Biotransformation of Alkenes by *Rhodococcus* OU

by

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Thesis submitted to the University of Warwick for the degree of Doctor of Philosophy

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Finally, I wish to thank my wife, Jenny, for her eternal patience, love and support throughout this project, and my parents for the many sacrifices made over the years for my education.

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DECLARATION

All of the work presented here was carried out wholly by myself. All other sources of information have been acknowledged within the bibliography. None of the material contained within this thesis has been used in any previous application for a degree.

Jonathan Ralph Hunt

LIST OF ABBREVIATIONS

$[\alpha]_D^x$	Specific rotation of plane-polarized light at 589nm (sodium D-line) and x°C.
A_x	Absorbance at x nm.
ATCC	American Type Culture Collection, Rockville, Maryland, USA.
d.o.t.	Dissolved oxygen tension.
EDTA	Ethylenediaminetetraacetic acid.
% ee	% Enantiomeric excess.
$FADH_2$/FAD	Flavin adenine dinucleotide (reduced/oxidized forms).
g	Acceleration due to gravity.
GLC	Gas-liqid chromatography.
GLC/MS	Gas-liquid chromatography/mass spectrometry.
h	Hour.
HPLC	High performance liquid chromatography.
IFO	Institute for Fermentation, Osaka, Japan.
IUPAC	International Union of Pure and Applied Chemistry.
Km	Michaelis constant.
NADH/NAD^+	Nicotinamide adenine dinucleotide (reduced/oxidized forms).
NADPH/$NADP^+$	Nicotinamide adenine dinucleotide phosphate (reduced/oxidized forms).
^{1}H-NMR	Proton nuclear magnetic resonance spectroscopy.
min	Minute.
MMO	Methane monooxygenase.
sMMO	Soluble methane monooxygenase.

pMMO	Particulate methane monooxygenase.
MS	Mineral salts medium.
NRRL	Northern Utilization Research and Development Division, Peoria, Illinois, USA.
PHB	Poly-ß-hydroxybutyrate.
q_p	Specific production rate (nmol min^{-1} mg^{-1} dry weight).
rpm	Revolutions per minute.
S_N2	Substitution nucleophilic bimolecular.
t_d	Culture doubling time (h).
μ_{max}	Maximum specific growth rate (h^{-1}).
% w/v	Concentration: weight per 100ml.
% v/v	Concentration: volume per 100ml.

SUMMARY

Epoxides are an important class of synthons, produced in large quantities (notably epoxyethane and epoxypropane) for the manufacture of polymers. Reaction of epoxides with nucleophiles is stereospecific, offering a route to homochiral pharmaceuticals and agrochemicals from homochiral epoxides. With few exceptions, production of homochiral epoxides is difficult to achieve by chemical syntheses alone. However, alkene epoxidation by monooxygenase enzymes has been shown to proceed with a high degree of stereoselectivity in many instances.

The aims of this project were to isolate microorganisms capable of converting alkenes to epoxides and to select the most suitable isolate for further characterization. Two Gram positive bacteria were isolated using α-methylstyrene (αMeS-1) and octane (*Rhodococcus* OU). The latter isolate was subjected to a more detailed study.

Rhodococcus OU were shown to convert a range of structurally diverse alkenes to their corresponding epoxides: aliphatic (1-alkenes from propene to 1-tetradecene and *cis*-2-butene), alicyclic (cyclopentene and cyclohexene) and aromatic (styrene, allylbenzene and allylphenylether) alkenes. Alcohols, aldehydes and ketones were produced from alkenes with sub-terminal double bonds, in addition to epoxides.

The stereoselectivity of alkene epoxidation was investigated by chiral HPLC. Partial resolution of (±)-1,2-epoxy-3-phenoxypropane was achieved, although assignment of the two peaks was not possible. Biotransformation of allyl phenyl ether to 1,2-epoxy-3-phenoxypropane was shown to proceed in a stereoselective manner. Problems associated with the chiral analysis of styrene oxide were not overcome, but preliminary results suggest that *Rhodococcus* OU is completely stereoselective for (R)-(+)-styrene oxide.

Alkene epoxidation was shown to occur by one or more monooxygenase enzymes, expression of which is inducible by growth on n-alkanes but not by growth on 1-hexanol or glucose. Catalytic activity was retained after freezing in liquid nitrogen and storage at -70°C, only diminishing after being stored in excess of two months.

Optimization of 1-alkene epoxidation was investigated, with particular reference to 1-hexene epoxidation. The specific rate of 1-alkene epoxidation (q_p) was shown to increase as chain length decreased, correlating with an increase in 1-alkene solubility in water. Increasing the biocatalyst concentration resulted in an increase in volumetric productivity, but a decrease in q_p. Epoxidation of 1-hexene showed saturable kinetics, q_p being maximal between 0.05% to 0.10% (v/v) 1-hexene, whilst the final concentration of 1,2-epoxyhexane attained was concentration-dependant up to 0.40% (v/v) 1-hexene (the maximum concentration tested). Addition of co-substrates was not shown to enhance q_p.

To Jenny, Dad and Mum

CHAPTER 1

INTRODUCTION

1.1 Biotransformations in context

A consequence of our need to compartmentalize science into physics, chemistry, biology and their subdivisions is that each division requires its own definition. This becomes a problem when one realises that science is a spectrum of disciplines which does not lend itself easily to being split into discrete categories. Biotransformations provide an example of this, since it is often easier to recognise an archetypal biotransformation than it is to define what constitutes a "biotransformation".

One definition of a biotransformation might be: the use of a biological catalyst (whole cells, isolated organelles or enzymes) to perform a selected modification on a compound whereby the product(s) or the process has some intrinsic value. Use of the term "value" is subjective, since a biotransformation without a commercial application could be argued to have no value. Although this definition is vague, the following examples illustrate the commercial utility of biotransformations:

1. **Synthesis.** One compound is converted by biotransformation into a synthetically more valuable product *e.g.* Conversion of glucose to fructose by glucose isomerase.

2. **Resolution.** Removal or modification of one stereoisomer in a racemic modification by biotransformation leaves the desired stereoisomer pure or allows it to be purified more easily *e.g.* Stereoselective[1] hydrolysis of chiral esters by lipases.

3. **Waste detoxification.** A toxic substrate is converted by biotransformation to a less toxic product. (The process rather than the product is of more value in this instance) *e.g.* Removal of toxic compounds from waste effluents.

4. **Bioanalysis.** The product(s) of a biotransformation can be used to quantitatively measure the concentration of the substrate *e.g.* Use of a glucose oxidase-based biosensor to monitor glucose concentrations.

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[1] "A stereoselective reaction is a reaction that yields predominantly one enantiomer of a possible pair, or one diasteromer (or one enantiomeric pair) of several possible diasteromers." Robert T. Morrison & Robert N. Boyd, Organic Chemistry. 5th ed. (Boston: Allyn and Bacon, 1987): p.345.

1:2 How to recognise an epoxide

Epoxides or oxiranes (IUPAC nomenclature) are compounds containing a three-membered heterocyclic ring, comprising two carbon atoms and one oxygen atom (Figure 1.1).

Nomenclature can be systematic using the "epoxy-" prefix or the "-oxirane" suffix (IUPAC nomenclature), or it can be trivial, the name being derived from the parent alkene (epoxides are readily prepared from alkenes) by adding the word "oxide" after the name of the alkene. This last method is more often reserved for the epoxides of well known alkenes.

FIGURE 1.1

The epoxide or oxirane ring

1:3 The role of epoxides as chiral synthons

The importance of epoxides can be appreciated by developing an appreciation of the inherent chirality of the world surrounding us. It could be argued that this began when Louis Pasteur chose to study the sodium ammonium salt of

racemic acid (a racemic modification of tartaric acid). This choice was serendipitous, since individual crystals of this compound are optically pure and dissymmetric, allowing Pasteur to resolve the mixture and demonstrate the relationship between optical activity and molecular geometry. From these observations in 1848, the concepts of chirality were cultivated, being of enormous importance to pharmaceuticals, agrochemicals, flavours and fragrances.

The human body is rich in chiral structures (enzymes, receptors, *etc.*), which will react to chiral compounds in a stereospecific[2] manner. Stereospecific interaction of carvone with receptors present in olfactory cells enables the brain to discriminate between the enantiomers of carvone: the odour of the levorotatory enantiomer is characteristic of oil of spearmint, whilst that of the dextrorotatory enantiomer is characteristic of essence of caraway. It is also not surprising that pharmacological studies with chiral drugs have shown that enantiomers can exhibit widely different pharmacokinetics and therapeutic effects. Occasionally, both enantiomers may exhibit therapeutic effects, as exemplified by the enantiomers of propoxyphene: dextropropoxyphene is marketed as an analgesic, whilst levopropoxyphene is marketed as an antitussive (Chafetz, 1991). However, the administration of racemic modifications is

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[2] "A stereospecific reaction is one in which stereochemically different molecules react differently." Robert T. Morrison & Robert N. Boyd, Organic Chemistry, 5th ed. (Boston: Allyn and Bacon, 1987): p.346.

potentially dangerous since the non-therapeutic enantiomer may exhibit toxic side effects.

The decision to market single enantiomers or racemic modifications is dependent upon many factors, based not only upon pharmacological activities but also on the technical feasibility of preparing homochiral compounds on a large scale (PMA Ad Hoc Committee on Racemic Mixtures, 1990). Increasingly however, regulatory authorities are placing more emphasis on the pharmacological characterization of drug enantiomers (Smith & Caldwell, 1988; de Camp, 1989), thus providing a strong incentive for the pharmaceutical industry to submit enantiomerically pure drugs for approval, rather than racemic modifications. To achieve this goal, the medicinal chemist will make use of stereoselective reactions, which in many instances can be fulfilled by the use of a homochiral epoxide intermediate or synthon.

The acute bond angles imposed during epoxide ring formation, make it highly stressed and very reactive. Opening of the epoxide ring can be achieved easily by nucleophilic substitution at one of the ring carbon atoms, allowing the addition of many different structural and functional groups. Gorzynski Smith (1984) alluded to the range of nucleophiles capable of ring-opening epoxides: "These include oxygen compounds (water, alcohols, phenols), nitrogen compounds (amines and derivatives of amines, azide, isocyanate), acids (hydrogen halides, hydrogen cyanide, sulfonic acids, and carboxylic acids), sulfur compounds (hydrogen sulfide, thiols,

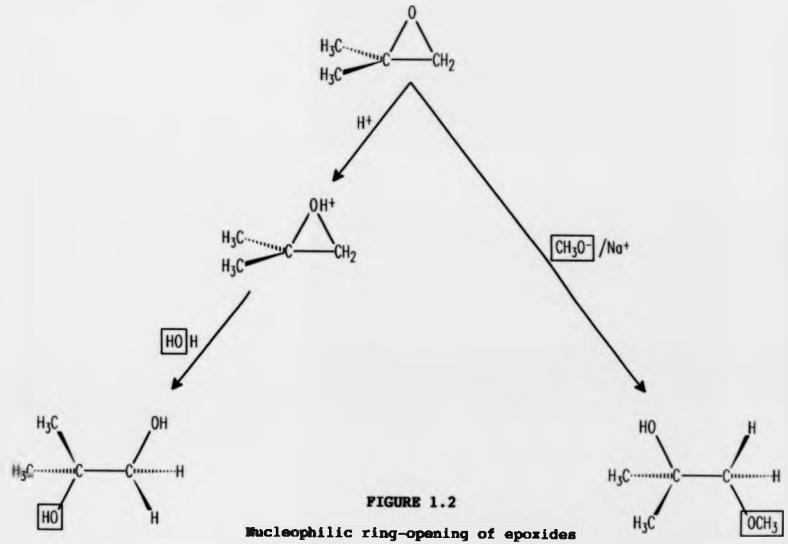


FIGURE 1.2

Nucleophilic ring-opening of epoxides

thiophenols, sulfides, thioacids, and several sulfur anions), and various carbon nucleophiles."

Nucleophilic ring opening of epoxides exhibits second-order kinetics, *i.e.* the reaction follows the S_N2 mechanism. The direction of nucleophilic attack with unsymmetrical epoxides is governed by both epoxide structure and reaction conditions (Figure 1.2).

Under acidic conditions the first step to ring-opening is protonation. A transition state is then formed with the incoming nucleophile, placing a positive charge on the carbon atom being attacked. Inductive and resonance effects can stabilize this positive charge, directing the nucleophile to the ring-carbon atom better able to support the positive charge. Ring-opening in neutral or basic conditions does not result in either ring-protonation or a positive charge residing on one of the ring-carbon atoms. The nucleophile will therefore prefer to attack the position that is sterically less hindered.

1.4 Chemical preparation of epoxides

To argue the validity of seeking biotransformation routes to epoxides without reference to what can be achieved by pure chemistry alone would be naive. This section is aimed at highlighting some of the chemical routes to epoxides, especially where epoxides are produced on a large scale or in enantiomerically enriched form.

1:4.1 Intramolecular ring closure of halohydrins

Epoxides may be prepared from alkenes indirectly from halohydrins (Figure 1.3). Formation of the epoxide ring from a halohydrin (usually the chlorhydrin or bromohydrin) is essentially a reversal of the ring-opening reaction described in section 1:3. A lone-pair of electrons on the oxygen atom displaces the halogen atom attached to the adjacent carbon atom. This reaction is base-catalyzed, following the S_N2 mechanism and therefore is completely stereoselective and stereospecific: enantiomerically pure halohydrins will react to give enantiomerically pure epoxides.

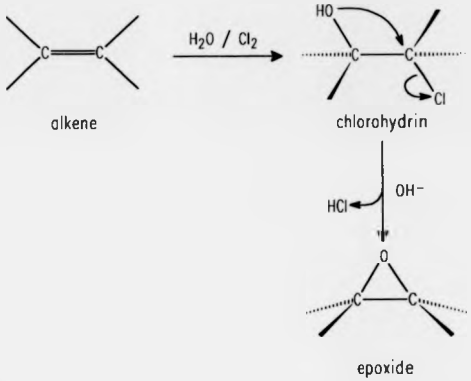


FIGURE 1.3

Formation of epoxides from alkenes via chlorohydrins

The Chlorohydrin process for propene epoxidation:

The Chlorohydrin process accounted for 55% of the epoxypropane produced in 1984 from the United States, Western Europe and Japan (Chem Systems, 1988). Chlorine is hydrated to form hypochlorous acid and hydrochloric acid. The hypochlorous acid is reacted with propene to give a mixture of chlorohydrins (90% 1-chloro-2-propanol, 10% 2-chloropropanol). Lime or caustic soda (preferentially) is used to catalyze the intramolecular displacement of chlorine as well as the neutralization of the hydrochloric acid produced during the chlorine hydration step and that produced on ring-closure. The epoxypropane is then distilled off from solution to avoid hydrolysis of the epoxide ring (Chem Systems, 1988).

1:4.2 Epoxidation of alkenes by peroxycarboxylic acids

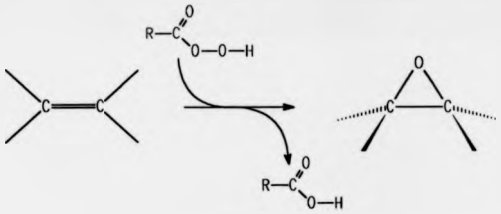


FIGURE 1.4

Epoxidation of alkenes with peroxycarboxylic acids

Peroxycarboxylic acids react with alkenes to yield the corresponding carboxylic acid and epoxide respectively (Figure

1.4). Unlike many peroxycarboxylic acids, 3-chloroperoxybenzoic acid is stable, making it the reagent of choice in most instances (section 2:1). This reaction is stereospecific (*cis*-alkenes react to give *cis*-epoxides and *trans*-alkenes react to give *trans*-epoxides).

Use of chiral peroxycarboxylic acids has been shown to give rise to enantiomerically enriched epoxides. Pirkle and Rinaldi (1977) carried out an epoxidation of *p*-bromobenzylidene-*N*-*tert*-butylamine with peroxycamphoric acid, achieving 40±1% enantiomeric excess (% ee) of the levorotatory enantiomer. The other alkenes tested were reacted with a much lower degree of enantioselectivity (<14% ee).

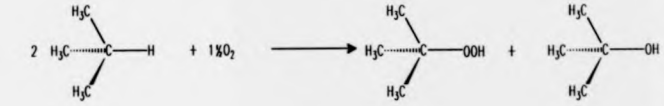
1:4.3 Epoxidation of alkenes with hydroperoxides

Reaction of molecular oxygen with hydrocarbons in the presence of a free radical initiator gives rise to hydroperoxides by a process known as autoxidation. The propensity towards autoxidation is increased in hydrocarbons that have stable free radicals (*e.g.* at tertiary and benzylic positions). These hydroperoxides, including hydrogen peroxide, can be used as an oxygen source for alkene epoxidation.

The Oxirane process for propene epoxidation:

Hydroperoxide epoxidation of propene, known as the Oxirane process, accounted for 45% of the epoxypropane produced in the United States, Western Europe and Japan during 1984 (Chem Systems, 1988). Co-products derived from the hydroperoxides used for the epoxidation, contribute

1. Autoxidation of isobutane and ethylbenzene



2. Epoxidation of propene by hydroperoxide

FIGURE 1.5

The Oxirane Process for propene epoxidation

significantly to the overall economics of the process. The major co-products produced are styrene (38%), used for the production of various polymers and t-butyl alcohol (62%), used as an antiknock agent in petrol, or dehydrated to isobutylene for polymer production.

The Oxirane process is performed in two stages: autoxidation of isobutane (TBA or t-butyl alcohol process) / ethylbenzene (Styrene process) followed by the epoxidation (Figure 1.5). The epoxidation is catalyzed by transition metal catalysts such as molybdenum, tungsten, vanadium or titanium salts (Chem Systems, 1988).

Sharpless epoxidation of allylic alcohols:

One of the most significant developments in the epoxidation of alkenes was the development of an asymmetric epoxidation catalyst by Sharpless and co-workers (Sharpless, 1986). The substrate range is limited to allylic alcohols, using t-butyl hydroperoxide as the oxygen source and titanium tetraisopropoxide ($Ti(OiPr)_4$) in association with diethyl tartrate (chiral chelating ligand) as the catalyst.

Subsequent development of a "catalytic" version of this process with the inclusion of molecular sieves afforded many advantages over the original "stoichiometric" version:

a. Greatly reduced levels of catalyst are required.

b. Higher reactant concentrations can be employed.

c. Higher yields are obtained.

The milder reaction conditions of this "catalytic" process allow production of epoxy alcohols that previously were susceptible to titanium-catalyzed ring-opening reactions in the "stoichiometric" process (Sharpless, 1988). Although this reaction is limited to allylic alcohols, both epoxide enantiomers are accessible depending on whether L-(+)-diethyl tartrate (naturally occurring isomer) or D-(-)-diethyl tartrate is used in the reaction.

Asymmetric epoxidation of alkenes, other than allylic alcohols, using hydroperoxides have proven to be less successful. Helder *et al.* (1976) converted α,β-unsaturated ketones to their corresponding epoxides in a two-phase system using chiral phase-transfer catalysts (quaternary ammonium salts of quinine and quinidine) and hydrogen peroxide or *t*-butyl hydroperoxide as the oxygen source. Yields were high but enantiomeric excess was mediocre (25% ee).

1:4.4 Other preparations of epoxides

Glycidic esters (α,β-epoxy esters) can be prepared by the Darzens Condensation of aldehydes/ketones with α-halo esters in the presence of a strong base (Figure 1.6). A modified form of the Darzens Condensation has been used to synthesize α,β-epoxy-sulphones and α,β-epoxy-nitriles with optical yields upto 23% (Colonna *et al.*, 1978). In common with the reactions carried out by Helder *et al.* (1976), these were performed in two-phase systems using chiral phase-transfer catalysts.

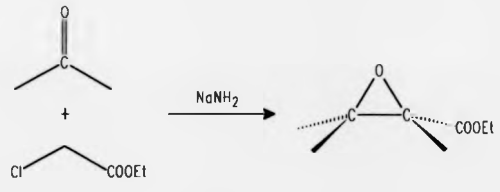


FIGURE 1.6

The Darzens Condensation

Commercial production of epoxyethane is achieved by direct addition of molecular oxygen (air or oxygen-enriched air) to ethene at 200°C to 300°C and 10 to 30 atmospheres pressure, using a silver catalyst. This is the only example of an epoxide prepared commercially by this method, other alkenes being less amenable to direct addition of molecular oxygen.

1.5 Biotransformation routes to epoxides

It is evident that in most instances, stereoselective epoxidation is difficult to achieve by chemistry alone. The use of enzymes for stereoselective and/or stereospecific catalysis has shown great potential, especially for oxidation and reduction reactions (Roberts, 1990). Stereoselective formation of epoxides by enzyme catalysis has also proven to be a realistic substitute for, or an extension of conventional chemistry.

Examples of epoxide formation in plant and animal metabolism include the formation of: 18-hydroxy-9,10-epoxystearate in the biosynthesis of cutin (Kolattukudy *et al.*, 1973; Croteau & Kolattukudy, 1974), disparlure, the gypsy moth pheromone and squalene-2,3-oxide in the biosynthesis of cholesterol. Use of such biocatalysts would be limited, mainly because the controlled growth of plant and animal cells (a necessary prerequisite to the production of enzyme catalysts) is generally difficult to achieve and inherently very slow. In contrast, microorganisms are fast growing and far less fastidious, making such biocatalysts easier and cheaper to produce. Biotechnological routes to epoxides have therefore concentrated on microbial systems, an aspect which constitutes the remainder of this introduction.

1:5.1 Epoxides from halohydrins

A biotransformation route to epoxides was spawned from studies of the dehalogenation of 2,3-dibromopropanol by a *Flavobacterium* species by Castro and Bartnicki (1968). Crude cell-free extracts were shown to metabolize 2,3-dibromopropanol by the sequential formation of two epoxide intermediates (Figure 1.7) resulting from a halohydrin epoxidase activity.

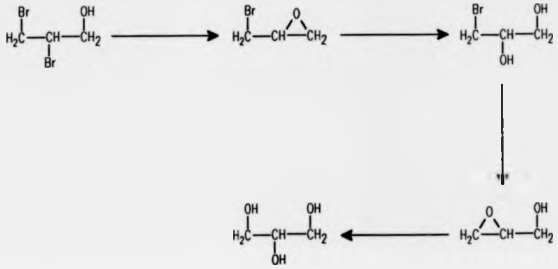


FIGURE 1.7

Metabolism of 2,3-dibromopropanol by a *Flavobacterium* species

The Cetus Process:

In 1979, it was announced that Standard Oil Company and Cetus Corporation were to build a $15,000,000 pilot plant for the conversion of ethene and propene to their corresponding epoxides and glycols (Chemical Week, 1979). This process (the Cetus Process) comprises three enzyme-catalyzed reactions (Figure 1.8), the final step of which makes use of the

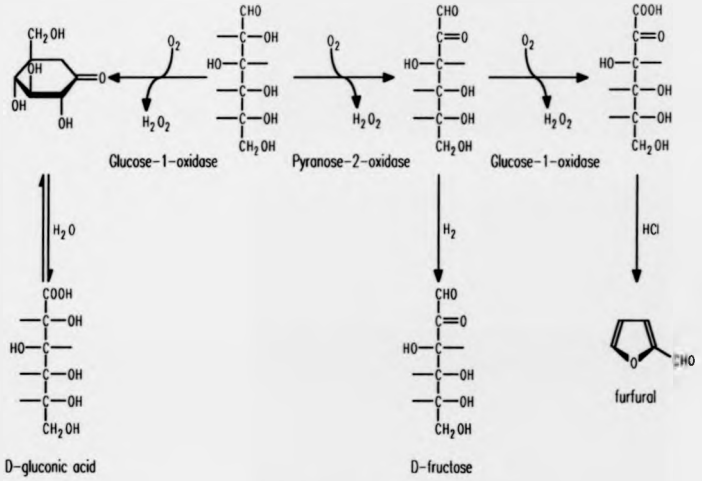


FIGURE 1.9

Formation of co-products from the Cetus Process

Flavobacterium species studied by Castro and Bartnicki (1968) and Bartnicki and Castro (1969).

In common with the Oxirane Process (section 1:4.3), co-product formation resulting from the biogeneration of hydrogen peroxide contributes significantly to the overall economics of the Cetus Process.

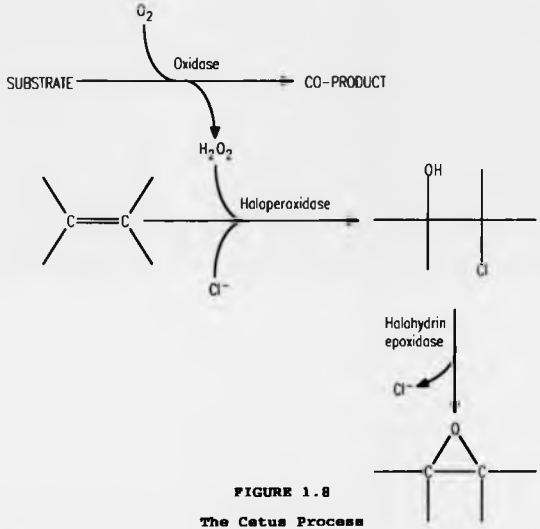


FIGURE 1.8

The Cetus Process

One possible co-product is formaldehyde, generated from methanol oxidation by alcohol oxidase. Using D-glucose as the

substrate, it is possible to produce three economically important co-products (Figure 1.9): D-fructose, D-gluconic acid and furfural (Neidleman, 1980). Pyranose-2-oxidase converts D-glucose to D-glucosone which can either be catalytically hydrogenated to D-fructose or converted to D-2-ketogluconic acid (a precursor of furfural) by glucose-1-oxidase. It is therefore possible to generate two moles of hydrogen peroxide from one mole of glucose. Since glucose-1-oxidase is able to convert D-glucose to D-glucono-δ-lactone (the lactone of D-gluconic acid), presumably the oxidation of D-glucose to D-2-ketogluconic acid would have to be operated so as to avoid premature termination of the reaction by D-glucono-δ-lactone formation.

The second step in the Cetus Process is the formation of halohydrin from the alkene, utilizing the hydrogen peroxide generated in the first step and halide ions. Various haloperoxidase enzymes capable of catalyzing this reaction have been used to produce bromohydrins from ethene, propene, 1-butene, 1,3-butadiene (including its corresponding monoepoxide & monobromohydrin), 2-butene (*cis*- & *trans*-), isobutene, allyl chloride, styrene and allene (Neidleman, 1980; Geigert *et al.*, 1983).

Halohydrins derived from asymmetric alkenes can exist as either of two positional isomers (*e.g.* the halohydrins of propene can exist as either: 1-halo-2-propanol or 2-halo-1-propanol). Neidleman (1980) reported that the ratio of bromohydrin isomers produced enzymatically from propene, 1,3-

butadiene and 1,3-butadiene monoepoxide was consistently 9:1 (the halogen atom residing predominantly at the end of the molecule). It was later shown that the ratio of products formed from various alkenes by chloroperoxidase (from *Caldariomyces fumago*) and lactoperoxidase (from milk), was similar to that observed when the same alkenes were treated with hypohalous acid (Geigert *et al.*, 1983). From these observations it was concluded that hypohalous acid, generated by the haloperoxidases, was likely to be the reacting species.

The final step in the Cetus Process is the displacement of the halogen atom from the halohydrin by intramolecular nucleophilic substitution (section 1:4.1). The halohydrin epoxidase responsible for this transformation is derived from a *Flavobacterium* species. In contrast to the oxidase and haloperoxidase enzymes used in the first two steps of the Cetus Process, halohydrin epoxidase is not used as a purified preparation, but is used *in vivo*.

Cetus Corporation predicted that the cost of producing epoxides by this route would be halved compared to other (then current) technologies (Chemical Week, 1979), based upon comparisons with the Chlorohydrin Process (section 1:4.1) (Chemical Week, 1980):

> The process [the Cetus Process] has at least three advantages over classical chemical methods of making the oxides, according to Neidleman. A variety of oxides, including those of ethylene and propylene, can be produced, perhaps with only modifications of the purification

equipment at the end of the process. The halide ion, which is inexpensive and recyclable, is used instead of a more-expensive and dangerous elemental halogen, such as chlorine. Finally, in the basic chlorohydrin process, propylene oxide is formed by alkali treatment, which results in large amounts of waste calcium chloride and, says Neidleman 30% of process investment is tied up in disposing of these wastes. Cetus's route, he says, would eliminate this cost "to a very considerable degree."

Although the Chlorohydrin Process utilizes lime as the base for ring-closure of the chlorohydrins and neutralization of the hydrochloric acid produced during chlorine hydration and ring-closure of the halohydrin, caustic soda can be used instead. Use of caustic soda results in the production of sodium chloride waste, which is cheaper to dispose of than calcium chloride waste (Chem Systems, 1988). Another factor to be considered is the market price of the co-products generated in the Cetus Process (*cf.* the Oxirane Process, section 1:4.2), which can greatly affect the final cost of the epoxide.

Subsequent studies at Cetus Corporation on the substrate specificity of chloroperoxidase (from *Caldariomyces fumago*), may help simplify the Cetus Process. Geigert *et al.* (1986) reported that chloroperoxidase could catalyze the halide-independent epoxidation of propene, allyl chloride, 1,3-butadiene, cyclopentene and styrene; of these, styrene was the most reactive. Halide-independent epoxidation of these substrates could not be demonstrated by lactoperoxidase (from bovine milk), horseradish peroxidase or myeloperoxidase (from canine leukocytes).

Stereoselective synthesis of halohydrins from alkenes would furnish a biosynthetic route to enantiomerically pure epoxides. However, if hypohalous acid is the reactive species in the reaction as predicted (Geigert *et al.*, 1983), then this possibility appears remote. This is supported by the lack of stereoselectivity observed by Kollonitsch *et al.* (1970) in the hydroxychlorination of *cis*- and *trans*-propenylphosphonic acid by chloroperoxidase.

