Slope} = 1.34 \times 10^{-8}\ M\ s^{-1}$$

$$r = 0.976$$

$$\bar{X} = 1.37 \pm 0.03 \times 10^{-8}\ M\ s^{-1}$$

Table 5. Calculation of the Rate of Sulphoxide Production

(p-Methylphenyl methyl sulphide, lb)

(x = 255, y = 236)

(Dilution = 1:5)

(A)

Time (hr)	OD_{236}(y)	OD_{255}(x)	Cso
3.0	0.489	0.564	4.40×10^{-4}
5.25	0.513	0.585	4.66×10^{-4}
6.76	0.541	0.595	5.06×10^{-4}
8.25	0.547	0.573	5.31×10^{-4}
9.0	0.568	0.605	5.45×10^{-4}
20.0	0.770	0.701	8.10×10^{-4}
21.5	0.772	0.638	8.64×10^{-4}

Slope = 6.5×10^{-9} M s^{-1}

r = 0.997

Table 5. Calculation of the Rate of Sulphoxide Production (p-Methylphenyl methyl sulphide, 1b)

(x = 255, y = 236)

(B)

Time (hr)	OD_{236}(y)	OD_{255}(x)	Dilution	Cso
1.5	0.114	0.283	1:5	1.30×10^{-6}
2.25	0.114	0.275	1:5	6.69×10^{-6}
3.0	0.142	0.295	1:5	4.01×10^{-5}
5.0	0.171	0.302	1:5	8.38×10^{-5}
6.5	0.188	0.302	1:5	1.12×10^{-4}
8.0	0.217	0.302	1:5	1.61×10^{-4}
25.5	0.213	0.175	1:10	4.78×10^{-4}
27.0	0.229	0.182	1:10	5.22×10^{-4}

Slope = 5.6×10^{-9} M s^{-1}

r = 0.99

$\bar{X} = 6.1 \pm 0.5 \times 10^{-9}$ M s^{-1}

Table 6. Calculation of the Rate of Sulphoxide Production (p-Ethylphenyl methyl sulphide, 1c)

(x = 257, y = 235)

(A)

Time (hr)	OD_{257}(x)	OD_{235}(y)	Dilution	Cso
2.75	0.310	0.275	1:5	1.27×10^{-4}
5.0	0.378	0.395	1:5	2.12×10^{-4}
6.0	0.372	0.403	1:5	2.22×10^{-4}
6.75	0.368	0.410	1:5	2.30×10^{-4}
9.25	0.385	0.501	1:5	3.10×10^{-4}
10.75	0.375	0.519	1:5	3.31×10^{-4}
12.25	0.427	0.612	1:5	3.97×10^{-4}
23.0	0.467	0.935	1:5	6.88×10^{-4}
24.75	0.383	0.779	1:6.25	7.20×10^{-4}
26.25	0.367	0.770	1:6.25	7.18×10^{-4}

Slope = 7.2×10^{-9} M s^{-1}

r = 0.997

Table 6. Calculation of the Rate of Sulphoxide Production (p-Ethylphenyl methyl sulphide, 1c)

(x = 257, y = 235)

(Dilution = 1:5)

(B)

Time (hr)	OD_{257}(x)	OD_{235}(y)	Cso
1.25	0.312	0.244	0.96×10^{-4}
2.75	0.324	0.288	1.33×10^{-4}
3.5	0.334	0.305	1.45×10^{-4}
5.0	0.344	0.340	1.74×10^{-4}
5.5	0.358	0.360	1.87×10^{-4}
6.0	0.380	0.382	1.98×10^{-4}
6.75	0.336	0.385	2.20×10^{-4}

Slope = 6.0×10^{-9} M s^{-1}

r = 0.997

$\bar{X} = 6.60 \pm 0.6 \times 10^{-9}$ M s^{-1}

Table 7. Calculation of the Rate of Sulphoxide Production (p-Fluorophenyl methyl sulphide, 1d)

(x = 249, y = 230)

(A)

Time (hr)	OD_{230}(y)	OD_{249}(x)	Dilution	Cso
2.0	0.440	0.660	1:5	2.83×10^{-4}
3.5	0.510	0.705	1:5	3.79×10^{-4}
4.5	0.539	0.720	1:5	4.22×10^{-4}
6.0	0.717	0.852	1:5	6.51×10^{-4}
7.0	0.235	0.330	1:20	6.81×10^{-4}
7.5	0.760	0.872	1:5	7.16×10^{-4}
13.0	0.942	0.938	1:5	10.09×10^{-4}
26.5	0.835	0.664	1:6.67	13.82×10^{-4}
29.0	0.886	0.685	1:6.67	16.46×10^{-4}

Slope = 1.3×10^{-8} M s^{-1}

r = 0.98

Table 7. Calculation of the Rate of Sulphoxide Production (p-Fluorophenyl methyl sulphide, 1d)

(x = 249, y = 230)

(B)

Time (hr)	$OD_{230}(y)$	$OD_{249}(x)$	Dilution	Cso
1.0	0.202	0.380	1:6.67	8.61×10^{-5}
2.0	0.232	0.418	1:6.67	11.98×10^{-5}
4.0	0.257	0.425	1:6.67	1.76×10^{-4}
5.25	0.302	0.447	1:6.67	2.66×10^{-4}
6.0	0.308	0.442	1:6.67	2.87×10^{-4}
6.75	0.305	0.445	1:6.67	2.76×10^{-4}
25.0	0.673	0.632	1:6.67	10.05×10^{-4}
26.5	0.708	0.655	1:6.67	10.69×10^{-4}

$$\text{Slope} = 1.1 \times 10^{-8}\ \text{M s}^{-1}$$

$$r = 0.99$$

$$\bar{X} = 1.2 \pm 0.1 \times 10^{-8}\ \text{M s}^{-1}$$

Table 8. Calculation of the Rate of Sulphoxide Production (p-Chlorophenyl methyl sulphide, le)

(x = 260, y = 242)

(A)

Time (hr)	OD_{242}(y)	OD_{260}(x)	Dilution	Cso
3.0	0.175	0.238	1:10	1.97×10^{-4}
4.75	0.197	0.265	1:10	2.24×10^{-4}
6.5	0.222	0.291	1:10	2.58×10^{-4}
7.5	0.242	0.310	1:10	2.87×10^{-4}
8.5	0.230	0.275	1:10	2.88×10^{-4}
12.5	0.282	0.298	1:10	3.84×10^{-4}
23.0	0.346	0.222	0.7:10	5.83×10^{-4}
27.0	0.422	0.284	0.7:10	7.01×10^{-4}
28.0	0.430	0.282	0.7:10	7.20×10^{-4}

Slope = 0.97×10^{-8} M s^{-1}

r = 0.992

Table 8. Calculation of the Rate of Sulphoxide Production (p-Chlorophenyl methyl sulphide, le)

(x = 260, y = 242)

(Dilution = 1:10)

(B)

Time (hr)	OD_{242}(y)	OD_{260}(x)	Cso
1.0	0.083	0.200	0.251×10^{-4}
5.0	0.152	0.255	1.33×10^{-4}
6.5	0.165	0.232	1.79×10^{-4}
9.0	0.213	0.248	2.72×10^{-4}
10.0	0.205	0.236	2.64×10^{-4}
13.25	0.280	0.205	4.52×10^{-4}
22.5	0.475	0.317	7.91×10^{-4}
24.0	0.502	0.328	8.41×10^{-4}

Slope = 1.0×10^{-8} M s^{-1}

r = 0.996

$\bar{X} = 9.9 \pm 0.15 \times 10^{-9}$ M s^{-1}

Table 9. Calculation of the Rate of Sulphoxide Production (p-Bromophenyl methyl sulphide, 1f)

(x = 262, y = 243)

(Dilution = 1:10)

(A)

Time (hr)	OD_{243}(y)	OD_{262}(x)	Cso
2.75	0.098	0.160	4.09×10^{-5}
4.5	0.059	0.120	1.43×10^{-5}
5.5	0.050	0.110	0.85×10^{-5}
6.25	0.072	0.123	2.77×10^{-5}
8.0	0.081	0.130	3.49×10^{-5}
11.0	0.105	0.130	6.20×10^{-5}
12.0	0.104	0.125	6.31×10^{-5}
23.0	0.238	0.184	1.89×10^{-4}
30.0	0.350	0.220	3.0×10^{-4}
32.0	0.350	0.210	3.04×10^{-4}
34.75	0.382	0.220	3.41×10^{-4}

Slope = 3.10×10^{-9} M s^{-1}

r = 0.996

Table 9. Calculation of the Rate of Sulphoxide Production (p-Bromophenyl methyl sulphide, lf)

(x = 262, y = 243)

(Dilution = 1:5)

(B)

Time (hr)	OD_{243}(y)	OD_{262}(x)	Cso
1.0	0.250	0.340	6.72×10^{-5}
3.0	0.265	0.355	7.24×10^{-5}
5.0	0.390	0.448	1.23×10^{-4}
7.0	0.442	0.472	1.47×10^{-4}
8.0	0.512	0.546	1.70×10^{-4}
11.0	0.480	0.463	1.71×10^{-4}
14.0	0.590	0.510	2.22×10^{-4}

Slope = 3.3×10^{-9} M s^{-1}

r = 0.971

$\bar{X} = 3.2 \pm 0.1 \times 10^{-9}$ M s^{-1}

Table 10. Calculation of the Rate of Sulphoxide Production (p-Nitrophenyl methyl sulphide, lg)

(x = 225, y = 248)

(Dilution = 1:10)

Time (hr)	OD_{248}(y)	OD_{225}(x)	Cso
0.5	0.089	0.110	4.94×10^{-5}
2.0	0.216	0.250	1.36×10^{-4}
3.0	0.219	0.260	1.32×10^{-4}
4.5	0.212	0.242	1.37×10^{-4}
5.5	0.229	0.283	1.27×10^{-4}
9.0	0.290	0.315	2.03×10^{-4}
10.5	0.325	0.358	2.23×10^{-4}
12.5	0.322	0.340	2.35×10^{-4}
13.5	0.330	0.357	2.32×10^{-4}
23.0	0.560	0.583	4.16×10^{-4}
24.0	0.510	0.488	4.20×10^{-4}
27.0	0.552	0.520	4.63×10^{-4}
29.0	0.569	0.540	4.73×10^{-4}
31.0	0.597	0.550	5.13×10^{-4}
34.0	0.635	0.612	5.19×10^{-4}

Slope = 4.0×10^{-9} M s^{-1}

r = 0.993

Table 11. Calculation of the Rate of Sulphoxide Production

(p-Methoxyphenyl methyl sulphide, 1h)

(x = 257, y = 243)

(Dilution = 1:10)

(A)

Time (hr)	OD_{243}(y)	OD_{257}(x)	Cso
1.0	0.605	0.810	3.58×10^{-5}
2.0	0.637	0.817	7.04×10^{-5}
3.5	0.662	0.801	1.17×10^{-4}
4.5	0.662	0.765	1.50×10^{-4}
6.0	0.785	0.801	2.75×10^{-4}
7.5	0.875	0.801	3.90×10^{-4}
8.5	0.931	0.794	4.68×10^{-4}
10.5	0.999	0.712	6.31×10^{04}

Slope = 2.1×10^{-8} M s^{-1}

r = 0.997

Table 11. Calculation of the Rate of Sulphoxide Production

(p-Methoxyphenyl methyl sulphide, 1h)

(x = 257, y = 243)

(Dilution = 1:10)

(B)

Time (hr)	OD_{243}(y)	OD_{257}(x)	Cso
2.25	0.562	0.60	1.72×10^{-4}
3.25	0.682	0.662	2.70×10^{-4}
4.25	0.690	0.545	3.87×10^{-4}
6.5	0.768	0.592	4.44×10^{-4}
8.5	0.876	0.550	6.21×10^{-4}
9.75	0.875	0.560	6.10×10^{-4}
12.5	1.15	0.501	10.16×10^{-4}
22.25	0.738	0.282	13.78×10^{-4}

Slope = 1.7×10^{-8} M s^{-1}

r = 0.983

$\bar{X} = 1.9 \pm 0.2 \times 10^{-8}$ M s^{-1}

Table 12. Calculation of the Rate of Sulphoxide Production

(Benzyl methyl sulphide, 3a)

(x = 247, y = 260)

(Dilution = 1:5)

(A)

Time (hr)	OD_{247}(x)	OD_{260}(y)	Cso
3.0	0.317	0.308	8.16×10^{-3}
6.5	0.315	0.308	8.22×10^{-3}
10.5	0.330	0.327	8.86×10^{-3}
12.0	0.355	0.350	9.43×10^{-3}
13.0	0.324	0.326	8.98×10^{-3}
24.0	0.320	0.343	10.08×10^{-3}
26.5	0.298	0.328	9.88×10^{-3}

Slope = 2.27×10^{-8} M s^{-1}

r = 0.940

Table 12. Calculation of the Rate of Sulphoxide Production

(Benzyl methyl sulphide, 3a)

(x = 247, y = 260)

(Dilution = 1:5)

(B)

Time (hr)	OD_{247}(x)	OD_{260}(y)	Cso
3.0	0.282	0.292	8.30×10^{-3}
5.5	0.293	0.301	8.49×10^{-3}
7.0	0.318	0.315	8.54×10^{-3}
10.0	0.324	0.323	8.82×10^{-3}
12.0	0.323	0.324	8.91×10^{-3}
13.0	0.340	0.337	9.14×10^{-3}
15.5	0.344	0.342	9.31×10^{-3}
22.0	0.373	0.365	9.76×10^{-3}
24.5	0.374	0.368	9.90×10^{-3}

