Biocatalytic Resolution of Substituted Styrene Oxides

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Summary

Stereochemistry and chirality are arguably two of the most important subjects pertaining to the development of new pharmaceutical drugs. Since enantiomers have the potential to encompass different pharmacological effects in biological systems, both enantiomers have to be tested for pharmacological activity. Not only has obtaining these single enantiomers become crucial, but formulation of the pure enantiomer of a drug also has the potential to contain advantages for both pharmaceutical formulation and therapeutic effect.

Epoxide hydrolase is an enzyme commonly found in nature that catalyses the hydrolysis of epoxides, resulting in the formation of the corresponding *vicinal* diol. Over the last few years a large amount of research has been completed on these enzymes from sources such as mammals, insects, bacteria and fungi. Micro-organisms especially have enjoyed ample attention because of their abundant supply. Recently it was found that certain yeasts contain this enzyme and have the ability to enantioselectively catalyse certain hydrolysis reactions. Styrene oxides are terminal epoxides that are, due to the reactivity of the epoxide ring, useful synthons in the organic synthesis of pharmaceutical products.

The first objective of this project was to synthesize three nitro derivatives of styrene oxide namely *para-, meta-, and ortho*-nitrostyrene oxide. Al three products were obtained from the corresponding nitrophenacyl bromide in yields of 52%, 90% and 57% respectively.

The second objective was to find a suitable yeast strain containing the epoxide hydrolase enzyme to enantioselectively hydrolyse the synthesised products and unsubstituted styrene oxide. A screening was completed during which 410 yeast strains from more than 44 genera were tested. Epoxide hydrolase activity was found to be widespread throughout the screened yeast domain, while the genera *Candida, Debaryomyces, Pichia, Rhodosporidium, Rhodotorula* and *Trichosporon* specifically were very successful in catalysing the hydrolysis of the substrates. *Rhodosporidium toruloides* UOFS Y-0471 and *Rhodotorula glutinis* UOFS Y-0653 were chosen for further studies because of their superior enantioselectivity.

The final objective was to optimise these reactions in terms of pH, temperature and substrate concentration. It was found that a pH value of 7,2 and a temperature of 45 °C yielded optimal

enzyme activity. Increased temperatures (45 °C), however, lead to a decrease in enantioselectivity and, in the case of *R. toruloides* together with the substrate *para*-nitrostyrene oxide, reversed enantioselectivity. Lower temperatures (15 °C) increased enantioselectivity, resulting in a remarkable improvement from a 10% yield of the single enantiomer (45 °C) to a 35% yield. Surprisingly this temperature decrease had a very small affect upon the reaction time.

Keywords: *Epoxide hydrolase, styrene oxide, kinetic resolution, biocatalysis.*

Opsomming

Stereochemie en chiraliteit is waarskynlik twee van die belangrikste faktore in die ontwikkeling van nuwe geneesmiddels. Aangesien enantiomere oor die potensiaal beskik om verskillende farmakologiese effekte in 'n biologiese sisteem te hê, moet beide enantiomere vir farmakologiese aktiwiteit getoets word. Die gebruik van suiwer enantiomere kan ook voordele inhou vir farmaseutiese formulering en kan verbeterde terapeutiese effekte tot gevolg hê.

Epoksiedhidrolase is 'n ensiem wat algemeen gevind kan word in die natuur en wat die hidrolise van epoksiede kataliseer. Hierdie reaksie het die vorming van die ooreenstemmende diol tot gevolg. Tydens die afgelope paar jaar is baie navorsing gedoen op ensieme vanaf bronne soos soogdiere, insekte, bakterieë en fungi. Mikro-organismes het veral baie aandag geniet as gevolg van hulle oorvloedige beskikbaarheid. Dit is onlangs ontdek dat sekere giste ook die ensiem bevat en oor die vermoë beskik om die hidrolisereaksie enantioselektief te kataliseer. Stireenoksiede is terminale epoksiede wat, as gevolg van die reaktiwiteit van hulle drielidring, nuttige substrate is in die sintese van geneesmiddels.

Die eerste doelstelling van hierdie projek was om drie nitrostireenoksiede te sintetiseer, naamlik *para*-, *meta*- en *orto*-nitrostireenoksied. Al drie die produkte is verkry met opbrengste van 52%, 90% en 57% onderskeidelik.

Die tweede doelstelling was om 'n toepaslike gis te vind wat die ensiem bevat en wat oor die vermoë beskik om die hidrolise van al drie die nitroderivate en ongesubstitueerde stireenoksied enantioselektief te kataliseer. In die studie is 410 verskillende giste vanuit meer as 44 genera getoets. Daar is gevind dat epoksiedhidrolaseaktiwiteit baie wyd verspreid in die betrokke gisversameling voorkom. Die genera *Candida*, *Debaryomyces*, *Pichia*, *Rhodosporidium*, *Rhodotorula* en *Trichosporon* was mees suksesvol. *Rhodosporidium toruloides* UOFS Y-0471 en *Rhodotorula glutinis* UOFS Y-0653 is gekies vir verdere studies aangesien hulle die hoogste aktiwiteit vir die substrate vertoon het.

Die finale doelstelling was om hierdie reaksies te optimiseer in terme van pH, temperatuur en substraatkonsentrasie. Optimale ensiemaktiwiteit is gevind by 'n pH-waarde van 7,2 en 'n

temperatuur van 45 °C. Hoë temperature (45 °C) het egter tot gevolg gehad dat enantioselektiwiteit verlaag, en in die geval van *R. toruloides* en *para*-nitrostireenoksied, dat die enantioselektiwiteit omkeer. Laer temperature (15 °C) het egter gelei tot hoër enantioselektiwiteit, wat 'n sterk verhoging in opbrengs van 10% (45 °C) tot 35% van die suiwer enantiomeer gehad het. Hierdie verlaging in temperatuur het egter nie 'n noemenswaardige effek op die reaksietyd gehad nie.

Sleutelwoorde: *Epoksiedhidrolase, stireenoksied, kinetiese resolusie, biokatalise.*

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Chapter 1

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1. Introduction

1.1 Background

The physicist Jean-Baptiste Biot first discovered optical activity in 1815. In 1848 the chemist and microbiologist Louis Pasteur made a set of observations, which led him a few years later to make a proposal that is the foundation of stereochemistry. He proposed the existence of isomers whose structures differ only in being mirror images of each other, and whose properties differ only in the direction of rotation of polarized light. These isomers were later named enantiomers [1]. This pioneering work by Pasteur marked the beginning of chiral separation; as well as further motivating Pasteur to continue his studies concerning the properties of different asymmetrical structures. For the first time the methodology of resolution via diastereomer formation was introduced [2].

For more than a century after Pasteur's discovery only three techniques for enantiomeric purifications would be used, i.e. [2]:

- Spontaneous resolution.
- Diastereomeric separation and
- Differential enzymatic reactivity.

Until recently it was common practice for a pharmaceutical company to market a chiral drug as a racemate. This approach in effect meant that each dose of a racemic drug contained an equal share of both enantiomers, which could have different effects. Both enantiomers could have similar effects and differ only in the magnitude of the effect (e.g. ibuprofen, an anti-inflammatory agent). Alternatively one enantiomer may have no pharmacological activity whatsoever (e.g. imipenem, an antibiotic). Finally one of the enantiomers could have no therapeutic value but have the potential to cause unsuspected deleterious side effects. For example the (R,R)-enantiomer of the tuberculostatic ethambutol **(1b)** can cause blindness (Figure 1-1) and the lethal side effects associated with the painkiller benoxaprofen might have been avoided had the drug been sold as a pure enantiomer [3].

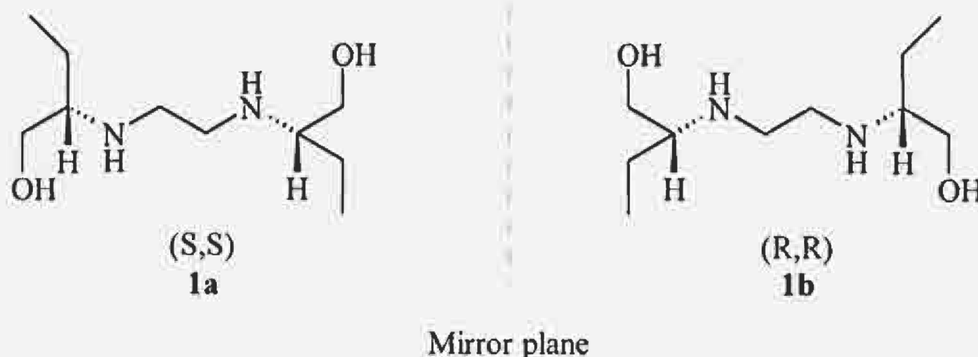

Figure 1-1 The two enantiomers of ethambutol.

These previously described differences in biological activity of drugs may result from differences in [4]:

- Protein binding and transport.
- Mechanism of action.
- Rates of metabolism.
- Changes in activity due to metabolism and
- Clearance rates.

Crosby [5] summarised the most important reasons for the production of optically pure materials:

- The biological activity of a drug is often associated with only one enantiomer.
- Production of only one enantiomer allows separation of the different activities exhibited by the enantiomers.
- The unwanted isomer is at best 'isomeric ballast' gratuitously applied to the environment.
- An optically pure compound may have more than double the activity when compared to the racemate because of antagonism.
- The use of the single enantiomer is now required by law in certain countries, the unwanted enantiomer being considered as an impurity.
- Where the switch from racemate to enantiomer is feasible, there is the opportunity to double the capacity of an industrial process; alternatively, where the optically active component of the synthesis is not the most costly, it may allow significant savings to be made in some other achiral but very expensive process intermediate.
- The physical characteristics of enantiomers versus racemates may present processing or formulation advantages.