1.5.2 Direct epoxidation of alkenes

Van der Linden (1963) can be credited with one of the most significant reports in the early history of microbial alkene epoxidation. Epoxidation of 1-heptene, 1-octene, and 1-nonene was shown to be catalyzed by heptane-grown *Pseudomonas* cells, a phenomenon which did not occur with peptone-grown cells. The involvement of epoxides in alkane metabolism was excluded because epoxides were never detected during n-alkane oxidation, nor could the *Pseudomonas* cells be encouraged to grow on epoxides. "Therefore, we must conclude that epoxides are formed by an enzyme system already present in the alkane-grown cell and closely related to - if not identical with - the alkane oxidizing system." This proposition that alkane hydroxylation and alkene epoxidation were likely to be catalyzed by the same enzyme, was later proven to be correct. The enzyme responsible for this biotransformation was an alkane monooxygenase.

Monooxygenase enzymes occur commonly in microorganisms growing at the expense of hydrocarbons. These enzymes initiate

hydrocarbon degradation (Figure 1.10) by the insertion of one atom of molecular oxygen into the substrate, the other atom of molecular oxygen being reduced to water.

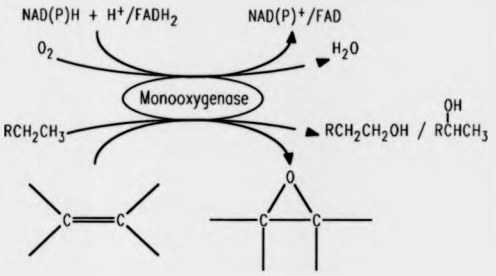


FIGURE 1.10

Epoxidation of alkenes by monooxygenase enzymes

The ability to insert molecular oxygen into carbon-hydrogen bonds has proven difficult by conventional chemistry. This goal is important to the chemical industry, heavily dependent upon petroleum-based starting materials for its industrial syntheses. Interest in monooxygenase enzymes has arisen from their ability to functionalize hydrocarbons in a single step in a regio- and stereoselective manner.

The relaxed substrate specificity exhibited by many monooxygenase enzymes is fortuitous to the field of biotransformations, allowing the production of epoxides from

alkenes. From the view point of whole-cell biotransformations, alkene epoxidation can be considered as a co-metabolic event, as defined by Dalton and Stirling (1982): "Co-metabolism - the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound." The non-growth substrate (or biotransformation substrate) is the alkene, whilst the transformable compound is any compound of endogenous or exogenous origin (section 5:6) whose oxidation sustains the supply of reduced cofactors ($NAD(P)H$ or $FADH_2$) necessary for monooxygenase activity.

1:6 Microbial epoxidation catalysts

Various microorganisms expressing monooxygenase activity have been studied as epoxidation catalysts, each with its own peculiar characteristics. The following discussion of these microorganisms will concentrate on their potential utility as biocatalysts: stereoselectivity, substrate and regiospecificity being a prime consideration.

1:6.1 *Pseudomonas oleovorans*

The catabolically versatility of the *Pseudomonas* genus is widely recognised; it is perhaps not surprising therefore, that a *Pseudomonas* species has been used in a biotransformation route to epoxides. At the time when studies of *Pseudomonas oleovorans* began, the monooxygenase (or ω-hydroxylase) it possesses was unusual since it did not contain cytochrome P-450 (McKenna & Coon, 1970). Its utility for alkene epoxidation did

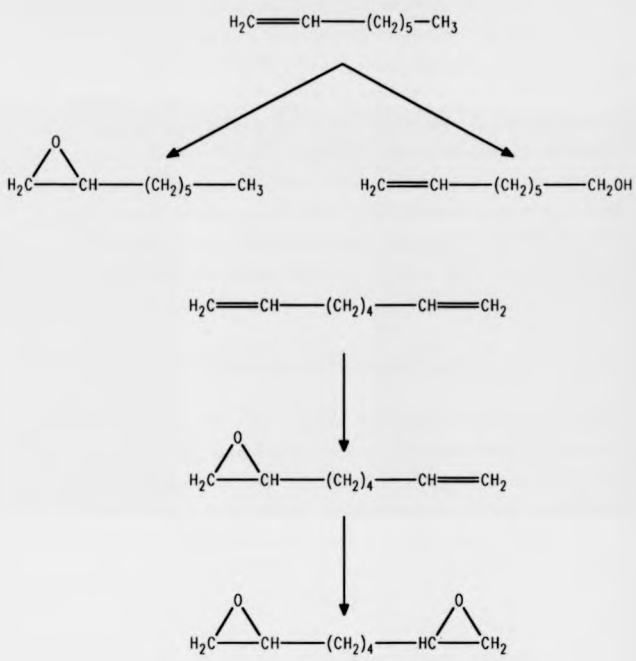


FIGURE 1.11

Oxidation of 1-octene and 1,7-octadiene

by *Pseudomonas oleovorans*

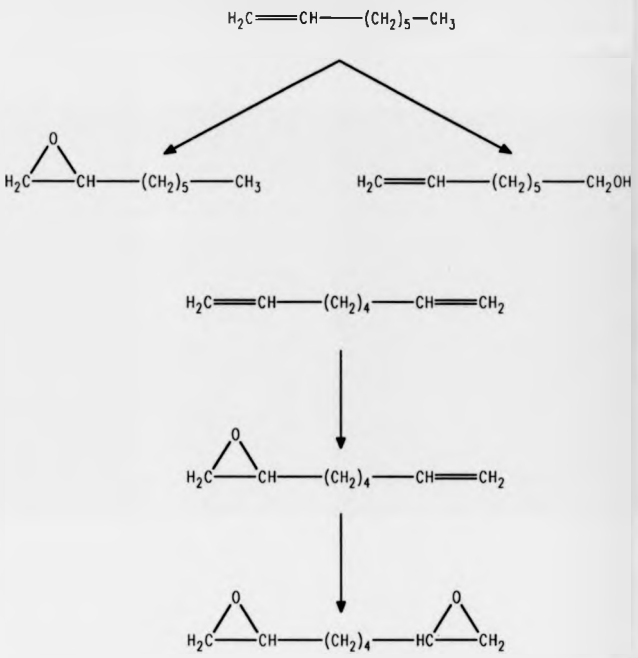


FIGURE 1.11

Oxidation of 1-octene and 1,7-octadiene

by *Pseudomonas oleovorans*

not become apparent until study of this enzyme was carried out at the Esso Research and Engineering Company (May and Abbott, 1972a).

The first studies of alkene epoxidation were carried out on 1,7-octadiene and 1-octene, using the purified ω-hydroxylase from *Ps. oleovorans* (May & Abbott, 1972b). Two products were identified from each of these substrates (Figure 1.11). It was obvious that this enzyme was capable of both hydroxylating terminal methyl groups and epoxidizing terminal double bonds.

Epoxidation and hydroxylation by the ω-hydroxylase of *Ps. oleovorans* are competitive reactions, catalyzed by the same enzyme. May *et al.* (1975) showed a relationship between the chain length of terminal dienes and their propensity towards epoxidation, comparing this to data on n-alkane hydroxylation reported previously (Peterson & Coon, 1968). Epoxidation and hydroxylation were both optimal with a chain length of eight carbon atoms.

The range of 1-alkenes tested on the purified ω-hydroxylase was extended beyond 1-octene (May & Abbott, 1972b); 1-decene was preferentially epoxidized (67% - 76%), whereas propene and 1-butene were exclusively hydroxylated, illustrating the dependency of reaction type on chain length (May *et al.*, 1975).

Alkenes with internal double bonds were also tested as substrates. All previous studies had shown that only the methyl groups ($-CH_3$), not the methylene groups ($-CH_2-$) of n-alkanes and fatty acids were hydroxylated by the ω-hydroxylase. Perhaps it was not surprising therefore, that *trans*-5-Decene only gave *trans*-5-decen-1-ol and not the corresponding epoxide (May *et al.*, 1975). Cyclohexene however, gave two unsaturated cyclic alcohols: 2-cyclohexen-1-ol and 3-cyclohexen-1-ol. The alicyclic ring was obviously accessible to the active site of the ω-hydroxylase so why should cyclohexene not be epoxidized? It appeared that internal double bonds were recalcitrant to epoxidation by this enzyme, contrasting with chemical epoxidation (the inductive effect of the alkyl substituents either side of the double bond enhance its reactivity to electrophilic reagents such as peracids). Obviously other factors were influencing enzymatic reactivity such as steric hindrance and substrate binding, the latter being supported by evidence from epoxidation inhibition studies with imidoesters (May *et al.*, 1975).

Many of the later studies on alkene epoxidation by *Ps. oleovorans* were carried out with whole cells instead of purified ω-hydroxylase. Schwartz (1973) isolated a cold-stable variant of *Ps. oleovorans*, designated TF4-1, which produced 1,2-epoxyoctane at five times the rate of the parent strain (*Ps. oleovorans* 1-RAM). Further strain improvements were achieved by Schwartz and McCoy (1973), by isolating *Ps. oleovorans* TF4-1L, derived from TF4-1, which produced 1,2-epoxyoctane at a rate nine times that of 1-RAM. It was this

latter strain, TF4-1L (ATCC 29347), which was utilized in all subsequent studies on *Ps. oleovorans*.

The ω-hydroxylase of this organism is transcribed from the *alkBFGHJKL* operon and the *alkST* locus on the OCT plasmid, indigenous to *Ps. oleovorans*. This enzyme is a three component monooxygenase comprising: rubredoxin reductase (AlkT), rubredoxin (AlkG) and alkane hydroxylase (AlkB). The last component is responsible for alkane/alkene binding and oxygen insertion, whilst the other two components serve to transfer reducing equivalents from NADH to the alkane hydroxylase to facilitate the reduction of an atom of oxygen to water. Expression of the *alkBFGHJKL* operon is positively regulated by AlkS (Witholt *et al.*, 1990). Growth on n-alkanes from pentane to dodecane, especially heptane to nonane, result in induction of the monooxygenase (Schwartz, 1973). Gratuitous inducers include: 1,7-octadiene (Schwartz & McCoy, 1973), dimethoxyethane (de Smet *et al.*, 1983) and diethoxymethane (Johnstone *et al.*, 1987).

The most significant discovery about alkene epoxidation by *Ps. oleovorans* TF4-1L was made by May and Schwartz (1974) who showed that enzymatically produced 7,8-epoxy-1-octene was enantiomerically enriched. The specific rotation of chemically synthesized (R)-(+)-7,8-epoxy-1-octene ($[\alpha]_D^{16}$=+12.4°) was very similar to that obtained for the microbially-produced epoxide ($[\alpha]_D^{25}$=+12.2°), the latter corresponding to an optical purity of 80% as determined by ^{1}H-NMR, in the presence of a chiral shift reagent. Such enantioselectivity demanded that attack of

the double bond of 1,7-octadiene be almost exclusively from the *si-si* face of the molecule.

A more detailed report of the stereochemical course of 1,7-octadiene epoxidation was offered by the same authors and their co-workers (May *et al.*, 1976). Use of 1,7-octadiene allowed the epoxidation reactions to be studied in terms of their stereoselectivity and stereospecificity. The enantioselective formation of (R)-(+)-7,8-epoxy-1-octene (84% ee) was in agreement with their previous report, whilst subsequent formation of (R,R)-(+)-1,2:7,8-diepoxyoctane from the monoepoxide, generated microbially from 1,7-octadiene, proceeded to 66% ee.

A racemic modification of 7,8-epoxy-1-octene, prepared chemically, was used to demonstrate enantiospecificity in *Ps. oleovorans* TF4-1L. It was argued that 50% ee of (R,R)-(+)-1,2:7,8-diepoxyoctane would suggest that *si-si* addition at the double bond had occurred irrespective of the configuration at the preformed chiral centre. Similarly, 0% ee would indicate that the configuration at the preformed chiral centre was influencing the stereochemical course of epoxidation. The diepoxide produced (18% ee of the (R,R)-configuration) and the remaining unreacted monoepoxide (6% ee of the (S)-configuration) demonstrated a degree of enantiospecificity not anticipated.

Further evidence of stereoselectivity was provided by de Smet *et al.* (1983) who showed that 1-decene epoxidation by *Ps.*

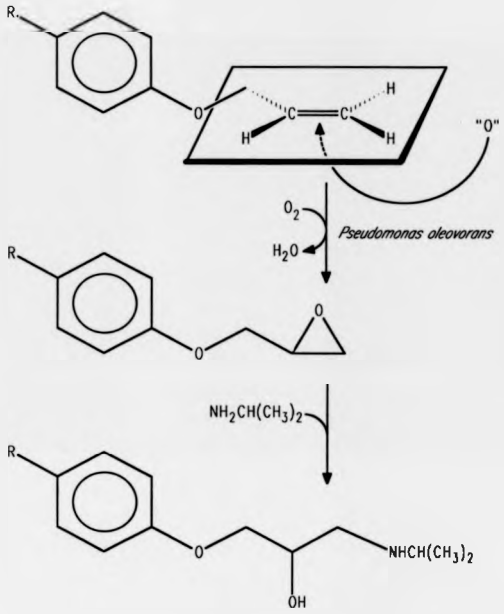


FIGURE 1.12

Production of ß-blockers using *Pseudomonas oleovorans*

oleovorans TF4-1L resulted in the preferential formation of (R)-(+)-1,2-epoxydecane ($[\alpha]_D^{20}$=+7.5°) with 60±2% optical purity. Epoxidation of allyl benzene was also enantioselective, with the preferential formation of the laevorotatory enantiomer of 1,2-epoxy-3-phenoxypropane, although the optical purity was not determined.

Exploitation of *Ps. oleovorans* TF4-1L at Shell Research Limited in collaboration with Gist-Brocades, to synthesize (S)-(-)-Metoprolol (95.4-100% ee) and (S)-(-)-Atenolol (97% ee), achieved some success. These compounds are used to block β-adrenergic receptors, for the treatment of angina, hypertension, irregular heart rhythms and associated problems, the therapeutic efficacy being substantially higher in the (S)-(-)-enantiomer (Johnstone *et al.*, 1986). The epoxides of allyl phenyl ether and para-substituted analogues were prepared using *Ps. oleovorans* TF4-1L and the epoxide ring opened with iso-propylamine (Figure 1.12). Because epoxide ring opening is completely enantioselective, the epoxide intermediates should have identical enantiomeric purities.

In the literature, the variety of epoxides formed by *Ps. oleovorans* TF4-1L is limited. On this basis, the versatility of this biocatalyst has to be questioned. However, the depth of understanding regarding its genetics, enzymology and utility in two-phase reactors (Witholt *et al.*, 1990) may in part compensate for its drawbacks.

1:6.2 *Corynebacterium equi*

Accumulation of 1,2-epoxyhexadecane and hexadecane-1,2-diol (mol ratio 95:5) by *Corynebacterium equi* IFO 3730 occurred when it was grown on 1-hexadecene as the sole source of carbon and energy. The purified epoxide and diol exhibited optical activity: $[\alpha]_D^{25}$=+9.64° (*c*=3.71, n-hexane) and $[\alpha]_D^{25}$=+1.47° (*c*=0.34, ethyl acetate) respectively, both being assigned the (R)-configuration. The optical purity of 1-methoxy-2-hexadecanol, derived enantioselectively from the epoxide was 100% as determined by ^{1}H-NMR in the presence of an europium chiral shift reagent. Because *C. equi* IFO 3730 was able to grow on 1-hexadecene, it was argued that enantiomerically pure epoxides could be generated either by asymmetric epoxidation or by selective degradation of the (S)-enantiomer from an enzymatically produced racemic modification. Epoxide and diol recovered from incubating *C. equi* IFO 3730 with (±)-1,2-epoxyhexadecane did not show any optical activity, suggesting that the epoxidation was asymmetric (Ohta & Tetsukawa, 1979).

The yield of epoxide was partially optimized by cultivation at an initial pH of 7.2, terminating the cultivation after two days and by the inclusion of hexadecane or octane (Ohta & Tetsukawa, 1979). It is not clear however, if the n-alkanes acted as co-substrates or provided an organic secondary phase, which has been shown to enhance epoxidation by *Pseudomonas oleovorans* TF4-1L (de Smet *et al.*, 1983).

The variety of different types of biotransformations performed by this organism is remarkable:

a. Oxidation of secondary alcohols to ketones, the oxidation of 2-dodecanol being slightly enantiospecific (Ohta *et al.*, 1984).

b. Oxidation of sulphides to sulphones and optically active sulphoxides (Ohta *et al.*, 1985; Yamazaki & Hosono, 1989).

c. Enantiospecific hydrolysis of sulfinyl esters (Ohta *et al.*, 1986).

One of the most elegant studies carried out with *C. equi* IFO 3730 was the demonstration that both enantiomers of 1-benzenesulphenyl-2-propanol could be obtained by manipulating the pH of cultivation so as to reverse the enantioselective reduction of 1-benzenesulphenyl-2-propanone (Ohta *et al.*, 1986).

Although this microorganism has not been studied in great detail as an epoxidation catalyst, its general utility towards a number of stereoselective and stereospecific reactions does warrant its inclusion here.

1:6.3 *Nocardia corallina*

As an epoxidation catalyst, this organism is well proven by the wide range of epoxides it is capable of forming and the high optical purities attained.

Strain B-276 from a collection of hydrocarbon-assimilating microorganisms was identified by Furuhashi and co-workers as *N. corallina* (Furuhashi *et al.*, 1981). From this point, the lineage of *N. corallina* B-276 (ATCC 31339) becomes uncertain, since no reference is made to the different strain phenotypes noted in early reports. It is also apparent that some of the claims made of *N. corallina* B-276 do not withstand close scrutiny!

Furuhashi *et al.* (1981) showed that *N. corallina* B-276 was able to grow on n-alkanes (methane to butane), 1-alkenes (ethene to 1-butene & 1-tridecene to 1-octadecene), butadiene, isobutene, 1,2-propanediol and 1,2-tetradecanediol. Growth on 1,2-epoxytetradecane occurred only after a long lag phase and therefore might have been at the expense of its hydrolysis product (1,2-tetradecanediol). An important observation was that apart from ethene, growth on the other 1-alkenes resulted in the accumulation of the corresponding 1,2-epoxides. Production of 1,2-epoxytetradecane was enhanced when growth was limited by allowing the culture pH to fall. Presumably when growth was limited, 1,2-epoxytetradecane production continued without being further degraded for biomass production, resulting in epoxide accumulation.

Growth on 1-tetradecene gave two products: 1,2-epoxytetradecane and 1,2-tetradecanediol, the former being the major product. Purified 1,2-epoxytetradecane showed optical activity ($[\alpha]_D^{20}$=+8.6° ≡ (R)-(+)-1,2-epoxytetradecane) corresponding to an optical purity of >86% (determined by ^{1}H-NMR in the presence of an europium chiral shift reagent) (Furuhashi *et al.*, 1981). Optimized conditions for the production of 1,2-epoxytetradecane produced a final epoxide concentration of 80 g l^{-1} (0.38M), with a yield of 54 mol % (based on the amount of alkene utilized) after six to seven days' growth (Furuhashi & Takagi, 1984). The (R)-(+)-1,2-epoxytetradecane produced ($[\alpha]_D^{25}$=+9.4°) had an optical purity of 88% ee, being determined as before (Furuhashi *et al.*, 1981).

Subculturing *N. corallina* B-276 on nutrient agar (both in the presence and absence of propene) resulted in a loss of 1-tetradecene epoxidation activity, which did not occur after subculturing on a semi-defined medium with propene as the main carbon source. Growth on propene appeared to redress partially the loss in activity of the subcultured low activity strain. *N. corallina* B-276 was found to be a mixture of two strains: one with a rough colony morphology (strain R), the other with a smooth colony morphology (strain S). Strain S, in contrast to strain R, did not grow on propene nor did it epoxidize propene; production of 1,2-epoxytetradecane occurred at a much reduced rate (2-3%) compared to strain R. The loss in 1-tetradecene epoxidation activity by subculturing on nutrient agar was attributed to an increase in the proportion of S-type cells in

the culture, which was reversed by pre-culturing on propene (Furuhashi *et al.*, 1981).

Little significance was placed on these observations, except to report that strain S was a spontaneous mutant. If this was indeed the case, and not the result of using a mixed culture, this would imply one of three scenarios:

1. Strain S possessed a monooxygenase catalytically less efficient than that of strain R, having a greater affinity for 1-tetradecene than propene (to the extent that propene is not a substrate at all).

2. Strain S did not express the monooxygenase as well as strain R, the rate of propene epoxidation being too low to be detected.

3. Strain R contains two monooxygenase enzymes; one that acts upon short-chain alkenes, the other acting upon the longer-chain alkenes. Therefore strain S had lost its ability to synthesize the short-chain monooxygenase.

The last two scenarios would confer a growth advantage on strain S, which would account for the increase in the proportion of S-type cells when subcultured on nutrient agar. Evidence that the last scenario might be true was provided five years later when the rate of 1-alkene epoxidation showed two optima (Furuhashi *et al.*, 1986). The specific epoxidation rate of 1-alkenes by pre-grown cells (on nutrient broth + 1%

glucose) decreased from ethene to 1-hexene, increased to 1-undecene and then tailed off again. It is unlikely that the medium chain-length epoxides (1,2-epoxypentane to 1,2-epoxynonane) caused higher product inhibition than the short-chain epoxides, as the latter are potent sterilizing agents.

Having described the two variants of *N. corallina* B-276, no reference is made in future reports as to which is used. Non-uniformity was again highlighted by Miyawaki *et al.* (1986) who used a mucopolysaccharide producing strain (S-1-1) and a mutant producing low levels of mucopolysaccharide (P-1-200) for epoxypropane production, the former having a higher specific activity.

Epoxidation of 1-tetradecene by propene-grown cells in the presence of 2mg ml^{-1} chloramphenicol (therefore inhibiting protein synthesis), reduced the activity by 20% (Furuhashi *et al.*, 1981). This was regarded as evidence that a single monooxygenase was responsible for propene and 1-tetradecene epoxidation: "Though no attempt was made to isolate and determine the absolute configuration of propyleneoxide, it will be identical with those of long chain 1,2-epoxides because propylene-grown cells produced R-(+)-1,2-epoxytetradecane in the presence of chloramphenicol." Coordinate induction of two monooxygenase enzymes by growing cells on propene was not considered.

Furuhashi *et al.* (1981) showed that chloramphenicol reduced 1-tetradecene epoxidation in glucose-grown cells by

96%, implying that the monooxygenase was not present in glucose-grown cells at the start, but was synthesized during the biotransformation. Since the cells were suspended in 0.05M phosphate buffer, nutrients essential for cell growth were absent, but protein synthesis could still have occurred with amino acids liberated during protein turnover in the resting cells.

Furuhashi (1986) later suggests that monooxygenase expression in *N. corallina* B-276 is constitutive: "Although most of the epoxide-producing microorganisms shows [sic] their epoxidizing activity only when they are grown on hydrocarbons (for instance, methans [sic], propane), in the case of *N. corallina*, glucose- or sucrose-grown cells have also the activity." Clearly this is not the case! This fallacy is further exacerbated by claims (Furuhashi & Takagi, 1984) that optimal production of 1,2-epoxytetradecane by glucose-grown "resting cells" occurred when the cells were suspended in 25mM phosphate buffer supplemented with $MgSO_4.7H_2O$ (750mg l^{-1}), $FeSO_4.7H_2O$ (30mg l^{-1}), $CaCl_2.2H_2O$ (4mg l^{-1}), $MnCl_2.4H_2O$ (60µg l^{-1}) and yeast extract (2g l^{-1}). To assign this as a "resting cell reaction" is an obvious misnomer, as the supplemented phosphate buffer could easily support cell growth and subsequent induction of a monooxygenase activity.

Media optimization studies carried out by Furuhashi and Takagi (1984) showed that yeast extract could enhance 1-tetradecene epoxidation without causing a significant increase in biomass concentration. The enhancement of epoxidation is

likely to result from the glucose present in yeast extract (Crueger & Crueger, 1984). Miyawaki *et al.* (1986) commented on the advantage of using glucose as a co-substrate during propene epoxidation, since in the absence of glucose some of the propene was converted to carbon dioxide to regenerate reduced cofactors.

The list of epoxides formed by *N. corallina* B-276 is impressive with optical purities ranging from 5% ee to 94% ee (Furuhashi, 1986). Although this list of epoxides is longer than those formed by many other microorganisms, it must be remembered that the catalytic versatility of many other microorganisms appears not to have been studied as extensively as it has with *N. corallina* B-276. Notable among the list of epoxides formed by *N. corallina* B-276 are those produced by epoxidation of sub-terminal carbon-carbon double bonds (*cis*- and *trans*-2-octene) (Furuhashi, 1986). Apart from *o*- and *p*-chlorostyrene oxides and 1-naphthyl glycidyl ether, all of the other epoxides exhibited a positive optical rotation (Furuhashi, 1986); the substrate has a marked effect on stereoselectivity, to the extent that it can be reversed.

Despite the ambiguity regarding which strain of *N. corallina* B-276 has been used and the misinterpretation of some of the data, this organism has been proven a very useful catalyst: epoxides generated by *N. corallina* B-276 have been used for the synthesis of chiral ferroelectric liquid crystals (Furuhashi, 1986) and prostaglandin ω-chains (Takahashi *et al.*, 1989).

1:6.4 Methanotrophs

Aerobic methanotrophs utilize methane as the sole source of carbon and energy during growth. Oxidation of methane by these microorganisms is initiated by the action of methane monooxygenase (MMO) which inserts molecular oxygen into methane to form methanol. In a manner similar to other hydrocarbon assimilating microorganisms, methanol is then sequentially oxidized to the corresponding carbonyl compound (formaldehyde), carboxylic acid (formic acid) and carbon dioxide. Assimilation of C_1-compounds into biomass necessitates an unusual biochemistry (Large, 1983). The broad substrate specificity exhibited by many MMO enzymes, together with the ubiquitous nature of methanotrophs, have led to the suggestion that their role in the co-metabolic mineralisation of environmental pollutants may be significant (Dalton & Stirling, 1982). Their ability to transform an enormous range of compounds has resulted in much effort being expended to realise their potential as industrial catalysts (Higgins *et al.*, 1980).

Two methanotrophs in particular have been studied in detail: *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b. In both of these methanotrophs, the cellular location of the MMO enzyme is controlled by the concentration of copper salts in the growth medium (Stanley *et al.*, 1983). Grown in Cu^{2+}-rich media, MMO activity is membrane associated and can be sedimented by centrifugation (particulate or pMMO). Growth in Cu^{2+}-poor media results in MMO activity that cannot be sedimented by centrifugation (soluble or sMMO). It is likely that these two forms of MMO are transcribed from

different genes (Stainthorpe *et al.*, 1990), manifest as differences in their substrate specificity and sensitivity to inibitors. Soluble MMO has a wider substrate specificity than pMMO, and because of the greater ease of purification, sMMO has been better characterized than pMMO.

Regeneration of the NADH used for resting-cell hydroxylation and epoxidation reactions is attained either by oxidation of internal reserves of poly-β-hydroxybutyrate (PHB), or by the addition of a co-substrate such as methanol or formate. The PHB content of cells increases when grown in media containing excess carbon source, being more pronounced in type II methanotrophs (*e.g. Mts. trichosporium* OB3b) than type I methanotrophs (*e.g. M. capsulatus* (Bath)) (Dalton & Stirling, 1982). Consequently, hydrocarbon biotransformations carried out by *Mts. trichosporium* OB3b are less dependent on the supply of exogenous co-substrates (Dalton & Stirling, 1982).

MMO enzymes are certainly versatile catalysts, appearing to have a propensity toward the epoxidation of the shorter 1-alkenes as well as some representative examples of subterminal n-alkenes, aliphatic and aryl alkenes (Table 1.1).

TABLE 1.1

Whole cell epoxidations by methanotrophs

Epoxides Produced	A	B	C	D
Epoxyethane	+		+	+
Epoxypropane	+	+	+	+
1,2-Epoxy-2-propene			+	
1,2-Epoxybutane	+		+	+
cis-2,3-Epoxybutane	+	+	+	+
trans-2,3-Epoxybutane	+	+	+	+
1,2-Epoxy-3-butene		+	+	+
1,2-Epoxy-3-methyl-3-butene			+	
1,2-Epoxypentane				+
1,2-Epoxyhexane				+
Cyclohexene oxide	+*			
1,2-Epoxy-3-methylcyclohexane	+*			
1,2-Epoxy-4-methylcyclohexane	+*			
Styrene oxide	+	+		

A = *Methylococcus capsulatus* (Bath) (Colby *et al.*, 1977; *Leak & Dalton, 1987).

B = *Methylosinus trichosporium* OB3b (Higgins *et al.*, 1979).

C = *Methylobacterium* CRL-26 (Patel *et al.*, 1982).

D = Methanotroph H-2 (Imai *et al.*, 1986).

The catalytic versatility exhibited by methanotrophs, exists at the expense of stereoselectivity. Subramanian (1986) reported that epoxypropane and 1,2-epoxybutane produced by *M. capsulatus* NCIB 11132 and *Mts. trichosporium* NRRL 11202 were

racemic modifications. It was later shown that epoxypropane, 1-chloro-2,3-epoxypropane and trans-2,3-epoxybutane produced by five methanotrophs also were formed essentially as racemic modifications (Weijers, van Ginkel *et al.*, 1988; Table 1.2).

TABLE 1.2

Enantiomeric composition of epoxides produced by methanotrophs[a]

	Epoxypropane		1-Chloro-2,3-epoxypropane		trans-2,3-epoxybutane	
Methanotroph	R	S	R	S	R	S
A	53[b]	47	46	54	50	50
B	56	44	52	48	51	49
C	54	46	50	50	50	50
D	54	46	48	52	52	48
E	54	46	48	52	50	50

A = *Methylomonas albus* BG8

B = *Methylococcus capsulatus* (Texas)

C = *Methylosinus sporium* 5

D = *Methylocystis parvus* OBBp

E = *Methylosinus trichosporium* OB3b

[a] = Data from Weijers, van Ginkel *et al.* (1988)

[b] = % Composition (± 2%)

These authors (Weijers, van Ginkel *et al.*, 1988) also compared the enantiomeric composition of epoxypropane produced by *Mts. trichosporium* OB3b when grown on agar plates containing 1μM Cu^{2+} and 25μM Cu^{2+}. Because a racemic modification of

epoxypropane was produced in both cases, it was argued that sMMO had a similar stereoselectivity to pMMO in this organism. However, no mention was made of whether the cells of *Mts. trichosporium* OB3b had been assayed for sMMO and pMMO activity. These conclusions are highly presumptive, since a significant proportion of cells harvested from agar plates containing 25μM Cu^{2+} may have been expressing sMMO resulting from diffusion gradients imposed by growth on solid media (see Wimpenny, 1990, for a discussion of diffusion-limited growth in bacterial colonies).