Slope = 2.1×10^{-8} M s^{-1}

r = 0.99

$\bar{X} = 2.19 \pm 0.09 \times 10^{-8}$ M s^{-1}

Table 13. Calculation of the Rate of Sulphoxide Production (p-Methylbenzyl methyl sulphide, 3b)

(x = 220, y = 232)

(Dilution = 1:10)

(A)

Time (hr)	OD_{220}(x)	OD_{232}(y)	Cso
1.5	0.511	0.208	6.0×10^{-5}
2.5	0.606	0.302	2.0×10^{-4}
3.0	0.475	0.232	1.46×10^{-4}
4.75	0.630	0.332	2.5×10^{-4}
6.00	0.634	0.394	3.9×10^{-4}
7.5	0.701	0.496	5.7×10^{-4}
9.5	0.925	0.715	9.0×10^{-4}

Slope = 2.2×10^{-8} M s^{-1}

r = 0.966

Table 13. Calculation of the Rate of Sulphoxide Production (p-Methylbenzyl methyl sulphide, 3b)

(x = 220, y = 232)

(Dilution = 1:10)

(B)

Time (hr)	OD_{220}(x)	OD_{232}(y)	Cso
1.5	0.255	0.128	8.6×10^{-5}
3.5	0.291	0.167	1.48×10^{-4}
4.5	0.363	0.223	2.19×10^{-4}
5.5	0.385	0.239	2.38×10^{-4}
6.5	0.432	0.285	3.05×10^{-4}
8.0	0.527	0.372	4.29×10^{-4}
10.0	0.705	0.544	6.83×10^{-4}
11.5	0.789	0.630	8.15×10^{-4}

$$\text{Slope} = 2.1 \times 10^{-8} \text{ M s}^{-1}$$

$$r = 0.972$$

$$\bar{X} = 2.15 \pm 0.05 \times 10^{-8} \text{ M s}^{-1}$$

Table 14. Calculation of the Rate of Sulphoxide Production

(p-Nitrobenzyl methyl sulphide, 3c)

(x = 267, y = 278)

(A)

Time (hr)	OD_{278}(y)	OD_{267}(x)	Dilution	Cso
3.0	0.502	0.520	0.7:10	7.23×10^{-4}
5.0	0.590	0.604	0.7:10	8.83×10^{-4}
7.0	0.709	0.719	0.7:10	10.92×10^{-4}
8.0	0.783	0.792	0.7:10	12.16×10^{-4}
9.0	0.731	0.742	0.7:10	11.23×10^{-4}
21.25	0.803	0.805	1:25	22.4×10^{-4}
22.0	0.805	0.805	1:25	22.6×10^{-4}

Slope = 2.26×10^{-8} M s^{-1}

r = 0.996

Table 14. Calculation of the Rate of Sulphoxide Production

(p-Nitrobenzyl methyl sulphide, 3c)

(x = 267, y = 278)

(B)

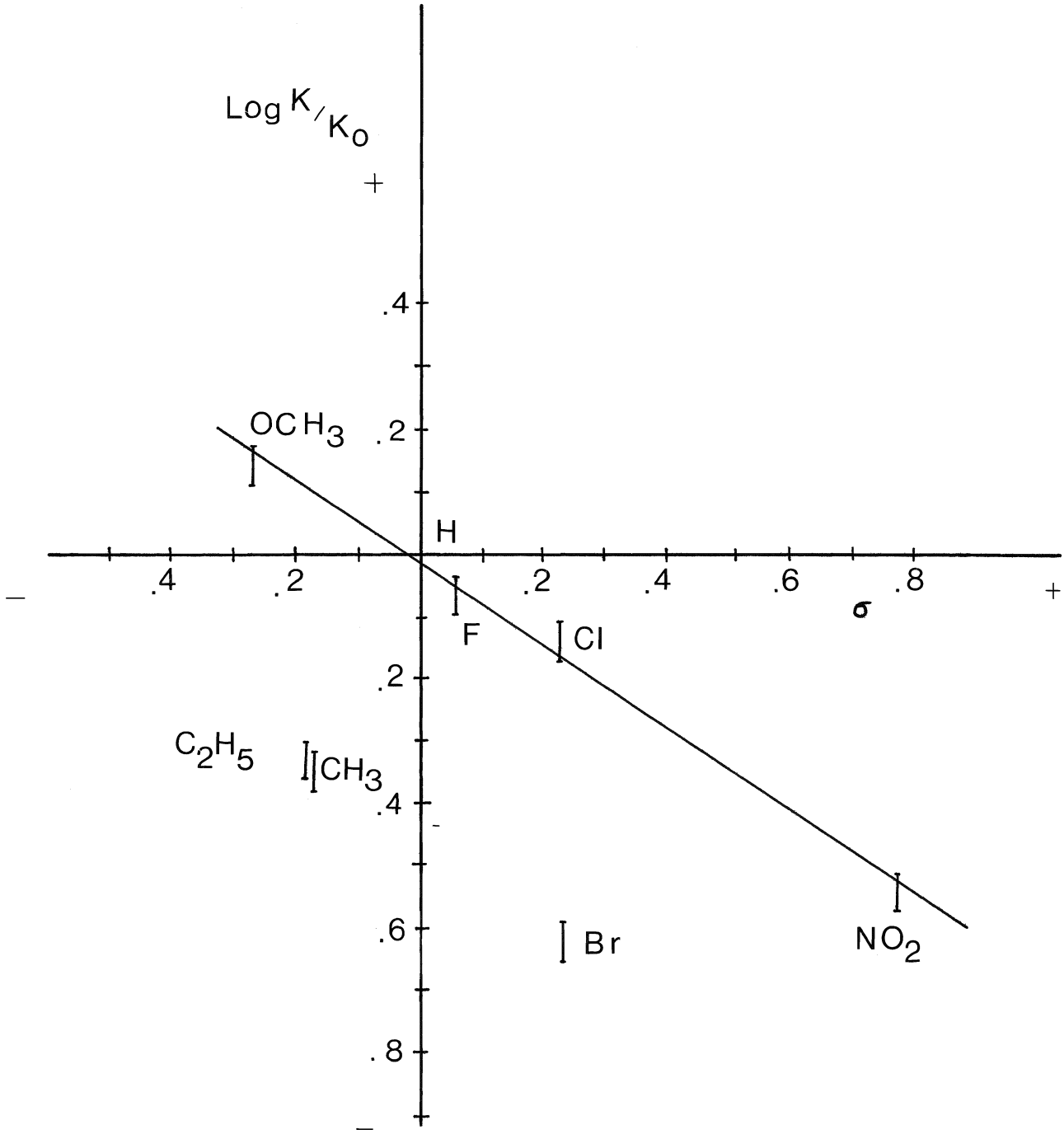
Time (hr)	OD_{278}(y)	OD_{267}(x)	Dilution	Cso
3.5	0.612	0.640	0.8:10	7.47×10^{-4}
5.5	0.709	0.732	0.8:10	9.03×10^{-4}
6.5	0.718	0.738	0.8:10	9.28×10^{-4}
8.5	0.840	0.856	0.8:10	11.16×10^{-4}
10.25	0.771	0.781	0.7:10	11.92×10^{-4}
11.5	0.790	0.801	0.7:10	12.18×10^{-4}
23.75	0.90	0.898	1:20	20.35×10^{-4}

$$\text{Slope} = 1.74 \times 10^{-8}\ M\ s^{-1}$$

$$r = 0.997$$

$$\bar{X} = 2.0 \pm 0.26 \times 10^{-8}\ M\ s^{-1}$$

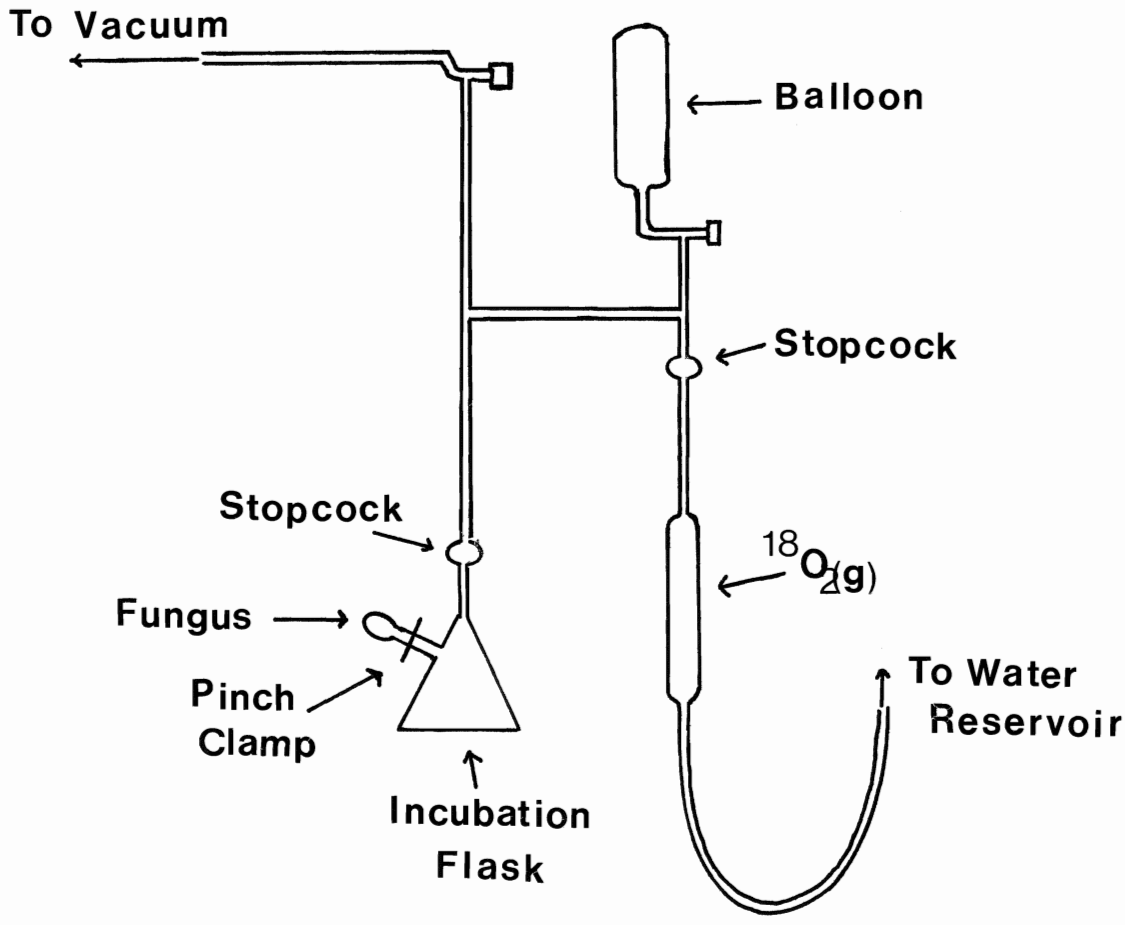
Figure 20. Hammett Plot for the Oxidation of Alkyl Aryl Sulphides (1,3)



Incubation under an ^{18}O labelled atmosphere

In order to remove all of the ^{16}O gas from the incubation flask and replace it with the isotope ^{18}O gas, the set up according to Figure 21 was required. A 250 ml erlenmeyer flask was first filled with 100 mL distilled water and then attached to a vacumn system. The flask was then evacuated, cooled with liquid nitrogen, warmed to room temperature and then cooled a second time *in vacuo*. After thawing, the enriched atmosphere (nominally 90% ^{18}O) was then placed inside the flask. Using water displacement, a balloon was filled with the enriched gas and by opening the valve to the incubation flask, the gas was drawn in. This was repeated twice more in order to bring the flask to normal atmospheric pressure. The fungus was then introduced via a side arm as well as the substrate, phenyl methyl sulphide (100 mg dissolved in 1.15 mL methanol). The sealed flask was then placed on an rotary shaker for a period of 36 hours, after filtering the mycelia was washed with distilled water and the medium was extracted with chloroform. The extract was dried (sodium sulfate) and evaporated to yield phenyl methyl sulphoxide. The residue, (42 mg) was sent for gas chromatography → mass spectrometry isotope analysis, the ratio of ^{18}O:^{16}O present in the sulphoxide was calculated as 78.5:21.5.

Figure 21. Apparatus for Introduction of $^{18}O_2(g)$ into incubation flask.