Today, an estimated 80% of all drugs in development are single enantiomers of chiral drugs [6]. Since the early 1970s there has been a dramatic increase in research on new methods for the preparation of chiral compounds [7]. Manufacture of chemical products applied either for the promotion of human health, or to combat pests which otherwise adversely impact on the human food supply, is now increasingly concerned with enantiomeric purity [5].

1.2 Obtaining pure enantiomers

The production of optically pure materials generally presents a challenge bearing in mind that, to be of practical large-scale use, the enantiomeric excesses ought to be at least 70% and preferably close to 100% for the crude material, which is initially produced [5].

Various different approaches have been applied to obtain pure enantiomers. Figure 1-2 illustrates these approaches divided into three groups, on the basis of the type of raw material used. These methods will be discussed further in chapter 2.

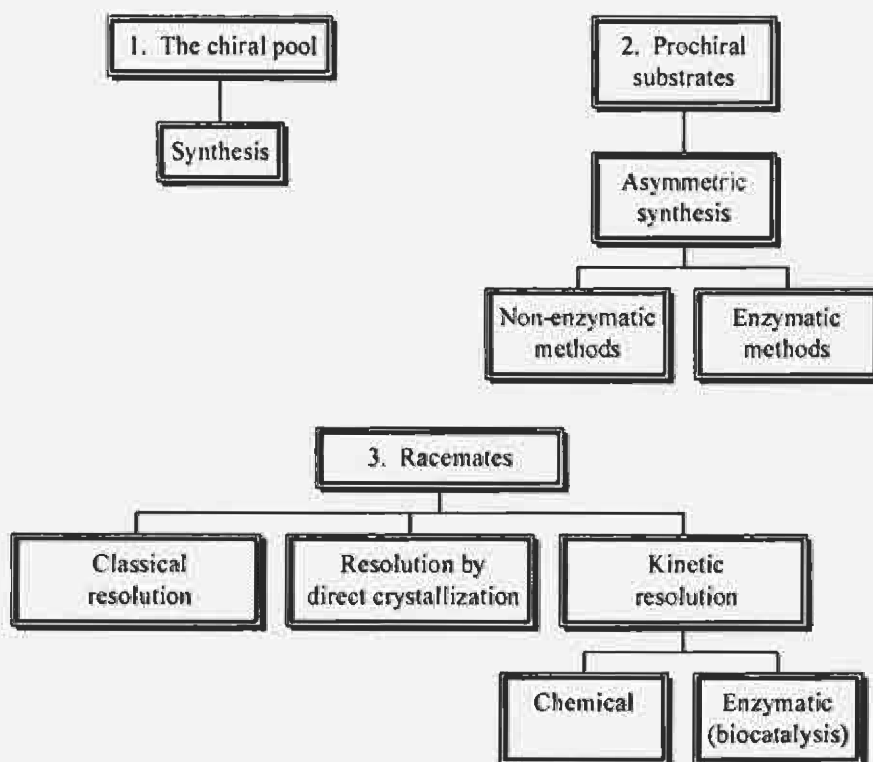

Figure 1-2 Different approaches to obtain optically pure compounds.

2. Motivation

Even though an extensive variety of methods exist to obtain pure enantiomers, each of these methods has its own distinctive disadvantages, generally making the resolution of racemates into their pure enantiomers a complicated and expensive task. Biocatalytic resolution is an inexpensive and relatively simple method with a very wide range of applications, making it an ideal alternative to conventional methods such as asymmetric synthesis. By applying this technology to phenyl-substituted styrene oxides it was envisaged that a simple, inexpensive and yet effective method for the resolution of the enantiomers of these important chiral building blocks could be found.

3. Aims and objectives

The biocatalytic resolution of chiral epoxides can be undertaken from a variety of different viewpoints. The aims of this study were firstly to synthesize three nitro derivatives of styrene oxide, namely *para*-nitrostyrene oxide, *meta*-nitrostyrene oxide and *ortho*-nitrostyrene oxide and secondly to find a suitable biocatalyst or biocatalysts within the yeast domain for their enantioselective hydrolysis. During this reaction (Figure 1-3) the racemate **(2a)** is hydrolysed enantioselectively, leading to the formation of the *vicinal* diol **(2c)**. The third objective was to optimise these reactions, determining the optimal pH, temperature and substrate concentration in order to increase the reaction rate, enantioselectivity and the yield of the enantiopure epoxides.

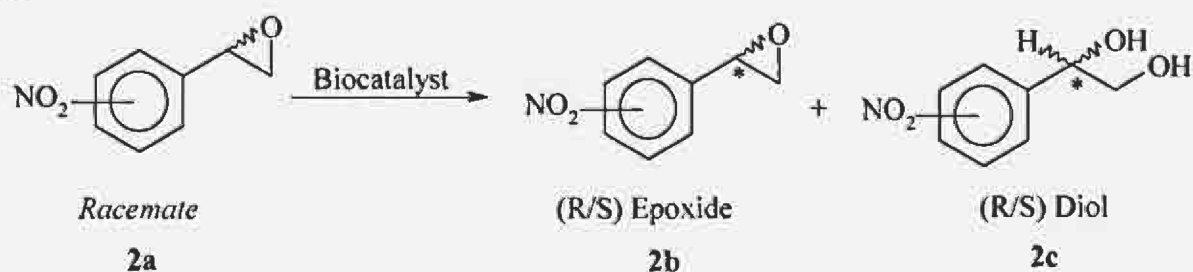

Figure 1-3 Biocatalytic enantioselective hydrolysis of the nitro derivatives of styrene oxide.

4. Outline of this dissertation

Biocatalytic resolution with the epoxide hydrolase enzyme is an actively emerging strategy to obtain the pure enantiomers of epoxides and their corresponding vicinal diols. An overview of the literature is presented (Chapter 2).

The syntheses of three of the four terminal epoxides that were not commercially available are reported together with all NMR and MS data (Chapter 3).

Testing four hundred and ten yeast strains for enantioselective activity completed a screening of the four chosen substrates. Two strains with the best activity were selected, their enantioselectivity investigated, and the influence of the position of nitro substitution on the phenyl ring of styrene oxide reported (Chapter 4).

Optimisation of the reactions involved determining the optimal pH, temperature and substrate concentration for each of the reactions. This was done with both whole cells as well as a cell free extract and the results were compared (Chapter 5).

The research is concluded with an overview of all the results that were obtained and the conclusions that were drawn, as well as the possible relevance of these results (Chapter 6).

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Chapter 2

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1. Background

Chirality is defined by Collet *et al.* [1] as the geometric property that is responsible for the non-identity of an object with its mirror image. A chiral object may exist in two enantiomorphic forms (enantiomers), which are mirror images of one another. Such forms lack inverse symmetry elements, that is, a centre, a plane, and an improper axis of symmetry. Objects that possess one or more of these inverse symmetry elements are superimposable on their mirror images and are said to be achiral.

Compounds that exist in two forms that are non-superimposable mirror images show optical activity meaning that they rotate the plane of polarised light in opposite directions. This property is shown not only by an asymmetric carbon molecule (i.e. one with four different substituents) (Figure 2-1), but also by other atoms such as sulphur, phosphorus, and some metal atoms. It can also occur when rotation around an atomic bond is hindered by large functional groups. Compounds differing only in their capacity to rotate plane-polarised light in opposite directions are known as enantiomers [2].

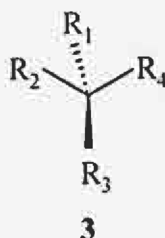


Figure 2-1 An asymmetric carbon atom where $R_1 \neq R_2 \neq R_3 \neq R_4$.

2. Racemates and racemisation

A racemate is defined by McMurry [3] as a 50:50 mixture of the two enantiomers of a molecule or compound. Such a mixture is denoted either by the symbol (±) or by the prefix *d,l* to indicate a mixture of dextrorotatory and levorotatory forms. Although the (±) and the *d,l* nomenclature is still frequently used, the Cahn-Ingold-Prelog convention is currently recommended for specifying the configuration of isomers. In this method, the ligands around the chiral centre are given a priority according to the IUPAC sequence rules. The molecule is then positioned with the ligand with the lowest priority away from the viewer. If the sequence of the remaining three ligands is arranged so that the highest to the lowest priority is in a clockwise manner, the molecule is assigned the *(R)* or *rectus* (**4b**); the counterclockwise

sequencing is given the (*S*) or *sinister* designation (**4a**) (Figure 2-2). Two other terms may be used to compare pharmacological activity of two enantiomers. It has been proposed that the isomer imparting the desired activity be called the eutomer and that the 'inactive' or unwanted isomer be labelled the distomer. From this comes the eudismic ratio, which is the ratio of the potencies of the two enantiomers [4]. These eudismic ratios can be used to describe *in vitro* or *in vivo* potency ratios of a drug or substance. Potentially more than one eudismic ratio could exist for a racemate, if the compound had more than one pharmacological effect [5].