1:6.5 *Nitrosomonas europaea*

Among the epoxidation catalysts discussed here, *Nitrosomonas europaea* ATCC 19178 is the most unusual, being a nitrifying bacterium responsible for the conversion of ammonia to nitrate III (nitrite). Ammonia oxidation is initiated by ammonia monooxygenase which converts it to hydroxylamine, analogous with the hydroxylation of methane to methanol by MMO (section 1:6.4). Suzuki *et al.* (1974) suggested that NH_3 rather than NH_4^+ is the preferred substrate of ammonia monooxygenase, as inferred from the observation that the *Km* decreased with increasing pH.

Nitrogen is sp^3-hybridized with three unpaired electrons, which in the case of ammonia are shared with the *s*-orbitals of three hydrogen atoms. Spatially the ammonia molecule has a tetrahedral arrangement of sp^3-orbitals, hydrogen nuclei occupying three corners of the tetrahedron whilst the fourth is occupied by the unshared sp^3-orbital. Although the H-N-H bond

angle in ammonia (107°) is smaller than that of the H-C-H bond angle in methane (109.5°) these two molecules are clearly similar. This is corroborated by findings that ammonia oxidizers (including *N. europaea*) are capable of methane oxidation (Jones & Morita, 1983) and methane oxidizers are capable of ammonia oxidation (O'Neill & Wilkinson, 1977).

In common with other monooxygenase-catalyzed reactions, ammonia monooxygenase requires a source of reductant (NADH) which in whole cells can be regenerated by the oxidation of co-substrates such as hydroxylamine, hydrazine or tetramethyl-hydroquinone (Shears & Wood, 1986). Apart from methane, ammonia monooxygenase is also capable of oxidizing other hydrocarbons: n-alkanes from ethane to octane, 1-alkenes from ethene to 1-pentene and 2-butene (Hyman *et al.*, 1988), cyclohexane (Drozd, 1980) and benzene (Hyman *et al.*, 1985).

The utility of *N. europaea* as an epoxidation catalyst is limited. Hyman *et al.* (1988) showed that in addition to the corresponding epoxide, ammonia monooxygenase also converted alkenes to unsaturated alcohols. In the case of 1-butene, 1-pentene and 2-butene, hydroxylation at a saturated carbon occurred preferentially to epoxidation. As is the case with *Pseudomonas oleovorans* (section 1:6.1) the yield of epoxide would therefore be low, since a significant proportion of the alkene could be converted to an unsaturated alcohol. However, *N. europaea* differs from *Ps. oleovorans* in that the latter appears only to hydroxylate terminal methyl groups whereas the

former can in addition affect subterminal hydroxylations (Hymans *et al.*, 1988).

It is perhaps easier to compare ammonia monooxygenase to MMO rather than the ω-hydroxylase of *Ps. oleovorans* since their natural substrates (ammonia and methane) closely resemble each other in size and shape, and because MMO is also capable of subterminal hydroxylation (Green & Dalton, 1989). The similarity between ammonia monooxygenase and MMO has already been alluded to by Hyman *et al.* (1988): "Ammonia monooxygenase is similar to membrane-bound methane monooxygenase in cellular location . . . the substrate range of ammonia monooxygenase would appear to be more similar to that of the soluble methane monooxygenase".

Weijers, van Ginkel *et al.* (1988) showed that *N. europaea* is far more stereoselective than methanotrophs (*cf.* Table 1.2) in the production of the following epoxides: epoxypropane (36% ee, S-enantiomer), 1-chloro-2,3-epoxypropane (72% ee, R-enantiomer) and 1,2-epoxybutane (42% ee, S-enantiomer). Although these enantiomeric purities are not exceptionally high, *N. europaea* is unique since the dominant enantiomers have the opposite absolute configuration to those produced by the other microorganisms cited in the same report. Based on this observation alone, *N. europaea* will undoubtedly remain one of the most important microbial epoxidation catalysts studied to date.

1:6.6 Alkene-utilizers

It is clear that in those organisms discussed above whose monooxygenase enzymes have been purified and characterized, the same enzyme is responsible for both alkane hydroxylation and alkene epoxidation. Such enzymes could be classed as alkane monooxygenases since their physiological role is that of alkane hydroxylation, alkene epoxidation being fortuitous. Many alkene-utilizing bacteria have been isolated by de Bont and co-workers over the past fifteen years, which are incapable of growth on alkanes (Hartmans, de Bont and Harder, 1989). Monooxygenase enzymes contained within these microorganisms can therefore be classified as alkene monooxygenases. Typically, members of the *Mycobacterium*, *Nocardia* and *Xanthobacter* genera have been isolated which catalyze the epoxidation of alkenes as the first step in alkene degradation.

Epoxidation by alkene-utilizers generally occurs with a high degree of stereoselectivity (Weijers, van Ginkel *et al.*, 1988, Archelas *et al.*, 1988). Based upon a restricted study of alkene- and alkane-grown bacteria, it has also been suggested that alkene-utilizers have an advantage in that they are less prone to inactivation by epoxides than are alkane-grown bacteria (Habets-Crützen & de Bont, 1985). This is a distinct advantage in a biotechnological process, the catalyst for which is highly susceptible to the toxic effects of the product. It was demonstrated that *in vivo*, epoxides exerted their effect by irreversibly inactivating the monooxygenase (Habets-Crützen & de Bont, 1985). The disadvantages of alkene-utilizers derive from their inherent ability to degrade the epoxides they

produce in many instances and their low growth rates (doubling times of 8 to 28 hours, Habets-Crützen *et al.*, 1984) although many of the *Xanthobacter* species do grow slightly faster (doubling times of 5 to 7 hours, van Ginkel & de Bont, 1986).

Mycobacterium E20

Ethene oxidation by this bacterium was shown to be initiated by an NADH-dependent monooxygenase, followed by conversion of the epoxide to acetyl-CoA which is oxidized to carbon dioxide via the glyoxylate cycle (de Bont & Albers, 1976; de Bont & Harder, 1978 and de Bont *et al.*, 1979).

Mycobacterium E20 is unusual because in addition to growth on 1-alkenes (ethene to 1-hexene) and 1,2-epoxides (epoxyethane to 1,2-epoxybutane) it is also able to grow on a wide range of n-alkanes (ethane to hexadecane) (de Bont, 1976 and Habets-Crützen *et al.*, 1984). Although this ability is shared with *Corynebacterium equi* (section 1:6.2) and *Nocardia corallina* (section 1:6.3), less is known regarding the enzymes involved in hydrocarbon degradation in these latter two organisms.

Simultaneous adaptation studies with succinate-, ethene- and ethane-grown cells showed that hydrocarbon degradation was inducible. The specific rate of 1-alkene and n-alkane oxidation was dependent upon the growth substrate: ethene-grown cells oxidized 1-alkenes faster than their corresponding n-alkanes, whereas ethane-grown cells showed a marked preference for ethane rather than ethene. This trend was reflected by cell-

free extracts of ethene-grown cells, but no cell-free activity could be detected with ethane-grown cells. It was concluded from these observations that *Mycobacterium* E20 contained two monooxygenase activities: an alkene monooxygenase induced by growth on ethene and an unstable alkane monooxygenase induced by growth on ethane (de Bont *et al.*, 1979).

Evidence in support of there being two monooxygenase activities present in *Mycobacterium* E20 was provided by Habets-Crützen *et al.* (1985). Degradation of epoxypropane and 1,2-epoxybutane by this strain was inhibited by the addition of 10mM 1,2-epoxybutane and epoxypropane respectively, allowing the enantiomeric composition of epoxides produced from propene and 1-butene to be measured by complexation gas-liquid chromatography. Stereoselectivity was much higher using ethene-grown cells (76% ee (R)-epoxypropane and 62% ee (R)-1,2-epoxybutane) than ethane-grown cells (6% ee (S)-epoxypropane and 18% ee (S)-1,2-epoxybutane).

Differences in enantiomeric composition of the epoxides produced can be explained by:

a. Growth on ethene induces a monooxygenase enzyme with a higher enantioselectivity than the monooxygenase induced by growth on ethane.

b. Cells grown on ethene contain an enantiospecific epoxide dehydrogenase (degrading the S-enantiomer faster than the R-enantiomer), whereas ethane-grown cells contain an epoxide

dehydrogenase that degrades R- and S-enantiomers at a similar rate.

c. Ethene- and ethane-grown cells contain an enantiospecific isomerase activity either augmenting a poorly enantioselective monooxygenase (ethene-grown cells) or working in opposition to an enantioselective monooxygenase (ethane-grown cells).

In order to determine which of these theories accounts for the observed enantiomeric compositions would require studies to be carried out with purified preparations of the monooxygenase and epoxide dehydrogenase enzymes.

Xanthobacter Py2

This bacterium was isolated on relatively high concentrations of propene (van Ginkel & de Bont, 1986), growing approximately five times faster on propene than *Mycobacterium* Py1 (also isolated on propene, de Bont *et al.*, 1980). In addition to growth on propane, *Xanthobacter* Py2 can grow at the expense of ethene, 1-butene, 1,3-butadiene, epoxypropane and 1,2-epoxybutane (van Ginkel *et al.*, 1987), but in contrast to *Mycobacterium* E20 it is unable to grow on ethane, propane or butane.

Epoxide accumulation was reported not to occur when propene-grown cells were incubated with propene or 1-butene (van Ginkel & de Bont, 1986; Weijers, van Ginkel *et al.*, 1988). A conflicting report by Habets-Crützen *et al.* (1987) implied that *Xanthobacter* Py2 is able to accumulate the corresponding

epoxides, and with high enantiomeric purities: (R)-epoxypropane (94% ee) and (R)-1,2-epoxybutane (88% ee). Stereoselectivity of 1-chloro-2-propene and *trans*-2-butene epoxidation was also shown to be high: (S)-1-chloro-2,3-epoxypropane (98% ee) and *trans*-(2R,3R)-2,3-epoxybutane (78% ee) (Weijers, van Ginkel *et al.*, 1988).

An alternative approach to the production of enantiomerically enriched epoxides has been explored by Weijers, de Haan and de Bont (1988). Incubation of *Xanthobacter* Py2 with a racemic modification of *trans*-2,3-epoxybutane, *trans*-2,3-epoxypentane and *cis*-2,3-epoxypentane resulted in the stereospecific degradation of the (2S)-isomers, leaving the (2R)-isomers enantiomerically pure. In the case of *trans*-2,3-epoxybutane, the 2R,3R-isomer did not appear to be degraded at all.

It is feasible that the accumulation of *trans*-(2R,3R)-2,3-epoxybutane (78% ee) from *trans*-2-butene (Weijers, van Ginkel *et al.*, 1988) may be accounted for (in part at least) by the stereospecific degradation of *trans*-(2S,3S)-2,3-epoxybutane. This is supported by the observation that the specific rate of *trans*-2-butene oxidation is almost 1.5 times faster than the specific rate of epoxide accumulation (van Ginkel *et al.*, 1987), suggesting that some of the epoxide is being degraded.

Resolution of epoxypropane by *Xanthobacter* Py2 was poor, since both enantiomers were degraded at similar rates

(approximately 42% ee (S)-epoxypropane at a yield of 17%). Better resolution of 1,2-epoxyalkanes was afforded by *Nocardia* H8 (Weijers & de Bont, 1991): >98% ee (S)-epoxypropane (13% yield), >98% ee (S)-1,2-epoxybutane (10% yield), >98% ee (S)-1,2-epoxyhexane (2% yield) and >98% ee (R)-1-chloro-2,3-epoxypropane (19% yield). The optical purity and yield of *trans*-(2R,3R)-2,3-epoxybutane produced by *Nocardia* H8 is very similar to that produced by *Xanthobacter* Py2: >98% ee *trans*-(2R,3R)-2,3-epoxybutane (49% yield).

Production of enantiomerically pure epoxides by the stereospecific degradation of one isomer cannot exceed 50% yield. This does not represent a significant drawback, since the price differential between racemic and optically pure epoxides is likely to be large in most instances. Also, it should not be forgotten that the degraded isomer can be utilized for the production and maintenance of the biocatalyst, which otherwise would have to be achieved by the supply of an alternative carbon substrate.

1:7 Miscellaneous biotransformation routes to chiral epoxides

Optically pure epoxides have been prepared by stereospecific ring-opening of the epoxide with epoxide hydrolases (microsomal enzymes) and glutathione S-transferases (cytosolic enzymes), derived from liver (Wistuba & Schurig, 1986; Bellucci *et al.*, 1989). In essence, these reactions are S_N2 catalyzed nucleophilic substitutions at one of the epoxide

ring-carbon atoms (section 1:3), using either water or glutathione as the attacking nucleophile.

Stereoselective reduction of prochiral carbonyl groups in α-haloketones, offers a route to enantiomerically pure halohydrins and consequently the corresponding epoxides by intramolecular displacement of the halogen atom (section 1:4.1). This can be achieved with a wide variety of microorganisms, but more typically with yeasts (Servi, 1990). Both enantiomers of a particular chiral halohydrin can be accessed by careful selection of biocatalysts (Fantin *et al.*, 1991) or by mutation of strains containing dehydrogenase enzymes with opposing stereoselectivity (Shieh *et al.*, 1985).

The stereoselectivity of lipase enzymes has been exploited in the resolution of esters of chiral epoxyalcohols (Ladner & Whitesides, 1984), chiral epoxy carboxylic acids (Mohr *et al.*, 1989) and chiral halohydrins (Hiratake *et al.*, 1988). An advantage of this approach is that access to both enantiomers is possible, either as the free alcohol or acid, or the ester. The commercial utility of microbial lipases for the manufacture of enantiomerically pure β-blockers has been reviewed by Kloosterman *et al.* (1988), illustrating the importance of epoxides as chiral synthons in the production of pharmaceuticals and agrochemicals.

An unusual application of lipases in the formation of epoxides is the catalytic perhydrolysis of octanoic acid in organic solvents (Björkling *et al.*, 1990). Lipase from *Candida*

antartica was used to recycle catalytic amounts of octanoic acid to peroxyoctanoic acid with hydrogen peroxide; the peroxycarboxylic acid then being used *in situ* to convert cyclohexene, 3-ethylpent-2-ene and tetramethylethylene to their corresponding epoxides (*cf.* section 1:4.2).

1:8 Project objectives

The strength of a biotransformation route to epoxides resides in its proven ability to form chiral epoxides of high optical purity. One of the best methods available is the monooxygenase-catalyzed conversion of prochiral alkenes to chiral epoxides, due to the broad substrate specificity and high stereoselectivity exhibited by many monooxygenase enzymes.

A phenomenon shown to occur in microbial epoxidation catalysts is that good stereoselectivity is generally antithetical to broad substrate specificity (section 1:6); consequently the choice of biocatalyst is very important. It could be argued that the desired stereoselectivity could be attained with most alkene substrates by selecting a biocatalyst from a range of well characterized microorganisms already available from commercial culture collections. From an industrial approach, in most instances this would be the best way to ensure that the time taken to develop an epoxidation process to a satisfactory stage is minimized. This is because a comprehensive knowledge of the substrate specificity, stereoselectivity, physiology and genetics of a microorganism

may help to discern its suitability for use in the bioreactors and downstream processing equipment available in any particular industrial production facility.

The question that was initially addressed in this project was asked from a more academic approach: is it possible to isolate a microorganism possessing the ability to perform a selected epoxidation with high stereoselectivity and productivity, by careful choice of the isolation substrate? It was based upon the supposition that any enzymic activity is almost certainly better expressed in a microorganism as yet undiscovered in nature's culture collection, than in any collection plagiarized and mutated by man. The objectives of this project were therefore to decide which epoxide to produce, select the most appropriate isolation substrates, isolate as many microorganisms as possible and select the most promising isolate for characterization.

CHAPTER 2

MATERIALS AND METHODS

2:1 Synthesis of epoxides

1,2-Epoxyheptane, 1,2-epoxynonane and 1,2-epoxyundecane were synthesized from their corresponding 1-alkenes by reaction with 3-chloroperoxybenzoic acid (see section 1:4.2).

2:1.1 Reaction procedure

A 100ml twin-necked round-bottomed flask was charged with 20ml of dichloromethane and slightly in excess of 0.01 mol 3-chloroperoxybenzoic acid. A condenser was attached to one neck, the other neck was sealed with a Suba-Seal (William Freeman & Co. Ltd., Suba-Seal Works, Staincross, Barnsley, S. Yorkshire. S75 6DH). The mixture was cooled in an ice-water bath, whilst being stirred from below with a magnetic stirrer. When cooled, 0.01 mol of 1-alkene was introduced drop-wise through the Suba-Seal. The reaction was allowed to proceed for 24 hours, maintaining the ice-water bath for the first hour then allowing it to warm up to room temperature slowly as the ice thawed.

2:1.2 Work-up procedure

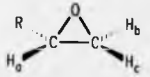
The contents of the flask was transferred to a 100ml separating funnel and extracted with ¼ volume of 10% (w/v) NaOH (aq.). The organic phase (lower layer) was separated from the aqueous phase and returned to the separating funnel to be washed with distilled water. The extraction and washing procedures were repeated, until the organic phase remained clear.

The organic phase was dried over anhydrous magnesium sulphate for 15 minutes before filtering through a fluted filter paper. The dichloromethane was evaporated under vacuum at 20°C to 35°C and the residue dissolved in diethylether for redistillation under vacuum in a Büchi kugelrÖhr (model: GKR-50). The resultant liquid was analyzed by capillary GC and by 250MHz ^{1}H-NMR (Bruker ACF250; Bruker Spectrospin Ltd., Banner Lane, Coventry). The coupling constants for the H_b and H_c protons (Figure 2.1) are given in Table 2.1.

TABLE 2.1

Coupling constants for H_b and H_c protons

	H_b		H_c	
Epoxide	J_{ab}	J_{bc}	J_{ac}	J_{bc}
1,2-Epoxyheptane	5.0 Hz	2.8 Hz	4.7 Hz	4.7 Hz
1,2-Epoxynonane	5.0 Hz	2.8 Hz	5.2 Hz	4.1 Hz
1,2-Epoxyundecane	5.0 Hz	2.8 Hz	4.5 Hz	4.5 Hz



$R = CH_3-(CH_2)_n-$

FIGURE 2.1

Assignment of the ring protons of 1,2-epoxyalkanes

Chemical shifts (δ) for the various protons were approximately: 0.8 ppm (methyl protons), 1.1-1.6 ppm (methylene protons), 2.35-2.40 ppm (H_b), 2.65-2.70 ppm (H_c) and 2.80-2.85 (H_a). Multiplets were observed for the methylene and H_a protons, triplets for the methyl protons and doublets of doublets for the H_b and H_c protons (doublets from the H_c protons of 1,2-epoxyheptane and 1,2-epoxyundecane overlapped to give triplets).

2:2 Growth media composition

All chemicals used for the preparation of media were of the highest grade and purity available from BDH or Fisons.

TABLE 2.2

Composition of Mineral Salts (MS) Medium

Part	Compound / Solution	Concentration
A	KH_2PO_4	2.0 g l^{-1}
	Trace Metal Solution	2.0 ml l^{-1}
B	NH_4Cl	3.0 g l^{-1}
	$MgSO_4.7H_2O$	0.4 g l^{-1}

1. All components were dissolved in distilled water.

2. Part A was adjusted to pH 7.0 with KOH (aq.), the pH of part B was not altered prior to autoclaving. The pH was not adjusted in medium reservoirs used for continuous cultures, as the pH was controlled automatically within the fermenter.

3. Volumes were adjusted to a ratio of 8 (part A):2 (part B), except for medium reservoirs which were adjusted to 35 (part A):1 (part B).

4. Parts A and B were sterilized separately by autoclaving at 121°C for 15 minutes. Fermenters and medium reservoirs were sterilized at 121°C for 50 minutes.

5. Bacto-Agar (Difco Laboratories, Detroit, Michigan, USA.) was added to part A prior to autoclaving to achieve a final concentration of 1.5% (w/v).

TABLE 2.3

Composition of Trace Metal Solution*

Compound	Concentration
Na_2EDTA	50.0 g l^{-1}
$ZnSO_4.7H_2O$	2.20 g l^{-1}
$CaCl_2$	5.54 g l^{-1}
$MnCl_2.4H_2O$	5.06 g l^{-1}
$FeSO_4.7H_2O$	5.00 g l^{-1}
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.10 g l^{-1}
$CuSO_4.5H_2O$	1.57 g l^{-1}
$CoCl_2.6H_2O$	1.61 g l^{-1}

1. All components were dissolved in distilled water.

2. The pH adjusted to pH 6.0 with KOH (aq.).

* As described by Vishniac & Santer (1957) with the following modifications: zinc sulphate was reduced from 22.0 g l^{-1}; iron (II) sulphate was increased from 4.99 g l^{-1}.

2:3 Isolation procedures

2:3.1 Inoculum preparation

All soil and water samples were collected and processed within one day for the majority of samples, or as quickly as was possible.

Soil samples were suspended in MS-medium and a small amount of the suspension transferred to fresh MS-medium, whilst water samples were supplemented with salts sufficient to achieve concentrations equal to that of MS-medium. An attempt was made to further enrich soil samples for microorganisms capable of degrading 1,3,5-triethylbenzene by passing water-saturated air through glass wool soaked in 1,3,5-triethylbenzene, then up through a column of soil; samples of the enriched soil were removed at intervals for inoculum preparation as outlined above.

2:3.2 Isolation methodology

Batch enrichment:

Batch isolations were performed in 250ml conical flasks containing 50ml of supplemented water sample or 50ml of MS-medium inoculated with up to 5ml of soil suspension. The hydrocarbon was added at various concentrations: 0.1% (v/v), 0.05% (v/v) or in a centre well to provide a vapour. The flasks were stoppered with Suba-Seals and incubated with shaking at 30°C.

Continuous enrichment:

A 100ml aspirator bottle was used as a culture vessel for continuous enrichment on MS+1,2,4-trivinylcyclohexane initially, then MS+n-octane. Sterile MS medium was added to achieve a dilution rate of approximately 0.05 h^{-1}. The hydrocarbon reservoir (a 100ml glass measuring cylinder) was placed in series with the air pump to deliver the hydrocarbon as a vapour. Both the culture vessel and the hydrocarbon reservoir were placed within a thermostatically-controlled water bath (30°C). Agitation was achieved by means of a magnetic stirrer located beneath the water bath. Culture volume was regulated by the gas outlet port, acting as an overhead weir. The culture vessel was inoculated at regular intervals to replenish that which had been washed out.

2:3.3 Purification procedure

Samples were withdrawn at intervals for microscopic examination and for plating out on MS-agar. Agar plates were incubated in an inverted position in sealed polypropylene boxes. Filter papers soaked with the appropriate hydrocarbon were placed in the lid of petri dishes to generate a vapour.

2.4 Biochemical characterisation of *Rhodococcus* OU

Most of the biochemical tests were performed in triplicate using an API 20B test kit (API-bioMérieux (UK) Ltd., Grafton Way, Basingstoke, Hampshire. RG22 6HY) incubated at 30°C for one, two and three days.

TABLE 2.4

Biochemical tests

Test	Principle
Glucose fermentation	Fermentation of glucose under liquid paraffin leads to a drop in pH, changing the medium from blue-green to yellow (indicator: bromothymol blue).
Catalase	Catalase activity is detected by the formation of oxygen bubbles after addition of hydrogen peroxide.
Cytochrome oxidase	A violet complex results with tetramethylparaphenylene-diamine OX reagent in the β-galactosidase or thiosulphate reduction tubes.
Gelatinase	Proteolysis of Kohn gelatin releases a black pigment.
Urease	Liberation of ammonia from urea increases the pH of the medium, changing it from yellow to red (indicator: phenol red).
Tryptophanase	Deamination of tryptophan to indole results in a violet colouration with Kovács' reagent.

TABLE 2.4 - *continued*

β-Galactosidase	Release of o-nitophenol from o-nitrophenyl β-D-galactose turns the medium yellow.
Nitrate reductase	Reduction of nitate V to nitrate III (nitrite) results in a red colouration with Griess reagents.
$S_2O_3^{2-}$ --> H_2S	Reduction of thiosulphate to H_2S results in a black precipitate in the presence of an iron salt.
Pyruvate --> acetoin	A pink-red colouration is seen by reaction of acetoin with naphthol in an alkaline medium.
Acid production during carbohydrate utilization	Production of acidic metabolites from carbohydrates turns the medium from red to yellow (indicator: phenol red).
Citrate utilization	Utilization of citrate causes the pH of the medium to drop, especially in aerobic regions, turning it from green to blue (indicator: bromothymol blue).
Acetate & propionate utilization	Growth on these carbon sources was measured by an increase in absorbance at 600nm when grown in MS-medium.

2.5 Growth of Rhodococcus OU

Rhodococcus OU was maintained by subculturing on nutrient agar (Oxoid Ltd., Wade Road, Basingstoke, Hampshire. RG24 0PW), which was used as an inoculum source for shake-flask cultures. Growth in liquid culture (MS-medium supplemented with the appropriate carbon source) was monitored by changes in

absorbance at 600nm (unless otherwise stated). Culture purity was checked by microscopic examination (x 1000 magnification) and by plating onto nutrient agar.

2.5.1 Growth in shake-flask culture

Shake-flask cultures were set-up in 250ml conical flasks containing 50ml MS-medium. The flasks were stoppered with Suba-Seals for volatile carbon sources, or with foam bungs for non-volatile carbon sources. Gaseous hydrocarbons were added through the Suba-Seal with a syringe and needle to achieve a head-space concentration of approximately 25% (v/v), whilst all other carbon sources were added to a final concentration of 0.1%. When growth was not detected within two weeks of incubation, substrate toxicity was suspected; in these instances lower concentrations of the carbon substrate were tested.

2.5.2 Growth in continuous culture

Rhodococcus OU was grown continuously in a LH-2000 Series fermenter (L. H. Fermentation Ltd., Unit 10, Nimrod Industrial Estate, Elgar Road, Reading, Berkshire. RG2 0EB) with a 2 litre working volume at a dilution rate of 0.02 h^{-1}, pH 7.0 and 30°C. The carbon source (n-hexane or n-heptane) was supplied as a vapour in the gas inlet stream.

2:6 Biotransformations

2:6.1 Preparation and storage of cell suspensions

All biotransformations were carried out with resting cell suspensions. Cells were harvested from the culture broth by centrifugation at 6,370 x g, 4°C for 15 minutes. The cell pellets resuspended in ice-cold 20mM potassium phosphate buffer pH7.0 and kept on ice until required. Cell preparations that were to be used on more than one day were drop-frozen into liquid nitrogen and stored at -70°C; these frozen cell preparations were then thawed at room temperature and kept on ice until they were to be used.

2:6.2 Biomass dry weight analysis

Cell pellets obtained by centrifugation (see section 2:6.1) from a known volume of cell suspension in buffer were transferred to pre-dried and pre-weighed aluminium foil boats. The boats were then dried overnight in an oven at 105 - 110°C and re-weighed to calculate the biomass concentration: g (dry weight) l^{-1}.

2:6.3 Biotransformation procedures

Small scale biotransformations were carried out in 250ml conical flasks containing 10ml of cell suspension, and stoppered with Suba-Seals. The flasks were pre-warmed to 25°C (unless otherwise stated) for 2 minutes in a Gyrotory Water Bath Shaker (model G76; New Brunswick Scientific (UK) Ltd., Edison House, 163 Dixons Hill Rd., North Mymms, Hatfield, Hertfordshire. AL9 7JE), shaking at 70% of maximum speed.

Unless otherwise stated, biotransformations were started by addition of 0.1% (v/v) liquid hydrocarbon or 25% (v/v) (in the head-space) gaseous hydrocarbon. When co-substrates were used, these were added at the same time as the 1-hexene. Samples were taken using a 1ml plastic syringe and 23-guage needle through the Suba-Seal and processed for analysis by capillary GLC.

2:7 Analyses

2:7.1 Gas-liquid chromatography

Samples for analysis by capillary GLC and GLC/MS were extracted into an equal volume of diethylether. Whenever possible, authentic standards of all possible biotransformation products were used to determine the GLC conditions necessary for the analysis. Products were identified by co-elution with the authentic standards and by comparison of their mass spectra to those of authentic standards. For quantitative analysis, peak areas were converted to concentration by reference to a calibration curve generated with external standards.

The majority of samples were analysed in a Philips PU 4500 chromatograph/FID on a BP1 (25 QC 3/BP1-2.0) non-polar capillary column (Scientific Glass Engineering Pty. Ltd., 1 Potters Lane, Kiln Farm, Milton Keynes. MK11 3lA). Nitrogen carrier gas was applied at a pressure of 12 psi, and the column operated isothermally (injector: 250°C, detector: 250°C). Peak areas were determined with a Hewlett-Packard 3390A Integrator (Hewlett-Packard Ltd., King Street Lane, Winnersh, Wokingham,

Berkshire. RG11 5AR). Injection volumes were less than 1.0µl (splitter ratio was varied).

Biotransformations of alkenes less than four carbon atoms in length were analysed in a Philips GVC chromatograph/FID on a Porapak Q (80-100 mesh) (Waters Chromatography Division. supplied by: Phase Separations Ltd., Deeside Industrial Park, Deeside, Clwyd. CH5 2NU) packed GC column, again operated isothermally (injector: 250°C, detector: 300°C). The reaction mixture (5µl) was injected directly onto the column without prior extraction into an organic solvent.

2:7.2 Mass spectrometry

Identification of products was carried out by GLC/MS (Carlo Erba MFC500, FSA Laboratory Supplies, Bishop Meadow Road, Loughborough, Leicestershire. LE11 0RG; Kratos MS25RFA, Kratos Ltd., Barton Dock Rd., Urmston, Manchester. M31 2LD) using a BP1 capillary column, comparing spectra to authentic standards (where available) or to spectra contained within the data base.