An Investigation of the Biotransformation of Organic Selenides by Fungi

Experimental II

Phenylselenol[113]

For the preparation of the title compound, the procedure reported by Foster[144] was employed. To a 500 mL round-bottom flask fitted with a refl x condensoe, 200 mL of dry diethyl ether, 26 mL bromobenzene (.25 mole) and 6.0 grams (.25 mole) magnesium turnings (finely cut up) were added. The flask was heated to a gentle reflex and maintained until most of the metal had reacted. At this point 19.0 grams of grey selenium powder (.25 mole, dried over H_2SO_4 for 24 hours *in vacuo*), was added slowly over a period of half an hour. After all the selenium was added, the solution was refluxed for an additional hour and then cooled to room temperature. The contents of the flask were then poured onto 300 g of ice and 20 mL of concentrated HCl were added. This solution was subsequently filtered and extracted three times with 125 mL portions of diethyl ether. The organic layer was then dried (sodium sulfate) and evaporated *in vacuo*. From the yellow solution, 18.95 g (73%) of phenylselenol was obtained following a bulb-to-bulb distillation (100^{o}/15 mm, lit[144] b.p. 84-86/25 mm) and this solution was then stored under nitrogen for future use. The ^{1}H NMR spectrum showed signals at δ 1.64(1H,s), 7.35(5H,m). The mass spectrum m/e (%): 155(12), 154(100, 153(35), 152(24), 151(9), 128(9), 78(10), 76(16), 63(9), 51(11).

Phenyl methyl selenide 9

The procedure of Foster and Brown[145] was then followed for the synthesis of this thioether. To a solution of sodium hydroxide (1.52 g in 20 mL water), one equivalent of phenylselenol 5.0 g (0.038 mole) was added, and this solution was then mixed with 50 mL of absolute ethanol containing 2.0 mL (0.038 mole) of iodomethane. After refluxing for 5 hours, the solution was cooled to room temperature, 200 mL of distilled water was added and the mixture was extracted three times with 50 mL portions of diethyl ether. The extract was then washed twice with 75 mL of a 10% sodium hydroxide solution and once with 100 mL distilled water. The ether layer was then dried (sodium sulfate) and evaporated in vacuo. The pure selenide, 4.0 g (62%) was obtained following a bulb-to-bulb distillation b.p. 97-100/15 mm (lit[145] 202-204). The ^{1}H NMR spectrum showed signals at δ 2.34 (3H,s), 7.33 (5H,M). The mass spectrum m/e (%): 174(18, 172(100), 170(45), 169(18), 168(18), 159(15), 157(90), 155(44), 144(18), 143(18), 117(9), 91(43), 78(15), 77(53), 65(12), 51(41), 50(22), 39(12).

Phenyl methyl selenoxide 14

Phenyl methyl selenoxide was synthesized using a standard oxidizing reagent, sodium meta-periodate. Into a 500 mL round-bottom flask, 200 mL of ice cold methanol and 1.5 g (8.7×10^{-3} mole) of phenyl methyl selenide were placed.

Added to this flask was, 2.06 g (9.6×10^{-3} mole) of sodium metaperiodate, in 20 mL of water and the resulting solution was allowed to stir overnight (14 hours). This solution was then filtered and the volume of methanol was reduced in vacuo. When approximately 30 mL of solution remained, 100 mL of distilled water was added and the solution was extracted with chloroform three times (75 mL). The organic layer was then dried (sodium sulfate) and evaporated, leaving the crude phenyl methyl selenoxide. Following column chromatography, .85 g (52%) of the pure selenoxide was isolated (45-49, lit[146] 53-54). An infrared scan of the title compound produced a characteristic peak at 800 cm.[147] The ^{1}H NMR spectrum showed signals at δ 2.68(3H,S), 7.43(5H,m). The mass spectrum contained peaks at m/e (%): 191(19), 189(100), 187(50), 186(18), 185(20), 172(21), 170(11), 91(12), 69(14), 57(16), 55(26), 42(28), 40(33), 38(16).

[Methyl^{14}-C]-Methyl phenyl selenide

Using the same procedure as previously described,[145] the labeled selenide was synthesized using [Methyl^{14}-C]-methyliodide (0.248 mg, 100 µCi). The labeled iodide was diluted to 1.48 g (1.77×10^{-2} mole) with cold material and this was then added to a solution of 0.69 g sodium hydroxide in 10 mL water, (1.73×10^{-2} mole) 2.72 g phenylselenol (1.73×10^{-2} mole) and 70 mL

of ethanol. This mixture was then refluxed for 1 hour, with a cold trap to prevent the loss of any volatile radioactive material into the atmosphere. The solution was cooled to room temperature, diluted with 150 mL distilled water and extracted with three 100 mL portions of diethyl ether. The organic layer was then washed three times, twice with 75 mL of a 10% sodium hydroxide solution, once with 100 mL water, dried (sodium sulfate) and evaporated to produce 2.15 g (73%) [Methyl-^{14}C]-phenyl methyl selenide, S.A. 7.8 μCi/nmole. The ^{1}H NMR spectrum of this product showed signals identical to that of an authentic sample, and because of the absence of any phenylselenol a distillation of this crude product was omitted. In order to confirm the presence of the label only in phenyl methylselenide, radioscanning of a thin layer chromatography plate of the product was carried out and indicated that PhSeMe was the only radioactive component.

Cyclohexylmethyl phenyl sulphide 6

A solution of 3.3 g (3.0×10^{-2} mole) thiophenol with 1.1 g (2.7×10^{-2} mole) sodium hydroxide in 10 mL distilled water, was added to 100 mL of acetone containing 4.84 g (2.7×10^{-2} moles) of cyclohexylmethyl bromide. The solution was cooled to 0^{o}C and dry nitrogen was swept through the system for a period of 20 hours. The acetone was evaporated *in vacuo* and 100 mL of distilled water was added to the residue. The resulting solution was then extracted with three 50 mL portions

of chloroform, washed twice with a 75 mL sodium hydroxide solution (10%) and once with a 75 mL portion of distilled water. The organic layer was dried (sodium sulfate), evaporated and the pure sulphide (2.4 g, 38% yield) was isolated following the bulb-to-bulb distillation b.p. 178/13 mm (lit[148] 19-21°). The ^{1}H NMR spectrum showed signals at δ 2.1-1.0 (11H,m), 2.25(2H,d), 7.2(5H,m). The mass spectrum of a sample m/e (%): 207(8), 206(43), 124(12), 123(21), 111(11), 110(100), 97(24), 81(15), 67(12), 55(82), 41(19). The ^{13}C NMR values are contained in Table 4.

Cyclohexylmethyl phenyl sulphoxide 15

Using the same procedure as previously described for phenyl methyl selenoxide, 1 g (4.85×10^{-3} mole) cyclohexylmethyl phenyl sulphide was initially added to 200 mL of ice cold methanol. A solution of sodium metaperiodate 1.35 g, dissolved in 20 mL distilled water (6.3×10^{-3} mole) was then added drop-wise over the period of an hour and this mixture was stirred overnight. After 20 hours, the solution was filtered, the volume of methanol was reduced *in vacuo* and 75 mL of distilled water was added. This solution was then extracted with three 80 mL portions of chloroform, the extract was dried (sodium sulfate) and then evaporated. Both a ^{1}H NMR spectrum of the isolated product and thin layer chromatography showed a mixture of sulphide and sulphoxide present. Using column chromatography, 06g (50% yield) of the pure sulphoxide was isolated, m.p.

49-52° lit[148] 56-57°. The ^{1}H NMR spectrum showed signals at δ2.1 - 1.0 (11H,M), 2.4 - 2.75 (2H,M), 7.55 (5H,m). The mass spectrum showed signals m/e (%): 222(4), 207(5), 206(35), 126(82), 123(18), 110(90), 109(12), 97(46), 96(12), 81(26), 78(28), 77(16), 69(12), 67(22), 55(100), 54(11). The ^{13}C NMR values are contained in Table 4.

Cyclohexylmethyl phenyl selenide 5

A solution of phenylselenol 5 g (3.18×10^{-2} mole) and 1.2 g sodium hydroxide (3.0×10^{-2} mole) in 20 mL of water was added to 200 mL of ice cold acetone containing 5.3 g (3.0×10^{-2} mole) of cyclohexylmethyl bromide. The solution was cooled with an ice bath, swept with dry nitrogen and stirred for 20 hours. The solution was then warmed to room temperature, the acetone was evaporated and 100 mL of distilled water was added to the remaining contents of the flask. This solution was extracted with three 50 mL portions of chloroform, and then the extract was washed twice with a 75 mL solution of 10% sodium hydroxide followed by 100 mL of water. The chloroform was dried and evaporated to leave 6.2 g of a crude product. Following bulb-to-bulb distillation 4.2 g (62% yield) of the pure selenide b.p. 190-195°/18 mm (lit[149] 52/0.3 mm) was isolated. The ^{1}H NMR spectrum showed peaks at δ 2.1 - 1.05 (11H,m), 2.78 (2H,d), 7.20 (5H,m). The mass spectrum showed peaks at m/e (%) 245(20), 252(10), 158(42), 156(21), 155(12), 154(18), 97(61), 77(16), 69(13), 55(100). The ^{13}C NMR values are contained in

Table 4.

Crotyl phenylsulphide 8

For the preparation of the title compound the following reagents were added to 200 mL of ice cold acetone: 3.9 g (9.8×10^{-2} mole) of sodium hydroxide in 30 mL water, 12.8 g (1.1×10^{-1} mole) thiophenol and 9.6 mL (9.7×10^{-2} mole) crotyl chloride. The solution was stirred for 20 hours at $0^{\circ}C$ and the resulting precipitation was removed by filtration. The filtrate was reduced in volume in vacuo, diluted with 100 mL water and then extracted with 75 mL of chloroform three times. The extract was then washed twice with 75 mL of a 10% sodium hydroxide solution and once with 50 mL water. The extract was dried with sodium sulfate and evaporated to yield 12.3 g (67% yield) of crude sulphide. With a bulb-to-bulb distillation, 9.8 g of the pure sulphide was obtained (54% yield, b.p. 115-117/11 mm, lit[150] $82-83^{\circ}/0.9$ mm). The ^{1}H NMR showed signals at δ 1.7 - 1.3 (4H,m), 4.6 - 4.4 (1H,m), 5.7 - 5.5 (2,3H,m), 7.5 - 7.1 (5H,m). The mass spectrum of the product included ions m/e (%) 164(40), 111(11), 110(100), 109(17), 85(20), 83(20), 66(10), 65(13), 55(84). The values for the ^{13}C NMR can be found in Table 4.

Crotyl phenyl sulphoxide 16

Following the usual oxidation procedure with sodium meta-

periodate, 1.5 g (9.15×10^{-3} mole) crotyl-phenyl-selenide, 2.35 g (1.1×10^{-2} mole) sodium metaperiodate in 50 mL water and 200 mL ice cold methanol were required to produce 0.92 g (61% yield) of the sulphoxide. The pure sulphoxide (.68 g) was isolated from column chromatography as an oil (lit[151] oil. The ^{1}H NMR spectrum showed signals at δ 1.67 (3H,d), 3.50 (2H,d), 5.7 - 5.24 (2H,m) 7.6 (5H,S). The mass spectrum included ions m/e (%): 180(2), 164(11), 126(38), 110(100), 109(26), 105(12), 78(31), 77(26), 66(28), 65(17), 55(86), 54(17), 53(17), 51(25).

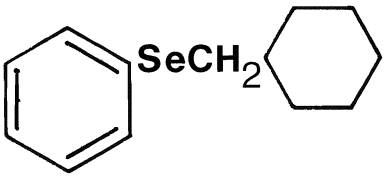
Crotyl phenyl selenide 7

This compound was synthesized using 10 g phenyl-selenol (6.4×10^{-2} mole), 2.12 g sodium hydroxide (5.3×10^{-2} mole) dissolved in 20 mL of water and 5.2 mL crotyl chloride (5.3×10^{-2} mole). The solution of the selenol and sodium hydroxide was first added to the solution of crotyl chloride in acetone (200 mL) and the resulting mixture was stirred for 20 hours at 0°C while dry nitrogen was passed through the reaction mixture. The solution was then filtered, the acetone evaporated *in vacuo* and diluted to 100 mL with H_2O. The filtrate was then extrated with three 75 mL portions of chloroform, washed twice with 75 mL of a 10% sodium hydroxide solution and once with 100 mL of water. The chloroform layer was then dried (sodium sulfate) and evaporated to leave the crude selenide 9.7 g (87%).[152] From a bult-to-bulb distillation of the crude product, a mixture of two products were obtained and from

which, the pure selenide was isolated using column chromatography 2.3 g (76% from 3 grams). The mass spectrum contained peaks at m/e (%) 314(1), 312(1), 236(4), 235(4), 234(22), 232(13), 231(6), 230(6), 212(4), 210(2), 159(5) 158(26), 157(23), 156(16), 155(26), 154(100), 153(25), 152(18), 117(9), 115(8), 78(34), 77(62), 76(9), 75(7), 74(8), 55(75), 53(7), 51(64), 50(25). Elemental analysis: calculated 56.88%C, 5.73%H, observed 56.75%C 5.69%H. The ^{1}H NMR contained signals at δ 1.58(3H,d), 3.38(2H,d), 5.48(2H,m), 6.90 - 7.63(5H,m).