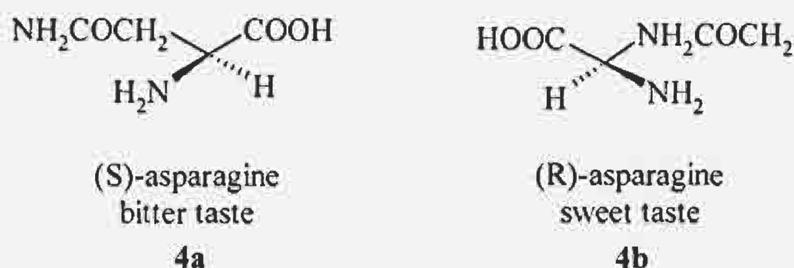
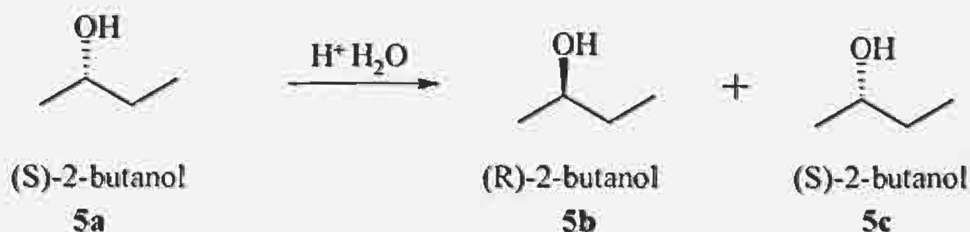


Figure 2-2 The two enantiomers of asparagine.

The process by which racemates are formed from an optically pure substrate is known as racemisation. This situation occurs when a planar intermediate is formed, and the consequential approach to the planar intermediate by a reactant molecule results in racemisation, i.e. the racemisation of 2-butanol (Scheme 2-1). The formation of carbocation intermediates involves a sp^2 carbon of which the three groups attached will be in a trigonal planar arrangement. Since access from the top and the bottom of the planar carbocation is equal, the nucleophile approaches 50% of the time from below the plane and 50% of the time from above the plane thus producing the two enantiomers. Such a mixture would be optically inactive [6].



Scheme 2-1 Racemisation of (S)-2-butanol

3. Chirality and its consequences

The chiral nature of living systems has obvious implications on biologically active compounds interacting with them. On a molecular level, chirality is an inherent property of the "building blocks of life", such as amino acids and sugars, and therefore, of peptides, proteins and polysaccharides. As a result, metabolic and regulatory processes mediated by biological systems are sensitive to stereochemistry and dissimilar activities can be observed when comparing the activities of two different enantiomers [7].

3.1 Pharmacological and pharmaceutical implications

Drug action is the result of pharmacological and pharmacokinetic processes. Various examples exist where the enantiomers of drugs show differences in their bioavailibility, distribution, and metabolism and excretion behaviour and where stereochemical parameters have a fundamental significance in their action. One example is that of the β-blocker propranolol (Figure 2-3), commonly used for the treatment of hypertension. The less active (R)-enantiomer **(6b)** is more susceptible to first-pass metabolism than the 100 fold more active (S)-enantiomer **(6a)** [7].

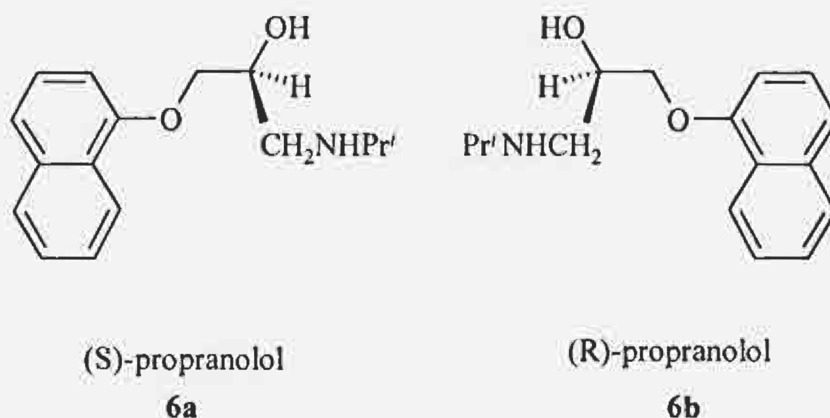


Figure 2-3 The two enantiomers of propranolol

Pharmaceutical drug development is often considered to be challenging and complex when chiral molecules are involved. Synthetic and purification methods for compounds of this nature are generally regarded as being difficult and time consuming. This situation however is now changing. New methods are becoming available for chiral separation for example; high throughput screenings that can be used in the selection of suitable systems for biosynthetic processes [8].

The Food and Drug Administration (FDA) has divided the enantiomers of chiral drugs into three distinct groups i.e.[9, 4]:

- Both enantiomers have similar desirable effects that could be identical, or could differ in the magnitude of effects, e.g.
    - Both enantiomers of dobutamine are positive inotropes;
    - Both ibuprofen enantiomers are anti-inflammatory agents;
    - Both enantiomers of warfarin and phenprocoumon are anti-coagulants;
    - The enantiomers of bupivicaine both produce local anesthesia, and it is therefore desirable to have both enantiomers present.
- One enantiomer is pharmacologically active and the other is inactive, e.g.
    - The enantiomers of the quinolones and the β-lactam antibiotics are all antibacterial substances in which one enantiomer is pharmacologically active and the other is inactive.
- Each enantiomer has a completely different activity, e.g.
    - (+)-sotalol is a type 3 antiarrhytmic while (-)-sotalol is a β-blocker.
    - (+)-ketamine is a hypnotic, while (-)-ketamine is responsible for undesired side-effects.

3.2 Economic consequences

Economic interests are obvious and essential driving forces in the development of new substances and technological improvements. Chiral drugs continue to be a significant force in the global pharmaceutical market. Worldwide sales of single-enantiomer drugs surged 21% in 1997 over 1996 (Table 2-1) to almost R830 billion (1,00 US $ = 9,43 ZAR) [10] and were predicted to increase steadily over the following years (Figure 2-4). Interestingly, 269 of the top 500 selling drugs are marketed as the single enantiomers [7].

Another consequence is that of the so-called racemic switch. In 1997 the FDA proposed a five-year marketing exclusivity to developers of single enantiomers of previously racemic drugs. In the case of bisoprolol for example (a beta-adrenergic blocking agent used for hypertension), the current $100 million per year (US sales alone), could justify the cost of racemic switch. This would be to the advantage not only of the drug manufacturers but also the chiral intermediate suppliers [11].

Table 2-1 Worldwide sales of chiral drugs during 1996 and 1997 [10].

	Total drug sales 1997 (US $ millions)	Sales of single-enantiomer chiral drugs (US $ millions)	
		1996	1997
Antibiotic	26 752	19 527	20 064
Cardiovascular	36 580	17 530	20 064
Hormones/endocrinology	14 703	8 006	8 528
Oncology	11 558	6 803	7 513
Hematology	14 970	5 199	6 033
Antiviral	13 630	1 815	5 537
Central nervous system	36 069	4 065	5 231
Respiratory	29 088	1 201	2 327
Immunosuppressant	3 386	1 475	1 998
Anti-	18 309	895	1 483
inflammatory/analgesic	6 432	675	1 087
Ophthalmic	14 789	545	843
Dermatology	60 818	--	669
Gastrointestinal	8 026	450	626
Benign prostate hyperplasia	15 000	3 790	5 934
Other			
TOTAL	310 110	72 900	87 919

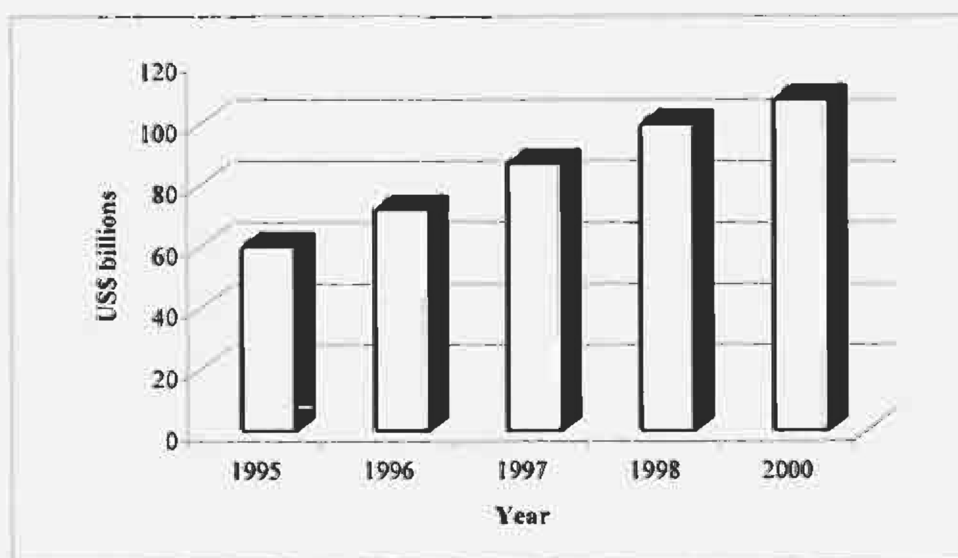


Figure 2-4 Chiral drug sales worldwide until 1998 and expected for the year 2000 [7].

3.3 Official regulation

Recognition of potential pharmacological activity differences of pharmaceutical enantiomers has led to increased attention by regulatory authorities. In the United States, the FDA released a policy statement for the development of new stereoisomeric drugs [12] and during 1994 the Drugs Directorate of the Health Protection Branch (HPB) (in Canada) set out guidelines to sponsors of new drug submissions on specific areas to be addressed during the development of chiral drugs. The European Union (EU) Committee on Proprietary Medicinal Products (CPMP) released its final guidelines on the investigation of chiral substances in December 1993 [9]. In contrast to this, the South African Medicines Control council has set specific guidelines, but no official regulation has been endorsed as of yet [13].