2:7.3 Chiral HPLC

A Beckman System Gold HPLC (solvent delivery module 110B, analogue interface 406 & programmable detector module 166) or Gilson HPLC (pump module 305, manometric module 806 & dynamic mixer 811B; supplied by: Anachem, Charles St., Luton, Bedfordshire. LU2 0EB) connected to an Applied Biosystems 1000S Diode Array Detector (supplied by: Anachem) were used. Chiral resolution of epoxides was determined on a Chiralcel OB (Figure

2.2) HPLC column (Diacel Chemical Industries Ltd. supplied by: J. T. Baker B. V., P. O. Box 1, 7400 AA Deventer, Holland) with a mobile phase of hexane (HiPerSolv, BDH) and propan-2-ol (HPLC Solvent, Fisons) at a flow rate of 0.25ml to 0.75ml min^{-1}.

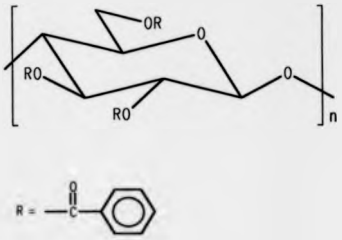


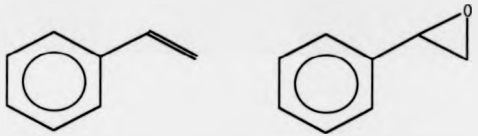
FIGURE 2.2

Chiralcel OB stationary phase

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF BACTERIA

In the 1980's, the literature was dominated by research into the formation of epoxides from simple aliphatic alkenes. Relatively little effort had been expended on the epoxidation of aryl alkenes. For this reason styrene was chosen as the target compound for this study, as styrene is structurally the most simple of the aryl alkenes (Figure 3.1).



Styrene (Vinylbenzene) Styrene oxide (Phenyloxirane)

FIGURE 3.1

Styrene and styrene oxide

It was decided therefore, to isolate a microorganism that would be suited for aryl alkene epoxidation, and in particular the epoxidation of styrene. In order to increase the likelihood of isolating such an organism, three areas were considered:

a. Inoculum source

b. Substrate choice

c. Isolation regime

3.1 Inoculum source

The autochthonous microbial community in any niche may contain microorganisms expressing the desired biocatalytic activity, but the number of these microorganisms may be very low and therefore difficult to isolate.

In a recent review on hydrocarbon degradation, Leahy and Colwell (1990) summarize the phenomenon of selective enrichment from the findings of others '. . . that the numbers of hydrocarbon-utilizing microorganisms and their proportion in the heterotrophic community increase upon exposure to petroleum or other hydrocarbon pollutants and that the levels of hydrocarbon-utilizing microorganisms generally reflect the degree of contamination of the ecosystem.' Since the capacity for alkene epoxidation is frequently expressed in microorganisms able to degrade hydrocarbons, selective enrichment for hydrocarbon degrading microorganisms is likely to yield a rich source of epoxidation activity. This illustrates the reasoning behind the choice of inoculum sources:

i. Soil samples around chemical dump sites.

ii. Sludge from industrial waste treatment plants.

iii. Soil surrounding hollow plastic golf balls containing glass wool soaked in chlorobenzene which were buried just below the soil surface for numerous months. These had remained from previous projects aimed at the isolation of chlorobenzene degrading microorganisms.

iv. Soil contaminated with oil from heavy duty farm machinery, around the bases of oil storage tanks and kerb-side soil near roads, lay-bys and car-parks.

v. Leaf litter (from pine and deciduous woodland).

vi. Dirty stream and canal water.

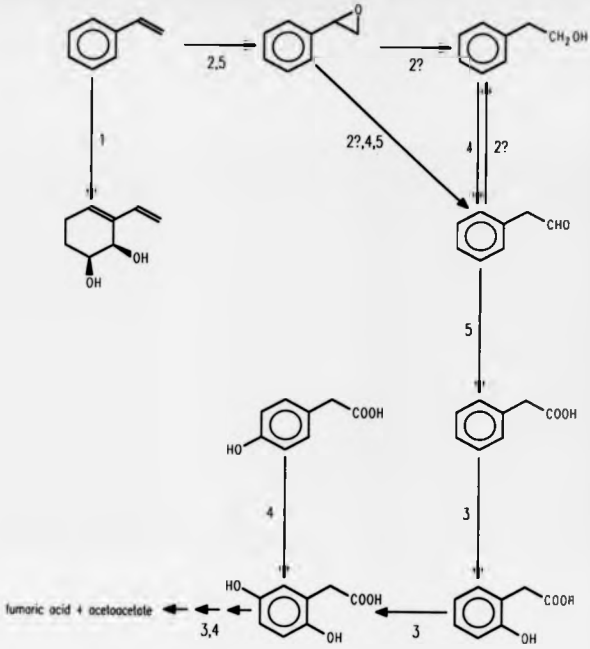
All of these sites were likely to contain hydrocarbons of anthropogenic (accidental or deliberate spillages of fuel and lubricant oils), or natural origin (resinous materials found in leaf litter). These inocula were used individually or in combination during the isolation studies. With one exception, no further treatment following collection was given to the inocula samples and all were used in as fresh a state as possible. In the case of the soil sample originating from a chemical dump site, a portion of this was further enriched by exposure to 1,3,5-triethyl benzene before being used in liquid culture for isolation purposes (see section 2:3.1).

3.2 Substrate choice

The choice of which hydrocarbons to use as the isolation substrates was influenced by the findings of Hou *et al.* (1983). In their report, the specific rate of epoxidation of ethene, propene, 1-butene, 1,3-butadiene, 1-pentene and 1-hexene was measured for 27 strains of propane-grown bacteria. Of these, 19 showed optimal activity towards propene epoxidation. It could be argued that since propene resembles the growth substrate more closely in structure than any of the other alkenes, it would fit the active site of the propane monooxygenase better than the other alkenes and consequently be converted to the epoxide at a faster rate than the other alkenes. This provided the impetus to select isolation substrates showing structural similarities to styrene.

Careful consideration was given to the current knowledge of microbial degradation of hydrocarbons, when choosing isolation substrates. The need for care in selecting the isolation substrate is illustrated by studies of microbial styrene degradation. This molecule possesses an aromatic ring and a vinyl substituent, each moiety being a potential target for microbial transformation and degradation (Figure 3.2).

In some organisms, degradation of styrene is initiated on the vinyl substituent. Growth on styrene by *Pseudomonas* 305-STR-1-4 (Shirai & Hisatsuka, 1979) resulted in the accumulation of styrene oxide and 2-phenylethanol in the culture broth. It was thought that the latter product arose from reduction of the



1 *Pseudomonas putida* (MST) (Bestetti et al., 1989)

2 *Pseudomonas* 305-STR-1-4 (Shira & Hisatsuka, 1979)

3 *Pseudomonas fluorescens* (ST) (Baggi et al., 1983)

4 *Xanthobacter* 124X (Hartmans, Smits et al., 1989)

5 S5 (Hartmans et al., 1990)

FIGURE 3.2

Pathways of styrene degradation

epoxide ring of styrene oxide. An alternate fate for styrene oxide was discovered in *Xanthobacter* 124X (Hartmans, Smits *et al.*, 1989) and a number of other styrene utilizers, including S5 (Hartmans *et al.*, 1990). These bacteria are able to catalyze the isomerization of styrene oxide to phenylacetaldehyde by styrene oxide isomerase. In the case of S5, phenylacetaldehyde is further oxidized to phenylacetate. Degradation of phenylacetate by *Pseudomonas fluorescens* (ST) was shown to proceed by sequential mono-hydroxylation to yield homogentisic acid, followed by oxidative and hydrolytic cleavage to fumaric acid and acetoacetate (Baggi *et al.*, 1983; *cf.* degradation of phenylalanine and tyrosine).

Although styrene-grown cells of *Xanthobacter* 124X were shown to oxidize styrene oxide, 2-phenylethanol, phenylacetaldeyde and phenylacetate, it could not be proven that styrene was metabolized via these intermediates (Hartmans, Smits *et al.*, 1989), supporting the idea that styrene degradation in this bacterium may be initiated by ring hydroxylation. In *Pseudomonas putida* MST, growth on styrene led to the accumulation of 1,2-dihydroxy-3-ethenyl-3-cyclohexene (Bestetti *et al.*, 1989). Styrene degradation therefore was not initiated at the vinyl moiety, rather the aromatic ring was subjected to reduction and dihydroxylation (presumably by a dioxygenase) to form a *cis*-diol.

The dichotomy of styrene degradation exhibited in the microorganisms described above, illustrate the potential difficulties with using a styrene utilizer for the production

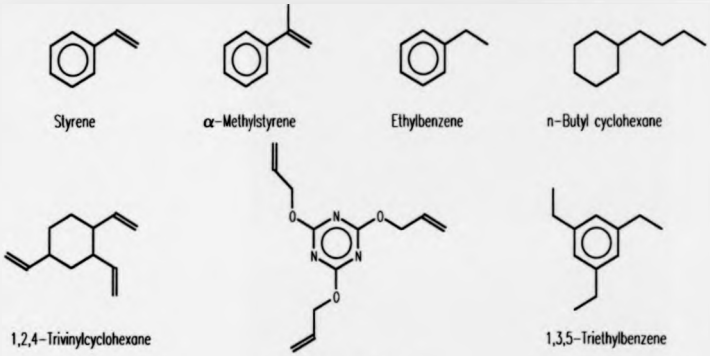


FIGURE 3.3

Styrene and its analogues used as isolation substrates

of styrene oxide. This is primarily due to their inherent ability to degrade styrene and any styrene oxide they may produce. Those organisms initiating styrene degradation at the aromatic ring would have to be identified and excluded. A process based upon the use of the remaining organisms would probably necessitate the use of metabolic inhibitors or the generation of mutants defective in the degradation of styrene oxide to prevent the desired product from being further metabolized. It was argued that a better approach to obtaining a suitable biocatalyst would be to use styrene analogues as isolation substrates (Figure 3.3). This should endow the isolates with the ability to recognise styrene as a substrate (and hopefully exhibit good stereoselectivity in styrene epoxidation) whilst being unable to degrade styrene oxide.

3.2.1 1,3,5-Triethylbenzene

Aerobic degradation of aromatic compounds normally requires that a catechol be formed as an intermediate prior to ring cleavage (see Gibson and Subramanian, 1984, for a review of microbial aromatic ring cleavage). By substituting ethyl groups in the 1, 3 and 5 positions on the benzene ring, catechol formation is prevented without prior degradation of the ethyl side chains. This should force any isolates to metabolize ethyl substituents before the benzene nucleus. The ethyl side chains should also allow these isolates to recognise the vinyl moiety of styrene due to the similarity in size of the ethyl and vinyl substituents.

3:2.2 1,2,4-Trivinylcyclohexane

This compound possess the vinyl side chains, common to styrene, but in this instance the benzene ring of styrene is mimicked by a six-membered alicyclic ring. The alicyclic ring is more difficult to degrade than the aromatic ring and is usually degraded to the exclusion of aliphatic compounds (see Trudgill, 1984, for a review of microbial aliphatic ring degradation). 1,2,4-Trivinylcyclohexane should therefore select for microorganisms able to degrade either aliphatic or alicyclic compounds, the former being more likely to predominate.

3:2.3 2,4,6-Triallyloxy-1,3,5-triazine

The benzene ring of styrene is replaced in this molecule by a nitrogen-containing heterocyclic ring. Aromaticity is still retained but because of the valency of nitrogen, a vicinal dihydroxylation on the ring cannot occur without loss of the conjugation present in the substrate molecule; therefore a catechol equivalent is not attainable in this case. Also, substitution in the 2, 4 and 6 positions, again makes access to the ring more difficult. The allyloxy ring substituents are longer than the ethyl or vinyl substituents of 1,3,5-triethylbenzene or 1,2,4-trivinylcyclohexane respectively, which may make the terminal unsaturated bond more accessible to enzymatic attack.

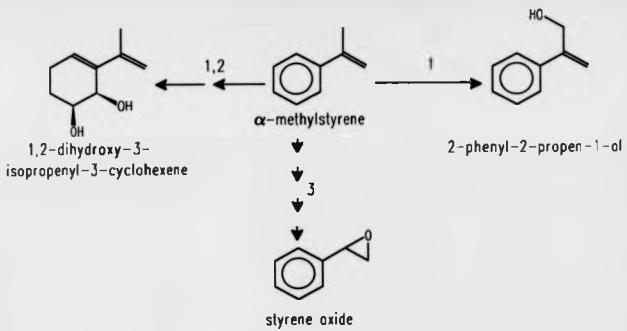
3:2.4 n-Butyl cyclohexane

As was explained earlier for 1,2,4-trivinylcyclohexane, the alicyclic ring of this compound should be more resistant to

attack than a benzene ring, leaving the n-butyl side chain the more favourable moiety for microorganisms to degrade. The basic shape of the styrene molecule is retained, replacing the benzene ring with a cyclohexane ring and the ethenyl side chain with a butyl substituent.

3:2.5 α-Methylstyrene

As its name suggests, this compound has very close structural similarity to styrene. At the time when this work was performed, there were no reports of α-methylstyrene degradation being initiated on the isopropenyl side chain, it was however a plausible route. Bestetti *et al.* (1989) later demonstrated this mode of α-methylstyrene metabolism in *Pseudomonas putida* (MST). Two pathways exist for the degradation of α-methylstyrene in this bacterium (Figure 3.4): transformation of the aromatic ring producing a substituted cyclohexene *cis*-diol (1,2-dihydroxy-3-isopropenyl-3-cyclohexene) and oxidation of the isopropenyl substituent resulting in 2-phenyl-2-propen-1-ol formation. Srivastava (1990) reported the isolation of two *Bacillus* strains which accumulate styrene oxide when grown on α-methylstyrene (Figure 3.4), again demonstrating the susceptibility of the side chain as a site for initiating degradation. Although many of the potential problems associated with using styrene as an isolation substrate are shared by α-methylstyrene, it was hoped that a microorganism might be isolated that only degrades the isopropenyl substituent, whilst possessing the ability to co-metabolize styrene to styrene oxide.



1 *Pseudomonas putida* (MST) (Bestetti et al., 1989)
2 *Pseudomonas convexa* S1078I (Omori et al., 1974)
3 *Bacillus* 51283 & FH90 (Srivastava, 1990)

FIGURE 3.4

α-Methylstyrene degradation pathways

3.2.6 Other compounds

Compounds such as ethene, propene, 1-butene and 1,7-octadiene were used for the isolation of alkene utilizers, as well as some n-alkanes for the isolation of alkane utilizers. Aliphatic compounds such as these are generally easier to degrade than the more exotic substrates described previously.

3.3 Isolation regime

This programme was aimed at isolating organisms capable of aerobic growth at 30°C, pH 7, in a minimal salts medium. It

was appreciated that such constraints automatically restricted the types of microorganisms likely to be isolated, but in order to compensate for this, both batch and continuous culture regimes were employed.

3:3.1 Batch culture

The substrate was presented at three concentrations to overcome any potential problems of substrate toxicity. This well tried and tested approach has the advantage of being simple to perform. It has the disadvantage that during a batch culture, a succession of microorganisms is likely to occur since the culture environment changes as cell growth proceeds. Sampling of such cultures is therefore very context sensitive, so the most desirable microorganism may not necessarily be in abundance when the sample is taken.

3:3.2 Continuous culture

Continuous culture has been used for ecological studies to study the interactions between members of stable microbial communities. This approach has shown that continuous culture is an important tool for isolations. In a review by Veldkamp and Jannasch (1972) the authors describe the advantages of using chemostat cultures over batch cultures:

> . . . the chemostat offers the advantage that high population densities can be obtained at very low substrate concentrations, which are the rule rather than the exception in many natural environments. . . . At low substrate concentrations, in the chemostat, organisms other than those encountered in batch cultures (growing on the

same type of substrate, but at higher concentrations) become predominant.

One of two situations can occur when setting up a continuous enrichment chemostat: either a stable community of microorganisms can develop, or a single species may predominate to the extent that a monoculture results.

3.4 Isolated organisms

This initial stage of the project proved to be problematic and as a consequence, unduly time consuming. A number of bacteria were isolated initially which appeared to be scavenging trace amounts of carbon from the growth medium rather than utilizing the hydrocarbons added as the intended carbon substrate.

Growth of these isolates was meagre, being slightly enhanced by the addition of 0.05% yeast extract (Table 3.1). In most cases, liquid cultures showed significant growth inhibition by the hydrocarbon when compared to the controls on MS+0.05%YE alone. Growth was also poor on MS+0.05%YE agar; microscopic examination of colonies grown under a vapour of the appropriate hydrocarbon showed them to be rhizoidal, lying below the surface of the agar in the most part. Colony pigmentation was either white, yellow or pink.

TABLE 3.1

Growth of isolates in shake flask culture

ISOLATE	SUBSTRATE	% (v/v)	*A_{540}
O17DE-1	1,7-Octadiene	0.1	0.333
O17DE-2	1,7-Octadiene	0.1	0.303
O17DE-3	1,7-Octadiene	0.1	0.303
αMeS-1	α-Methylstyrene	0.1	1.71
αMeS-2	α-Methylstyrene	0.1	0.423
αMeS-3	α-Methylstyrene	0.1	0.510
TEB-SS	1,3,5-Triethylbenzene	0.05	0.471
TEB-CS	1,3,5-Triethylbenzene	0.05	0.336
TVCH-1	1,2,4-Trivinylcyclohexane	0.05	0.417
TVCH-2	1,2,4-Trivinylcyclohexane	0.05	0.432

*Absorbance measured at 540nm after five day's growth in MS+0.05%YE Medium.

3.4.1 αMeS-1

αMeS-1 was the only isolate able to grow to any significant extent, being a small Gram positive rod. A yellow pigment was excreted when grown on α-methylstyrene, which was not produced when grown on styrene or in the absence of any hydrocarbons. This yellow pigment proved too polar to extract into ethylacetate or diethylether for analysis by GLC-MS. Broth supernatant from a five day culture of αMeS-1 grown on α-methylstyrene was adjusted to pH 2 and pH 12 with a few drops of 2M HCl (aq.) or 2M NaOH (aq.). Spectral scans of these samples showed a pH-dependent shift (Figure 3.5a) with

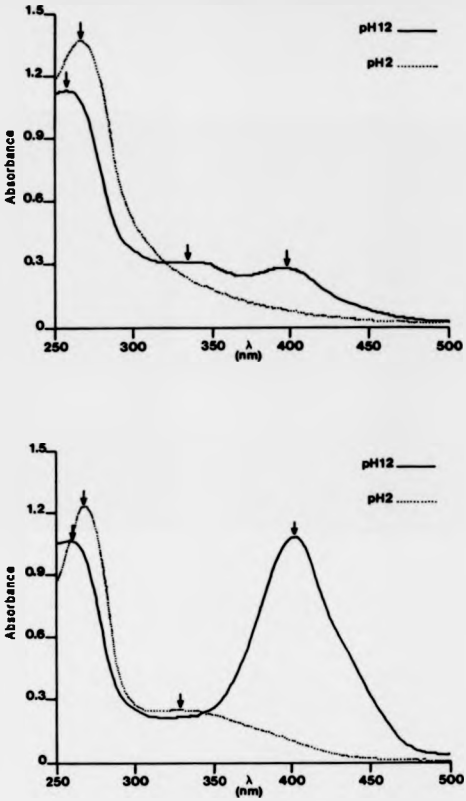


FIGURE 3.5

Spectral scans αMeS-1 broth supernatants from cultures grown on α-methylstyrene (a) and 1-phenylethanol (b)

absorbance maxima at 258nm, 335nm and 397nm (pH 12) and 266nm (pH 2).

Similar pH-dependent spectral shifts have also been reported by Dagley *et al.* (1960) for 2-hydroxy-4-carboxymuconic semialdehyde and 2-hydroxymuconic semialdehyde, produced by microbial transformation of protocatechuic acid and catechol respectively. Cripps *et al.* (1978) showed that *Nocardia* T5 grown on 1-phenylethanol also excreted a yellow pigment (2,7-dihydroxy-6-oxoocta-2,4-dienoate) into the broth, exhibiting a pH-dependent spectral shift. These products are characteristic of the *meta*-fission pathway of aromatic ring degradation, which may account for the pathway for α-methylstyrene degradation by αMeS-1 (Figure 3.6). This was more strongly implicated by analysis of the broth supernatant from a three day culture of αMeS-1 grown on 1-phenylethanol (Figure 3.5b). The spectra obtained are almost identical to those reported by Cripps *et al.* (1978) from *Nocardia* T5 grown on the same compound.

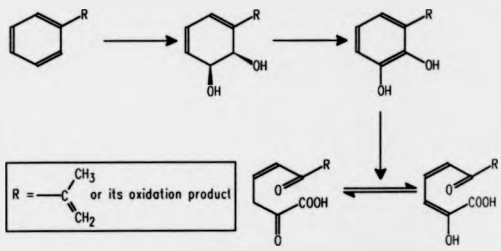


FIGURE 3.6

Possible pathway of α-methylstyrene degradation by αMeS-1

3:4.2 OU

The continuous enrichment chemostat gave rise to TVCH-1 and TVCH-2 when 1,2,4-trivinylcyclohexane was used as a carbon substrate. Approximately a week after replacing the 1,2,4-trivinylcyclohexane with n-octane, the culture vessel turned orange. Microscopic examination of the culture showed it to contain a monoculture of Gram positive pleomorphic rods. This organism was initially called OU since it was an Octane Utilizer. Although other n-alkane utilizers were isolated easily from soil and water samples after this event, the ability of OU to grow to high cell densities in continuous culture made it the preferred organism for further study.

3:5 Growth and characterization of *Rhodococcus* OU

3:5.1 Taxonomy and phenotyping

A full taxonomic characterization of OU was not an intention of this project as this could have been very time consuming and detracted from the main aims of the project. However, it was important to be able to draw comparisons between OU and taxonomically related organisms to be able to predict how genera-specific characteristics might affect the use of such an organism in a biotransformation process.

On the basis of its morphological characteristics, OU was identified as a nocardioform bacterium. A slope of OU was sent for typing to Microcheck, Inc. (P. O. Box 456, 48 South Main St., Northfield, Vermont 05663, USA). Based upon fatty acid

analysis, it was identified as a *Rhodococcus* species with a similarity index of 0.386 with *R. rhodochrous* (Appendix 3). Since the similarity index was not above 0.500, nor was it a single match comparison above 0.300, the species may not have been present in the then current version of the TSBA (Trypticase Soy Broth Agar) database. Which species of *Rhodococcus* this isolate is therefore cannot yet be assigned. Phenotypic and biochemical analysis of OU was compared to what was expected of the *Rhodococcus* type species, *R. rhodochrous* as described by Lechevalier (1986) (Table 3.2).

TABLE 3.2

Phenotypic and biochemical typing of OU

Test	OU	R. rhodochrous
Morphology	Pleomorphic	Pleomorphic
Gram Stain	+	+
Motility	-	-
Glucose fermentation	-	-
Catalase	+	+
Cytochrome oxidase	-	
Gelatinase	-	
Urease	-	
Tryptophanase	-	
β-Galactosidase	-	
Nitrate reductase	-	
$S_2O_3^{2-}$ --> H_2S	-	
Pyruvate --> acetoin	-	

TABLE 3.2 - *continued*

Acidification during carbohydrate utilization	OU	R. rhodochrous
Glucose	+	+
Fructose	+	+
Mannitol	+	+
Sorbitol	+	+
Saccharose	-	
L(+)Arabinose	-	-
Maltose	-	+
Rhamnose	-	-
Galactose	-	-
Mannnose	-	+
Starch	-	
Glycerol	-	+
Carbon source utilization	**OU**	**R. rhodochrous**
Citrate	+	
Acetate	+	+
Propionate	+	+

Refer to section 2:4 for methods.

Growth on agar plates is distinctive (Table 3.3 and Figure 3.7a). When grown on nutrient agar the elevation is conical and radially segmented, especially when the colonies are young (2-5 days). Grown on MS agar with a hydrocarbon, the surface becomes heavily lobed as the colony ages.

FIGURE 3.7a

Colonies of *Rhodococcus* OU grown for two weeks on MS + n-octane (4.5 x magnification)

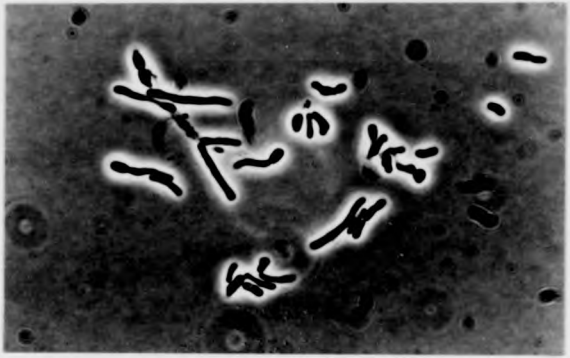


FIGURE 3.7b

Cells of *Rhodococcus* OU grown for two days in nutrient broth (2,143 x magnification)

TABLE 3.3

Colony morphology

Property	Nutrient Agar	MS + Octane
Colour	Orange	Orange
Shape	Rhizoid	Rhizoid
Elevation	Conical/Lobed	Umbonate/Lobed
Margin	Undulate	Undulate
Opacity	Opaque	Opaque
Surface texture	Matt	Matt
	Butyrous	Butyrous
	Segmented/Lobed	Lobed

During the first few months after isolating OU, a small number of pale orange-coloured colonies appeared on nutrient agar after plating-out serially diluted liquid cultures. These colour variants also appeared when single dark-orange coloured colonies were subcultured, indicating that the culture was not mixed but prone to the spontaneous generation of colour variants. Subculturing of the pale orange colonies did not show reversion to the darker phenotype, whilst repeated subculturing of the predominant dark orange colonies selected against the appearance of pale orange variants.

When viewed under the microscope, the cells are very variable in size and shape from coccoidal rods to round-ended rods to filaments, characteristic of nocardioforms (Figure 3.7b).

3:5.2 Growth on hydrocarbons and their oxidation products

Rhodococcus OU is able to grow on a wide range of hydrocarbons including n-alkanes, branched alkanes and aromatic compounds (Table 3.4) (Refer to section 2:5 for methods).

TABLE 3.4

Hydrocarbons tested as growth substrates

n-Alkane	Growth	n-Alkane	Growth
Methane	-	Nonane	+
Ethane	-	Decane	+
Propane	+	Undecane	+
Butane	+	Dodecane	+
Pentane	+	Tridecane	+
Hexane	+	Tetradecane	+
Heptane	+	Pentadecane	+
Octane	+	Hexadecane	+
Alicyclics/Other	**Growth**	**Aromatics**	**Growth**
Cyclohexane	-	Benzene	-
Cyclohexene	-	Toluene	+
1,3-Cyclohexadiene	-	Ethyl benzene	-
1-Hexene	-	Styrene	-
1,7-Octadiene	-	α-Methylstyrene	-
2,3-Dimethyl butane	+	Naphthalene	-

+/- : Growth observed/not observed.

When grown on the n-alkanes from propane to dodecane, the maximum specific growth rate (μ_{max}) exhibited an unusual trend

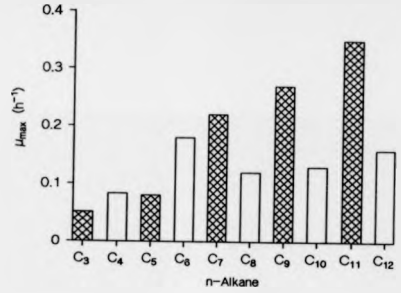


FIGURE 3.8a

Effect of n-alkane chain length on the maximum specific growth rate (μ_{max}) of *Rhodococcus* OU

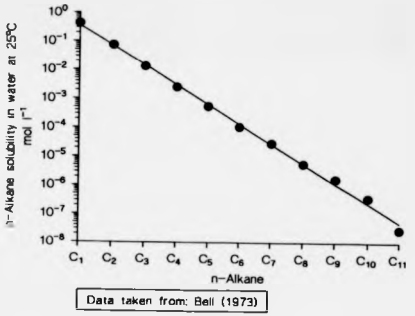


FIGURE 3.8b

n-Alkane solubility

(Table 3.5, Figure 3.8a). As the n-alkane chain length was increased, μ_{max} also increased. This cannot be accounted for in terms of substrate solubility since the longer n-alkanes are far less soluble than the shorter n-alkanes (Figure 3.8b).

What is also noticeable is a distinct pattern of higher maximum specific growth rate on n-alkanes with an odd number of carbon atoms per molecule when the cells are grown on heptane or the longer n-alkanes. This pattern is reversed for hexane and the shorter n-alkanes. Such a dichotomy is likely to be a biochemical phenomenon. It is possible that growth on n-alkanes from propane to hexane induces the expression of a different suite of hydrocarbon-degrading enzymes than is induced by growth on the longer n-alkanes.

TABLE 3.5

Maximum specific growth rates and doubling times when grown on n-alkanes

n-Alkane	μ_{max} (h^{-1})	t_d (h)	n-Alkane	μ_{max} (h^{-1})	t_d (h)
Propane	0.051	13.6	Octane	0.12	6.0
Butane	0.083	8.4	Nonane	0.27	2.6
Pentane	0.080	7.5	Decane	0.13	5.2
Hexane	0.18	3.8	Undecane	0.35	2.0
Heptane	0.22	3.2	Dodecane	0.16	4.4

Growth on n-alkanes longer than dodecane was too floccular to measure accurately by spectrophotometry.

All of the potential metabolites of hexane, arising from hydroxylation in the 1- or 2- position served as substrates for growth (Table 3.6). Neither 1-hexene nor 1,2-epoxyhexane supported growth, a situation desirable in a biotransformation process where the substrates and products are not subject to degradation.

TABLE 3.6

Growth on hexane, 1-hexene and their metabolites

Substrate	g dry weight l^{-1}
Hexane	0.540
Hexan-1-ol	0.698
Hexan-2-ol	0.548
Hexanal	0.982
Hexan-2-one	0.665
Hexanoic acid	0.451
1-Hexene	0
1,2-Epoxyhexane	0
1,2-Hexanediol	0.211

Cells were harvested seven days after inoculation with a hexane-grown culture (10% v/v). Substrates were added to a final concentration of 0.1% (v/v).