Crotyl pheny selenoxide

With the corresponding selenide (0.8 g, 37×10^{-3} mole) and a solution of sodium metaperiodate (.969, 4.8×10^{-3} mole) in water (10 mL), the synthesis of the title compound was attempted. The sodium metaperiodate solution was added drop-wise over a 1 hour period to an ice cold solution of the selenide in methanol (100 mL). This mixture was stirred for 16 hours at $0^{o}C$ and the resulting precipitate was removed by filtration. The filtrate was reduced in volume in vacuo, 100 mL of water was added and this mixture was then extracted with chloroform (three 50 mL portions). The extract was dried



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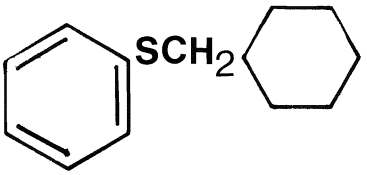

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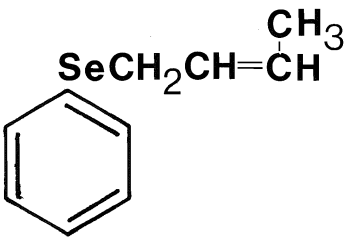

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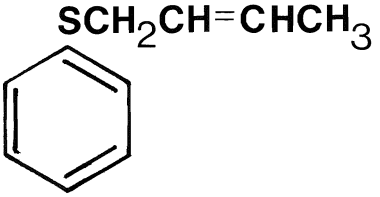

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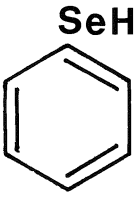

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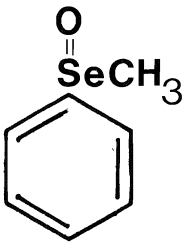

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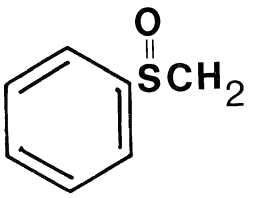

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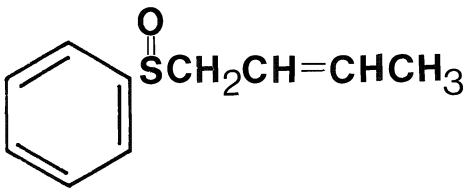

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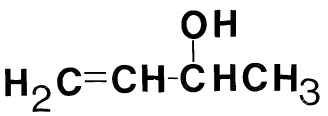

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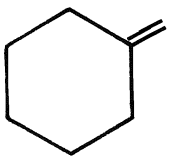

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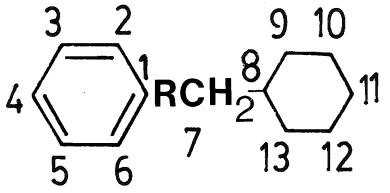
Table 4

^{13}C Chemical Shifts for alkyl aryl sulphides, sulphoxides and selenides.

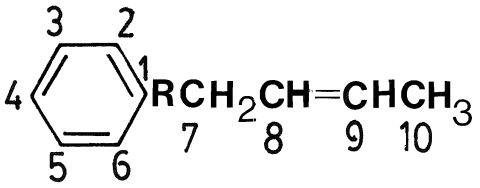
A.



Chemical Shift (ppm)

Coupound	C_1	$C_{2,6}$	$C_{3,5}$	C_4	C_7	C_8	$C_{9,13}$	$C_{10,12}$	C_{11}
6,R=S	137.9	128.8	128.7	125.5	41.0	37.6	32.9	26.1	26.4
15,R=S(=O)	145.2	131.1	129.5	124.1	66.5	33.4	32.3	25.7	25.9
5,R=Se	129.3	132.3	128.9	126.4	44.2	33.7	31.3	23.6	23.9

B.



Chemical Shift (ppm)

Compound	C_1	$C_{2,6}$	$C_{3,5}$	C_4	C_7	C_8	C_9	C_{10}
8,R=S	136.6	129.7	128.9	126.8	36.4	127.8	127.6	17.65
7,R=Se	129.3	133.6	128.8	128.2	30.0	127.1	126.9	17.59

(sodium sulfate) and evaporated to yield an oily residue (0.6 g) which was analyzed by gas chromatography-mass spectrometry. The only major volatile component identifiable was 3-butene-2-ol 17, by a comparison with an authentic sample. The mass spectrum showed ions at m/e (%): 72(14), 71(15), 58(4), 57(100), 55(12), 54(6), 53(7), 45(34).

Incubations

A. *Aspergillus niger* and *Aspergillus foetidus*

Both fungi are capable of growth in a Czapek Dox medium, utilizing 1-L Erlynmeyer flasks containing 150 mL of medium. Prior to inoculation with either of the fungi, the medium was sterilized and cooled to room temperature. The flasks were then placed on the rotary shaker for 48 hours, then filtered, the mycelia was washed with distilled water and then resuspended in 150 mL of distilled water. A solution of phenyl methyl selenide (60 mg dissolved in 1 mL of methanol per flask) was then added to the flasks. The incubation with the substrate was carried out for a further 48 hours at which time the flasks were then filtered, mycelia was washed and the medium was extracted with chloroform. The chloroform extract was then dried (sodium sulfate) and evaporated *in vacuo* to leave an oily residue. Using gaschromatography and ^{1}H NMR, no phenyl methyl selenoxide was detected in the medium extract of *A. niger*. The only major volatile component present was the starting material in 50% recovery.

With A. foetidus, the same results were obtained as no phenyl methyl selenoxide was detected in either the medium extract or mycelia. Starting material was the only major volatile component identified in a 61% recovery.

B. Helminthosporium species

The same procedure was followed for this fungus as that utilized for A. niger and A. foetidus. Helminthospoium sp. was however initially grown in a medium composed of V-8 tomato juice (200 mL), and calcium carbonate (3 g) per Liter of distilled water, pH adjusted to 7.2. Following an initial 3 day growth period, phenyl methyl selenide (60 mg in 1 mL MeOH per flask) was added to the fungus resuspended in 150 mL of distilled water. After the standard 48 hour incubation period, the flasks were filtered and worked up as previously described. An analysis of the residue extracted from the incubation medium showed only starting material as the major volatile component (50% recovery).

Prior to the incubation with phenyl methyl selenide, the corresponding sulphide was used as a substrate with some success. Following exactly the same procedure as previously reported, the sulphide was added to the incubation flasks (60 mg in 1 mL MeOH/flask) containing Helminthosporium sp. The residue from the chloroform extract of the medium, after the 48 hour incubation period, contained both starting material and phenyl methyl sulphoxide. The ^{1}H NMR spectrum of the

residue showed a definite shift in the resonance position of the methyl group, corresponding to the presence of phenyl methylsulphoxide.

C. Mortierella isabellina

The growth of the fungus and the initial set up of the various incubations were followed according to previous instructions. The results of these various incubations are contained in Tables 15 and 16 (pages 125-6). In all of the incubations, the products and recovered starting material were identified by comparison to authentic samples.

Table 16 contains the experiments which were used as controls. Runs 12 to 14 were set up in pairs as the fungus from the first incubation was resuspended in 120 mL of distilled water, following filtration and washings, and the second substrate was then added. In all three of these runs, phenyl-methyl-sulphide was the second substrate to be metabolized by the fungus. The same procedure for the work-up of the incubation medium was followed and the extracts were then analyzed by gas chromatography-mass spectrometry.

Table 15

Incubations of Substrates with *M. isabellina*

	Substrate	Wt(mg) per flask	Time(hr)	Compounds isolated (%)
1.	Phenyl methyl sulphide	100	24-36	phenyl methyl sulphoxide (40-60)
2.	Phenyl methyl selenide, 9	60	24	starting material (50)
3.	Phenyl methyl selenide, 9	60	48	starting material (62)
4.	Phenyl methyl selenide, 9	80	48	starting material (54)
5.	Phenyl methyl selenide, 9	150	36	see Tables 17 and 18
6.	[Methyl-^{14}C]-methyl phenyl selenide, 9	120	39	see Tables 19 and 20
7.	Crotyl phenyl sulphide, 8	50	48	crotyl phenyl sulphoxide (52)
8.	Crotyl phenyl selenide, 7	50	48	starting material (40)
9.	Cyclohexylmethyl phenyl sulphide, 6	50	48	cyclohexylmethyl phenyl sulphoxide (62)
10.	Cyclohexylmethyl phenyl selenide, 5	60	48	starting material (50)

Table 16

Control Experiments for the Incubations with *M. isabellina*

	Substrate	Wt(mg) per flask	Time(hr)	Compounds isolated (%)
11.	Phenyl methyl selenoxide	70	24	starting material (50)
12.	9	20	2	-
	Phenyl methyl sulphide	75	75	phenyl methyl sulphoxide (50)
13.	7	60	48	-
	Phenyl methyl sulphide	50	24	phenyl methyl sulphoxide (42)
14.	5	60	72	-
	Phenyl methyl sulphide	60	48	phenyl methyl sulphoxide (40)
15.	Methylene cyclohexane 18	50	48	starting material (gas phase only)
16.	3-butene 2-ol 17	70	48	3-butene-2-ol (74)

Selenium Analysis

The usual procedure for the growth of M. isabellina was followed in preparation for the selenium analysis of the medium and mycelia. A solution of phenylmethylselenide (150 mg) in methanol (1mL) was added to a 72 hour old growth of fungus resuspended in water (150 mL). The incubation was continued for another 48 hours, the flask was then removed from the rotaryshaker and the various dilutions were made from the medium and mycelia. These samples were then analyzed for selenium employing the DC Plasma emission spectrometer, the values are listed in Tables 17 and 18.

^{14}C-Analysis

[Methyl-^{14}C]-methyl phenylselenide was incubated with M. isabellina according to the procedure previously described. To this incubation 120 mg of the labeled substrate in methanol (1 mL) was added to 160 mL of medium and after 39 hours had elapsed, the various dilutions were made.

For all of the various solutions, two samples were prepared, the DPM were then measured on 10 separate runs then these values were averaged and corrected. For the selenide, three samples were accurately measured and each sample was then counted ten times. All of these results are contained in Tables 19 and 20.

Table 17

Distribution of Selenium following incubation with *M. isabellina*

	mg Selenium
Medium blank	.79
Medium before extraction	7.17
Mycelia before extraction	60.53
Mycelia after extraction	31.89
Extracted from mycelia (60.53-31.89) =	28.64
Selenium added to flask	68.6

Table 18

Distribution of selenium following incubation with *M. Isabellina*

	% of selenium (based on added substrate)
Total Medium	11
Mycelia after extraction	46
Extract of mycelia	41
Total Recovered	98

Table 19

[Methyl-^{14}C]-Methyl phenyl selenide

incubated with M. isabellina

Fraction	$\bar{x}$ (corrected)
Selenide	4,356,005
Medium	17,250
Medium extracted	14,675
Alcohol wash [methyl-^{14}C]-methyl iodide	18,903
Mycelia before extraction	2,629,578
Mycelia extract	1,563,095

Table 20

Distribution of Radio-activity following incubation of [Methyl-^{14}C]-methyl phenyl selenide with M. isabellina.

Sample	%total activity of substrate
Medium	.40
Medium extract	.34
Mycelia before extraction	60.4
Extract of mycelia	35.9
Total recovered activity 60.4 + .4 =	60.8

An Investigation into the Hydroxylation of Ethylbenzene and p- Ethyltoluene by *Mortierella isabellina* NRRL 1757 and their Enantiomeric Purity

Experimental III

Incubations

For the initial set up and incubation of Mortierella isabellina, the same procedure was followed as stated in the previous experimental sections.

A. para-Ethylbenzene

Following the growth of Mortierella isabellina over the usual initial growth period and resuspension into H_2O (120 mL per flask), 100 mg of substrate (dissolved in 1.2 mL MeOH per flask) was added to each 1-L erlynmeyer flask. After 96 hours the solution was filtered. The usual extraction procedure of the medium was then completed and 2.36 g of crude product was obtained from 5.0 g of starting material. Column chromatography was performed on this residue and 0.620 mg (12%) of the pure alcohol was obtained. The ^1H NMR contained signals at δ:1.50(3H,d), 1.98(1H,s), 4.9s(1H,m), 7.35(5H,s). The mass spectrum contained peaks at m/e(%):124(3), 122(24), 108(7), 107(100), 104(25), 91(24), 79(92), 78(33), 77(69), 63(7), 51(41), 50(18), 43(51), 39(18), 36(27). This incubation therefore resulted in the production and subsequent isolation of 1-phenylethanol 19.

B. para-Ethyltoluene

Following the same set up as that employed for para-ethylbenzene, the substrate was allowed to incubate with the

fungus for 96 hours, at which time, the usual extraction procedure was performed. Starting with 2.0 g of para-ethyltoluene, 1.1 g of the crude alcohol was obtained. The crude mixture was then purified using column chromatography and 0.073 g of the product was obtained (4% yield).

The ^{1}H NMR of the pure microbial product showed signals at δ :1.38 (3H,d), 2.30 (3H,s), 4.68 (1H,8) 7.01 (4H,s). The mass spectrum contained signals at m/e (90): 136(24), 121(100), 119(35), 118(32), 117(36), 115(16), 93(93), 92(24), 91(94), 89(10), 77(50), 65(32), 63(14), 51(18), 43(83), 36(81). The spectral data corresponds to the production of p-methyl-1-phenylethanol 20.