3.4 Optically pure epoxides

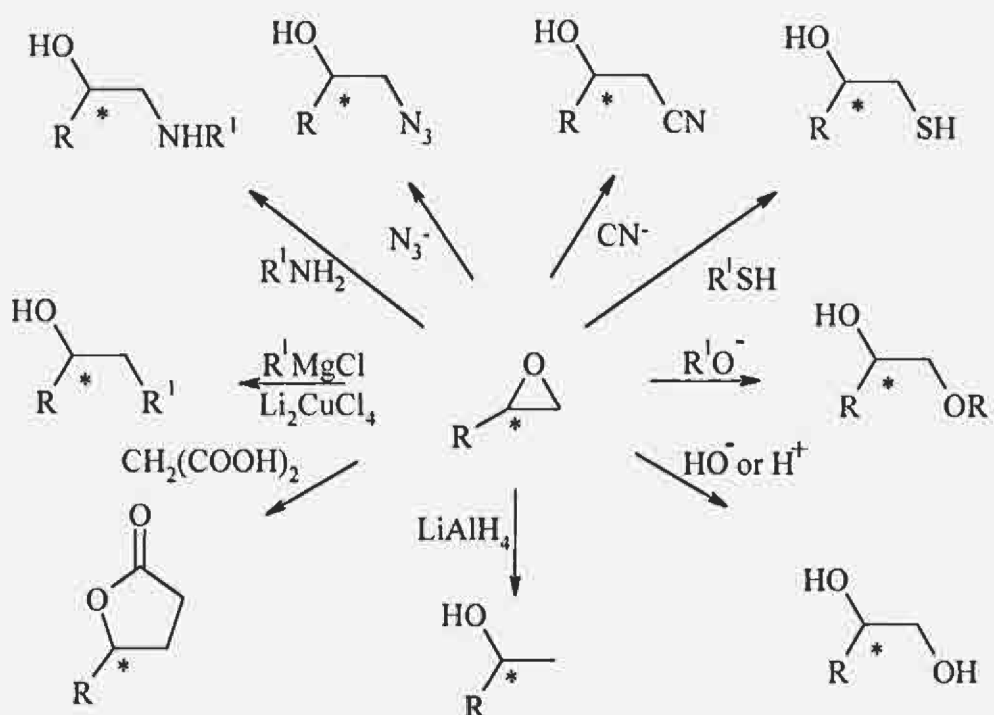
Epoxides are organic three-membered oxygen containing compounds that, in nature, arise from the oxidative endogenous metabolism, as well as from xenobiotic compounds via chemical and enzymatic oxidation processes, including the cytochrome P-450 monooxygenase system [14]. These epoxides are versatile intermediates in organic synthesis because they are reactive molecules. The development of efficient and practical methods for the synthesis of chiral nonracemic epoxides using asymmetric chemical catalysis based on heavy-metal catalysts is one of the hallmark achievements of the past decade [15].

Epoxide rings may easily be opened by a variety of nucleophiles yielding a broad range of valuable products. Even though certain epoxides have a long history as bulk chemicals, optically pure epoxides are attracting more and more attention as high value chiral intermediates. Optically pure propylene oxides, glycidols, and styrene oxides are examples of compounds now commercially available as chiral building blocks [16]. These aforementioned terminal epoxides are arguably one of the most important subclasses of the epoxide compounds, and yet no general and practical method exists for their production in enantiomerically pure form [17].

3.4.1 Uses and industrial applications

Historically, chiral drugs were developed and marketed as racemates because suitable single-isomer manufacturing technology was lacking. In 1996, of the approximately 400 drugs in the development pipeline at phase II human clinical trials, nearly 60% were chiral, of which 40% were being developed in single enantiomer form [18]. As mentioned before epoxides

are highly reactive electrophiles because of the strain inherent in the three-membered ring and the electronegativity of the oxygen atom. They react readily with various O-, N- S-, and C-nucleophiles, acids, bases, and reducing and oxidising agents, allowing access to bifunctional molecules [19] (Scheme 2-2).



Scheme 2-2 Reaction of epoxides with nucleophiles, acids, bases and reducing and oxidising agents [19, 20].

As a result of their high reactivity, chiral epoxides have been used for the synthesis of various different pharmaceutical drugs, e.g. [19]

- β-adrenergic agonist and antagonists.
  (S)-propranolol, (S)-atenolol, (S)-metoprolol, and (S)-timolol.
- Antibiotics.
  Cloramphenicol, (+)-aspicillin, azole antifungals and antiviral (R)-tritylglycidol derivatives.
- Anti-tumour pharmaceuticals.
  (2R,3S)-taxol and taxotère (Paclitaxel) and pancratistatin.
- HIV-1 protease inhibitors.
  Indinivar, ritonavir and saquinavir.

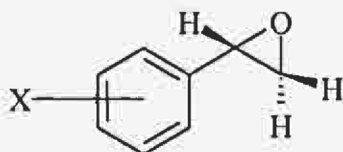
- Ca^{2+}- and K_{ATP} –channel agonists and antagonists.
  (2R,3S)-diltiazem and cromakalin.

A few more diverse uses and applications that exist for optically pure epoxides and diols have recently been described, i.e.

- Two routes for the manufacture of the novel scalemic β-amino disulfide were described by Fulton and Gibson [21], utilising (S)-phenylglycine and (R)-styrene oxide. The β-amino disulfide was used as a catalyst in the enantioselective addition of diethylzinc to aldehydes providing (R)-secondary alcohols in 39-80% enantiomeric excess *(ee)*.
- Carbon dioxide fixation has recently received much attention owning to environmental concerns while the reaction of carbon dioxide with epoxides has been of interest as a useful catalytic fixation method. Yano *et al.* [22] found that carbon dioxide could effectively undergo carboxylation with an epoxide using magnesium oxide as a catalyst. In their paper they presented a novel method for the fixation of carbon dioxide by metal oxide to (R)-styrene oxide with retention of stereochemistry.

3.4.2 Optically pure styrene oxides

During the last decade a multitude of work has been completed on the enantioselective hydrolysis of phenyl-substituted styrene oxides (Figure 2-5), probably because of the large amount of synthetic possibilities. This class of substrate is also known as substituted phenyl oxiranes (IUPAC) but since they are more commonly referred to as styrene oxides, they will henceforth be referred to as such.



7

Figure 2-5 General structure of phenyl-substituted styrene oxides.

Table 2-2 summarizes the biocatalysts, with reference to the species, that have been found to have enantioselective activity towards phenyl-substituted styrene oxides.

Table 2-2 Biocatalysis of various phenyl-substituted styrene oxides.

X = H						
Species	**Epoxide (*E*) (%*ee*)**	**Diol (*D*) (%*ee*)**	**Yield (*E*) (%)**	**Yield (*D*) (%)**	**E**	**Reference**
Aspergillus niger	S(99)	R(65)	28	50	20,5	[23], [24], [25], [34], [61], [65]
Beauveria sulferescens	R(98)	R(45)	34	45	49	[23], [25], [34], [61], [65]
Agrobacterium radiobacter	S(99)	R(49)	33		12	[26]
Beauveria densa	R(>95)	R(78)	18	34	29,5	[27]
Streptomyces antibioticus	R(99)	S(72)			31	[28]
Streptomyces fradiae	R(70)	S(23)			4	[28]
Streptomyces arenae	R(52)	S(38)			3	[28]
Syncephalastrum racemosum MUCL 28766	S	S				[24], [29]
Beauveria bassiana	R(>99)	R(50)			12	[24], [30]
Aspergillus terreus	R	R				[24]
Chaetomium globosum LCP 679	S	R				[24]
Cunninghamella elegans LCP 1543	S	R				[24]
Mortierella isabellina	R	R				[24]
Rhodotorula glutinis	S(>98)	R(48)	18		11,7	[31]
Nocardia corallina B-276	R(85)					[16]
X = *p*-Me						
Aspergillus niger	S(96)	R(66)	34	40	18,3	[25], [61]
Beauveria sulferescens	R(>98)	R(76)	30	45	33	[25], [61]
Agrobacterium radiobacter	S(>99)		36			[26]
Beauveria densa	R(>95)	R(61)	21	48	14,5	[27]
Syncephalastrum racemosum	S	S				[29]
X = *m*-Me						
Beauveria densa	R(50)	R(67)	19	44	8,2	[27]

X = *o*-Me						
Beauveria densa	R(12)	R(5)	24	36	1,2	[27]
X = *p*-F						
Aspergillus niger	S(98)	R(81)	35	47	43	[25], [61]
Beauveria sulferescens	R(96)	R(78)	25	50	31	[25], [61]
Beauveria densa	R(>95)	R(77)	18	36	28	[27]
Syncephalastrum racemosum	S	S				[29]
X = *p*-Cl						
Aspergillus niger	S(98)	R(79)	33	49	38	[61]
Beauveria sulferescens	R(54)	R(72)	54	66	10,5	[61]
Agrobacterium radiobacter	S(>98)		36			[26]
Beauveria densa	R(>95)	R(82)	17	36	37	[27]
Syncephalastrum racemosum	S	S				[29]
X = *m*-Cl						
Beauveria densa	0	21	60	10		[27]
Agrobacterium radiobacter	S(>99)					[26]
X = *o*-Cl						
Beauveria densa	0	0	77			[27]
Agrobacterium radiobacter	S(>99)		35			[26]
X = *p*-Br						
Aspergillus niger	S(98)	R(79)	34	51	40	[25], [61]
Beauveria sulferescens	R(96)	R(72)	33	52	33	[25], [61]
Beauveria densa	R(>95)	R(87)	19	41	53	[27]
Syncephalastrum racemosum	S	R				[29]
X = *p*-CN						
Aspergillus niger	S(98)	R(76)	38	42	33	[25], [61]
Beauveria sulferescens	R(15)	R(50)	15	25	3,5	[25], [61]
Syncephalastrum racemosum	S	R				[29]
X = *p*-NO_2						
Aspergillus niger	S(>98)	R(70)	37	54	25	[25], [32], [33], [61]
Beauveria sulferescens	S(50)	R(49)	50	36	3,5	[25], [61]
Syncephalastrum racemosum	S	R				[29]

4. Obtaining optically active compounds

As mentioned in Chapter 1, various methods exist for producing pure enantiomers. The different approaches (Figure 2-6) are subsequently discussed together with some of their advantages and disadvantages.