CHAPTER 4

BIOTRANSFORMATION OF ALKENES AND OTHER COMPOUNDS

The decision to use cells of *Rhodococcus* OU grown in continuous culture was taken to help reduce variability problems associated with batch culture and to ensure that the cells were in an actively growing state. The amount of hydrocarbon (n-hexane or n-heptane) supplied to the culture was prone to variation as the ambient temperature around the hydrocarbon reservoir and connecting tubing changed (seasonally and diurnally); as a result, steady state conditions were not controlled accurately.

It must be remembered that the biotransformations discussed in this chapter are performed with whole cells of *Rhodococcus* OU. The advantage of using whole cells for alkene epoxidation is that regeneration of reduced co-factors (NADH, NADPH or $FADH_2$) is carried out *in vivo*. The disadvantage of whole cell studies is that of interpretation:

1. Transport of substrates and products into and out of the cell may restrict the apparent range of biotransformations catalyzed by the monooxygenase enzyme(s) of *Rhodococcus* OU.

2. Products may result from the action of one or more enzymes and not necessarily from a monooxygenase enzyme.

3. Absence of any products may mean that the substrate is fully metabolized to biomass and/or carbon dioxide.

4. In most cases, biotransformations were analyzed after extraction into an organic solvent. This method may prevent detection of some compounds, especially the more polar products, which remain in the aqueous phase.

Great care was taken to ensure that all potential products of a biotransformation could be analyzed under the assay conditions employed. Any unexpected products therefore may not be detected.

Despite these restrictions on what information can be gleaned from whole cell studies, it would be premature to attempt a study the monooxygenase enzyme(s) of *Rhodococcus* OU without a prior knowledge of its capabilities as a whole cell biocatalyst. This is further justified since a biotechnological process would be unlikely to utilize a purified monooxygenase, mainly due to the difficulties of *in vitro* cofactor regeneration. A list of substrates tested on *Rhodococcus* OU and the products detected is given in Table 4.1; discussion of each class of compounds will be dealt with in a separate section.

TABLE 4.1

Biotransformations catalyzed by *Rhodococcus* OU

1-Alkenes	Products detected
Propene	Epoxypropane
1-Butene	1,2-Epoxybutane
1-Pentene	1,2-Epoxypentane
1-Hexene	1,2-Epoxyhexane
1-Heptene	1,2-Epoxyheptane
1-Octene	1,2-Epoxyoctane
1-Nonene	1,2-Epoxynonane
1-Decene	1,2-Epoxydecane
1-Undecene	1,2-Epoxyundecane
1-Dodecene	1,2-Epoxydodecane
1-Tridecene	1,2-Epoxytridecane
1-Tetradecene	1,2-Epoxytetradecane
2-/3-Alkenes	**Products detected**
(*Z*)-2-Butene	(*Z*)-2,3-Epoxybutane, Butanone, Crotonaldehyde
(*E*)-2-Butene	none
2-Hexene	none
3-Hexene	none

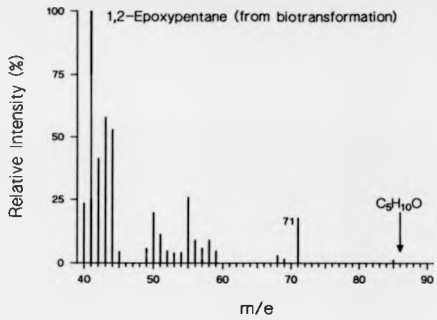
TABLE 4.1 - *continued*

Alicyclic alkenes	Products detected
Cyclopentene	Cyclopentene oxide, 2-Cyclopenten-1-one, Cyclopentanone
Cyclohexene	Cyclohexene oxide, 2-Cyclohexen-1-one, Cyclohexanone, Cyclohexanol*
1,3-Cyclohexadiene	none
Aryl alkenes	**Products detected**
Styrene	Styrene oxide
α-Methylstyrene	none
(*E*)-β-Methylstyrene	Cinnamyl alcohol, Cinnamaldehyde, Unknown
Allylbenzene	2,3-Epoxypropylbenzene
Allyl phenyl ether	1,2-Epoxy-3-phenoxypropane, Unknown
(*Z*)-Stilbene	none
(*E*)-Stilbene	none
Other compounds	**Products detected**
Benzene	none
Ethylbenzene	3- or 4-Ethylphenol, Acetophenone (trace)

*Observed only on one occasion.

When the configuration is known, internal alkenes are designated as either *cis* (*Z*) or *trans* (*E*), otherwise they are a mixture of both isomers.

Refer to sections 2:6 and 2:7 for methods.



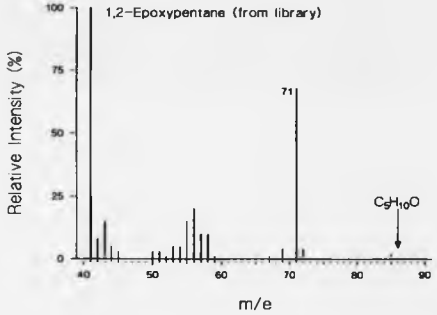


FIGURE 4.1

Mass spectrum of 1,2-epoxypentane produced by biotransformation (a) compared to the library spectrum (b)

4:1 1-Alkenes

The corresponding 1,2-epoxyalkanes of all of the 1-alkenes tested, with the exception of 1,2-epoxypentane, were commercially available or synthesized from 1-alkenes by reaction with 3-chloroperoxybenzoic acid (section 2:1). Confirmation of epoxide production from 1-pentene was obtained by comparison of the product mass spectrum to that of 1,2-epoxypentane contained within the mass spectra data base (Figure 4.1). With all of the 1-alkenes tested, only the corresponding epoxide was detected. This is in contrast to the ω-hydroxylase of *Pseudomonas oleovorans* which produces 7-octen-1-ol in addition to 1,2-epoxyoctane from 1-octene (May & Abbott, 1972a), 9-decen-1-ol and 1,2-epoxydecane from 1-decene, but only 3-buten-1-ol from 1-butene and 2-propen-1-ol from propene (May *et al.*, 1975). Since *Rhodococcus* OU is able to grow on 5-hexen-1-ol, it is likely that the products arising from hydroxylation of the methyl group of 1-alkenes are degraded by *Rhodococcus* OU, which would not be observed with a purified enzyme such as the ω-hydroxylase of *Pseudomonas oleovorans*.

4:2 2- and 3-Alkenes

The majority of organisms capable of producing epoxides from alkenes are highly regioselective, expressing a general preference for double bonds located terminally on the molecule.

Of those 2- and 3-alkenes tested, only *cis*-2-butene appeared to be transformed to any products (Figure 4.2).

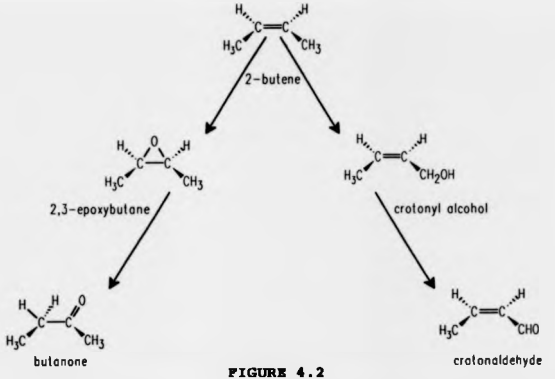


FIGURE 4.2

Proposed pathways of *cis*-2-butene oxidation by *Rhodococcus* OU

The appearance of crotonaldehyde may be accounted for by the sequential hydroxylation of the methyl group (to yield crotyl alcohol) and oxidation to the corresponding aldehyde, presumably by an alcohol dehydrogenase. Production of butanone is likely to result from an enzyme-catalyzed rearrangement of *cis*-2,3-epoxybutane as has been observed for phenylacetaldehyde formation from styrene oxide (Hartmans, Smits *et al.*, 1989), since injection of a standard of *cis*-2,3-epoxybutane on the GLC column at the temperatures employed during the analysis, only gave rise to a single peak with no apparent shoulders. An alternative explanation for the appearance of butanone is by

hydration of the double bond (to yield 2-butanol) and subsequent oxidation to the corresponding ketone; this is unlikely for the reasons explained in section 4:3.1.

It was anticipated that *cis*- and *trans*-2-butene might react stereospecifically because of differences in the accessibility of the double bond. Such differences were evident using purified sMMO from *Methylococcus capsulatus* (Bath) (Green & Dalton, 1989). The *cis*-isomer favoured production of the epoxide (53% epoxide, 47% crotyl alcohol), whereas the *trans*-isomer favoured production of crotyl alcohol (27% epoxide, 73% crotyl alcohol). It is easy to envisage that when the methyl groups of 2-butene are on the same side of the molecule (*cis*-configuration) that the double bond is easily accessible to the active site of sMMO, whereas access to the double bond in the *trans*-isomer is hindered by the methyl groups.

4:3 Alicyclic alkenes

In common with the alkenes discussed in the last section, alicyclic alkenes have carbon-carbon double bonds located internally. Both cyclopentene and cyclohexene proved to be good substrates, giving rise to a similar range of products in both cases. Biotransformation of cyclohexene was investigated further, allowing similar arguments to be proposed for the formation of the corresponding products from cyclopentene.

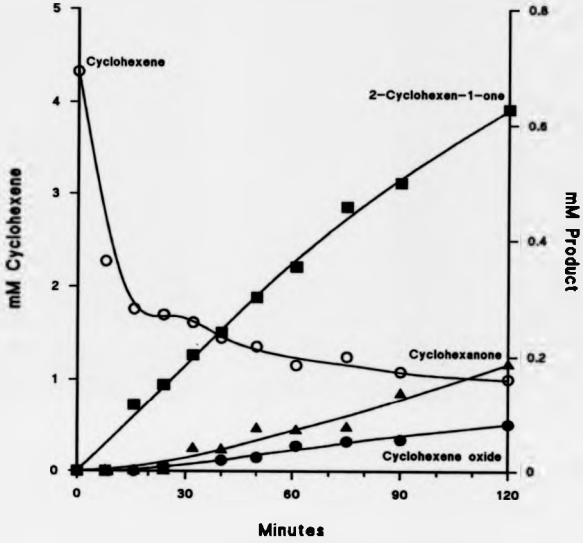


FIGURE 4.4

Biotransformation of cyclohexene by *Rhodococcus* OU

(Refer to sections 2:6 and 2:7 for methods)

Analysis of cyclohexene metabolites by capillary GLC did not allow the resolution of 2-cycohexen-1-ol, cyclohexanone and cyclohexanol when operated isothermally (Figure 11.4), unless the column temperature was lowered to 50°C when cyclohexanone could be resolved from the other two compounds. Better resolution was achieved using the capillary GLC column from the GLC/MS also operated isothermally at 50°C (Figure 4.3).

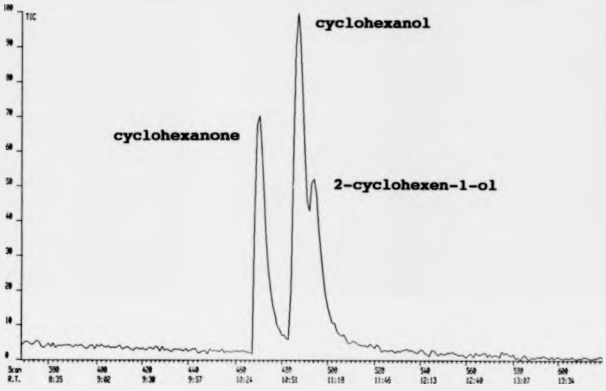


FIGURE 4.3

Resolution of 2-cyclohexen-1-ol, cyclohexanone and cyclohexanol by GLC/MS

Up to three other products apart from the epoxide were detected. Production of these metabolites was monitored over time (Figure 4.4) which showed that cyclohexene oxide was only a minor component of the reaction and that the products were

not produced sequentially. The sequence of reactions leading to the appearance of these products was addressed in two ways: considering how cyclohexene oxidation could be initiated and by using the products as biotransformation substrates.

4:3.1 Initiating oxidation of cyclohexene

There are three ways an oxygen atom could be inserted into cyclohexene: epoxidation of the double bond, hydroxylation at a saturated carbon and hydration of the double bond.

Hydroxylation at a saturated carbon:

This could give rise to two possible products: 2-cyclohexen-1-ol and/or 3-cyclohexen-1-ol. The ω-hydroxylase of Pseudomonas oleovorans was shown to produce both of these unsaturated alcohols from cyclohexene, but none of the epoxide (May *et al.*, 1975). Studies with sMMO from *Methylococcus capsulatus* (Bath) suggest that the oxidation of substrates occurs by a nonconcerted mechanism: hydrogen abstraction preceding hydroxylation (Green & Dalton, 1989). If a similar mechanism occurs in the monooxygenase enzyme(s) of *Rhodococcus* OU, production of 2-cyclohexen-1-ol would be favoured. This is because the homolytic bond dissociation energy for hydrogen removal from the allylic position is lower than that from the non-allylic position, due to resonance stabilization of the resultant allylic radical (Figure 4.5).

None of the unsaturated alcohols were detected but hydroxylation at the allylic position followed by oxidation of the hydroxyl group to the corresponding ketone could account

for the appearance of 2-cyclohexene-1-one. It is also plausible that oxidation of a 3-cyclohexen-1-ol intermediate to 3-cyclohexen-1-one could rearrange to 2-cyclohexen-1-one, achieving stability through conjugation of the carbonyl and carbon-carbon double bonds (Figure 4.6).

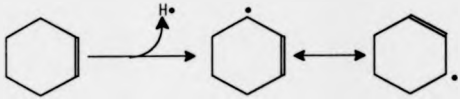


FIGURE 4.5

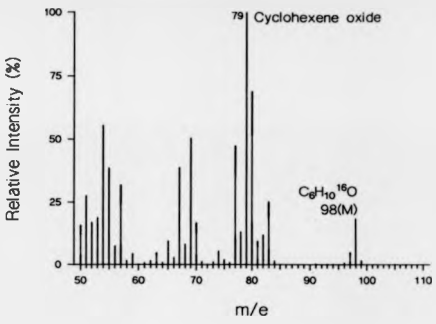
Resonance stabilization of the cyclohexene allylic radical

FIGURE 4.6

Rearrangement of 3-cyclohexen-1-one to 2-cyclohexene-1-one

Hydration of the double bond:

Hydration of carbon-carbon double bonds is not an uncommon biochemical phenomenon (*e.g.* conversion of fumarate to malate in the tricarboxylic acid cycle and during β-oxidation of fatty acids). Degradation of hydrocarbons by hydration of



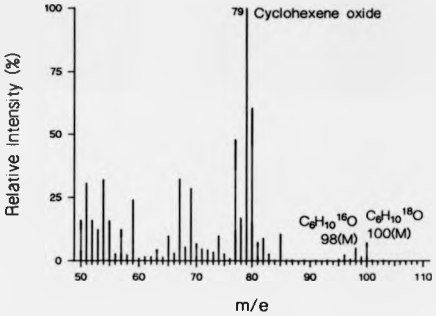
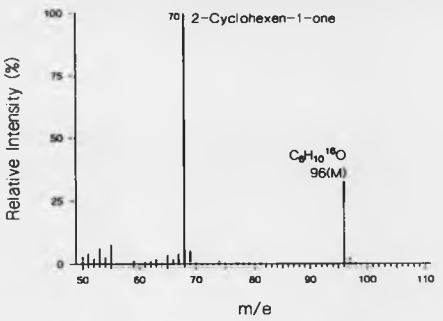


FIGURE 4.7

Mass spectra of cyclohexene oxide produced by biotransformation in the absence (a) and presence (b) of $^{18}O_2$



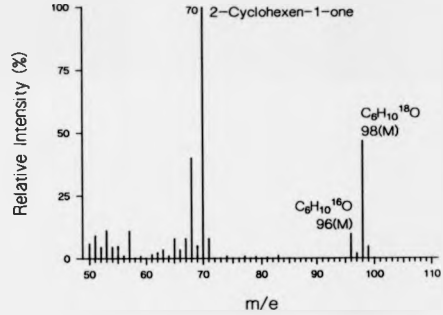
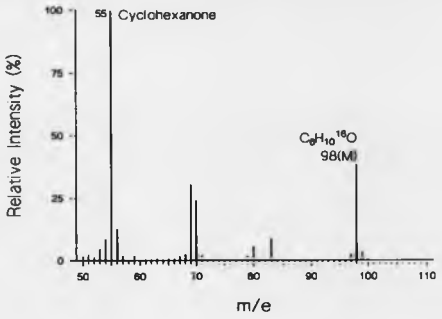


FIGURE 4.8

Mass spectra of 2-cyclohexen-1-one produced by biotransformation in the absence (a) and presence (b) of $^{18}O_2$



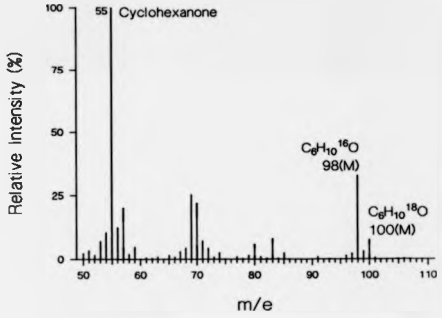


FIGURE 4.9

Mass spectra of cyclohexanone produced by biotransformation in the absence (a) and presence (b) of $^{18}O_2$

unsaturated carbon-carbon bonds generally occurs under anaerobic conditions for aromatic compounds (Young, 1984) and has been noted in only a few instances for simple aliphatic compounds (Hartmans, de Bont & Harder, 1989).

If a hydratase activity were responsible for initiating the biotransformation of cyclohexene, the first product would be cyclohexanol. Products derived from cyclohexanol would therefore contain an oxygen atom derived from water, not from molecular oxygen. Biotransformation of cyclohexene in the presence of $^{18}O_2$ / $H_2{}^{16}O$ showed incorporation of ^{18}O into cyclohexene oxide (Figure 4.7), 2-cyclohexen-1-one (Figure 4.8) and cyclohexanone (Figure 4.9), suggesting that hydration of the carbon-carbon double bond is not significant. Although molecular oxygen is the likely terminal electron acceptor in *Rhodococcus* OU, the concentration of $H_2{}^{18}O$ produced by respiration will be insignificant when compared to the concentration of $H_2{}^{16}O$; this argued against the need for a control experiment using $^{16}O_2$ / $H_2{}^{18}O$. Despite flushing the head space within the reaction flask with nitrogen gas prior to addition of the $^{18}O_2$, absence of $^{16}O_2$ during the biotransformation could not be ensured, resulting in the appearance of ^{16}O mass ions.

4:3.2 Rearrangements of cyclohexene oxide

2-Cyclohexen-1-one and cyclohexanone could have arisen from rearrangements of the epoxide ring of cyclohexene oxide (Figure 4.10). In the case of cyclohexanone, rearrangement of epoxide rings to carbonyl compounds is a reaction often

encountered in chemistry (Gorzynski Smith, 1984) and recently reported as an enzyme-catalyzed mechanism for the degradation of styrene oxide (Hartmans, Smits *et al.*, 1989).

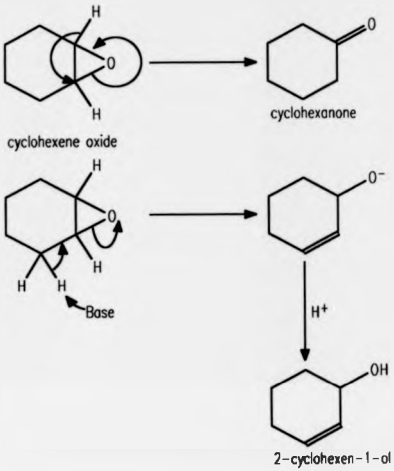


FIGURE 4.10

Rearrangement of cyclohexene oxide to cyclohexanone and 2-cyclohexen-1-ol

Deprotonation of cyclohexene oxide by lithium amide bases, yielding 2-cyclohexen-1-ol, is known (Whitesell & Felman, 1980). It is feasible that such a rearrangement could be enzymatically catalyzed within *Rhodococcus* OU, the resultant

allylic alcohol being oxidised to 2-cyclohexen-1-one so quickly that it could not be detected.

Such a mechanism has already been proposed for the appearance of 2-cyclohexen-1-ol from cyclohexene by sMMO from *Methylococcus capsulatus* (Bath) (Leak & Dalton, 1987). Later studies (Leak *et al.*, 1988) on the biotransformation of 3-methylcyclohexene by sMMO derived from *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* OB3b and *Methylosinus sporium* (strain 5) showed that the allylic alcohol (4-methylcyclohex-2-enol) did not arise by rearrangement of the epoxide (3-methylcyclohexene epoxide).

4:3.3 Feeding of cyclohexene oxidation products

TABLE 4.2

Feeding cyclohexene oxidation products to whole cells of Rhodococcus OU

Substrate added	Products detected
Cyclohexene oxide	none
2-Cyclohexen-1-ol	2-Cyclohexen-1-one
2-Cyclohexen-1-one	Cyclohexanone
Cyclohexanone	none
Cyclohexanol	Cyclohexanone

Refer to sections 2:6 and 2:7 for methods.

Interconversion of the various products may be achieved by simple oxidation and reduction reactions. Studies on the conversion of allylic alcohols by cell-free preparations of *Pseudomonas oleovorans* led to the resolution of two enzymic activities: an alcohol dehydrogenase and an olefin reductase, both active on 2-cyclohexen-1-ol (May *et al.*, 1979).

The most likely pathway of cyclohexene oxidation by *Rhodococcus* OU, based upon these studies and those on MMO and *Ps. oleovorans* ω-hydroxylase is depicted in Figure 4.11.

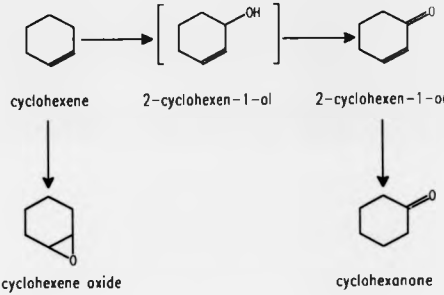


FIGURE 4.11

Proposed pathways of cyclohexene oxidation by *Rhodococcus* OU

4:4 Aryl alkenes

4:4.1 Styrene

Although *Rhodococcus* OU was able to epoxidize styrene without difficulty, analysis of styrene oxide was fraught with problems (see section 6:3 and the discussion below).

Analysis of styrene oxide by capillary GLC gave rise to two peaks. The peak eluting first was identified by GLC/MS as phenylacetaldehyde, the second peak as styrene oxide. The origins of the phenylacetaldehyde were not clear even though it was predicted to be a likely rearrangement product of styrene oxide. Analysis of the styrene oxide (supplied by Aldrich) by infra-red and ^{1}H-NMR spectroscopy showed that the sample was of high purity, in contrast to the much lower purities observed by capillary GLC. Thermal rearrangement on the capillary GLC column was suspected. This was confirmed by studying the effect of column temperature on the ratio of peak areas of styrene oxide to phenylacetaldehyde (Figure 4.12).

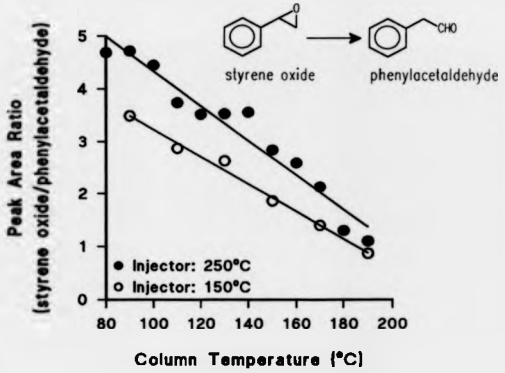


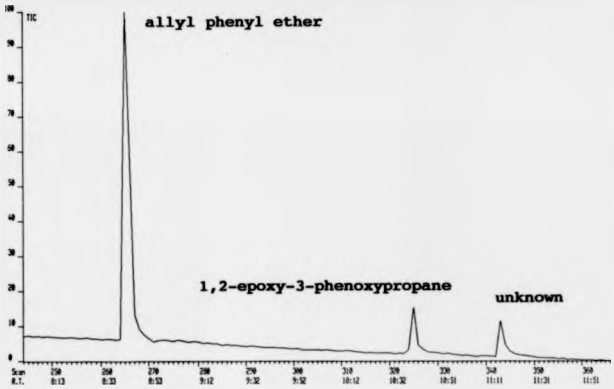
FIGURE 4.12

Thermal rearrangement of styrene oxide by capillary GLC

4:4.2 Allyl phenyl ether

Johnstone *et al.* (1987), reported the use of microbially-produced epoxides in the synthesis of β-blockers (drugs that are antagonists of the sympathetic nervous system, acting specifically on the β-receptors). Apart from a number of target β-blockers, the authors also chose to study the epoxidation of allyl phenyl ether as a β-blocker analogue precursor.

Allyl phenyl ether proved to be a suitable substrate for epoxidation by *Rhodococcus* OU, but an unidentified co-product was also produced during the biotransformations (Figure 4.13).



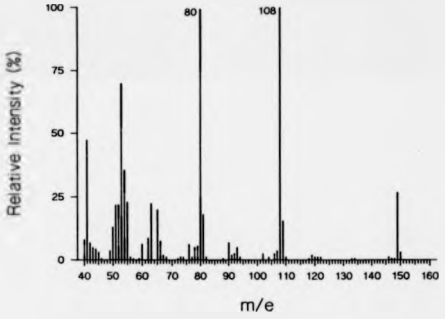


FIGURE 4.13

Chromatogram of allyl phenyl ether biotransformation extract and mass spectrum of the unknown co-product

That the co-product was *ortho*-allylphenol, a Claisen Rearrangement of allyl phenyl ether (Figure 4.14), was dismissed because:

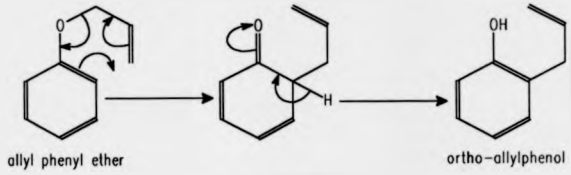


FIGURE 4.14

The Claisen Rearrangement of allyl phenyl ether to *ortho*-allylphenol

a. Claisen Rearrangements typically require the allyl aryl ethers to be refluxed at temperatures of approximately 200°C, which was never attained during the biotransformation.

b. When injected onto the capillary GLC column (operated at the same temperatures used for analyzing the biotransformation), a standard of allyl phenyl ether gave a single peak whose retention time was much shorter than that of the co-product. This eliminates the possibility of the Claisen Rearrangement occurring on the capillary GLC column.

c. The mass spectrum obtained for the co-product shows a potential molecular ion of m/e 150, larger than that expected

for *ortho*-allylphenol. The discrepancy (m/e 16) is likely to be due to addition of ^{16}O.

The possibility that a rearrangement of 1,2-epoxy-3-phenoxypropane was occurring on the capillary GLC column was excluded for the same reason as b. above. A comparison of the mass spectrum of the unidentified co-product to those stored in the mass spectra data base failed to suggest any plausible identity for the co-product.

4.4.3 *trans*-β-Methylstyrene

Inspired by the success of using microbially produced epoxides to synthesize β-blockers (Johnstone *et al.*, 1987), a novel route to ephedrine was devised utilizing the epoxide of *trans*-β-methylstyrene (Figure 4.15).

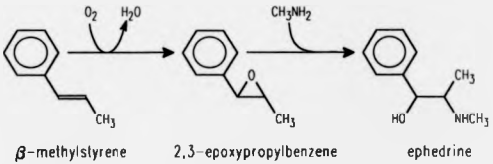
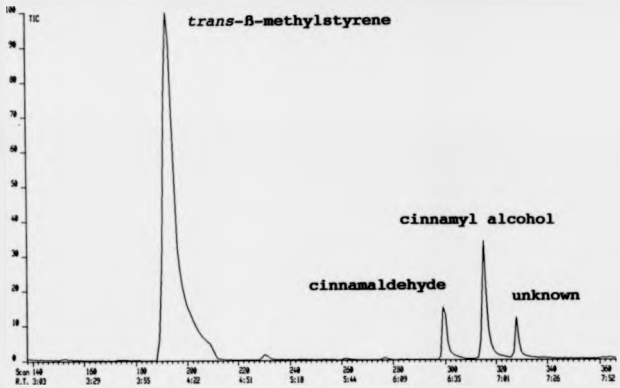


FIGURE 4.15

Synthesis of ephedrine from *trans*-β-methylstyrene



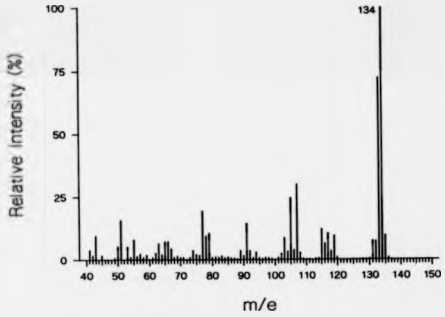


FIGURE 4.16

Chromatogram of trans-ß-methylstyrene biotransformation extract and mass spectrum of the unknown co-product

Ephedrine is an agonist of the sympathetic nervous system, stimulating the secretion of noradrenaline (norepinephrine). It is prescribed as a bronchodilator and decongestant. In common with the β-blockers, pharmacological activity resides in a single enantiomer, the αS,βR-enantiomer in the case of ephedrine (Bowman & Rand, 1980).

Nucleophilic substitution at the β-carbon atom of (1S,2S)-(-)-1-phenylpropylene oxide with methylamine would yield αS,βR-ephedrine. Unfortunately *Rhodococcus* OU did not produce 1-phenylpropylene oxide, preferring to transform the terminal methyl group instead. The appearance of cinnamaldehyde suggests that cinnamyl alcohol is the product of a monooxygenase-catalyzed biotransformation, which is oxidized to the corresponding aldehyde (cinnamaldehyde) by an alcohol dehydrogenase (*cf.* biotransformation of *cis*-2-butene, section 4:2).