Optical Rotation Data

Following the isolation of 1-phenylethanolfrom M. isabellina, a sample was sent to McMaster University, Hamilton, for an optical rotation measurement. Into a 1 mL volumetric flask filled with MeOH, 17.2 mg of the alcohol was added. (C = 0.85) This sample produced an optical rotation

$$[\alpha]_D^{22} = + 0.060 \pm .002^{o}$$

A second flask (5 mL) was then filled with MeOH and 19.1 mg of a hexane washing (C = 0.38) (1-phenylethanol) was added.

$$[\alpha]_D^{22} = + 0.032 \pm 0.002$$

$$[\alpha]_{actual} = \frac{100\ [\alpha]_{observed}}{lc} \qquad (16)$$

Where c = g/100 mL

l = cell length in dcm (l = 1)

For the (1) enantiomer $[\alpha]_D$ =-45.5 (MeOH, c = 5).
If the value obtained for 1-phenylethanol is due to only this alcohol and no lipid involvement,
the maximum enantiomeric enrichment = 15%

Procedure for Shift Reagents

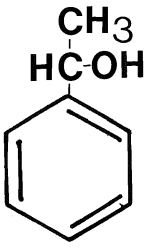
A. 1-Phenylethanol 19

A solution of 30.5 mg of 1-phenylethanol (in .5 mL of reagent grade carbontetrachloride) was added to 75 mg of the shift reagent Eu $(hfbc)_3$, in a NMR tube. This mixture was then assayed by a NMR scam in the the range of 15-6 ppm. The spectrum showed two signals that were coupled together δ 12.58 (1H, m) and 8.15 (3H, t). These values were obtained relative to spectroscopic grade benzene as an internal standard at δ7.3. One signal was assigned as a split quartet and the other a split doublet. These assignments were based on decoupling of the various signals. By irradiating at the doublet, the quartet collapsed to a triplet. The quartet signal appeared as though it was composed of two overlapping quartets. The doublet appeared to be a triplet but was believed to be composed of two doublets superimposed. By decoupling at the quartet signal, the doublet signal collapsed to two signals of

different peak areas. An integration of these two signals produced a value of 47:53 for a ratio of the two isomers, corresponding to an enantiomeric excess of 6%. The integration of the two peaks was carried out ten times and these values were averaged to obtain the ratio.

B. para-Methyl-1-phenylethanol 20

Following the same procedure as previously described, 39.8 mg of the alcohol in 0.6 mL of carbontetrachloride (.55M) was placed in an NMR tube. To this solution, 90.5 mg of the Europium shift reagent was added and the resulting mixture was scanned from 15-6 ppm. Using spectroscopic grade benzene as an internal standard, the NMR spectrum contained signals at 8.5 (3H, d), 8.75 (3H, d) and 12.48 (1H, t). Decoupling of the triplet resulted in the collapse of doublet signal into two singlets, one of which was poorly resolved and of low intensity (8.75 ppm). An integration of these two signals resulted in values of 10.4:89.6, corresponding to an enantiomeric excess of 80%.



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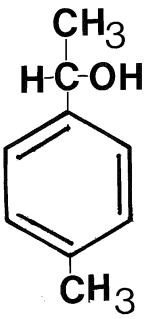

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Table 21. The Enantiomeric Purity of Products Isolated following Incubations with *M. isabellina*.

Substrate	Product	Enantiomeric Purity
p-Ethylbenzene	1-Phenylethanol	6%
p-Ethyltoluene	p-Methyl-1-phenylethanol	80%

The mechanism of Sulphide Oxidation by *Mortierella isabellina* NRRL 1757

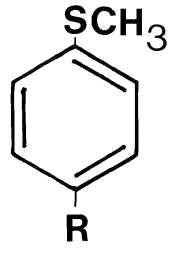
Discussion I

In attempting to study the enzymic oxidation of various sulphides 1 and 3, control experiments were performed prior to the start of the work presented in this project. These experiments need only a brief mentioning in order to obtain a clear understanding of Mortierella isabellina and why it was chosen for this study.

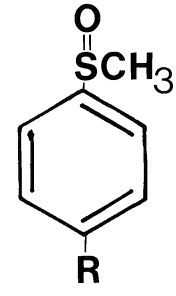
An ultraviolet scan of the aqueous replacement culture medium, containing the growing fungus, in the absence of sulphide was monitored over a period of 36 hours. This was done in order to find out if any ultraviolet absorbing compounds were present that would interfere with the analysis. However none was discovered. Various mixtures of sulphides and sulphoxides were prepared and an ultraviolet spectral analysis of these mixtures gave reliable measurements of the concentration of these solutions. In order to prove that Mortierella isabellina was capable of producing a range of sulphoxides, the same synthetic sulphoxides were characterized and compared to the microbial products. An ultraviolet scan at the termination of each run was taken and compared to that of an authentic sample. From this data there was no evidence to suggest the possibility of the formation of an ultraviolet absorbing compound other than the sulphoxide. It appears that, on the addition of phenyl methyl sulphoxide to the aqueous medium of Mortierella isabellina, is not taken up by the fungus, meaning that the association with the cell membrane and the active site is low.

These measurements support the assumption that the sulphoxide once released from the active site rapidly passes from the interior of the cell into the incubation medium. If this were not true, then the measurements may relate to the passage of sulphoxide from inside the cell membrane, a diffusion property, and not the kinetic effect of the various R groups on the sulphide 1 and 3. The rates for the Hammett plot were determined by monitoring the release of sulphoxide 2 and 4 into an aqueous medium by ultraviolet spectral analysis. In order to carry out the analysis, various extinction coefficients for the sulphides 1 and 3 and sulphoxide 2 and 4 were required at suitable wavelengths. Using a two component analysis and these extinction coefficients, the rate of sulphoxide production was obtained.

Both the sulphides and sulphoxides needed for the completion of this work were obtained prior to the start of this thesis.



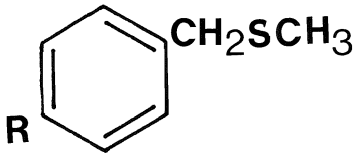
1



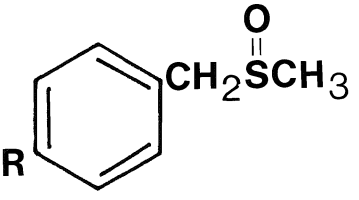
2

1,2

a R= H
b R=CH
c R=C_2H_5
d R= F
e R= Cl
f R= Br
g R=NO_2
h R=OCH_3



3



4

3,4

a R= H
b R=CH_3
c R=NO_2

The oxidative enzymes employed by Mortierella isabellina, as determined by $^{18}O(g)$ labeled experiments, show one of the major characteristics pertaining to monooxygenases. The oxygen entering the sulphide is obtained from the atmosphere rather than the aqueous medium. Other work demonstrated that utilizing ^{18}O labeled water there was no incorporation of the label in the isolated sulphoxide product.[64] Other useful experiments would be the isolation of the enzyme, and its characterization. However, the time involved for such projects is beyond that available for this study.

In dealing with any membrane bound system, such as the cytochrome P-450 dependant oxygenases present in Mortierella isabellina, the complexity of these biological environments makes the interpretation of various correlation studies difficult. Other factors such as membrane transport, steric problems and cofactor oxidation or co-oxidation could effect the value of rho and the proposed mechanism. The problem then is whether or not a linear relationship actually gives some mechanistic information pertaining to the question being studied, in this case the active species involved with cytochrome P-450. As with most biological systems, the problems of other factors influencing the reactions may be reduced by forseeing some of these problems and setting up suitable controls. In an attempt to show that the variation in reaction rate is correlated to the electronic, and not steric, properties of the para substituent, a study of three benzyl species 3a-3c was performed. In this case, the para R

groups should not be able to stabilize the reaction intermediate and the rates of sulphoxidation should be equivalent.

From the various kinetic measurements (3a-c), the rate of sulphoxide production for both the benzyl and aryl sulphides produced reasonable results. The rate observed for the oxidation of the benzyl sulphides confirms the fact that the phenemenon which is being measured probably can be attributed to an electronic effect of the para substituent and not the difference in size or the polar nature of the groups involved.

For the sulphides, a total of eight sulphides were employed to produce the Hammett plot as seen in Figure 20. From this group of eight sulphides only five produced a linear relationship with a rho value of -0.67. The data did not however correlate well with σ^+ values unlike the results obtained by previous researchers.[66] Those species that were capable of releasing electrons casued the rates to increase while electron withdrawing groups retarded the rate of sulphoxidation. The other three sulphides 1b (R = Ch_3), 1c (R = C_2H_5) and 1f (R = Br) did not correlate well with the data and were not used in the calculation of the rho value. The deviation in the rates for these three compounds may be a result of a change in the rate limiting step of the reaction. This however does not appear to be a plausible explanation, in relation to other enzymic reactions. Why should the fungus employ a different mechanism for these three compounds and not for the other five? In examining the benzyl compounds, all three species, 3a-c, seemed to produce reasonable results, there was no

deviation in 3b in comparison to the other two. Another explanation for this deviation could be a hydrophobic interaction between the substrate and the fungal membrane. An experiment to prove this interaction would be to study the benzyl oxidation rates of the corresponding eight alkyl aryl sulphides. If this interaction is present, then there should be similar deviations in the benzyl oxidation rates. In examining the oxidation rates pertaining to the benzyl sulphides 3a-3c, it appears that 3b does not show any appreciable change when compared with the other two. One further explanation for the problem encountered with 1b and 1c is that the oxidation of the methyl and ethyl groups is occurring. If the two groups were being oxidized, then there would be an uptake of sulphide but no sulphoxide production. It would appear as though the rate of sulphoxidation was slower than it actually was. For the benzyl compounds 3b and 3c, this hydroxylation should also occur. If this is true then their rate of sulphoxidation should also decrease, but this was not observed. This irregularity therefore requires further investigation as this phenomenon does not occur when dealing with the benzyl sulphides. The problem with compounds 1 & 3 can not adequately be explained at this time and requires further experimentation.

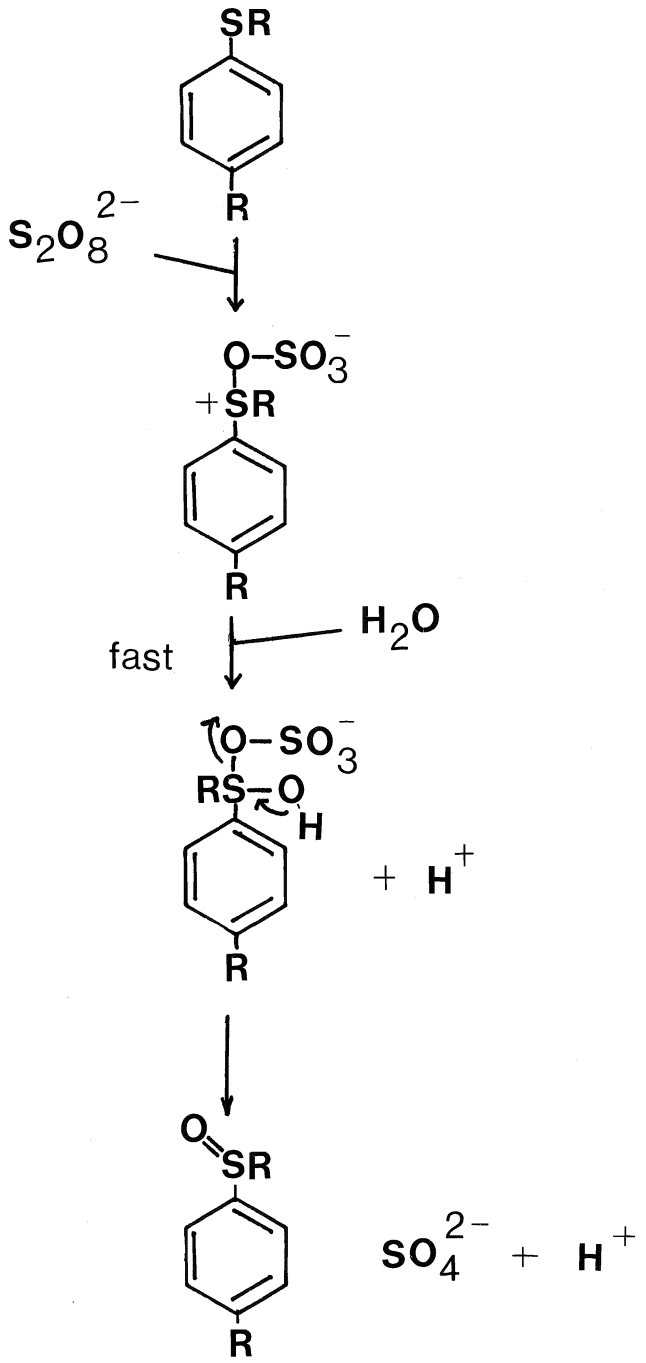
The rho value of -0.67 obtained in this study can be compared to those in Table 2. This value seems reasonable as it is within the range of both the chemical and enzymic reactions (except for the dopamine β-hydroxylase). All of the enzymic reactions present in Table 2, except for the dopamine β-hydroxylase ($p = 3.6$)[71] show a low negative value of rho and

the mechanisms based on these values, for these enzymes, seem to involve an intermediate at the transition state where there is very little electron demand on the substrate. Where there is an hydroxylation at a saturated carbon[87] (rho = -1.6), a carbon radical intermediate has been proposed and with the same enzymic preparation, the oxidation of sulphur was proposed to occur via a radical cation species (ρ = -0.16).[66]

In comparison to those values from chemical and enzymic reactions, the ρ value of -0.67 differs significantly from some studies using peroxide oxidants, for instance: tert-butyl hydroperoxide[75] (-1.97), hydrogen peroxide[77] (-1.13) and isoalloxazine hydroperoxide[79] (-1.67). There are, however, three instances where the rho values are similar; the oxidation of various phenyl methyl sulphides with H_2O_2 and a metal catalyst produced a rho value of -0.7.[78] Oddly enough these researchers were employing similar alkyl aryl sulphides in their study. The oxidation of sulphoxide to sulphones using m-chloroperoxybenzoic acid resulted in a rho value of -0.54. The final group was Srinivasan et al.[155] who reported a rho value of -0.87 (ρ^+ = -0.56) for the oxidation by potassium persulphate. These authors reported that the slow step in the reaction, the rate determining step, is the electrophilic attack at sulphide sulphur by the persulphate ion to form an intermediate sulphur cation, which in the presence of water produces the sulphoxide (Figure 22).