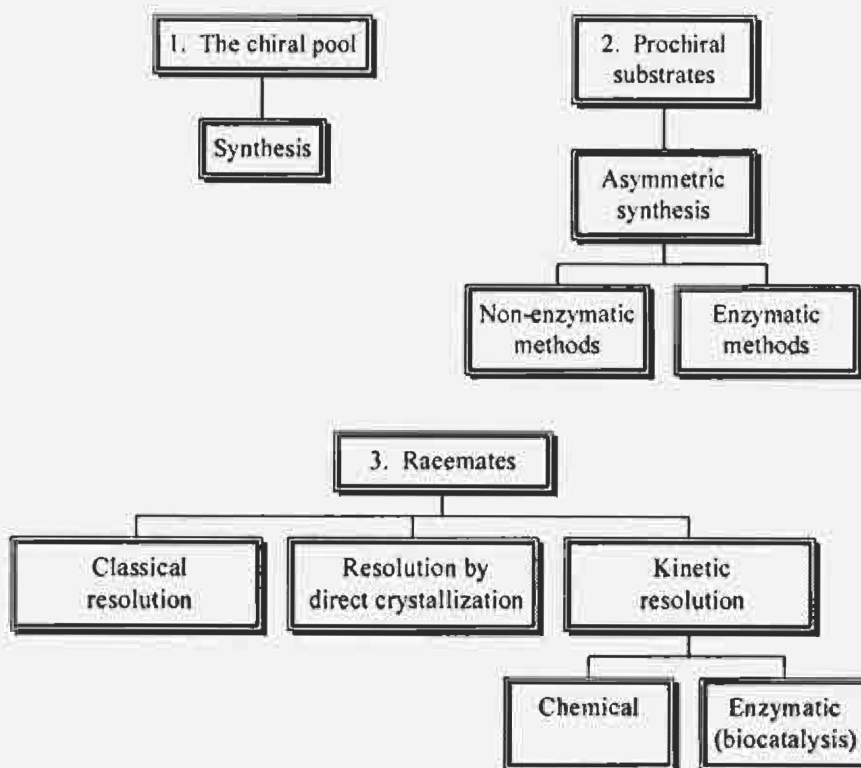
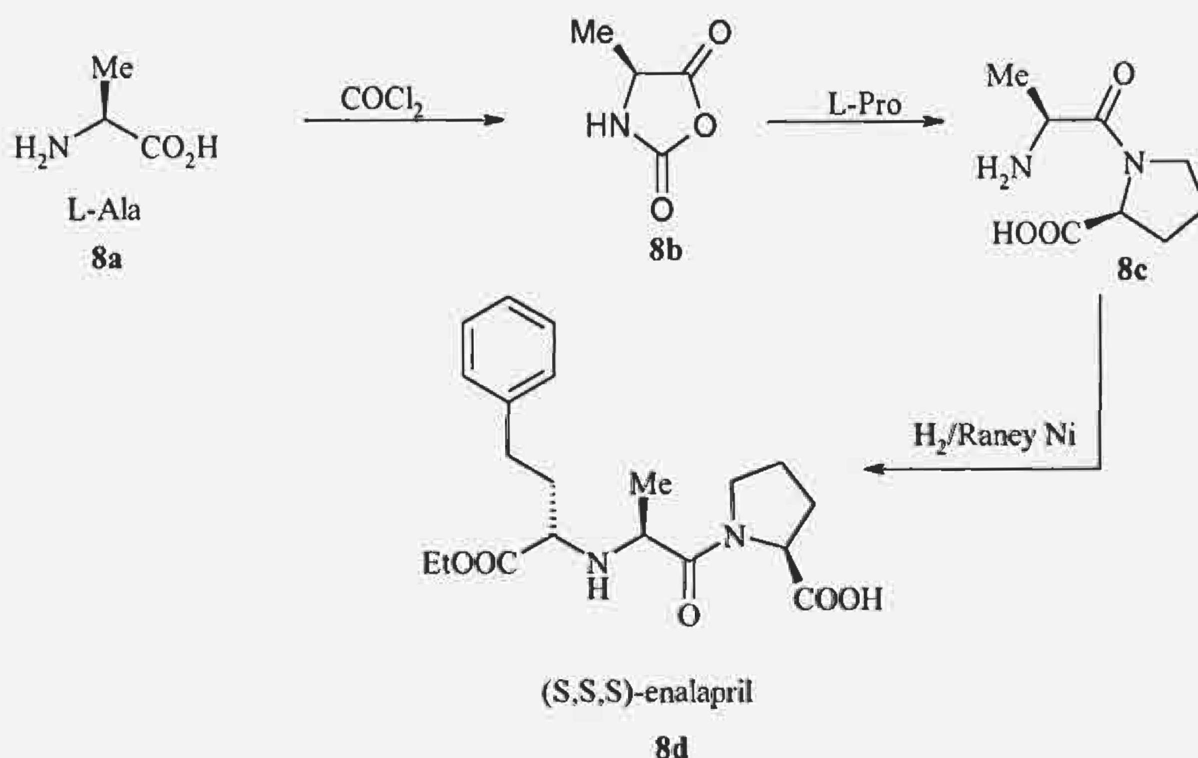


Figure 2-6 Different approaches to produce optically pure compounds

The preparation of enantiopure epoxides and of the corresponding *vicinal* diols is a very actively explored area, since these compounds are highly valuable synthons. These can be prepared *via* various multistep ways. Two direct chemical procedures (Figure 2-6, 2 – non-enzymatic methods) allowing for the synthesis of enantiopure epoxides have been particularly developed during the last decade: the Sharpless stereoselective epoxidation of olefins – which is restricted to allylic alcohols, and the Jacobsen/Katsuki procedure which gives mainly good results with some *cis*-substituted olefins. On the other hand the Sharpless osmium catalysed dihydroxylation approach, which allows for the direct preparation of enantiomerically enriched *vicinal* diols, has been proven to be essentially efficient for *trans*-disubstituted olefins. One drawback of these procedures is the fact that they are based on the use of heavy metal catalysts, which may be sources of industrial pollution [34]. These and other methods will be discussed hereafter.

4.1 Obtaining chiral compounds from the chiral pool

Even though nature does not always make just one enantiomer [35], there are many instances in which single enantiomers can be isolated from nature. The chiral pool refers to readily available optically active natural products and includes amino acids, hydoxy acids, carbohydrates, terpenes and alkaloids. During organic synthesis they can be incorporated into the target structure with the necessary modifications in order to achieve the desired chiral features. One example is the synthesis of enalapril, an angiotensin-converting enzyme (ACE) inhibitor used for the treatment of hypertension, which involves the use of L-proline (L-Pro) to yield the required (S,S,S)-isomer (**8d**). This synthesis is shown in Scheme 2-3 [36, 37].



Scheme 2-3 The synthesis of enalapril.

Other pharmaceuticals synthesised from chiral pool compounds include the peptide drug buserelin (Suprefact®), used for the treatment of prostatic cancer, the carbapenem β-lactam antibiotic imipenem (Primaxim®) and the coronary vasodilator isosorbide dinitrate (Isordil®) [13].

4.2 Resolution of racemates

Since the early 1970s the theory and practical aspects of resolution methods have been

painstakingly organised, analysed, expanded, and explained so that rational approaches likely to be successful can be identified and understood [35].

4.2.1 Classical resolution and chromatographic enantioseparation

Classically, forming diastereomeric salts or derivatives with enantioenriched chiral pool reagents has separated enantiomers. Since these diasteromeric derivatives are no longer enantiomers, they can be separated by conventional separation methods such as crystallization, or chromatography on silica or other conventional stationary phases. Another alternative is using a chiral discriminator or selector during chromatographic enantioseparation. Two types of selectors can be distinguished: a chiral additive in the mobile phase or a chiral stationary phase [38].

4.2.1.1 Diastereomer crystallisation

This method generally involves reaction of the racemate with an optically pure acid or base to give a mixture of two diastereomeric salts whose physical properties are different [46]. Thus, when a racemic acid A is combined with an optically pure base B, a mixture of two diastereomeric salts is formed (Scheme 2-4) which can be separated by crystallisation.

Scheme 2-4 The formation of diastereomers from a racemic mixture.