A co-product was again produced (Figure 4.16), whose identity could not be determined by comparison of its mass spectrum to those held in the mass spectra data base. If the base peak (m/e 134) is also the mass ion, addition of a single ^{16}O atom by epoxidation or ring hydroxylation might be suspected. The co-product did not co-elute with an authentic standard of 1-phenylpropylene oxide, but the possibility of an enzyme-catalyzed rearrangement of the epoxide to a ketone cannot be excluded (*cf.* biotransformation of *cis*-2-butene, section 4:2).

4:4.4 Allylbenzene

2,3-Epoxypropylbenzene was the only observed product during biotransformations of allylbenzene. It is interesting to note that *Rhodococcus* OU forms the epoxide of allylbenzene but hydroxylates the terminal methyl group of its structural isomer, *trans*-β-methyl styrene (section 4:4.3).

4:4.5 Other aryl alkenes

The lack of observable products from biotransformations with *cis*- and *trans*-stilbene and to a lesser extent with α-methylstyrene, may be the result of steric hindrance about the carbon-carbon double bond.

Dalton *et al.* (1981) noted that a ring hydroxylation product of α-methylstyrene was produced almost exclusively by the MMO of *Methylococcus capsulatus* (Bath), whereas styrene yielded a mixture of styrene oxide and *para*-hydroxystyrene. This reluctance to form the epoxide of α-methylstyrene was attributed to a greater degree of steric hindrance about the carbon-carbon double bond in α-methylstyrene when compared to styrene.

trans-Stilbene is solid at the temperatures employed in the biotransformations (melting point: 122-124°C) which may have limited its availability to the cells during the biotransformation. In order to minimise this problem, a 0.1% (w/v) solution in ethanol was added to a final concentration of 0.9% (v/v). Epoxidation of *cis*-stilbene should be easier than *trans*-stilbene due to the greater accessibility of the double

bond (*cf.* biotransformation of *cis*-2-butene, section 4:2), but no epoxide was detected. Transport of such large molecules into and out of the cell may be difficult, which may account for the observed recalcitrance of these compounds.

4:5 Other compounds

4:5.1 Benzene

Benzene was tested as a substrate because it represents the last member of the series: cyclohexene, 1,3-cyclohexadiene and benzene. In common with 1,3-cyclohexadiene, no products were detected. This may reflect a reluctance of *Rhodococcus* OU to act upon conjugated alkenes.

The mechanism of hydroxylation proposed for sMMO of *Methylococcus capsulatus* (Bath) (Green & Dalton, 1989) requires hydrogen abstraction to precede hydroxylation. Assuming such a mechanism is applicable to *Rhodococcus* OU, then this might explain the lack of benzene oxidation products, since removal of hydrogen by homolytic fission from benzene (460kJ mol^{-1}) is more difficult to achieve than from ethene (451kJ mol^{-1}) and 1-alkenes.

4:5.2 Ethylbenzene

Both ring hydroxylation and side-chain hydroxylation of ethylbenzene has been observed using whole cells of *Methylosinus trichosporium* OB3b (Higgins *et al.*, 1980; Burrows *et al.*, 1984) and crude preparations of sMMO from *Methylococcus*

capsulatus (Bath) (Dalton *et al.*, 1981). Similarly, hydroxylation of the ring and the side chain was also observed using *Rhodococcus* OU.

In common with the reports by Burrows *et al.* (1984) and Dalton *et al.* (1981), side-chain oxidation by *Rhodococcus* OU occurs at the benzylic position, although only in trace amounts. Formation of acetophenone probably results from a monooxygenase-catalyzed hydroxylation of ethylbenzene and subsequent oxidation to the corresponding ketone by an alcohol dehydrogenase. It has been proposed that hydrocarbon oxidation by sMMO from *Methylococcus capsulatus* (Bath) occurs by a nonconcerted mechanism (hydrogen abstraction preceding hydroxylation), involving a radical intermediate (Green & Dalton, 1989). Such a mechanism would favour hydroxylation of ethylbenzene at the benzylic position rather than hydroxylation at the terminal methyl group, due to resonance stabilization of the benzyl radical (Figure 4.17).

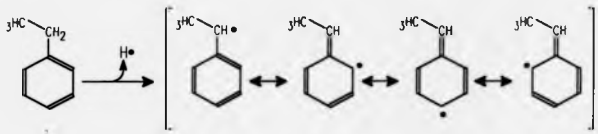


FIGURE 4.17

Resonance stabilization of the benzyl radical of ethylbenzene

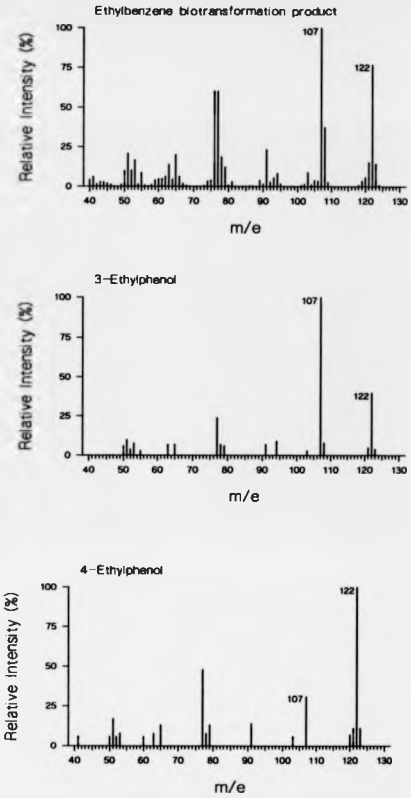


FIGURE 4.19

Mass spectra of ethylphenol produced by biotransformation (a), compare to library spectra for 3- (b) and 4-ethylphenol (c)

The phenolic product was probably derived from an arene oxide intermediate which can undergo an acid-catalyzed rearrangement with a concomitant N.I.H. shift, to yield the more stable phenol (Figure 4.18).

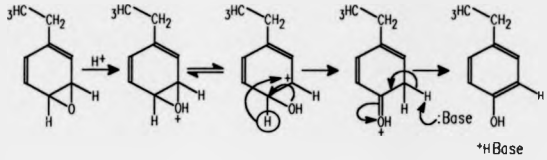


FIGURE 4.18

Acid-catalyzed rearrangement of ethylbenzene-3,4-oxide to 4-ethylphenol

It is uncertain whether the phenolic product was 3- and/or 4-ethyl phenol since these two compounds are not resolved by capillary GLC, even at a column temperature of 100°C. The mass spectrum of the product has a base peak of m/e 107, showing greater similarity to 3-ethyl phenol than 4-ethyl phenol (Figure 4.19). From this evidence, it is not possible to say that 3-ethyl phenol is the sole product of ring hydroxylation, as the mass spectrum generated may be a composite of both 3- and 4-ethyl phenol.

Based on chemical reactivity, alkyl substituents on benzene rings are activating groups, directing a second substitution in either the *ortho*- (2-) or *para*- (4-) positions, as has been observed for *Methylosinus trichosporium* OB3b (Higgins *et al.*, 1980), with a preference for *para*-hydroxylation (Burrows *et al.*, 1984). Hydroxylation in the *meta*-position has been observed using crude preparations of sMMO from *Methylococcus capsulatus* (Bath), although in very small (≤3%) quantities (Dalton *et al.*, 1981). Production of 3-ethylphenol (*meta*-position) by *Rhodococcus* OU was not therefore expected.

CHAPTER 5

OPTIMIZATION STUDIES

In his discussion of the "Effects of environmental conditions on formation of microbial products" Pirt (1975) suggested that:

> The influence of environmental factors on product formation in fermentations has, with few exceptions, been expressed in terms of the final or maximum product concentration. However, this overall effect is determined by the following independent factors: (i) biomass concentration; (ii) the specific production rate, q_p; (iii) the product yield from the substrate, $Y_{p/s}$; (iv) the duration of the synthetic activity; and (v) the rate of decomposition of the product. Ultimately, optimization must be analysed in terms of these five factors.

Although this was designed to offer guide lines for more traditional fermentation systems, the same factors can be applied to a biotransformation process which utilizes non-growing cells, as was the case in this study.

Studies presented here concentrate on a range of factors considered likely to affect the specific rate of epoxidation (q_p). It was hoped that by discerning which factors enhance production of one epoxide, the same rules could be applied to

optimizing production of any other epoxide. Whilst styrene epoxidation would be an attractive proposition for optimization as has been discussed earlier, it has certain disadvantages as a model system. Apart from the difficulties associated with the analysis of styrene oxide (section 4:4.1) the rate of epoxidation is relatively slow (Figure 5.1). This slow rate of epoxidation is certainly a challenge for optimization, but may be indicative of a system that is insensitive to small changes in process conditions.

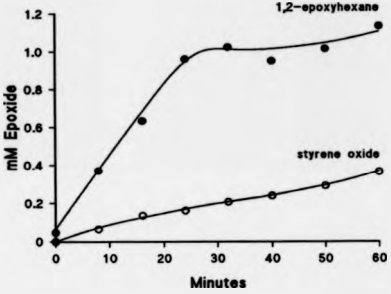


FIGURE 5.1

Comparison of styrene and 1-hexene biotransformation profiles

Growth substrate: n-heptane

Biocatalyst concentration: 10.1 g dry weight l^{-1}

Alkene concentration: 0.1% (v/v)

Biotransformation temperature: 25°C

Epoxidation of 1-hexene was chosen in preference to other alkenes because:

a. All of the potential metabolites of 1-hexene were commercially available and of high purity.

b. All of the potential metabolites of 1-hexene were easily resolved by capillary GLC, allowing rapid quantification (Figure 11.5).

c. Being a liquid at room temperature, 1-hexene is easier to handle than a gaseous or solid alkene, with the degree of accuracy required.

5:1 Effect of cell storage

Testing the effect of varying a single parameter requires that all other parameters remain constant. The concentration and physiological status of the biocatalyst was one of those parameters. One way of ensuring that the physiological status of cells used in comparative experiments is the same would be to use chemostat cultures. However, problems associated with maintaining a constant supply of hydrocarbon to the culture prevented steady state conditions being established. To overcome this problem, cell preparations were drop frozen in liquid nitrogen and stored at -70°C. The stability of the cells with regard to catalyzing alkene epoxidation was investigated by performing a 1-hexene biotransformations on a sample of

cells suspended in buffer, both before and after freezing in liquid nitrogen.

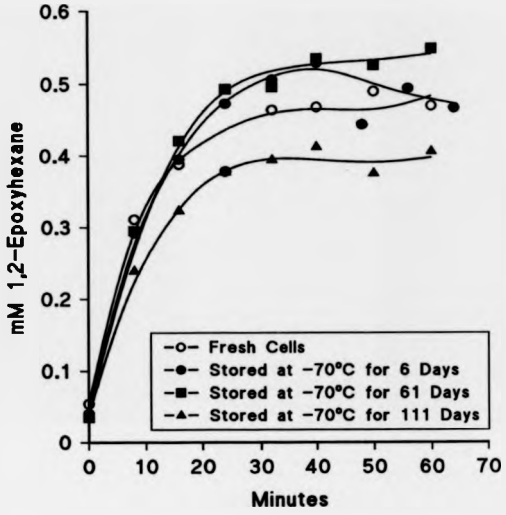


FIGURE 5.2

Effect of freezing and storage on catalytic activity

Growth substrate: n-heptane

Biocatalyst concentration: 10.1 g dry weight l^{-1}

Alkene concentration: 0.05% (v/v)

Biotransformation temperature: 25°C

The epoxidation activity decayed only after storage for more than three months at -70°C (Figure 5.2). Cells stored for up to two months appeared very stable, showing no appreciable change in q_p, although the concentration of 1,2-epoxyhexane reached after forty minutes incubation was higher using frozen cells. Cell stored in this manner therefore provide a useful way of maintaining biocatalyst preparations in an active state for long periods of time.

5:2 Effect of biotransformation temperature

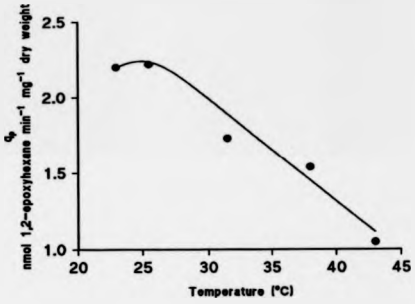


FIGURE 5.3

Effect of biotransformation temperature on 1-hexene epoxidation

Growth substrate: n-hexane
Biocatalyst concentration: 34.9 g dry weight l^{-1}
Alkene concentration: 0.1% (v/v)

Because of the availability of incubators, *Rhodococcus* OU was grown at 30°C. This of course may neither be the optimal temperature for growth nor for epoxidation. The q_p for 1-hexene epoxidation was maximal at around 25°C (Figure 5.3).

5.3 Effect of biocatalyst concentration

Biomass concentration and q_p were shown not to be independent as had been suggested by Pirt (1975), but were in part linked (Figure 5.4). Above a biomass concentration of 7g dry weight l^{-1} the q_p was fairly constant, whereas below 7g dry weight l^{-1} the q_p was inversely proportional to the biomass concentration. 1-Hexene epoxidation by *Methylococcus capsulatus* (Bath) showed a similar inverse relationship (Figure 5.5).

For *Rhodococcus* OU, volumetric productivity was directly proportional to biomass concentration (Figure 5.4), whereas no discernable trend was observed for *Methylococcus capsulatus* (Bath) (Figure 5.5).

5.4 Effect of substrate concentration

A saturable dependency of reaction rate on substrate concentration is characteristic of enzymes exhibiting Michaelis-Menten kinetics. When the effect of 1-hexene concentration on q_p was investigated, this too showed a saturable dependency on alkene concentration (Figure 5.6).

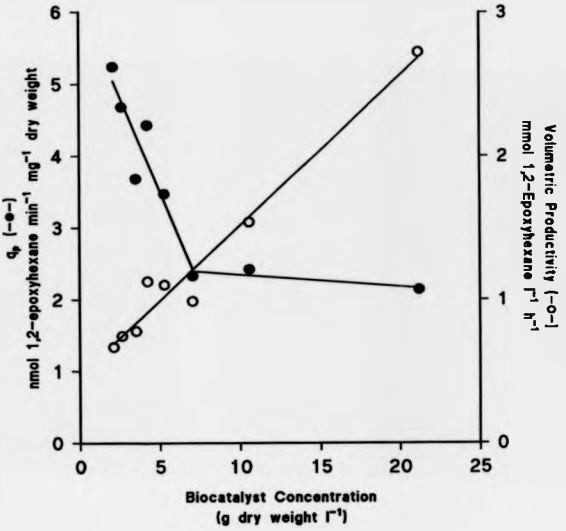


FIGURE 5.4

Effect of biocatalyst concentration on q_p and volumetric productivity: *Rhodococcus* OU

Growth substrate: n-hexane

Alkene concentration: 0.1% (v/v)

Biotransformation temperature: 30°C

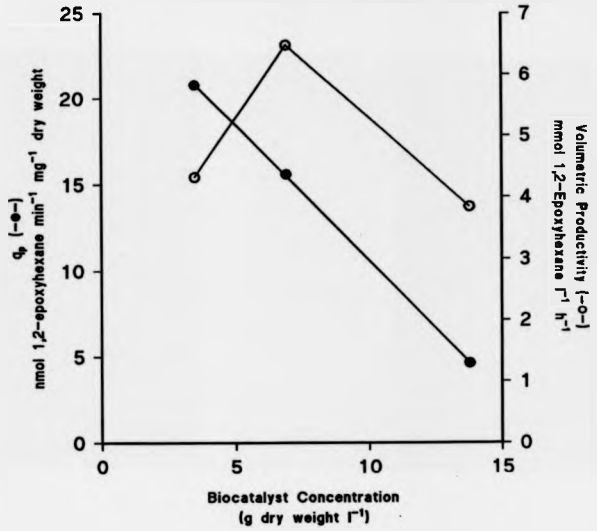


FIGURE 5.5

Effect of biocatalyst concentration on q_p and volumetric productivity: *Methylococcus capsulatus* (Bath)

Growth substrate: methane

Alkene concentration: 0.1% (v/v)

Co-substrate: 100mM potassium formate

Biotransformation temperature: 45°C

However, it would be dangerous to draw too many comparisons between the two systems because whole cell epoxidation of 1-hexene is complicated by a number of additional factors:

i. Whole cell epoxidation may involve more than one monooxygenase enzyme.

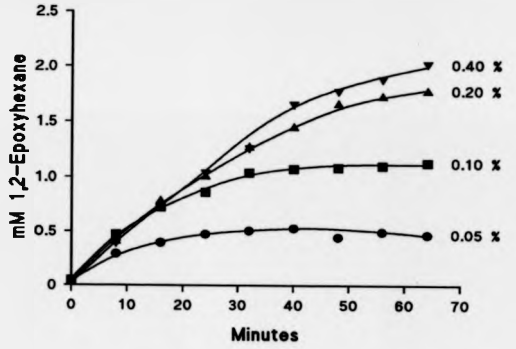
ii. Transport of the alkene/epoxide to/from the enzyme(s) performing the epoxidation may be the rate limiting step and might also show a saturable dependency on alkene concentration.

iii. Alkene and epoxide may be toxic to the cells. Foster (1962) reported the findings of Ishikura that 1-alkenes from 1-pentene to 1-nonene were very toxic to Gram-positive bacteria as well as yeasts and other fungi, whereas Gram-negative bacteria were far more resistant. The toxicity of epoxides is clearly illustrated by their use as sterilizing agents (Habets-Crützen & de Bont, 1985).

iv. The maximum solubility of 1-hexene is only 0.60mM in water at room temperature (McAuliffe, 1966).

v. If 1-hexene uptake only occurs from the aqueous phase, mass transfer limitations may result in 1-hexene epoxidation occurring faster than it can dissolve.

vi. If 1-hexene uptake only occurs at the interface between cells and liquid hydrocarbon, the interfacial area may be limiting.


mM 1,2-Epoxyhexane
2.5
2.0
1.5
1.0
0.5
0
0.40 %
0.20 %
0.10 %
0.05 %
0
10
20
30
40
50
60
70
Minutes

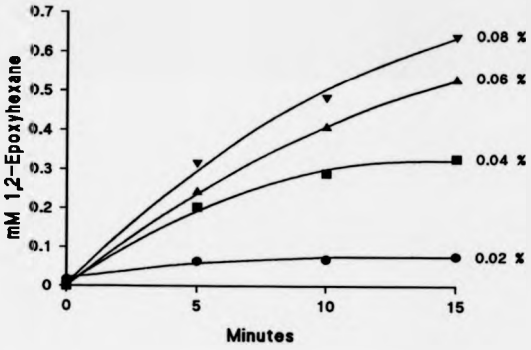

mM 1,2-Epoxyhexane
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.08 %
0.06 %
0.04 %
0.02 %
0
5
10
15
Minutes

FIGURE 5.6

Effect of 1-hexene concentration (% v/v)

on q_p and volumetric productivity

Growth substrate: n-heptane

Biocatalyst concentration: 10.1 g dry weight l^{-1}

Biotransformation temperature: 25°C

Studies on the uptake of n-alkanes from hexane to octadecane by *Candida tropicalis* suggested that they were transported solubilized from the aqueous phase, not by direct contact with the hydrocarbon (Yoshida *et al.*, 1971). Comparisons can be drawn to studies on the effect of tributyrin concentration its rate of hydrolysis by liver carboxylesterase (Dixon & Webb, 1979). Michaelis-Menten kinetics were followed up to the solubility limit of tributyrin; whilst further additions of tributyrin did not increase the rate of enzymatic hydrolysis. Liver carboxylesterase therefore will only hydrolyse esters dissolved in an aqueous phase.

A contrasting situation was observed for pancreatic lipase which would only hydrolyze methylbutyrate when the concentration had exceeded its maximum solubility (Sarda & Desnuelle, 1958). This correlated well with the enzyme acting at interfaces between the aqueous medium and the undissolved ester droplets.

It would at first appear that, in common with ester hydrolysis by pancreatic lipase, cells of *Rhodococcus* OU catalyze 1-hexene epoxidation only at their interface with 1-hexene droplets, since epoxidation occurred at 1-hexene concentrations above its maximum solubility in water. However it is quite probable that mass transfer limitations would be a problem within a shake flask, requiring that large quantities of alkene are added to increase the interfacial area necessary for good dissolution into the buffer. In order to minimize any mass transfer limitations in the shake flasks it would have

been better to have added 1-hexene as a fine emulsion in buffer; this may have resulted in the maximum epoxidation rate occurring at concentrations lower than were observed here.

Whatever the limitation in the system, the most useful finding of this investigation was that the final epoxide concentration could be increased to at least 2mM simply by increasing the alkene concentration. This suggests a potential for using these cells in a two-liquid-phase reactor system for enhancing epoxide formation, where the alkene could be dissolved at high concentrations in a metabolically inert organic phase. Alternatively, the alkene could be added as the second phase. Such an approach has been successful for alkene epoxidation by *Pseudomonas oleovorans* (Schwartz & McCoy, 1977; de Smet *et al.*, 1981) and *Mycobacterium* E3 (Brink & Tramper, 1985; 1987).

5.5 Effect of growth substrate

Drozd (1987) discussed the advantages and disadvantages of using hydrocarbon and carbohydrate feedstocks in biotechnological processes, concluding that hydrocarbons are suitable only when used as a starting material or as the non-polar phase in two-phase systems, and not as a growth substrate in the present economic climate.

Political volatility throughout the world, especially in oil or sugarbeet/sugarcane producing regions, will undoubtedly

affect the price of petroleum and carbohydrate feedstocks making one more attractive than the other, in an unpredictable manner. However, such considerations will be more prevalent in the manufacture of high volume, low cost biotechnological products where the cost of the feedstock represents a major proportion of the overall production costs. A biotransformation route to enantiomerically enriched epoxides would conform to being a low volume, high cost biotechnological product and therefore the feedstock cost would not be as significant compared to other processes (*e.g.* single cell protein production).

Hydrocarbon feedstocks are poorly soluble in aqueous growth media and are intrinsically flammable. If it could be proven that the ability of *Rhodococcus* OU to convert alkenes to epoxides was retained when the cells were grown on a carbohydrate, greater control over the fermentation could be achieved whilst eradicating the problem of substrate flammability. Another disadvantage of hydrocarbon feedstocks would be encountered if cell growth and the biotransformation were to be carried out simultaneously. In this instance, the growth substrate (alkane) and biotransformation substrate (alkene) would compete for the same enzyme(s), so reducing the q_p. This effect was demonstrated during 1-hexene epoxidation by *Rhodococcus* OU; heptane inhibited the q_p in a concentration dependent way (Figure 5.7).

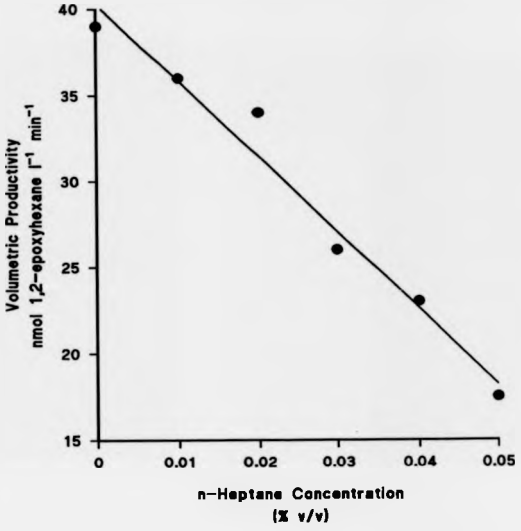


FIGURE 5.7

Inhibition of 1-hexene epoxidation by n-heptane

Growth substrate: n-heptane

Biocatalyst concentration: 10.1 g dry weight l^{-1}

Alkene concentration: 0.05% (v/v)

Biotransformation temperature: 25°C

Two alternative carbon substrates to alkanes were tested: glucose and hexan-1-ol. When presented with a mixture of carbon substrates, microorganisms will utilize them in a strict order (manifest as diauxie), carbohydrates generally being utilized preferentially to hydrocarbons. This is achieved by sequential expression of the enzymes required to metabolize each substrate, known as catabolite repression. Growing an organism on glucose is likely to prevent the expression of inducible monooxygenase enzymes and consequently their ability to convert alkenes to epoxides.

Expression of enzymes involved in alkane metabolism is often subject to coordinate regulation, such that growth on an alkane metabolite (*e.g.* hexan-1-ol) may induce expression of the monooxygenase enzyme(s) physiologically responsible for its production from the alkane. Hexan-1-ol is considerably cheaper than hexane, hexan-2-ol, hexanal, hexan-2-one or hexanoic acid), is more soluble than hexane and should not inhibit alkene epoxidation by competing for the monooxygenase active-site.

5:5.1 Glucose-grown cells

The highest rate of 1-hexene epoxidation observed using glucose-grown cells was 0.50 nmol 1,2-epoxyhexane formed min^{-1} mg^{-1} dry weight, at a biomass concentration of 4.9 g dry weight l^{-1}. This rate is low compared to hexane-grown cells which at the same biomass concentration showed a rate of 4.0 nmol 1,2-epoxyhexane formed min^{-1} mg^{-1} dry weight, indicating that the enzymes of alkane metabolism in *Rhodococcus* OU are inducible.

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It was hoped that a gratuitous inducer could be found for *Rhodococcus* OU, a precedent for which is provided by the induction of the -hydroxylase system of lactate- or glycerol-grown *Pseudomonas oleovorans* by 0.05% diethoxymethane, for the epoxidation of 4-(2-methoxyethyl)phenylallyl ether (Johnstone *et al.*, 1987). However, studies of octane- and hexane-stimulated oxygen uptake in a Clark-type oxygen electrode (Rank Brothers Ltd., High St., Bottisham, Cambridge CB5 9DA) by *Rhodococcus* OU grown on glucose in the presence of various concentrations (0.1mM, 1.0mM & 10mM) of formamide and 1,2-dimethoxyethane were inconclusive.

5:5.2 Hexan-1-ol-grown cells

At a biomass concentration of 4.3 g dry weight l^{-1}, the rate of 1-hexene epoxidation by hexan-1-ol-grown cells was 0.60 nmol 1,2-epoxyhexane formed min^{-1} mg^{-1} dry weight, compared to 3.9 nmol 1,2-epoxyhexane formed min^{-1} mg^{-1} dry weight for hexane-grown cells at the same biomass concentration. This level of expression is similar to glucose-grown cells. Growth on hexan-1-ol therefore does not result in the coordinate induction of any monooxygenase enzymes.

5:6 Effect of co-substrate addition

Based upon observations of methanotrophs, Dalton and Stirling (1982) defined co-metabolism as having an obligate requirement for the presence of "a growth substrate or another transformable compound", whilst fortuitous metabolism does not.

Regeneration of reduced cofactor (NAD(P)H or $FADH_2$), necessary for monooxygenase-catalyzed reactions, is achieved by oxidation of a co-substrate. If the co-substrate had to be supplied exogenously (*e.g.* methane metabolites such as methanol or formate), then the phenomenon was classified as co-metabolism. If the co-substrate was endogenous in origin (*e.g.* carbon storage polymers such as poly-β-hydroxybutyrate), then the phenomenon was classified as fortuitous metabolism. Clearly, the distinction between co-metabolism and fortuitous metabolism was entirely dependent upon the location of the co-substrate. In this thesis a broader interpretation of the term "co-metabolism" is adopted (see section 1:5.2), encompassing the term "fortuitous metabolism".

Rhodococcus OU is able to perform biotransformations in the absence of any exogenously added co-substrates, indicating that there are ample reserves of energy storage compounds within the cells. The endogenous oxidation rate of a sample of glucose-grown *Rhodococcus* OU cells (washed rigorously in buffer to remove any residual glucose adhering to the cell surface) was monitored using a Clark-type oxygen electrode to see how long such reserves would last over a 24 hour period at various temperatures (Figure 5.8).

The endogenous oxygen uptake rate remained stable even when the cells were incubated for 23 hours at 30°C, suggesting that *Rhodococcus* OU is able to store energy rich compounds in times of nutrient excess which it is then able to utilize in

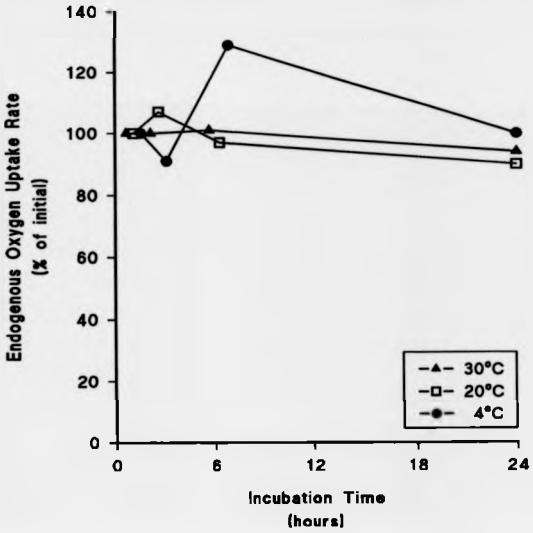


FIGURE 5.8

Effect of temperature on the endogenous oxygen uptake rate of glucose-grown cells of *Rhodococcus* OU

times of nutrient starvation. This could account for the grainy appearance of the cells under the microscope (x1000 magnification) which may be due to the presence of internal storage granules.

It is possible that q_p may be limited by the rate of supply of reduced cofactor. Oxidation of internal storage

compounds may be slower than the oxidation of exogenously supplied co-substrates, since the former would require depolymerization prior to oxidation. Various co-substrates were tested for their effects on 1-hexene epoxidation (Table 5.1; Figure 5.9).

TABLE 5.1

Co-substrates tested in 1-hexene biotransformations

	Final concentration	
Co-substrate	**%**	**mM**
Sodium acetate (anhydrous)	2.25	280
Sodium pyruvate (anhydrous)	2.25	210
Sodium succinate ($6H_2O$)	2.25	84
Ethanol (99.8%)	2.25	390

Instead of stimulating q_p, all of the co-substrates reduced the q_p relative to the control flask. This inhibition could be because:

a. Such compounds may be toxic to cells at the concentrations employed. Ethanol, for example, has been shown to inhibit the growth of cells (Aiba *et al.*, 1968) by virtue of it chaotrophic effects on cell membranes.

b. Addition and/or oxidation of these compounds could overcome the buffering capacity of the phosphate buffer used, adversely altering pH.