The results obtained from this study do not however reinforce the one electron route proposed for the sulphoxida-

Figure 22. Proposed Mechanism for the Oxidation of Alkyl Aryl Sulphides by a Peroxy-disulphate Ion.



tion by rat liver microsomes[66,67] (Figure 15); the value of rho, was larger than that using rat liver microsome preparation. The magnitude of rho infers that there must be a greater electron demand on sulphur at the transition state than that proposed by Watanabe et al.[85] The electrophilic attack by an iron-oxygen enzymic complex on sulphur appears to be a most reasonable mechanism, analagous to the persulphate mechanism.

In attempting to look at the various enzymic reactions, in terms of similar mechanisms for the sulphoxidation, some of these comparisons may not be directly relevant. For instance the dopamine β-hydroxylase enzyme is a copper containing enzyme and in one instance the site of oxidation was a carbon rather than sulphur. The difference in the site of attack often results in a different mechanism as stipulated by May et al.[71] Another potential problem lies in studying a dioxygenase and[154] attempting to relate its mechanism to a monooxygenase. There could however be some similarities between the two mechanisms and this is why they are studied. Also, some of these studies use σ^+ values to produce a better correlation coefficient. Thus it depends on whether or not the authors are utilizing a p^+ value to explain their mechanism.

The last study in Table 2, involves the use of a rat liver microsome preparation, however the source of oxygen for the hydroxylation reaction is from cumenehydrophoxide. Due to this change in source of oxygen, the mechanism involved with this species could possibly be different, unless of course the mechanism involves a peroxide and the variation of

rho is due to other factors. In order for the enzyme to work efficiently the cumenehydroperoxide has to be able to reach the active site and react with the substrate to form products. There are now two species which have to reach the active site and this could complicate the reaction mechanism. Thus this added problem makes a direct comparison of this enzyme system with cytochrome P-450 difficult.

The scope of the study of enzymic reactions utilizing both sulphur and carbon is apparent from the various entries in Table 2. Both these substrates are suitable for attempting to discover the active species present in monooxygenase such as cytochrome P-450 (or dioxygenase). However, it is apparent that the two mechanisms have different electronic considerations when looking at the rho values contained in Table 2. It has not been established that the elucidation of the sulphoxidation mechanism will in any way shed some light on the carbon hydroxylation. Only when a similar study relating to the carbon hydroxylation has been completed (under similar conditions) can a direct comparison be made between the two. Continued research is still required regarding the mechanism of cytochrome P-450. The value of -0.67 obtained in this study is another indication that it is an electrophilic attack by an iron-oxygen enzymic complex on sulphur similar to that of the persulphate mechanism.[155]

Future work pertaining to this study could involve an investigation into the oxidation of various benzylmethylsulphides in order to discover why the para-ethylbenzylmethyl-

sulfide did not exhibit the same behaviour as the corresponding aryl alkylsulfides, or the examination of different alkyl substituents on the sulphur (for example ethyl phenyl sulphide or butyl phenyl sulphide). In order to discover if the reaction was sensitive to steric congestion. Due to the wide range of specificity of cytochrome P-450, the last suggestion regarding steric congestion may not provide any new information regarding this enzyme.

An Investigation of the Biotransformation of Organic Selenides by Fungi

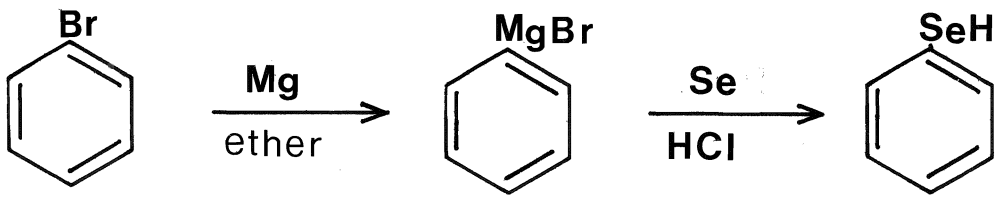
Discussion II

In order to discover whether or not the various fungui were capable of oxidizing the selenides 5, 7, it was decided that an incubation with phenyl methyl selenide 9 would be attempted first, since M. isabellina was capable of oxidizing the corresponding sulphide.[64] Following the incubation with 9, the remainder of the work was directed towards the syntheses of the selenides 5, 7, and the corresponding sulphides 6, 8. The incubation of these sulphides was attempted prior to the selenides in order to discover if the active site could accommodate these compounds.

For the synthesis of the selenides 5 and 7, phenyl selenol 13 was required for both species as a starting material. The procedure for its synthesis involved the production of the grignard reagent phenylmagnesium bromide in dry diethyl ether and the subsequent addition of selenium power and acid produced the selenol (Figure 23). A distillation of the product produced an adequate quantity of phenyl selenol. Extreme care was required due to the possible production of hydrogen selenide (H_2Se) during the final stage of its production. Also, once phenyl selenol was obtained, the product needed to be stored under nitrogen to prevent the formation of diphenyldiselenide(Ph_2Se_2). In any subsequent reactions, care was exercised so that the compound was stored under nitrogen for future use.

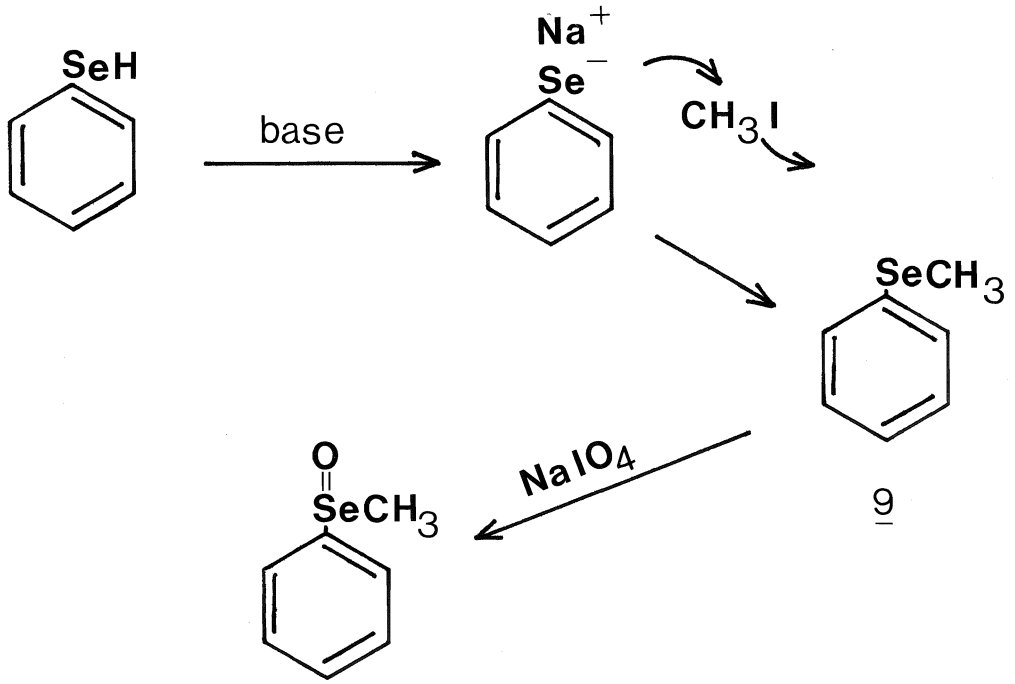
The production of phenyl methyl selenide 9 was accomplished through a standard procedure. An S_N2 reaction involving

Fibure 23. Synthesis of Phenylselenol 13.



13

Figure 24. Production of Phenyl methyl selenide 9 and Phenyl methyl selenoxide 14.



9

14

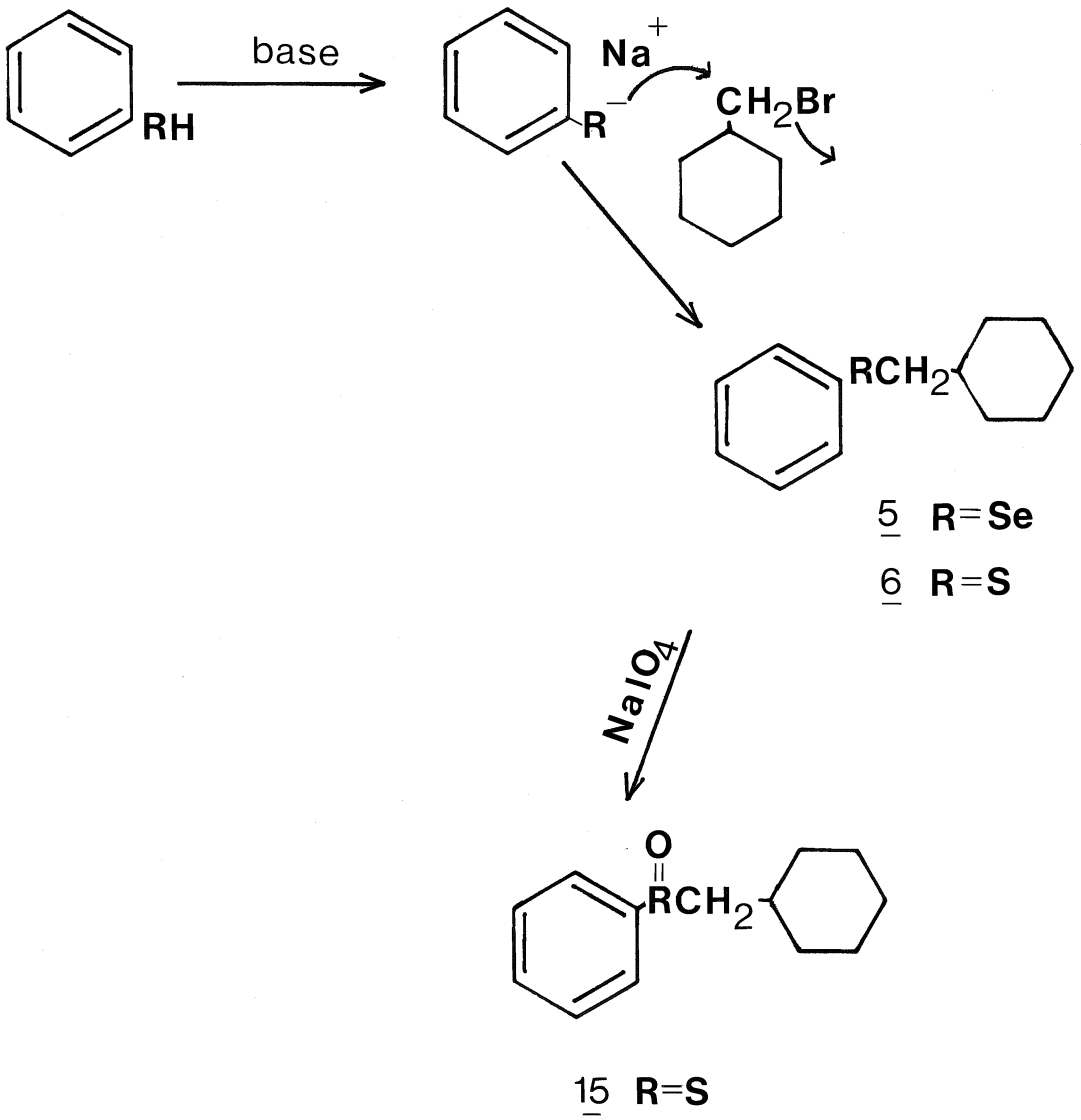
the selenol, base and iodomethane(Figure 24).

Since phenyl methyl selenide 9 was the control species, the corresponding selenoxide was synthesized chemically in order to have a standard from which to compare the putative microbial products. Phenyl methyl selenoxide 14 (Figure 24) was then synthesized using a standard oxidizing reagent sodium metaperiodate (NaIO4), this species was chosen due to its ability to oxidize sulphides to sulphoxides without the production of sulphones[64] which were often difficult to separate. There is very little mention of phenyl methyl selenoxide in the literature except for a chemical communication regarding its melting point.[146] Both the infrared and ^{1}H NMR spectra agreed with the proposed structure, but the (electron impact) mass spectrum however was not helpful. The mass spectrum contained a molecular ion for diphenyl diselenide. It was only when the FAB source was used that a molecular ion was detected for phenyl methyl selenoxide 14.

The mechanism involved for the synthesis of cyclohexylmethyl phenyl sulphide 6 and the corresponding selenide 5 was identical, as illustrated in Figure 25. A nucleophilic attack of the thiol (selenol) on cyclohexylmethyl bromide in an S_N2 reaction. The ^{1}H NMR, mass spectra and the physical characteristics of both samples indicated that in fact these species were obtained (6, 5).