4.2.1.2 Chromatographic enantioseparation

During the last few years preparative chromatographic resolution of racemates has been developed extensively, providing an alternative for access to pure enantiomers [39]. Some major advances have emerged in the understanding of solute-solvent interactions, and many successful separations by gas (GC) and by high performance liquid chromatography (HPLC) have been reported. Lochmüller and Souter have presented a selective review, mainly focussing on the type of chiral stationary phases [40].

Although chiral GC separation often results in low separation factors, quantitative resolution is often achieved due to the large number of theoretical plates available in capillary GC [39]. Following this, a number of racemates, with pharmaceutical and industrial applications, have

been separated into their enantiomers on a preparative scale e.g. inhalation anaesthetics enflurane, isoflurane and desflurane, methyl 2-chloropropionate (used for the synthesis of certain herbicides) [41] and *all-trans*-perhydrotriphenylene, a versatile synthon [42]. Schomburg [43] recently presented a short review concerning the principles, necessary instrumentation and applications of two-dimensional gas chromatography and furthermore described the application to enantiomer separation.

HPLC has recently received a lot of attention as a large-scale preparative method for the resolution of racemates. The advantages of this method are its high selectivity, simple product recovery, ease of further purification and short product recovery time [44]. The beta blocker propranolol, barbiturates (e.g. hexobarbital), diazepine derivatives (e.g. oxazepam), imidazole derivatives (e.g. miconazole) and dihydropyridine derivatives (e.g. nicardipine) are all examples of important pharmaceuticals that have been obtained in their enantiopure form through the use of HPLC [45].

4.2.2 Resolution by direct crystallisation

Direct preferential crystallisation of one enantiomer is possible only with conglomerates, defined as mechanical mixtures of crystals of the two enantiomers [46]. It is dependent on differences in rates of crystallisation of the two enantiomers and on the correlation between the melting point and the solubility phase. By seeding a supersaturated solution of the racemate with crystals of one enantiomer it is, in some cases, possible to achieve preferential crystallisation [46].

4.2.3 Kinetic resolution

Kinetic resolution depends on the fact that the rates of reaction of two enantiomers with an optically active agent are different. According to Sheldon [46], the optically active agent should preferably function in catalytic quantities and may be an enzyme or a chemical catalyst. The enantiomeric ratio E is a measure of the efficiency of a particular kinetic resolution and is characteristic of a process, therefore better describing the reaction with a certain substrate and a specific enzyme [47]. The enantiomeric excess (*ee*) is a property of the product alone. Chen *et al.* [48, 49] have previously described a method to calculate the E value (Equation 2-3) of irreversible reactions, using both the calculated *ee* values (Equation 2-1) and the degree of conversion (ξ) (Equation 2-2).

$$ee = \frac{([A]-[B])}{([A]+[B])}$$

Equation 2-1 Determining the enantiomeric excess. [A] and [B] represent the concentrations of the two enantiomers [48].

$$\xi = 1 - \frac{([A]+[B])}{([A_0]+[B_0])}$$

Equation 2-2 Determining the conversion. [A_0] and [B_0] represent the initial concentrations of the two enantiomers [48].

$$E = \frac{\ln[(1-\xi)(1-ee_S)]}{\ln[(1-\xi)(1+ee_S)]}$$

Equation 2-3 Sih's equation involving the *ee* of the substrate (ee_s) [48].

More recently Rakels *et al.* [50] described a modification of the aforementioned method that allows for the direct determination of E from ee_s and ee_p (*ee* product) measurements. With this method ξ measurements are no longer required (equation 2-4).

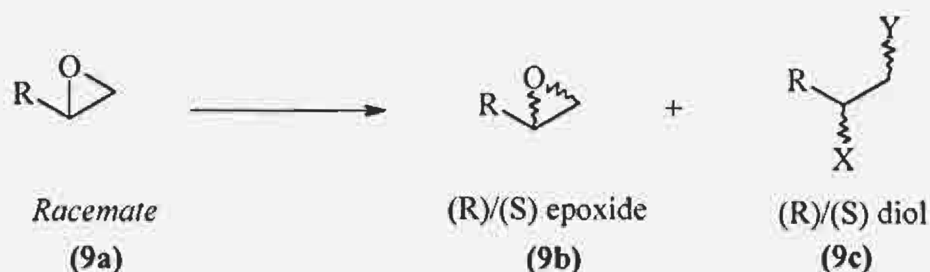
$$E = \frac{\ln\left({(1-ee_S)} \Big/ {\left(1+\left({ee_S}/{ee_P}\right)\right)} \right)}{\ln\left({(1+ee_S)} \Big/ {\left(1+\left({ee_S}/{ee_P}\right)\right)} \right)}$$

Equation 2-4 Determining E without having to determine ξ [50].

In favourable cases (E > 50), the reaction rates of the two enantiomers are substantially different, resulting in a virtually enantiospecific reaction. A high E value for a given reaction is crucial for the success of a kinetic resolution because it ensures a high *ee* as well as a high

yield. In principle, it is possible to always achieve a very high *ee* if a low yield is acceptable [51].

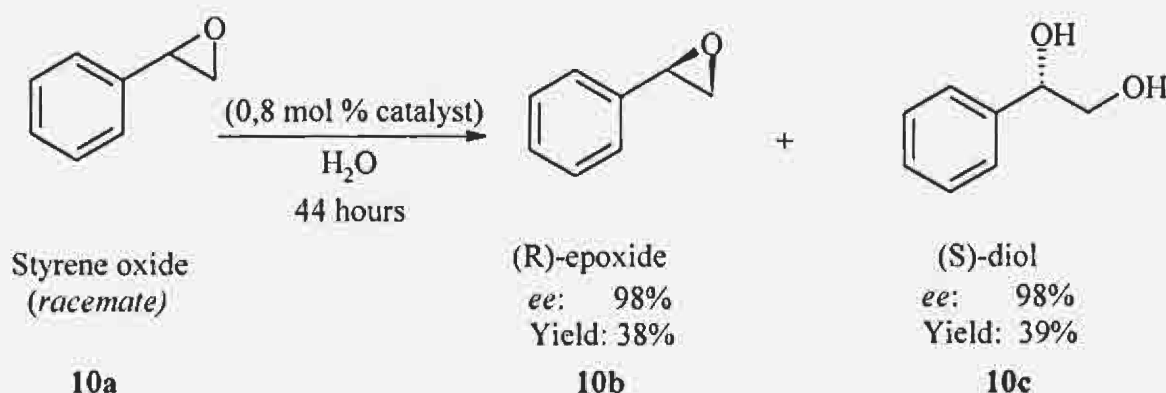
There are a very large number of examples of kinetic resolutions (biochemical and chemical) in which an enantioselective reaction takes place, for example the various epoxides, halohydrins, and diols [52, 53, 54, 55] obtained in their enantiopure form through biocatalysis. The efficiency of such a process depends on the relative rates of reaction of the two enantiomers with a chiral reactant, where the maximum yield of one isomer cannot be greater than 50% [35]. Scheme 2-5 depicts the resolution, chemical or biological, of a racemic terminal epoxide. The one enantiomer is hydrolysed, resulting in the formation of the corresponding *vic* diol **(9c)**, while not affecting the second enantiomer **(9b)**.



Scheme 2-5 General reaction for the resolution of a racemic terminal epoxide

4.2.4.1 Chemical kinetic resolution

Resolution strategies are especially viable when the racemic starting compound is inexpensive, readily available and when the enantiopure material is hard to access. One example of chemical kinetic resolution is the hydrolytic resolution of terminal epoxides with Jacobsen-metalloporphyrins (i.e. homochiral Co(III) Schiff base complex) to yield the corresponding *vic*-diols [56, 57]. This kinetic resolution presents an attractive method for accessing terminal epoxides in high enantiomeric purity and has previously been employed for the resolution of styrene oxide **(10a)** (Scheme 2-6).



Scheme 2-6 Kinetic resolution of styrene oxide using Jacobsens Co(III) catalyst [56].

4.2.4.2 Enzymatic kinetic resolution

The field of biocatalysis (i.e. the use of enzymes and micro-organisms for organic synthesis) is now at an exciting phase in its development. Biocatalytic resolutions utilise the selectivity of enzymes for one of the enantiomers of a chiral molecule. One enantiomer of the racemate remains unchanged whilst the other enantiomer is converted into the desired enantiomerically pure product or intermediate [58].

During the past 20 years there has been an increasing awareness of the opportunities for using enzymes to effect stereo-, regio- and chemo-selective transformations on non-natural organic substrates [59]. Epoxide hydrolases are very interesting enzymes which have been detected in organisms as diverse as mammals, plants, and microorganisms. About two-thirds of bioformations reported during the last two decades used hydrolase enzymes. The main reasons for this are reported as being [60]:

- Hydrolases do not require cofactors other than water.
- They are available from a variety of sources.
- They remain active in non-aqueous media, for example giving rise to ester formation rather than cleavage reactions.
- They frequently show remarkable chemo, regio- and stereoselectivity whilst accepting a wide range of substrates.

Besides biological studies, at least two other aspects of these enzymes are of importance, i.e. [61],

- Their use as asymmetric biocatalysts for organic chemistry applications and

- The determination of their catalytic mechanism.