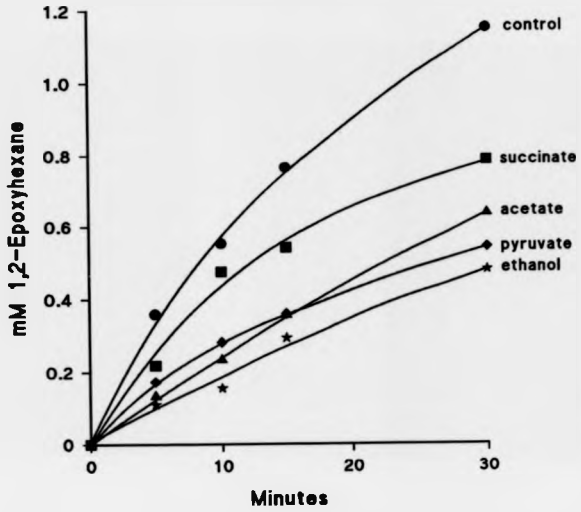


FIGURE 5.9

Effect of co-substrate addition on 1-hexene epoxidation by *Rhodococcus* OU

Growth substrate: n-hexane

Biocatalyst concentration: 31.4 g dry weight l^{-1}

Alkene concentration: 0.1% (v/v)

Biotransformation temperature: 30°C

c. Rapid oxidation of the co-substrates could remove oxygen from solution faster than it could be replaced by dissolution from the headspace. Inhibition of q_p would occur since molecular oxygen is required for alkene epoxidation.

It is possible that lower co-substrate concentrations and/or the use of higher buffer concentrations could alleviate the inhibition of, and possibly stimulate q_p.

5:7 Effect of 1-alkene chain length

Studies of 1-alkene epoxidation by "glucose-grown cells" of *Nocardia corallina* B-276, showed that the amount of epoxide accumulated after 24 hours incubation was dependent upon the alkene chain length (Furuhashi *et al.*, 1986). The quantity of epoxide formed from ethene to 1-hexene dropped with increasing chain length, increased up to 1-undecene after which it again fell as the 1-alkene was lengthened. A number of factors may have contributed to this pattern: substrate/product toxicity, substrate solubility and the binding avidity of different 1-alkenes to the active site of the participating monooxygenase enzyme(s). Another important consideration is that *N. corallina* B-276 is able to grow on ethene to 1-butene and 1-tridecene to 1-octadecene (Furuhashi *et al.*, 1981), suggesting that the observed rate of epoxide accumulation is dependent upon the epoxidation rate and alkene/epoxide degradation rate.

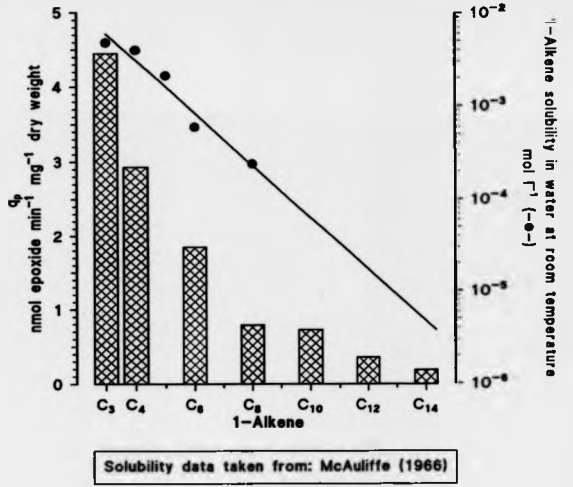


FIGURE 5.10

Effect of 1-alkene chain length on q_p

Growth substrate: n-heptane

Biocatalyst concentration: 27.5 g dry weight l^{-1}

Alkene concentration: C_3 & C_4: 25% (v/v) / C_6 - C_{14}: 0.1% (v/v)

Biotransformation temperature: 25°C

Similar studies with heptane-grown cells of *Rhodococcus* OU, performed over thirty minutes, showed that the rate of 1-alkene epoxidation was faster for shorter than longer 1-alkenes, which correlated well with 1-alkene solubility in water (Figure 5.10).

5:8 Scale-up of 1-hexene biotransformations

Performing biotransformations in a shake flask is a convenient and rapid way of varying reaction conditions systematically. However, shake flasks have the inherent problem of having poor mass transfer characteristics and do not lend themselves easily to continuous monitoring of parameters such as pH and dissolved oxygen tension.

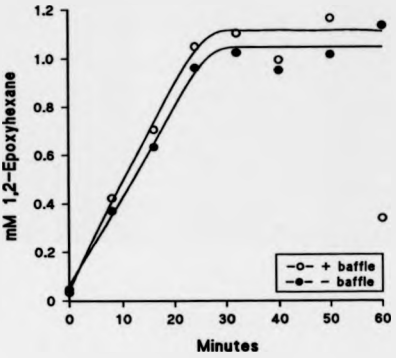


FIGURE 5.11

Biotransformation of 1-hexene by *Rhodococcus* OU in a baffled and non-baffled shake-flask

Growth substrate: n-heptane
Biocatalyst concentration: 10.1 g dry weight l^{-1}
Alkene concentration: 0.1% (v/v)
Biotransformation temperature: 25°C

An attempt was made to improve the mass transfer characteristics encountered in the shake flask by insertion of a stainless steel spring into the bottom of the flask to act as a baffle. The effect on 1-hexene epoxidation was to increase marginally the q_p and the final concentration of 1,2-epoxyhexane (Figure 5.11), which could have resulted either from improved aeration of the cell suspension, better mixing of the alkene, or a combination of both factors.

Initial attempts at a 1-hexene biotransformation in a fermenter proved to be difficult because the high aeration rates employed gave rise to excessive foam production, which could not be controlled by addition of antifoam agents. The high aeration rate also resulted in the epoxide being stripped from solution (Figure 5.12). Pulses of 0.5M sodium succinate and heptane vapour had no effect on the rate of epoxide loss.

A later attempt at 1-hexene biotransformation in the fermenter was carried out without aeration, relying on gas transfer across the head space-broth interface, which did not result in foam formation (Figure 5.13).

Dissolved oxygen tension (d.o.t.) and pH were monitored, the latter showing little variation. Over the first twenty minutes d.o.t. dropped quickly then stabilized for the next seventy minutes after which it again fell rapidly. As the d.o.t. approached zero percent, the broth was pulsed with air for approximately ten minutes until the d.o.t. attained saturation.

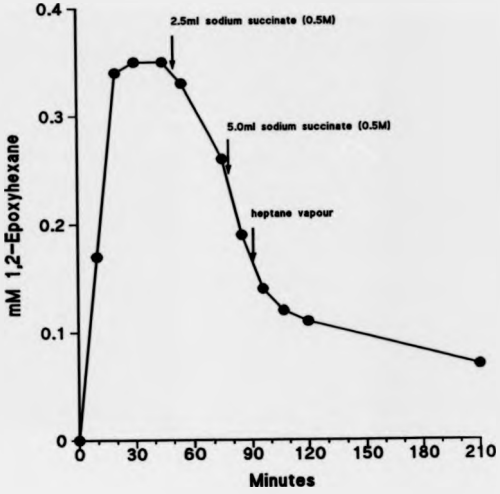


FIGURE 5.12

Large-scale biotransformation of 1-hexene by *Rhodococcus* OU (1)

Growth substrate: n-heptane

Cell suspension volume: 1.5 litres

Alkene concentration: 0.1% (v/v)

Biotransformation temperature: 25°C

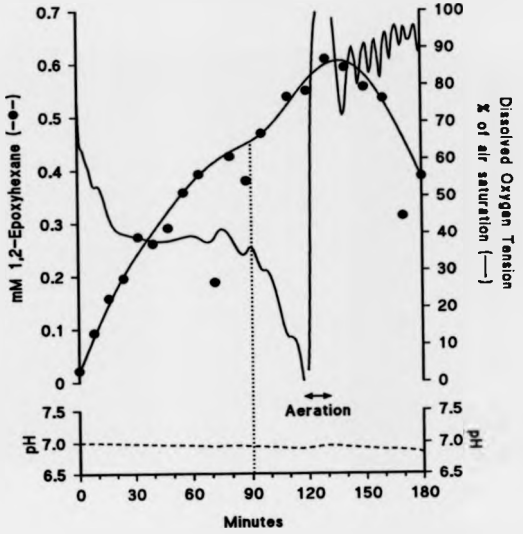


FIGURE 5.13

Large-scale biotransformation of 1-hexene by *Rhodococcus* OU (2)

Growth substrate: n-heptane

Biocatalyst concentration: 8.5 g dry weight l^{-1}

Cell suspension volume: 0.75 litres

Alkene concentration: 0.05% (v/v)

Biotransformation temperature: 25°C

Production of 1,2-epoxyhexane over the first ninety minutes showed a profile typical of shake flask experiments, but at the point when the d.o.t. began to fall sharply (after 90 minutes), a second phase of epoxidation occurred.

It may be possible that *Rhodococcus* OU deposits two types of energy storage compounds during growth. During the first phase of epoxidation, storage compound **A** was utilized to provide the co-substrate for driving the epoxidation. Within ninety minutes, storage compound **A** was depleted forcing the cell to switch to using storage compound **B**, leading to a second phase of epoxidation and a concomitant fall in d.o.t. This scenario is analogous to glycogen and trehalose metabolism in yeast (Gancedo & Serrano, 1989). Yeast deposit reserves of trehalose and glycogen as energy storage compounds, during growth when carbon substrates are in excess. The onset of starvation conditions stimulates glycogen and trehalose degradation, but the latter is degraded only when all of the glycogen has been utilized.

A portion of the cells used in this latter large-scale experiment were used in a shake flask biotransformation, employing the same concentration of 1-hexene as used in the fermenter. The profiles compared in Figure 5.14 clearly show that epoxidation in the shake flask supported a higher q_p. Since mixing of cell suspension and 1-hexene was almost certainly better in the fermenter, the rate of oxygen transfer in the fermenter was suspected to be the limiting factor.

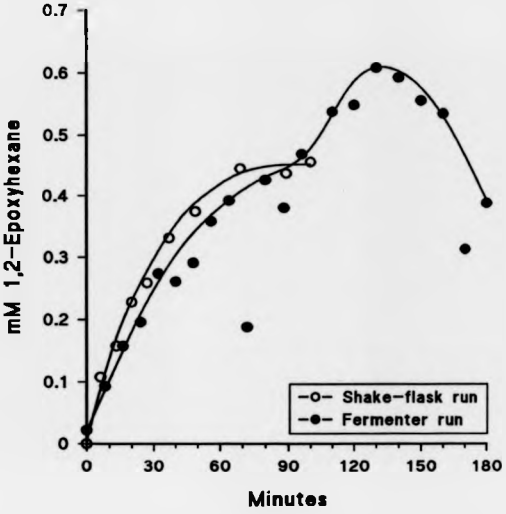


FIGURE 5.14

Comparison of large-scale (2) to shake-flask biotransformations of 1-hexene using the same cell preparation of *Rhodococcus* OU

This view was supported by monitoring oxygen absorption into 20mM K_2HPO_4 / KH_2PO_4 buffer pH 7.0 sparged with nitrogen gas (Figure 5.15). All of the conditions were identical to that employed during the biotransformation: same bioreactor, same volume of buffer (750 ml), temperature controlled to 25°C, stirred at 750 rpm and reliant on oxygen transfer from the head space. The surface area to volume ratio within the fermenter was much smaller than in the shake flask biotransformations which would account for the poor oxygen transfer rate observed in the fermenter.

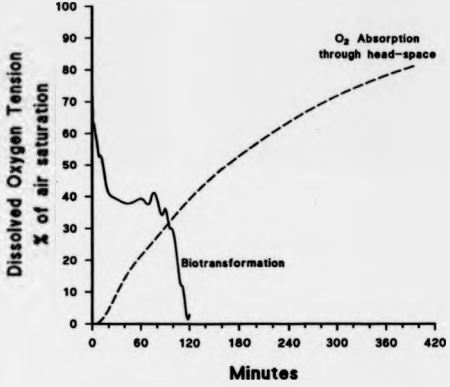


FIGURE 5.15

Oxygen absorption through the head-space within the fermenter compared to oxygen consumption during the large-scale (2) biotransformation of 1-hexene by *Rhodococcus* OU

CHAPTER 6

STEREOSELECTIVITY OF EPOXIDE FORMATION

The carbon-carbon double bond of an alkene molecule is planar. For prochiral alkenes, preferential addition of oxygen to one face of the double bond will result in a stereoselective epoxidation. This was recognised by May and Schwartz (1974): "the formation of (R)-(+)-7,8-epoxy-1-octene [by *Pseudomonas oleovorans*] must involve attack at the *si-si* face of the prochiral octadiene molecule".

Determination of enantiomeric excess still retains an element of empiricism. Apart from optical rotation, chiral analysis requires that the compound is complexed in a non-covalent manner with an enantiomerically enriched chiral auxiliary reagent. Chiral shift reagents are employed for ^{1}H-NMR analysis, whilst chiral HPLC and GLC rely upon stereospecific interactions with a homochiral stationary phase.

An alternative approach would be to introduce a second chiral centre to form diastereomers by reaction with an enantiomerically pure reactant. Unlike enantiomers, diastereomers possess different chemical properties in an achiral environment, allowing then to be separated and

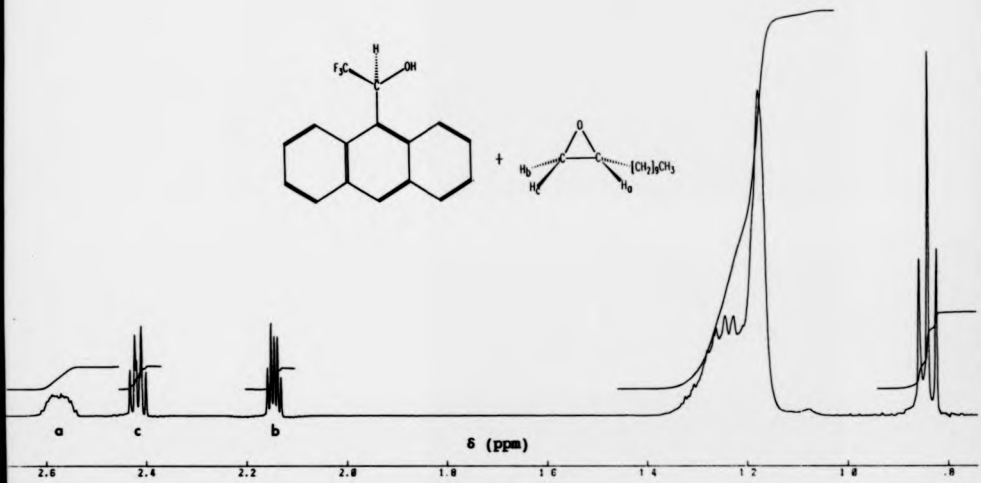


FIGURE 6.1

^{1}H-NMR spectrum of (±)-1,2-epoxydodecane in the presence of 3 mol equivalents of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol

quantified by standard procedures employed for mixtures of compounds. This method is problematical if the reaction is stereospecific, resulting in kinetic resolution and erroneous figures for enantiomeric composition if the reaction is terminated before completion.

6:1 Analysis of 1,2-epoxydodecane by ^{1}H-NMR

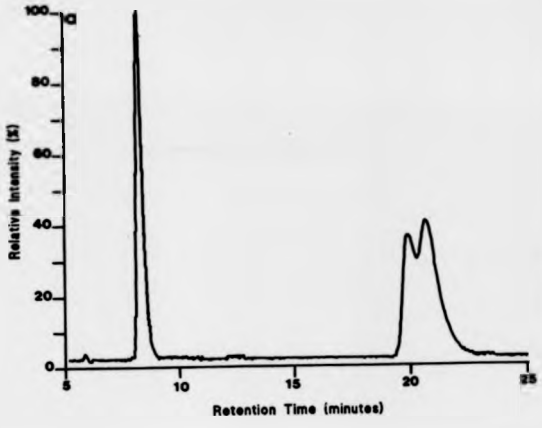
Application of ^{1}H-NMR spectroscopy in the presence of chiral shift reagents (especially europium-based chiral shift reagents) has been used to determine the enantiomeric composition of various microbially-produced 1,2-epoxyalkanes (*e.g.* Habets-Crützen *et al.*, 1985). An attempt was made to resolve a racemic modification of 1,2-epoxydecane in the presence of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol by 400MHz ^{1}H-NMR (Bruker WH400) (Figure 6.1).

The chemical shifts (δ) of protons attached directly to the epoxide ring were: H_a 2.58 ppm, H_b 2.15 ppm and H_c 2.42 ppm. These values are approximately 0.24 ppm upfield from those exhibited by the synthesized 1,2-epoxyalkanes (section 2:1) in the absence of a chiral shift reagent. Spin-spin splitting of the H_b and H_c protons by each other and by the H_a proton of 1,2-epoxyalkanes result in a doublet of doublets, although the doublets of H_c partially overlap, giving a triplet. In the presence of the chiral shift reagent the H_b and H_c peaks showed only slight resolution, insufficient for the purposes of enantiomeric composition analysis.

Better resolution could have been attained using different chiral shift reagents. However, this method was not further developed because routine analysis of epoxides by ^{1}H-NMR would require that microbially produced epoxides were prepared in relatively large quantities and in a purified form. In contrast, chromatographic quantification of enantiomers is far less exacting.

6.2 Analysis of 1,2-epoxy-3-phenoxypropane by chiral HPLC

Partial resolution (α = 1.1; R_s = 0.4) of the enantiomers of 1,2-epoxy-3-phenoxypropane was achieved on the Chiralcel OB column (mobile phase: 85% n-hexane / 15% propan-2-ol; flow rate: 0.5ml min^{-1}; detector wavelength: 254nm; Figure 6.2a). Assignment of each peak was not possible as standards of either resolved enantiomer were not commercially available. Although the resolution was poor, it was sufficient to demonstrate that the epoxide produced from allylphenylether by *Rhodococcus* OU is enriched for the enantiomer eluting first (Figure 6.2b).


a
100
80
60
40
20
0
Relative Intensity (%)
5
10
15
20
25
Retention Time (minutes)

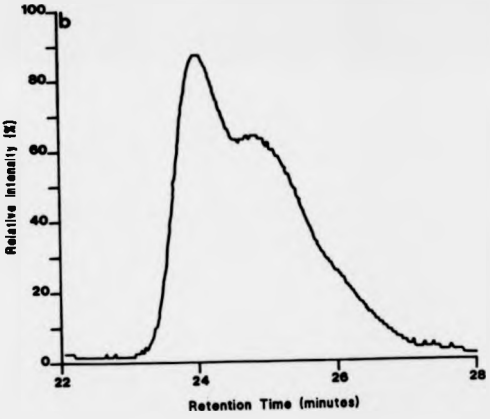

b
100
80
60
40
20
0
Relative Intensity (%)
22
24
26
28
Retention Time (minutes)

FIGURE 6.2

Analysis of allylphenylether and 1,2-epoxy-3-phenoxypropane by chiral HPLC

a. Chromatogram of allylphenylether + (±)-1,2-epoxy-3-phenoxypropane.
Mobile phase: 15% propan-2-ol / 85% n-hexane
Mobile phase flow rate: 0.5 ml min^{-1}

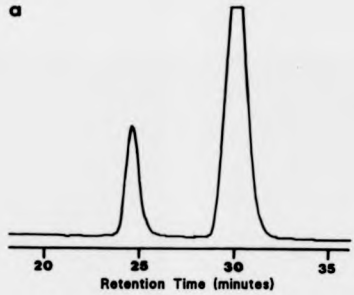
b. Chromatogram of 1,2-Epoxy-3-phenoxypropane produced by *Rhodococcus* OU from allylphenylether.
Mobile phase: 15% propan-2-ol / 85% n-hexane
Mobile phase flow rate: 0.25 ml min^{-1}

6:3 Analysis of styrene oxide by chiral HPLC

The analysis of styrene oxide was problematic both by capillary GLC analysis (section 4:4.1) and by chiral HPLC. Two preparations of styrene oxide were commercially available: R-(+)-styrene oxide (98% purity) and an unresolved preparation (99+% purity), both purchased from Aldrich. These were analysed on the Chiralcel OB column (mobile phase: 90% n-hexane / 10% propan-2-ol; flow rate: 0.5ml min^{-1}; detector wavelength: 245nm). The unresolved preparation gave two peaks (α = 1.4; R_s = 2.5) whilst the R-(+)-enantiomer gave a single peak (Figure 6.3).

From these observations, it was assumed that the enantiomers of styrene oxide had been resolved, the R-(+)-enantiomer eluting second. However, the peak areas of the R- and the S-enantiomer were significantly different: the S-enantiomer had a peak area approximately 17% that of the R-enantiomer (Figure 6.4). Why should this be so?

a. Was it because the unresolved preparation contained an impurity which either co-eluted with the R-enantiomer, or the enantiomers of styrene oxide were not resolved at all and the "S"-peak is the impurity? Analysis of the unresolved preparation by ^{1}H-NMR spectroscopy at the University of Warwick Chemistry Department and infra-red spectroscopy at Aldrich showed it to be of high purity, discounting this argument.


a
20
25
30
35
Retention Time (minutes)

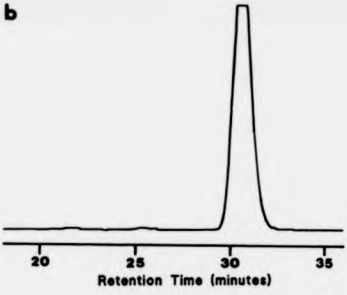

b
20
25
30
35
Retention Time (minutes)

FIGURE 6.3

Analysis of (±)-styrene oxide and R-(+)-styrene oxide by chiral HPLC

a. Chromatogram of (±)-styrene oxide.
Mobile phase: 10% propan-2-ol / 90% n-hexane
Mobile phase flow rate: 0.5 ml min^{-1}

b. Chromatogram of R-(+)-styrene oxide.
Mobile phase: 10% propan-2-ol / 90% n-hexane
Mobile phase flow rate: 0.5 ml min^{-1}

b. Is it because the S-enantiomer has a lower molar extinction coefficient at 254nm than the R-enantiomer? Since enantiomers have the same physical properties in an achiral environment (except for optical rotation), the molar extinction coefficients should be identical.

c. Was it because the unresolved preparation was not a racemic modification as had been assumed, but was enriched for the R-enantiomer? Polarimetric analysis in an AA-1000 polarimeter (Optical Activity Ltd., Industrial Estate, Bury Rd., Ramsey, Huntingdon, Cambridgeshire. PE17 1NA) of the two preparations showed the specific rotation of the R-(+)-enantiomer was $[\alpha]_D^{20}$ = +33° (as quoted in the Aldrich catalogue), whilst the unresolved preparation was optically inactive. It was concluded therefore, that the unresolved preparation was indeed a racemic modification.

d. Was it because the S-enantiomer was binding stereospecifically to the chiral stationary phase? Separation of enantiomers on a chiral stationary phase relies upon a stereospecific interaction, retarding the elution of one enantiomer more than the other. One would expect therefore, that the R-enantiomer would be more likely to bind to the stationary phase since it has the longer retention time.

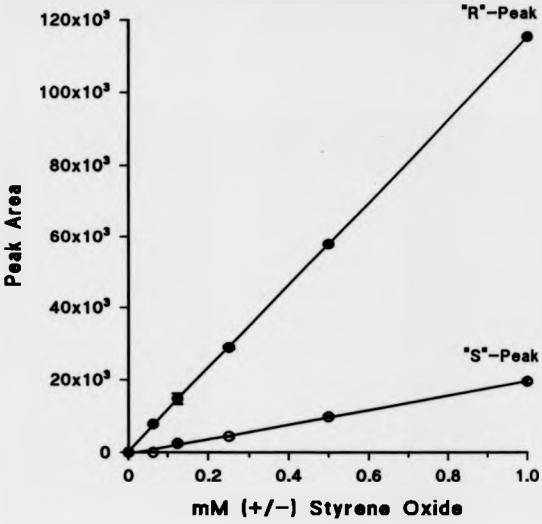


FIGURE 6.4

Peak area analysis of (±)-styrene oxide by chiral HPLC

In the absence of any other explanation to answer this anomaly, it was decided to investigate the stereoselectivity of styrene epoxidation by *Rhodococcus* OU and *Methylococcus capsulatus* (Bath). Biotransformations with the latter organism were performed at 45°C with 0.1M potassium formate as co-substrate. The reaction broths from both biotranformations were extracted into HPLC-grade n-hexane and the extract injected onto the Chiralcel OB column (Figure 6.5).

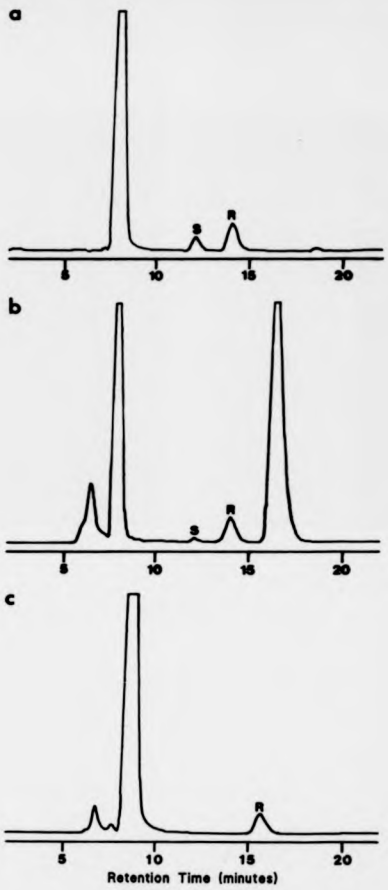

a
S
R
5
10
15
20
b
S
R
5
10
15
20
c
R
5
10
15
20
Retention Time (minutes)

FIGURE 6.5

Analysis of styrene biotransformation products by chiral HPLC

a. Chromatogram of styrene + (±)-styrene oxide.
Mobile phase: 10% propan-2-ol / 90% n-hexane
Mobile phase flow rate: 0.5 ml min^{-1}

b. Chromatogram of a styrene biotransformation by *Methylococcus capsulatus* (Bath).
Mobile phase: 10% propan-2-ol / 90% n-hexane
Mobile phase flow rate: 0.5 ml min^{-1}

c. Chromatogram of a styrene biotransformation by *Rhodococcus* OU.
Mobile phase: 10% propan-2-ol / 90% n-hexane
Mobile phase flow rate: 0.5 ml min^{-1}

The flow rate of mobile phase using the Beckman System Gold chromatograph (used to produce the chromatograms seen in Figure 6.5) was prone to slight variation, affecting retention times. Chromatograms **a** and **b** arise from consecutive injections, whereas chromatogram **c** was produced much later. The peak at 15.3 minutes in chromatogram **c** does correspond to the "R"-peak of styrene oxide.

It appeared that *Rhodococcus* OU was producing the "R"-enantiomer with absolute enantioselectively, whereas *M. capsulatus* (Bath) was producing some of the "S"-enantiomer as well. The large peak at a retention time of 16.3 minutes in chromatogram **b** is probably *p*-hydroxystyrene known to be produced in significant quantities from styrene by *M. capsulatus* (Bath) (Dalton *et al.*, 1981).

Chiral analysis of styrene oxide became more complicated when monitored with a diode array detector instead of a single wavelength detector (Figure 6.6). Absorption spectra (190nm - 350nm) generated from the two styrene oxide peaks were not identical (Figure 6.6b), indicating that the "R"- and "S"-peaks are different compounds, not enantiomers. It had already been shown that styrene oxide could rearrange to phenylacetaldehyde on the capillary GLC column (section 4:4.1). One possible explanation for the observations was that S-(-)-styrene oxide was rearranging stereospecifically to phenylacetaldehyde on the chiral stationary phase.

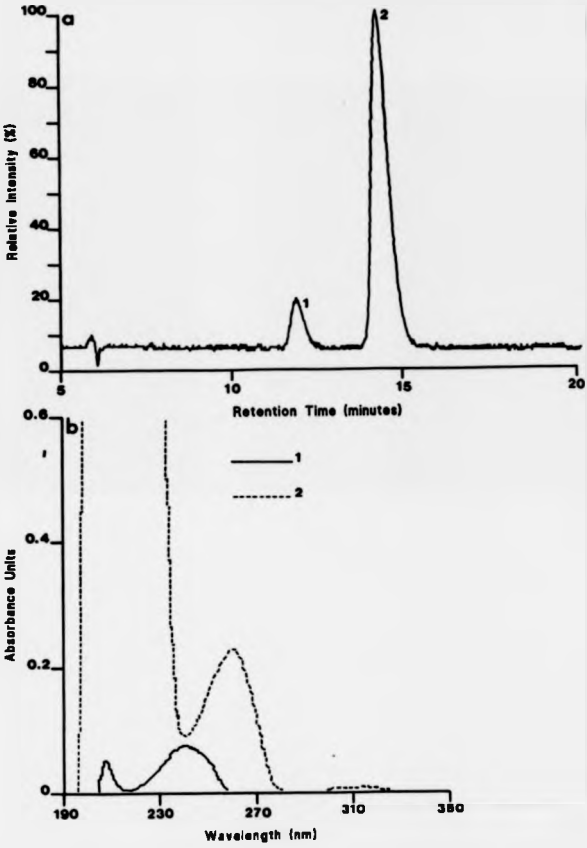

a
100
80
60
40
20
0
Relative Intensity (%)
1
2
5
10
15
20
Retention Time (minutes)
b
0.6
0.4
0.2
0
Absorbance Units
1
2
190
230
270
310
350
Wavelength (nm)

FIGURE 6.6

Diode array analysis of (±)-styrene oxide

a. Chromatogram of (±)-styrene oxide by chiral HPLC.
Mobile phase: 10% propan-2-ol / 90% n-hexane
Mobile phase flow rate: 0.5 ml min^{-1}

b. Absorption spectra of 'S'- and 'R'-styrene oxide peaks.