Since cyclohexylmethyl phenyl sulphide was a substrate in an incubation, then the corresponding sulphoxide (Figure 25) was also required for a comparison to the microbial

Figure 25. Syntheses of Cyclohexylmethyl phenyl sulphide 6, Cyctohexylmethyl phenyl sulphoxide 15 and Cyclohexylmethyl phenyl selenide 5.



extract. Using sodium metaperiodate and following the usual procedure for the oxidation reaction, the isolation of cyclohexylmethyl phenyl sulphoxide 15 was confirmed through ^{1}H NMR mass spectroscopy and ^{13}C NMR.

The synthesis of crotyl phenyl sulphide 8 and crotyl phenyl selenide 7 occurred through the standard procedure of the reaction of a nucleophile with crotyl chloride in an S_N2 mechanism as seen in Figure 26. The physical and spectral properties of both compounds seem reasonable with no major irregularities. The mass spectrum of the selenide did however cause some problems as the molecular ion discovered through electron impact corresponding to the presence of diphenyl diselenide. Even though there was a molecular ion for diphenyl diselenide (of low intensity), there was also a peak present around the 211 region which could be the molecular ion for crotyl phenyl selenide 7. The low intensity of the ion at 314 (m/e) was sufficient to warrant further investigation.

In order to resolve this problem, a library spectrum of diphenyl diselenide was obtained and compared to the one obtained from the synthesized selenide. The fragmentation patterns were different as well as their intensities, it was therefore decided they did not belong to the same compound. These variations could however be due to the differences in the method of obtaining the spectra. An elemental analysis of this compound was sufficient to prove that crotyl phenyl selenide 7 was the product.

The synthesis of phenyl methyl selenoxide 14, cyclohexyl-

Figure 26. The Preparation of Crotyl phenyl sulphide 8, Crotyl phenyl sulphoxide 16 and Crotyl phenyl selenide 7.

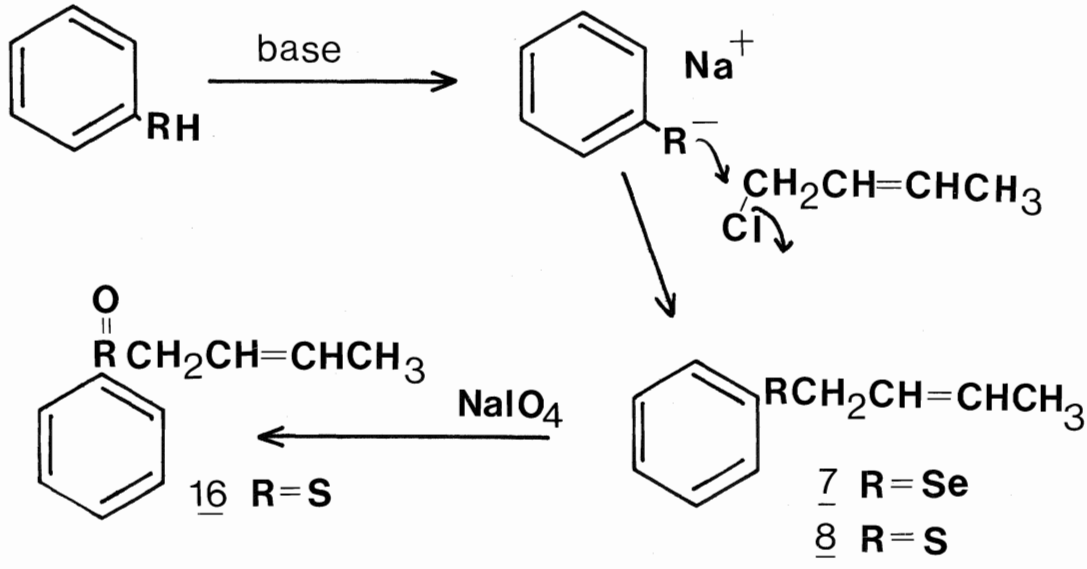
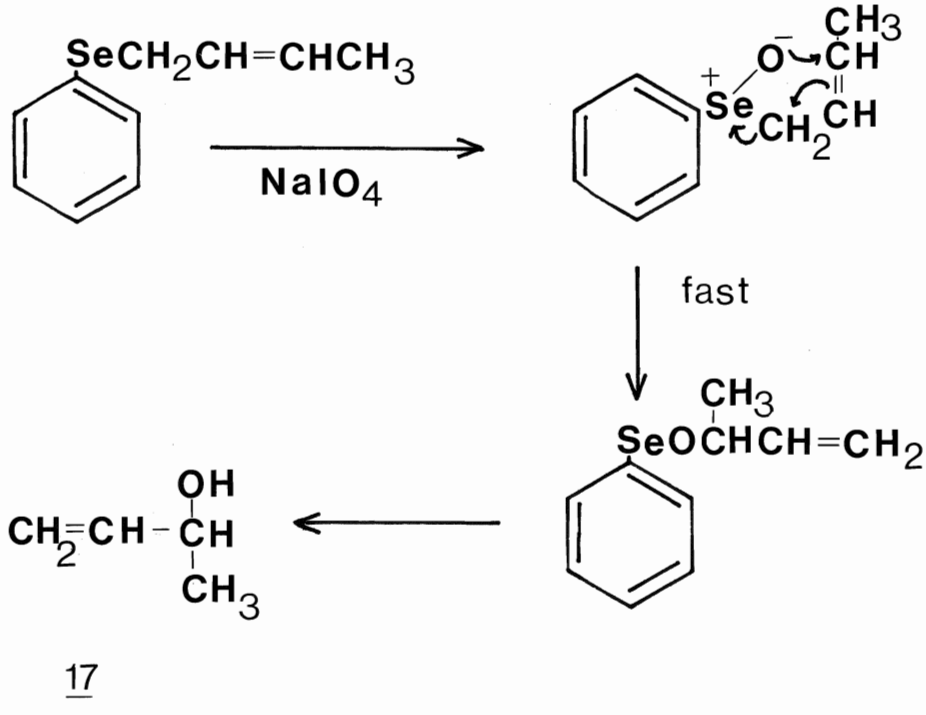


Figure 27. The Production of 3-Butene-2-ol 17 from Crotyl phenyl selenide 7.



17

methyl phenyl sulphoxide 15 and crotyl phenyl 16 sulphoxide were completed in order to obtain standards for the incubations. The oxidation of the corresponding selenides 5, 7 with sodium metaperiodate were also attempted with some minor success. The oxidation of crotylphenylselenide resulted in the isolation of a residue, which upon gas chromatography-mass-spectrometry contained a volatile component, 3-butene-2-ol 17 (based on a commercially obtained sample) (Figure 27). The oxidation of cyclohexyl methyl phenyl selenide, did not, however produce any detectable methylene cyclohexane 18 (Figure 28). Due to its low molecular weight and its low vapour pressure any sample produced due to the syn elimination may have been lost into the head space of the incubation flasks.

Incubations.

The three fungi; A. niger, A. foetidus and Helminthosporium sp. are all capable of performing the efficient asymmetric oxidation of alkyl aryl sulphides.[64,70] The incubation of A. niger and A. foetidus with phenyl methyl selenide however failed to produce the corresponding selenoxide of the substrate 14. In all three cases, only starting material was recovered (50-60%). Due to this lack of selenoxide formation from the reaction with these fungi, it seemed futile to try to oxidize the remaining selenides by further incubations. There are three possibilities that may explain why problems were

encountered with the selenide 9. The first possibility is that the substrate was unable to cross the cell membrane. The second is that the substrate could not enter the active site of the enzyme and the third, that the substrate was toxic to the fungi.[156]

The remainder of the time available for this investigation was spent examining the possibilities of substrate oxidation with M. isabellina.

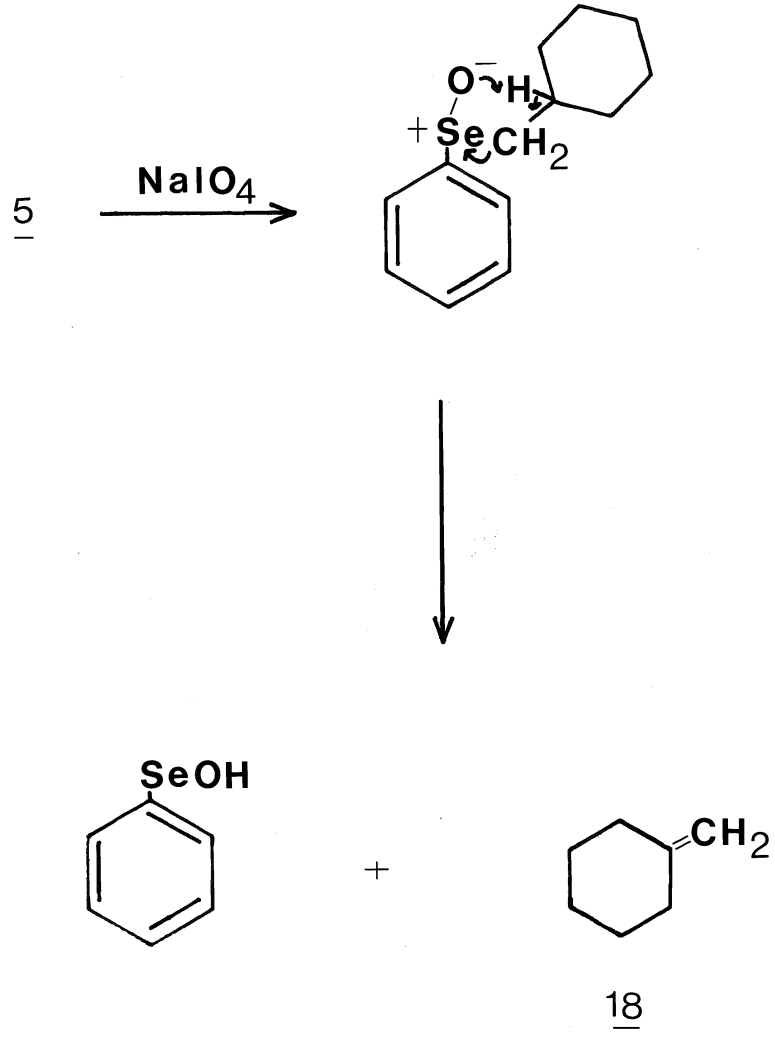
The results obtained from these incubations are contained in Table 15 and 16. It appears the M. isabellina is capable of oxidizing phenyl methyl sulphide 1a, crotyl phenyl sulphide 8 and cyclohexylmethyl phenyl sulphide 6, but the incubations with the corresponding selenides 5, 7 and 9 resulted in the recovery of only starting material. The substrate concentration of phenyl methyl selenide was varied in order to discover if the initial addition of 100 mg of substrate per flask was the reason for the lack of oxidase activity by M. isabellina. The subsequent reduction of the quantity of substrate by 40% followed by the results obtained through the control experiments dictate that the concentration of substrate was not the reason for the lack of selenoxide formation. The results contained in Table 16 represents the various control experiments. In these experiments the selenide was added first to the incubation medium and following the various incubation periods, the medium was filtered and a new substrate was then added to the resuspended mycelia. Runs 12-14 (see Table 16) illustrates that the fungus M. isabellina is not capable of carrying out

the oxidation of the alkyl aryl selenides 5, 7, and 9. However, the addition of these substrates does not damage the fungus as it is still able to oxidize phenyl methyl sulphide to the sulphoxide. The last two control experiments were completed in order to discover if it was possible to identify the oxidation products from the syn elimination (Figure 28) or the (2, 3)-sigmatropic rearrangement (Figure 27). One reason for the inability of the fungus to carry out the oxidation could be because of the processes involved in the isolation of these products. The fungus could be working effectively but the sensitivity was such that they could not be detected. In control experiments methylene cyclohexane 18 was isolated from the head-space of the flask while 3-butene-1-ol 17 was isolated from the extraction residue.

The remaining work was involved with attempting to discover what was happening to the selenide substrate, once added to the incubation medium. This was accomplished using two different methods of analyzing for the substrate. The first involved the use of a ^{14}C label in phenyl methyl selenide and the second was a selenium analysis using a plasma emission spectrometer.

The incubation of [methyl-^{14}C]-phenyl methyl selenide with M. isabellina and the subsequent assays of the medium, mycelia, extracts of both medium and mycelia produced some inconsistent results. Of the total selenide 9 added to the growing fungus in distilled water only 0.4% was located in the medium and 85% of which was extractable (refer to Table 20).

Figure 28. The Syn Elimination to Produce Methylene cyclohexane 18.



For the mycelia, 60.4% of the label was present in the mycelia and from which 60% was recovered in the mycelia extract. The only problem with this system of analysis was that only 60.8% of the ^{14}C label was recovered. The remaining 39.2% is unaccountable. The only explanation for this loss could be due to the loss of label into the atmosphere or possibly the scintilation measurements were unreliable due to quenching of the samples.[157]

The results obtained from the selenium analyses were however more encouraging (Tables 17 and 18). It seems that of the total selenide added to a flask of growing culture, approximately 11% is contained in the medium. This is however inconsistent with the ^{14}C assay. A total of 98% of the selenium added initially was accounted for versus 60.8% for the ^{14}C study. From the selenium analysis it appears that the mycelia contains the majority of the substrate and that from this 88%, it is possible to extract 53% of the label that was originally there. The only drawback with this experiment was that the medium was not extracted in order for a selenium analysis to be obtained.

The results obtained from these two studies do however reinforce one another in certain areas. A majority of the substrate was located in the mycelia and that through extraction, approximately half can be recovered.