4.2.4.2.1 Mammalian epoxide hydrolases

The two major mammalian epoxide hydrolases, microsomal epoxide hydrolase (mEH) and soluble epoxide hydrolase (sEH), are present in the liver, brain, lungs etc. of many animals. Both enzymes have broad, partially overlapping substrate specifities, but their individual substrate preferences are still quite distinct. In general, it is assumed that *mono*- and *cis*-disubstituted epoxides bearing a lipophilic substituent are good substrates for mEH. Important clinical drugs metabolised by mEH include epoxide derivatives of anticonvulsant drugs, phenytoin and carbamazepine [14]. For sEH also tri- and tetra-substituted epoxides and in particular, several *trans*-disubstituted epoxides have been found to be excellent substrates [62]. Recently mEH from rabbit liver has been shown to have the ability to enantioselectively hydrolyse *cis*-dialkyl substituted oxides [63] and *cis*-β-alkyl substituted styrene oxides [64] to their corresponding diols. The use of these enzymes during large-scale hydrolysis is however limited due to the obvious lack of supply of the enzyme.

4.2.4.2.2 Insect epoxide hydrolases

Even though substrate specific epoxide hydrolase enzymes have been found in various insects, including the gypsy moth *Lymantria dispar* and mite species like *Rhizoglyphus robini*, the large scale production of insect enzymes is still fairly difficult which strongly hampers biocatalytical applications for these enzymes [62].

4.2.4.2.3 Plant epoxide hydrolases

Plant epoxide hydrolases are specific for the hydrolases of *cis* fatty acid epoxides, resulting in *threo* diols. In principle they are useful for the synthesis of enantiopure epoxyfatty acids and dihydroxyfatty acids because of their stereochemical features and their relatively high activities [62].

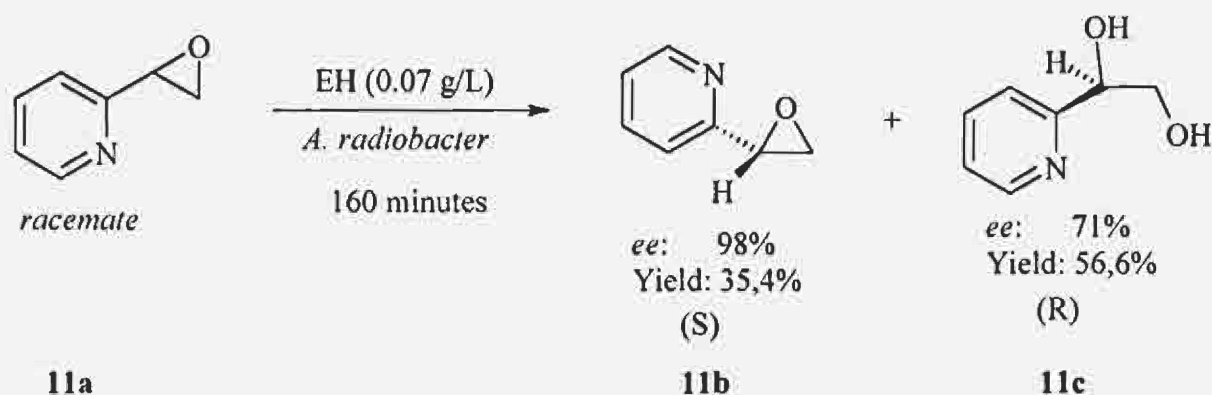
4.2.4.2.4 Bacterial epoxide hydrolases

Bacterial epoxide hydrolases can be divided into two groups:

- Constitutively produced enzymes and
- Enzymes involved in the metabolism of specific epoxides

The constitutively produced hydrolases are useful in the resolution of di-substituted epoxides only. In addition, their specific activities are in most cases not very high. On the contrary,

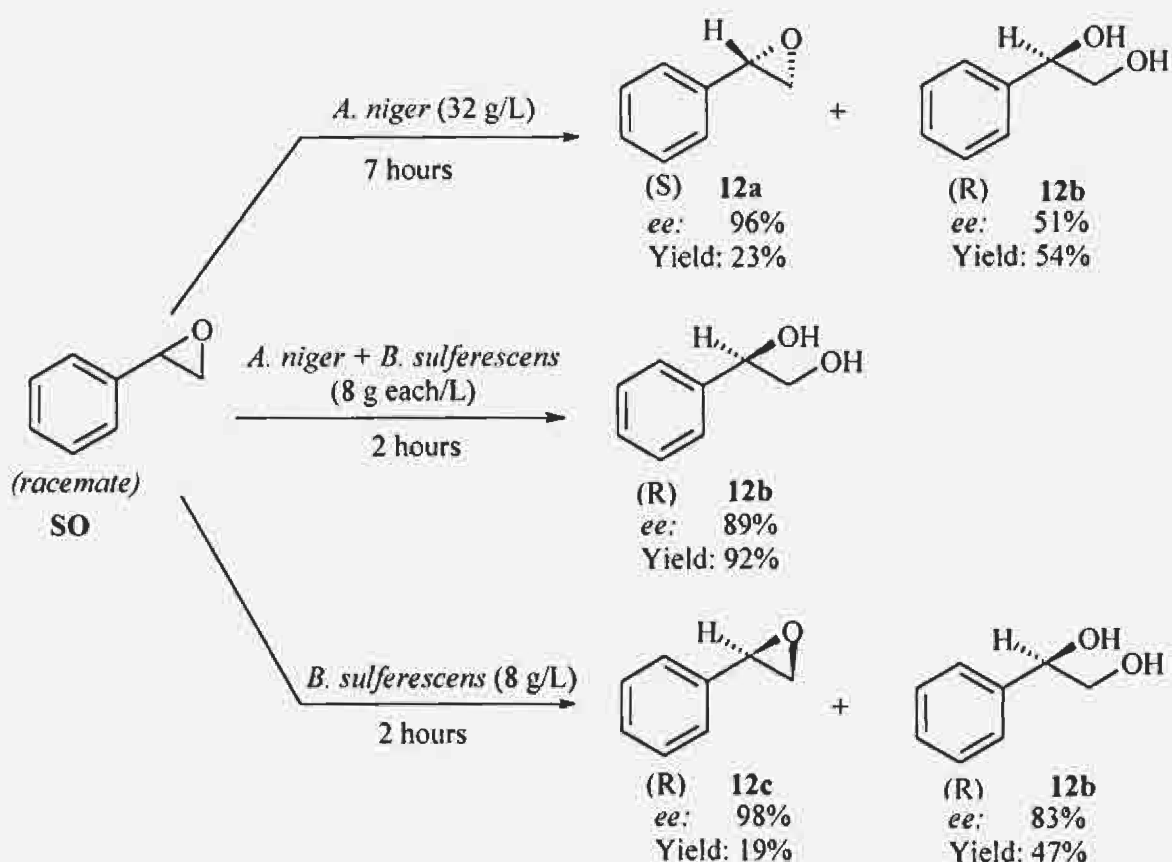
high specific activities are typical for the inducible enzymes, which regrettably only act on a limited range of substrates. It is however to be expected that, in the near future, some of these limiting features will be improved by genetic engineering [62]. Genzel *et al.* [65] have previously shown that the epoxide hydrolase enzyme from a bacterial strain *Agrobacterium radiobacter* and its Tyr215Phe mutant both have the ability to enantioselectively hydrolyse 2-, 3-, and 4-pyridyloxirane (Scheme 2-7), yielding the residual (S)-epoxide **(11b)** and the formed (R)-diol **(11c)**.



Scheme 2-7 Biohydrolysis of 2-pyridyloxirane

4.2.4.2.5 Fungal epoxide hydrolases

The use of fungal epoxide hydrolases seems to be a very promising method to prepare optically pure epoxides [66]. Furstoss *et al.* previously described the hydrolysis of styrene oxide by cells of two fungal strains *via* two distinct mechanisms [67]. Hydrolysis of racemic styrene oxide **(SO)** by *Aspergillus niger* proceeded with retention of configuration at the chiral centre, resulting in the (S)-residual epoxide **(12a)** and the (R)-diol **(12b)**. With cells of *Beauveria sulferescens* hydrolysis of **SO** resulted in the (R)-residual epoxide **(12c)** while the (S)-epoxide was converted with inversion of configuration to **12b** (Scheme 2-8). They noted that these two microbial transformations could easily be carried out on large-scale quantities, thus allowing production of several grams of either enantiomer. By using the two strains in combination they could theoretically achieve a yield of 100% of the formed diol, since both organisms lead to the selective formation of the (R)-enantiomer.

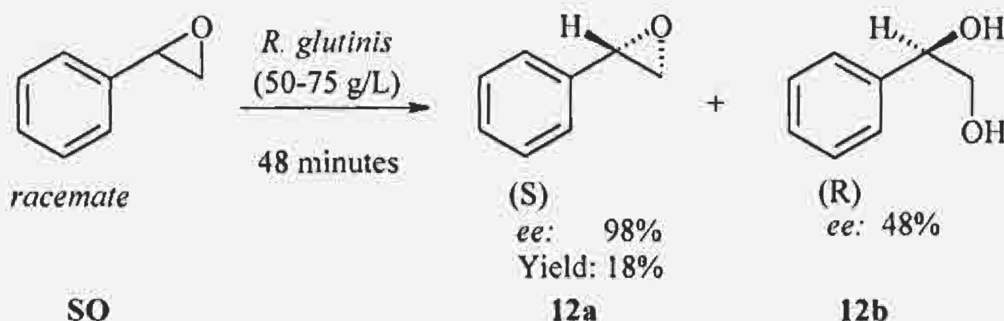


Scheme 2-8 Hydrolysis of racemic styrene oxide by fungal epoxide hydrolases [67].