This hypothesis was tested by injecting a sample of phenylacetaldehyde onto the same chiral HPLC column (Figure 6.7). In addition to a peak which co-eluted with the "S"-peak of styrene oxide, two other peaks were also produced! Being achiral, phenylacetalehyde should have only produced a single peak. The possibility of there being impurities present in the stock bottle of phenylacetaldehyde was dismissed on the basis of capillary GLC analysis and ^{1}H-NMR spectroscopy. Spectral scans of the "S"-peak of styrene oxide and the co-eluting phenylacetaldehyde peak showed identical absorption maxima (205nm & 243nm), but the relative intensity of the two spectral peaks were different (*cf.* Figure 6.6b and Figure 6.7b), indicating that they are not identical compounds.

The true identity of the "S"-peak of styrene oxide and the three peaks of phenylacetaldehyde is still elusive. If, however, the "S"-peak of styrene oxide is present when a racemic modification of styrene oxide is injected and not the pure R-(+)-enantiomer, it could still be used as a measure of enantiomeric composition.

Time constraints prevented collection of the two peaks of (±)-styrene oxide and the three peaks of phenylacetaldehyde for ^{1}H-NMR analysis and their subsequent identification.

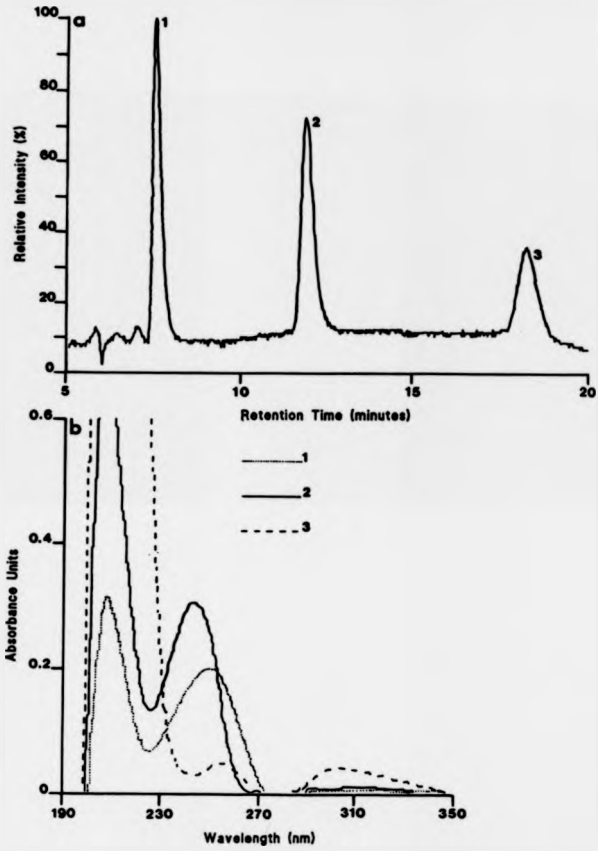

a
1
2
3
100
80
60
40
20
0
Relative Intensity (%)
5
10
15
20
Retention Time (minutes)
b
1
2
3
0.6
0.4
0.2
0
Absorbance Units
190
230
270
310
350
Wavelength (nm)

FIGURE 6.7

Diode array analysis of phenylacetaldehyde

a. Chromatogram of phenylacetaldehyde by chiral HPLC.
Mobile phase: 10% propan-2-ol / 90% n-hexane
Mobile phase flow rate: 0.5 ml min^{-1}

b. Absorption spectra of the three phenylacetaldehyde peaks.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The success of this project has to be determined from two aspects: the isolation of a suitable microorganism and its characterization as an epoxidation catalyst. Substrate toxicity hindered the isolation studies, to the extent that temporal constraints necessitated the use of less recalcitrant isolation substrates. The characterization studies were carried out using whole cells of *Rhodococcus* OU. Such an approach was justified because its application as an epoxidation biocatalyst would probably rely upon *in vivo* cofactor regeneration.

7.1 Isolation studies

The original objectives of this thesis were to isolate a microorganism using hydrocarbon analogues of styrene and test its ability to convert styrene to styrene oxide. It was hoped that the isolated microorganisms would catalyze the epoxidation of styrene better than microorganisms isolated on hydrocarbon substrates of a more disparate size and shape to styrene. This idea was based upon the supposition that the

environments sampled for the isolation studies contain a wide spectrum of catalytic activities. One would expect that under the defined set of conditions used during the isolations, that only those microorganisms best suited or able to adapt to growth on these substrates would be isolated. Extending this line of conjecture further, the first enzyme responsible for the degradation of the styrene analogue, presumed to be a monooxygenase, would have an active site closely fitting the styrene molecule conferring the ability to catalyze styrene epoxidation at a fast rate and with good stereoselectivity.

There are many criteria that have to be considered during an isolation programme which will affect the range of microorganisms isolated. Attempting to emulate in the laboratory the wide range of environmental variables found within soil and water ecosystems may have been profitable but also very time consuming. Typical of many isolation programmes, only those microorganisms able to adapt to growth under standard laboratory conditions (culture vessels, growth media, pH and temperature values) were ever likely to be isolated.

Few bacteria were isolated on the styrene analogues used in this study. Most of the isolates appeared to be scavenging carbon substrates rather than using those intended as the carbon source. It is likely that these exotic compounds presented a toxicity problem, even at the low concentrations used.

The inclusion of water-immiscible organic solvents into whole-cell alkene epoxidation reactions has proven successful in extending catalytic activity (Schwartz & McCoy, 1977; de Smet *et al.*, 1981; Brink & Tramper, 1985). This effect was attributed to the partial sequestration of the epoxides in the organic phase, thus lowering their concentration in the aqueous phase and alleviating their cytotoxic effects. Use of water-immiscible organic solvents in isolation flasks might also help alleviate the cytotoxic effects of isolation substrates, provided that the organic solvent was biologically inert.

A more sophisticated approach would have been to use a gradostat system as described by Wimpenny (1990). Establishing a gradient of the isolation substrate along a gradostat system inoculated with soil suspensions or mineral-ammended water samples would allow microorganisms to grow at the point along the gradient where the substrate concentration is just low enough not to be cytotoxic. Such a system would also enrich for microbes with a tolerance for higher substrate concentrations, resulting in the growth zone migrating further up the substrate gradient.

Of the styrene analogues employed, only α-methylstyrene yielded an isolate able to grow to any extent. Unfortunately, this isolate (αMeS-1) appeared to degrade the aromatic ring, reducing the likelihood of it being suitable for styrene oxide production. The ability of *Rhodococcus* OU to grow well on n-alkanes was favourable, since the ability to produce large

quantities of biocatalyst would be an important criterion in the selection of a microorganism for large scale epoxide production. For this reason effort was concentrated on the characterization of *Rhodococcus* OU rather than αMeS-1.

7:2 Catalytic versatility and regioselectivity of *Rhodococcus* OU

For a chemist, it would be desirable to develop a stereoselective catalyst that would perform the selected transformation on a broad range of substrates. The requirement for good stereoselectivity is normally obtained at the expense of catalytic versatility, especially for biological catalysts. For an enzyme-catalyzed epoxidation, one would expect that stereoselectivity could only be achieved if the alkene can only be oriented within the active site in a specific way so that oxygen is added to the same face of the double bond every time. Clearly this would require a close fit between alkene and active site, a situation improbable for more than a restricted range of alkenes.

The whole cell studies reported here show the diversity of reactions catalyzed by *Rhodococcus* OU, similar in range to those reported of *Nocardia corallina* B-276 (section 1:6.3) and the methanotrophs (section 1:6.4). Products isolated from a variety of alkene biotransformations reflect a tendency towards terminal oxidation, common to most microbial epoxidation catalysts, exemplified by the products of allyl

benzene and *trans*-β-methylstyrene biotransformations. These compounds are structural isomers, presenting *Rhodococcus* OU with a terminal and sub-terminal double bond respectively, yet only the terminal carbon in both cases is subjected to oxidation (sections 4:4.3 & 4:4.4).

The products from 2-butene biotransformations suggest that steric hindrance may account for the difficulty in sub-terminal oxidation (section 4:2), since the *trans*-isomer appeared unreactive, whereas the *cis*-isomer was subject to both terminal hydroxylation and sub-terminal epoxidation. This is supported by the ease with which cyclopentene and cyclohexene are oxidized, as these compounds have a *cis*-configuration about the double bond (section 4:3).

The differences in products derived from styrene (section 4:4.1) and ethylbenzene (section 4:5.2) was unexpected, since the former was subjected to side-chain epoxidation whereas the latter was ring-hydroxylated predominantly. Ethylbenzene was the only substrate that was proven conclusively to give rise to ring hydroxylation products, although such a biotransformation cannot be ruled out for either allylphenylether (section 4:4.2) or *trans*-β-methylstyrene (section 4:4.3).

The unknown products from allylphenylether and *trans*-β-methylstyrene biotransformations and the position of the ring-hydroxyl of the ethyl phenol produced during ethylbenzene biotransformations could be identified from their ^{1}H-NMR

spectra. However, this would require that the biotransformations be scaled-up and the products purified by preparative liquid chromatography.

It must be recalled that all of the biotransformations reported here were catalyzed by whole cells, not purified enzymes. In those instances where a single product was detected, it is probable that they were generated by the action of a monooxygenase. However, multiple products generated from *cis*-2-butene, cyclopentene, cyclohexene and *trans*-β-methylstyrene implicate the involvement of one or more alcohol dehydrogenase enzymes.

7:3 Optimizing alkene epoxidation by *Rhodococcus* 00

A number of parameters were tested for their effect on the specific rate of 1-alkene epoxidation (q_p), the results of which can be summarized as follows:

1. Cells were more active when grown using hexane or heptane as the carbon substrate compared to cells grown on hexan-1-ol or glucose, indicative of an alkane-inducible system.

2. Cells retained their alkene epoxidation activity after being drop-frozen in liquid nitrogen and stored at -70°C, for up to two months. This was particularly useful for experiments performed on more than one day.

3. The biotransformation temperature was optimal at 25°C.

4. Increasing biocatalyst concentrations resulted in a decrease in q_p, but a concomitant increase in volumetric productivity.

5. Increasing the concentration of 1-hexene up to 0.40% (v/v) resulted in a gradual increase in the final concentration of 1,2-epoxyhexane. The effect of 1-hexene concentration on q_p showed saturable kinetics, reaching saturation between 0.05% to 0.10% (v/v) 1-hexene. This study points towards the application of two-liquid-phase systems for enhancing productivity.

6. The q_p of 1-alkene epoxidation increased with decreasing chain-length, which correlated with 1-alkene solubility.

7.4 Stereoselectivity of epoxidation by *Rhodococcus* OU

Whilst some important factors for optimizing productivity in an epoxidation process have already been elucidated, the more important consideration is that of stereoselectivity. The latter is inherent to the monooxygenase and cannot easily be enhanced, whereas productivity is more amenable to improvement.

Chromatographic separation of enantiomers on chiral stationary phases was the preferred method of determining

enantiomeric composition, as this did not require work-up of the reaction mixture or derivatization to diasteromers. A detailed study of the stereoselectivity of epoxidation was hindered by problems encountered during chiral analyses. Despite these problems, preliminary results suggest that the epoxidation of styrene and allylphenylether by *Rhodococcus* OU were performed with a high level of stereoselectivity. However, to firmly establish the utility of *Rhodococcus* OU as a stereoselective biocatalyst, the enantiomeric composition of a range of structurally diverse epoxides produced by this microorganism needs to be ascertained.

7.5 Future prospects for *Rhodococcus* OU

Three aspects of characterizing *Rhodococcus* OU have been initiated: its catalytic versatility, its stereoselectivity and process optimization. Whilst the catalytic versatility has been extensively investigated, more work needs to be carried out on the other two areas.

It was difficult to justify time spent elucidating which enzymes were responsible for the observed biotransformations, without obtaining an overview of what *Rhodococcus* OU, as a whole-cell biocatalyst, was capable of performing. Now that this overview has been accomplished, a number of more fundamental questions can be addressed.

That a monooxygenase was responsible for initiating the biotransformations is certain from the studies on cyclohexene oxidation using $^{18}O_2$ (section 4:3), but it would be presumptuous to say that only one monooxygenase enzyme was involved. The distinct pattern of growth on different n-alkanes (section 3:5.2) might be indicative of there being at least two different monooxygenase enzymes capable of being expressed in this bacterium. If this were to be proven, then would this be reflected in the catalytic versatility, or more importantly the stereoselectivity? To answer these questions would require that the monooxygenase enzyme(s) be identified and purified.

Such an approach would also help to answer more specific questions raised during this study, such as: Does the monooxygenase specifically transform 1-alkenes to 1,2-epoxyalkanes or to a mixture of the epoxide and unsaturated alcohol? Does the lack of reactivity of certain compounds (*e.g.* 2-/3-hexene, 1,3-cyclohexadiene, benzene and stilbene) mean that they are poor substrates for the monooxygenase enzyme(s), or that they cannot gain access to the monooxygenase enzyme(s) in whole cells, or do the products get degraded by the bacterium? Purified *Rhodococcus* OU monooxygenase would also allow the elucidation of which alkene structural motifs are important for binding and catalysis.

Once the monooxygenase enzyme(s) have been identified, their expression could be monitored by polyacrylamide gel electrophoresis of cell-free extracts, so that a series of

potential gratuitous inducers could be tested and the effect on expression of various nutrient limitations during growth in chemostat culture, especially oxygen-limitation which has been shown to increase q_p in *Mycobacterium* E3 (Habets-Crützen *et al.*, 1987).

Improving expression of the monooxygenase enzyme(s) may not increase q_p if the supply of reduced cofactors is rate limiting. Initial investigations on the use of exogenous co-substrates suggest that they may offer no advantage over the internal supplies of energy-rich compounds. Ascertaining which energy storage compounds are present in these cells would facilitate studies on the growth conditions required for their deposition, so as to ensure an ample supply of endogenous co-substrate during prolonged biotransformations.

One might question the value of developing a new biological epoxidation catalyst, such as *Rhodococcus* OU, since there are a number of well characterized biocatalysts already available. If one required a process for the production of epoxides from alkenes with little or no consideration for enantiomeric composition, methanotrophs would be the obvious choice in most instances. However, if stereoselectivity is of prime importance then the choice would probably be between *Pseudomonas oleovorans* TF4-1L, *Corynebacterium equi* IFO 3730 or *Nocardia corallina* B-276. *Rhodococcus* OU could easily be placed among these last three, having a broad substrate specificity and the promise of good stereoselectivity.

APPENDIX 1

SOURCES AND PURITIES OF CHEMICALS

TABLE 8.1

Sources and purities of chemicals

Chemical	% Composition	Supplier
Acetophenone	99	Aldrich[1]
Allyl benzene	98	Aldrich
Allyl phenyl ether	96	Aldrich
Benzene	99+	Aldrich
Butane	99.5	ECM[2]
2,3-Butanediol	99	Aldrich
Butanone	99.6	BDH[3]
cis-2-Butene	95	Aldrich
trans-2-Butene	99+	Aldrich
Butyl cyclohexane	99+	Aldrich
3-Chloroperoxybenzoic acid	50-55	Aldrich
trans-Cinnamaldehyde	99+	Aldrich
Cinnamyl alcohol	98	Aldrich
Crotonaldehyde	85	Aldrich
Crotyl alcohol	97	Aldrich
1,3-Cyclohexadiene	98	Aldrich
Cyclohexane	99.5	BDH
cis,trans-1,2-Cyclohexanediol		Koch-Light[4]
Cyclohexanol	98	BDH
Cyclohexanone	99	BDH
Cyclohexene	99	BDH
Cyclohexene oxide	98	Aldrich
2-Cyclohexen-1-ol	96	Aldrich
2-Cyclohexen-1-one	97	Aldrich

TABLE 8.1 - *continued*

Cyclopentanol	99	Aldrich
Cyclopentanone	99+	Aldrich
Cyclopentene	99	Aldrich
Cyclopentene oxide	98	Aldrich
2-Cyclopentenone	98	Aldrich
n-Decane	99	BDH
1-Decene	94	Aldrich
1,2-Dimethoxyethane	99	Aldrich
n-Dodecane	99	BDH
1-Dodecene	95	Aldrich
1,2-Epoxybutane	99	Aldrich
cis-2,3-Epoxybutane	97	Aldrich
trans-2,3-Epoxybutane	96	Aldrich
1,2-Epoxydecane	98	Aldrich
1,2-Epoxydodecane	98	Aldrich
1,2-Epoxyhexane	99	Aldrich
1,2-Epoxyoctane	99	Aldrich
(±)-1,2-Epoxy-3-phenoxypropane	99	Aldrich
(±)-(2,3-Epoxypropyl)-benzene	98	Aldrich
1,2-Epoxytetradecane	85	Aldrich
Ethane	99	Aldrich
Ethylbenzene	99	BDH
2-Ethyl phenol	99	Aldrich
3-Ethyl phenol	99	Aldrich
4-Ethyl phenol	98	BDH
Formamide	99	Aldrich
Heptane	99.6	Aldrich
1-Heptene	99+	Aldrich
n-Hexadecane	99	BDH
Hexanal	99	Aldrich
n-Hexane	99+	Aldrich
DL-1,2-Hexanediol	98+	Aldrich
n-Hexanoic acid	99.5	BDH
Hexan-1-ol	98	BDH
2-Hexanol	99	Aldrich
2-Hexanone	99+	Aldrich

TABLE 8.1 - *continued*

1-Hexene	99+	Aldrich
trans-2-Hexene	99+	Aldrich
trans-3-Hexene	99+	Aldrich
5-Hexen-1-ol	99	Aldrich
Methane	99.7	ECM
α-Methylstyrene		BDH
trans-β-Methylstyrene	99	Aldrich
Naphthalene	99+	Aldrich
n-Nonane	99	Aldrich
1-Nonene	99	Sigma[5]
$^{18}O_2$	97.6	MSD[6]
Octane	99+	Aldrich
1,7-Octadiene	99	Aldrich
Oct-1-ene	99	BDH
n-Pentadecane	98	Aldrich
Pentane	99+	Aldrich
1-Pentene	99+	Aldrich
Phenylacetaldehyde		Aldrich
Phenylacetic acid	98.5	Aldrich
1-Phenyl-1,2-ethanediol	97	Aldrich
DL-1-Phenylethanol	99	BDH
2-Phenylethanol	98	BDH
(1S,2S)-(-)-1-Phenylpropylene oxide	98	Aldrich
(1R,2R)-(+)-1-Phenylpropylene oxide	98	Aldrich
Propane		ECM
Propylene oxide	99.5	BDH
cis-Stilbene	97	Aldrich
trans-Stilbene	96	Aldrich
cis-Stilbene oxide	97	Aldrich
trans-Stilbene oxide	99	Aldrich
Styrene	99+	Aldrich
Styrene oxide	97	Aldrich
(R)-Styrene oxide	98	Aldrich
Tetradecane	99+	Aldrich
1-Tetradecene	≥95	Koch-Light
Toluene	99+	Aldrich

TABLE 8.1 - *continued*

2,4,6-Triallyloxy-1,3,5-triazine	97	Aldrich
Tridecane		Koch-Light
1,3,5-Triethylbenzene	98	Aldrich
(S)-(+)-Trifluoro-1-(9-anthryl) ethanol	98+	Aldrich
1,2,4-Trivinylcyclohexane	98	Aldrich
n-Undecane	99	Aldrich
1-Undecene	99	Aldrich

[1] Aldrich Chemical Company Ltd.,
The Old Brickyard,
New Road,
Gillingham,
Dorset. SP8 4JL

[2] Electrochem Ltd.,
Newfield Ind. Est.,
Tunstall,
Stoke-on-Trent,
Staffordshire. ST6 5PD

[3] BDH Laboratory Supplies,
Merck Ltd.,
Broom Road,
Poole,
Dorset. BH12 4NN

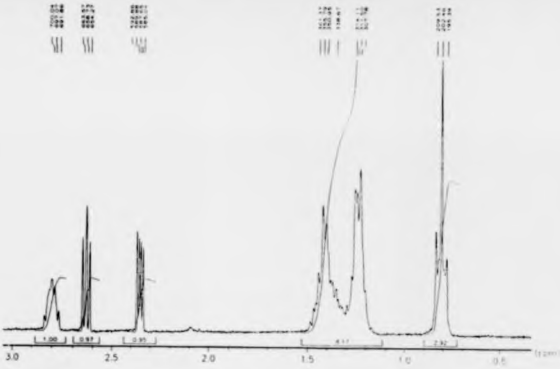
[4] Koch-Light Ltd.,
Rookwood Way,
Haverhill,
Suffolk. CB9 8PB

[5] Sigma Chemical Co. Ltd.,
Fancy Road,
Poole,
Dorset. BH17 7TG

[6] MSD Isotopes,
Cambrian Gases,
K&K-Greef Ltd.,
Suffolk House,
George Street,
Croydon. CR9 3QL

APPENDIX 2

^{1}H-NMR AND MASS SPECTRA OF CHEMICALLY-SYNTHESIZED 1,2-EPOXYALKANES



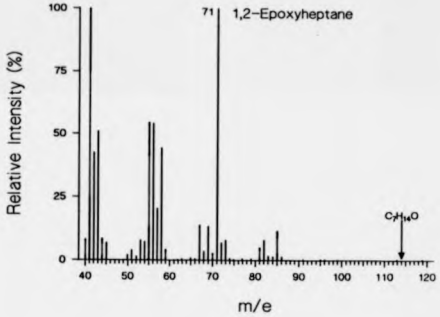
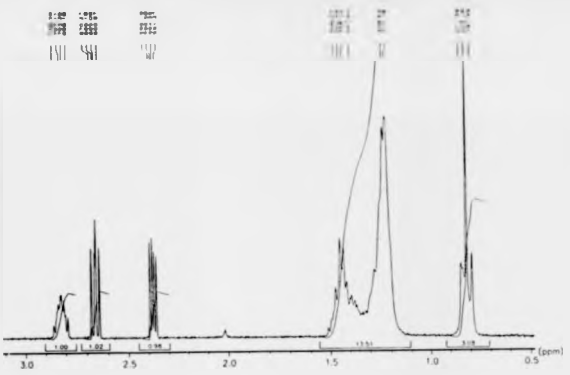


FIGURE 9.1

^{1}H-NMR spectrum (a) and mass spectrum (b) of synthesized 1,2-epoxyheptane



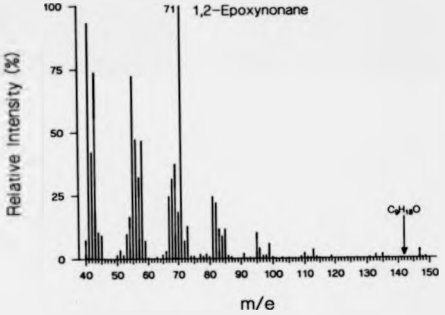
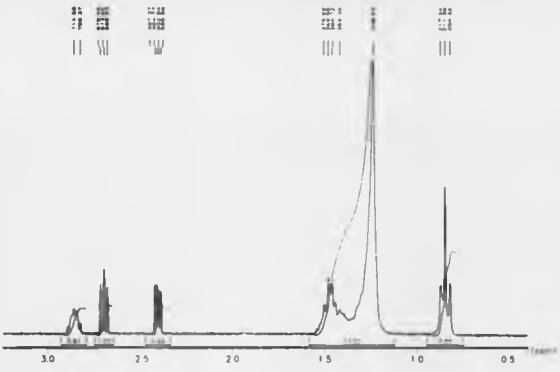


FIGURE 9.2

^{1}H-NMR spectrum (a) and mass spectrum (b) of synthesized 1,2-epoxynonane



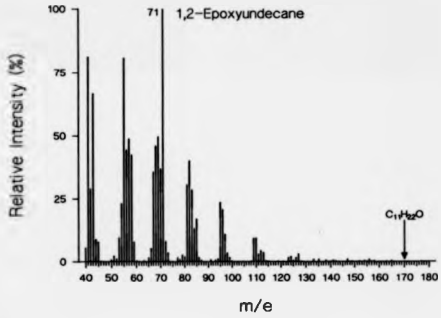


FIGURE 9.3

^{1}H-NMR spectrum (a) and mass spectrum (b) of synthesized 1,2-epoxyundecane

APPENDIX 3

FATTY ACID ANALYSIS OF *Rhodococcus* OU

MIS System Software 3.2 (Rev: 3.2) (Su s/n: 3008A6001 0) (Hw s/n: 2525054661) DATA:F91523699 24-MAY-91 12:53:46

ID: 6893 CEL-OU Date of run: 23-MAY-91 20:10:31
Bottle: 4 SAMPLE [ACROBE]

Rt	Area	Ar/Ht	Respon	ECL	Name	%	Comment 1	Comment 2
2.196	37510000	0.075	. . .	6.957	SOLVENT PEAK	(max rt		
2.358	858	0.024	. . .	7.360	. . . . .	(max rt		
7.933	1003	0.056	0.994	14.000	14:0	1.86	ECL deviates 0.000	Reference -0.007
9.475	1747	0.074	0.963	15.000	15:0	1.67	ECL deviates -0.000	Reference -0.000
10.050	13827	0.085	0.941	15.650	Sum In Feature 4 . .	12.93	ECL deviates 0.002	16:1 10ANS 9/15\20H
11.133	30948	0.061	0.930	16.000	16:0	28.05	ECL deviates -0.000	Reference -0.000
12.504	2952	0.070	0.921	16.795	17:1 0	2.70	ECL deviates 0.003	
12.859	2308	0.063	0.910	17.001	17:0	2.10	ECL deviates 0.001	Reference -0.007
14.213	46242	0.066	0.904	17.773	10:1 CIS 9	41.57	ECL deviates 0.004	
14.611	6329	0.063	0.901	18.000	18:0	5.66	ECL deviates -0.000	Reference -0.009
15.300	2095	0.066	0.895	18.395	10SA 10Me18:0 . . .	2.57	ECL deviates 0.003	
*******	13827	* * *	* * *	* * *	SUMMED FEATURE 4 . .	12.93	15:0 ISO 2OH/16:1W9	16:1 10ANS 9/15\20H

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	%Nr Ref	ECL Deviation	Ref ECL Shift
37510000	109206	109206	100.00	100599	5	0.002	0.000

TSBA (Rev 3.30) Rhodococcus 0.386
R. rhodochrous 0.386
Nocardia 0.375
N. asteroides 0.375

Comparison with TSBA (Rev 3.30): Rhodococcus-rhodochrous Distance: 9.450

	0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
14:0.	--a-----
15:0.	--a-------
16:1 0.	a*
16:1 CIS 9. . . .	a*----------
16:0.	----------*--------
17:1 ISO G. . . .	a*---
17:1 0.	---a------
17:0.	--a
10:1 ISO F. . . .	a*-
10:1 CIS 9. . . .	----------------------*-
19:0.	------a
10SA 10Me18:0 . . .	a----------
20:0.	a----
SUMMED FEATURE 4. .	------a-----
SUMMED FEATURE 8. .	a------

APPENDIX 4

GAS-LIQUID CHROMATOGRAMS

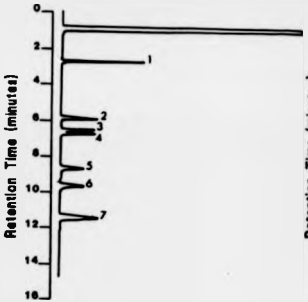


Figure 11.1

Column: BP1

Column temperature: 110°C

1. Ethylbenzene
2. Phenylacetaldehyde
3. 1-Phenylethanol
4. Acetophenone
5. 2-Phenylethanol
6. 2-Ethylphenol
7. 3-Ethylphenol/4-Ethylphenol

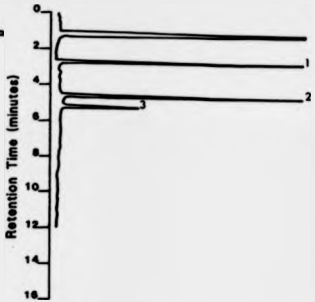


Figure 11.2

Column: BP1

Column temperature: 125°C

1. Styrene
2. Styrene oxide
3. Phenylacetaldehyde

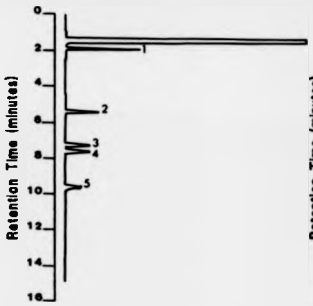


Figure 11.3

Column: BP1

Column temperature: 50°C

1. Cyclopentene
2. Cyclopentene oxide
3. Cyclopentanone
4. Cyclopentanol
5. 2-Cyclopenten-1-one

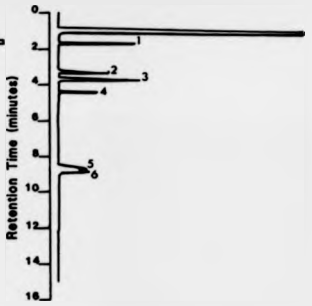


Figure 11.4

Column: BP1

Column temperature: 100°C

1. Cyclohexene
2. Cyclohexene oxide
3. 2-Cyclohexen-1-ol/ Cyclohexanone/Cylohexanol
4. 2-Cyclohexen-1-one
5. (*E*)-Cyclohexane-1,2-diol
6. (*Z*)-Cyclohexane-1,2-diol

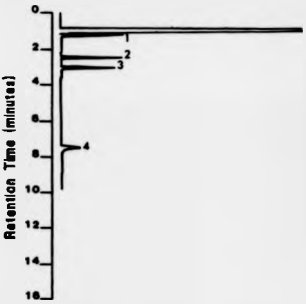


Figure 11.5

Column: BP1

Column temperature: 100°C

1. 1-Hexene
2. 1,2-Epoxyhexane
3. 5-Hexen-1-ol
4. 1,2-Hexanediol

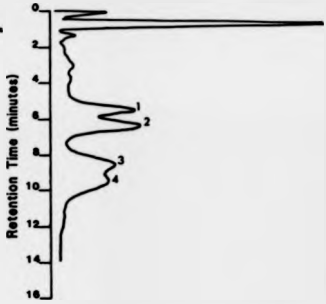


Figure 11.6

Column: Porapak Q

Column temperature: 180°C

1. (*S*)-2,3-Epoxybutane
2. Butanone
3. Crotonaldehyde
4. Crotyl alcohol

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