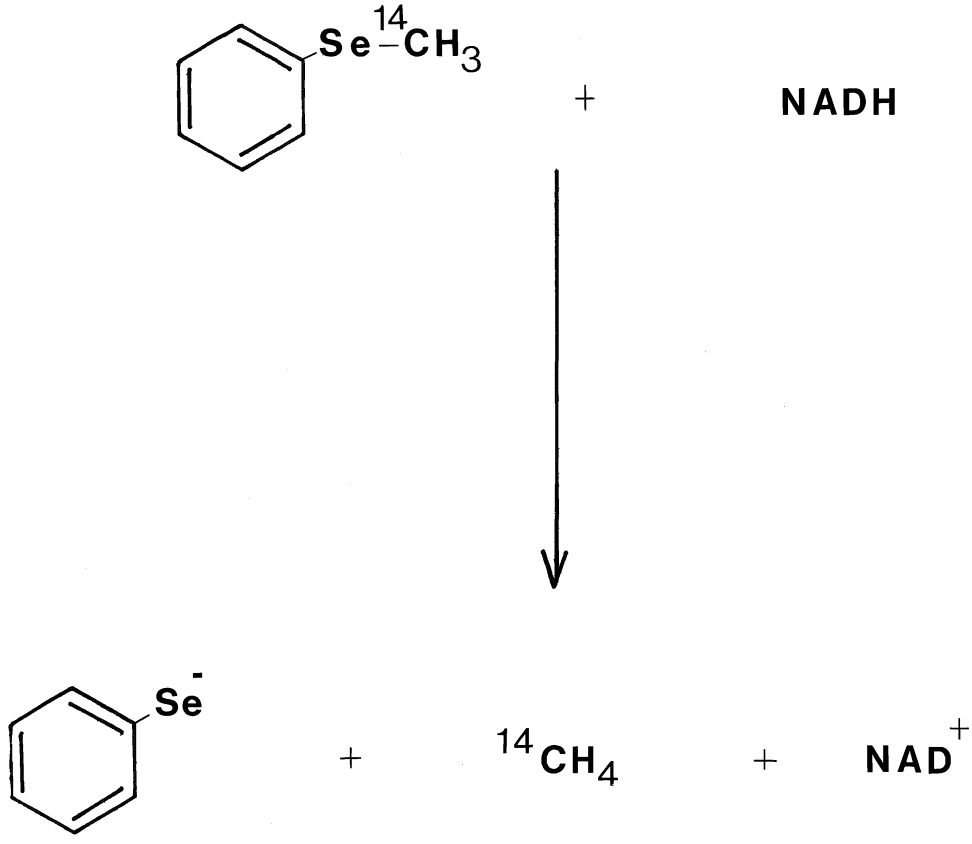
The problem of oxidation must be within the active site or possibly the substrate entering the active site, since the assays report that a majority of the labels (^{14}C, or selenium)

are present in the mycelia. Therefore the inactivity of the enzyme is not due to the inability of the substrate to enter the cell but with the enzyme itself.

Both these procedures are based on the notion that phenyl methyl selenide is being measured and not just their respective labels. One possibility is that the substrate is disassembled once inside the mycelia and the various portions are then utilized by the fungus. There is sufficient evidence to suggest that selenium and sulphur are metabolized through the same metabolic pathways.[158]

One further experiment would involve the synthesis of the corresponding sulphide with the ^{14}C label at the same site and monitor its uptake once incubated with M. isabellina. Would the distrubation of the label be the same? This probably need not be done as it has been documented that M. isabellina is capable of oxidizing phenyl methyl sulphide to the corresponding sulphoxide in a 60% yield.[70] The product usually is obtained from the medium. Using these results, the radioactive label should therefore be present in the medium greater than the 0.4% obtained from the ^{14}-C assay with 9. The incubation with the labelled sulphide should therefore resolve the problem if the scintillation technique is producing reasonable results. One further possibility for the loss of the ^{14}C-label and not the selenium could be due to a reductive cleavage of the selenium carbon bond, as illustrated in Figure 29. If this were the case, then the label would have been lost into the head space of the flask. A sample of the gas was removed,

Figure 29. Explaination for the Loss of Radioactive Label, Following an Incubation with M. isabellina.



however it was originally thought that carbon dioxide was present and the experiment was set up to trap this gas.

Chemical evidence for such a reaction is the increased stability of the selenides versus the corresponding sulphur ions. The pka of the selenides is usually lower than the same sulphides.[159] Also, reactions with n-butyllithium and phenyl methyl sulphide have resulted in the isolation of thiophenol.[160] In this case there was cleavage of the carbon-sulphur bond. Similar experiments have been attempted with phenyl methyl selenide 9, however with little success of isolating phenylselenol.[161]

The four fungi employed in this study, in spite of their ability to oxidize alkyl aryl sulphides,[70] were unable to carry out the oxidation of the corresponding alkyl aryl selenides. Attempts were made to discover the reason for this problem. It seems that the substrate is capable of entering the cell and the techniques utilized for the detection of these fragmentation products (17, 18) were adequate. The experiments performed in this section point to the conclusion that, the specificity of the monooxygenase enzymes present in the fungi (employed in this study) do not however, include alkyl aryl selenides. These four fungi are therefore unacceptable for the asymmetric microbial oxidation of selenides and the subsequent production of olefins and alkylic alcohols (Equations 5 and 8).

An Investigation into the Hydroxylation of Ethylbenzene and p—Ethyltoluene by *Mortierella isabellina* NRRL 1757 and their Enantiomeric Purity

Discussion III

Preliminary work with Mortierella isabellina, regarding the addition of ethylbenzene to growing cultures of the fungi, produced a hydroxylated species. Further studies discovered that in fact 1-phenylethanol was produced by this microorganism. The importance of this discovery is that no other microorganism (at this time) is known to be capable of carrying out this reaction. It is believed that Pseudomonas putida[135] is capable of this hydroxylation but no direct evidence is available for this phenomena. In most of the examples previously mentioned, the oxidation reactions were mainly with the aromatic ring (formation of diols)[124] and not the α carbon of a substituent. This reaction has however been monitored with various mammalian microsome preparations. It is therefore the purpose of this study to investigate this hydroxylation reaction with ethylbenzene and also para-ethyltoluene.

Preliminary experiments of various concentrations of 1-phenylethanol and the shift reagent were completed prior to these measurements of enantiomeric purity. The molar ratios were (substrate:shift reagent) 0.5M:0.125M, 0.5M:0.169 M and 0.5M:0.23M. Of the three solutions, the first two produced reasonable results in terms of the separation of the doublets and peak broadening. The third solution was unacceptable because of peak broadening. Due to the cost involved with the use of these shift reagents, the molar ratio decided for these compounds was 0.5M:0.125M.

Using the chiral shift reagent $Eu(hfbc)_3$, (Figure 19), the

enant omeric purity of the products obtained from the oxidation of p-ethylbenzene and p-ethyl-toluene was determined (Table 21). The approximate value obtained from the optical rotation measurements of 1-phenylethanol confirms the results obtained from this chiral shift reagent. The problem with these optical rotation measurements is that an initial ^{1}H NMR spec.of this product contains a peak due to the presence of lipid (from the mycelia) in the sample. Washing with hexane removed some of the lipid however this procedure was completed only twice for fear of losing most of the product in each successive washing. This lipid does have a positive optical rolation and since the amount present in 1-phenylethanol can not accurately be calculated, a maximum value was reported. Also, the value used in the calculation is based on the (l) enantiomer which was purely a random choice. Thus, the value obtained from the optical rotation measurements serve only as a rough check. An optical rotation measurement has to be obtained for p-methyl-1-phenylethanol to check against the chiral shift reagent.

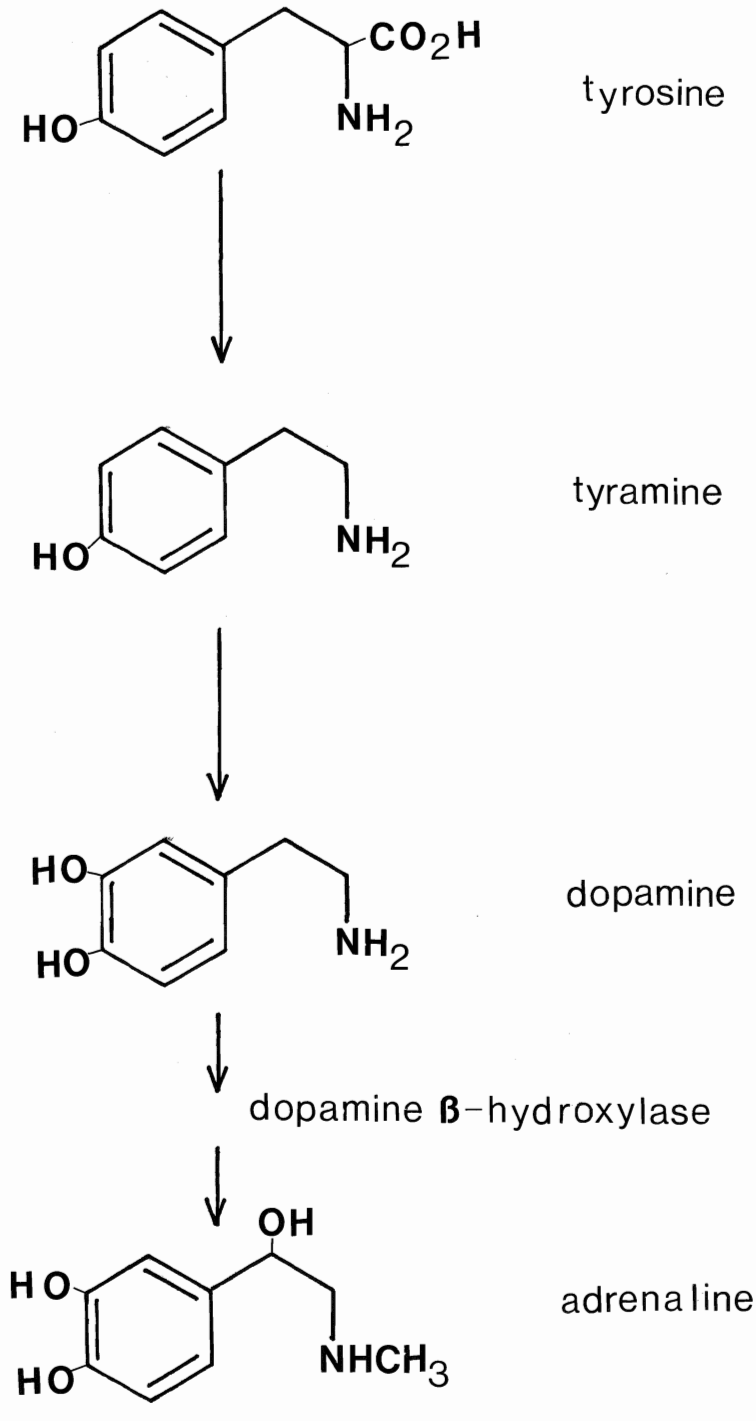
In order to discover which isomer is present in solution, one of them could be obtained (Aldrich) and added to the microbial product. By observing the change in the appropriate NMR peak height and following the same procedure, the configuration of the isomer can be resolved. This is true for 1-phenylethanol and could be used to relate to p-methyl-1-phenylethanol, based on the assumption that the enzyme has the same stereospecifity for both hydroxylations.

In looking at the values obtained for the two products (using the shift reagents, Table 21) some mechanistic information can be gained from these measurements. The enantiomeric purity of 1-phenylethanol is roughly 6% and for p-methyl 1-phenyl-ethanol 80%. The presence of the methyl group caused a large increase in the optical purity of the product. Assuming that when ethylbenzene is in the active site, it is not a tight fit and therefore free to move, resulting in a mixture of stereoisomers upon hydroxylation. For p-ethyltoluene, the fit in the active site has improved and there is more restriction in its movement. This could therefore account why the enantiomeric purity is large for p-methyl-1-phenyl ethanol when compared to 1-phenylethanol.

The chiral shift reagent employed in the study $Eu(hfbc)_3$, performed admirably in determining the enantiomeric purity of two microbial products. From these measurements, it appears that M. isabellina is capable of carrying out a stereospecific hydroxylation at the α position of a hydrocarbon and that further work with this system is still needed to discover its maximum potential in microbial transormations. Its present application in research has been involved with the efficient oxidation of alkyl aryl sulphides, of a high enantiomeric purity.[64,70]

One future possibility for this fungi is the production of adrenaline from tyrosine (Figure 30). In this reaction pathway, the hydroxylation reaction is usually carried out in the body by dopamine β-hydroxylase,[71] If M. isabellina is

Figure 30. Possible use for M. isabellina, in the Synthesis of Adrenaline.



capable of this same reaction, then it may serve as a relatively easy procedure for the commercial production of adrenaline and noradrenaline. Mechanistically it would also be important, where a direct comparrison between the two enzymes could be made. Besides M. isabellina, future research is required with Helminthosporium sp. This fungi is capable of the same oxidation reaction as M. isabellina,[70] however with the opposite configuration. If M. isabellina is unable to carry out the hydroxylation, due to the wrong stereochemistry, there is the possibility that Helminthosporium sp. could. The hydroxylation reaction with ethylbenzene and p-ethyltoluene would however have to be completed prior to this work.

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