Fungal epoxide hydrolases have also been shown to enantioselectively hydrolyse a wide variety of other substrates including *mono-*, *gem-*, *trans*, and *cis*-disubstituted alkyl epoxides [68], terminal epoxides and 2,2-disubstituted epoxides, 3,3-dimethyl-1,2-epoxybutane and certain *meso*-epoxides [69].

4.2.4.2.6 Yeast epoxide hydrolases

During a study conducted by Weijers [31], it was found that a strain of *R. glutinis* enantioselectively hydrolysed **SO**. Enantioselectivity was, without any exception, preferential towards hydrolysis of **12c** leading to the formation of the **12b** in excess (Scheme 2-9).



Scheme 2-9 Hydrolysis of racemic styrene oxide by yeast epoxide hydrolases [31].

In the following years various studies were conducted using EH from yeast origin for the enantioselective hydrolysis of epoxides. A few examples of these epoxides are 1,2-epoxides [70], *trans*-1-phenylpropene oxide, *trans*-ethyl-3-phenyl glycidate, indene oxide and (2,3-epoxypropyl) benzene [13]. Various other epoxides have however been accepted as substrates by yeast EH, demonstrating the broad range of yeast EH [31, 62]. Krieg *et al.* also successfully attempted the upscaling of one of these reactions to a continuous process through the use of a membrane bioreactor [71, 72].

4.3 Asymmetric synthesis from prochiral substrates

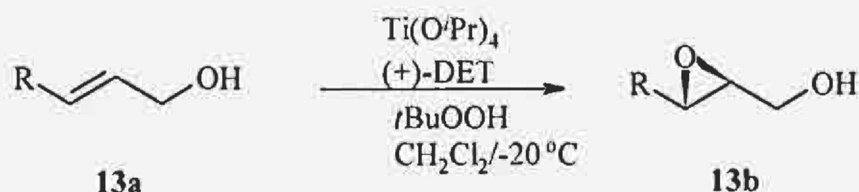
In a typical asymmetric synthesis, prochiral groupings are converted to chiral groupings [35]. Acquisition of enantiomerically pure materials through transformation of prochiral substrates necessitates the reaction with an optically active agent, used either stoichiometrically or catalytically, which expresses its chirality [36].

4.3.1 Non enzymatic methods

Various methods of non-enzymatic asymmetric synthesis exist, including asymmetric hydrogenation, hydroformylation, isomerization, oxidation, cyclopropanation, phase transfer catalysis, and cycloaddition [36]. A few examples of these routes will be discussed hereafter.

4.3.1.1 Sharpless asymmetric epoxidation

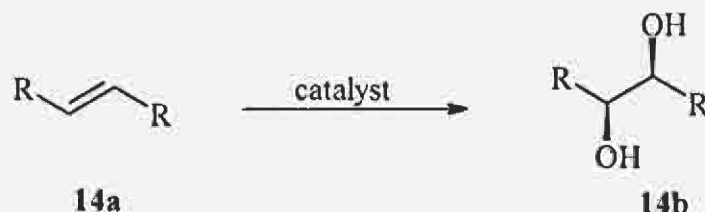
One example of how asymmetric synthesis can be applied to obtain optically pure epoxides is the asymmetric epoxidation developed by Katsuki and Sharpless. They reported a titanium-catalysed asymmetric epoxidation of a wide variety of allylic alcohols **(13a)** with *ee* values usually greater than 90% [60]. This epoxidation is shown in Scheme 2-10.



Scheme 2-10 Sharpless asymmetric epoxidation

4.3.1.2 Sharpless asymmetric dihydroxylation

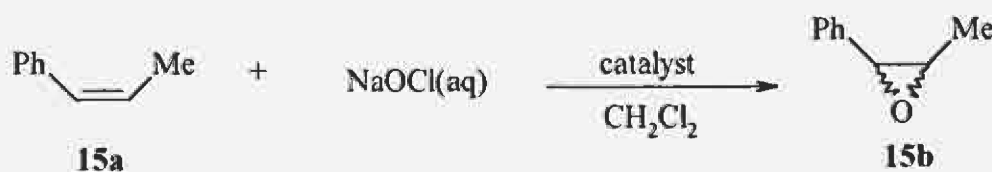
The osmium-catalysed asymmetric dihydroxylation developed by Sharpless (Scheme 2-11) is characterised by the requirement of ligand variation for the dihydroxylation of the 6 different structural classes of olefins **(14a)** to obtain high optical purity [73]. It has been applied to the synthesis of numerous enantiopure intermediates and bioactive compounds. The development of efficient methods to convert enantiopure *vic* diols **(14b)** to enantiopure epoxides, cyclic sulphates and sulphites, broadened the scope of asymmetric dihydroxylation reactions further [19].



Scheme 2-11 Sharpless asymmetric dihydroxylation

4.3.1.3 Jacobsen's asymmetric epoxidation

Jacobsen's catalyst for asymmetric epoxidation of unfunctionalised olefins is a Mn(III) complex of the chiral Schiff base of either (R,R)- or (S,S)-*trans*-1,2-diaminocyclohexane with 3,5-di-*tert*-butylsalicylaldehyde (Scheme 2-12) [74]. The amount of catalyst is dependent on the reactivity of the olefin, and ranges from 1.5 mol % to 16 mol %, [19].



Scheme 2-12 Asymmetric epoxidation of *cis*-β-methylstyrene **(15a)** [74].

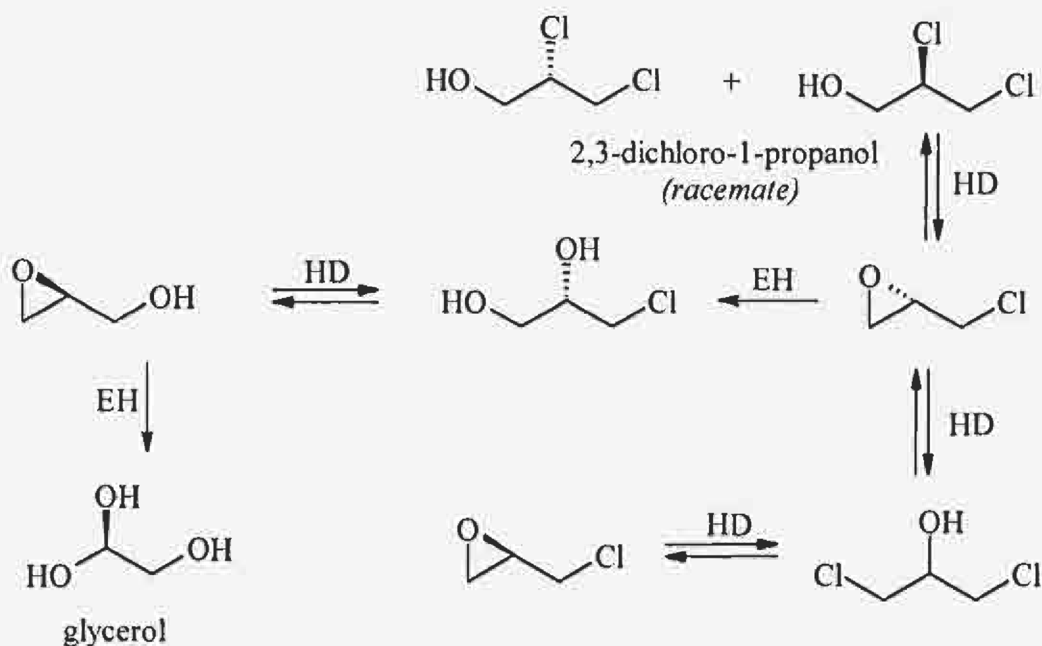
4.3.1.4 Other methods of asymmetric synthesis

- Kureshy reported the asymmetric epoxidation of styrene by chiral Ru(II) Schiff base complexes [75].
- Brunner recently described the asymmetric synthesis of the narcotic drug methohexital using palladium as catalyst [76]

4.3.2 Enzymatic methods

Enzymes are increasingly being used to prepare enantiomerically pure materials, showing an increasing recognition of the contribution that enzymes can make in the synthesis of optically pure materials [36]. The enzymes used during asymmetric synthesis can be divided into six main groups i.e. oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases [77]. The reactions catalysed by these enzymes include asymmetric oxidation, reduction, hydroxylation, reductive amination, ammonia addition, transamination, hydration and cyanohydrin formation [13, 36].

Asymmetric epoxidation by microorganisms was realised for the first time in 1973. Since then various organism have been found to have the ability to produce optically active epoxides [78] e.g. the epoxidation of styrene and its derivatives by chloroperoxidase [79] and a tandem enzyme reaction reported by Lutje Spelberg *et al.* [80]. They illustrated an enzymatic route to pure aromatic halohydrins and epoxides (Scheme 2-13). During this reaction 2,3-dichloro-1-propanol was converted by halohydrin dehalogenase and epoxide hydrolase, originating from *Agrobacterium radiobacter*, to various epoxides and halohydrins and finally to glycerol.



Scheme 2-13 Conversion of a halohydrin by epoxide hydrolase (EH) and halohydrin dehalogenase (HD) [80].

4.4 Asymmetric synthesis vs. kinetic resolution

Which is more attractive: asymmetric synthesis or kinetic resolution? Collet *et al.* [46] noted that superficially it would seem that asymmetric synthesis is more attractive since it has a theoretical yield of 100% compared to 50% for kinetic resolution. However, further inspection reveals several advantages compared to asymmetric synthesis. They name the following two examples:

- The optical purity can, in principle, be tuned to any required value by adjusting the degree of conversion.
- Kinetic resolutions tend, in general, to be simpler chemical processes than asymmetric synthesis.

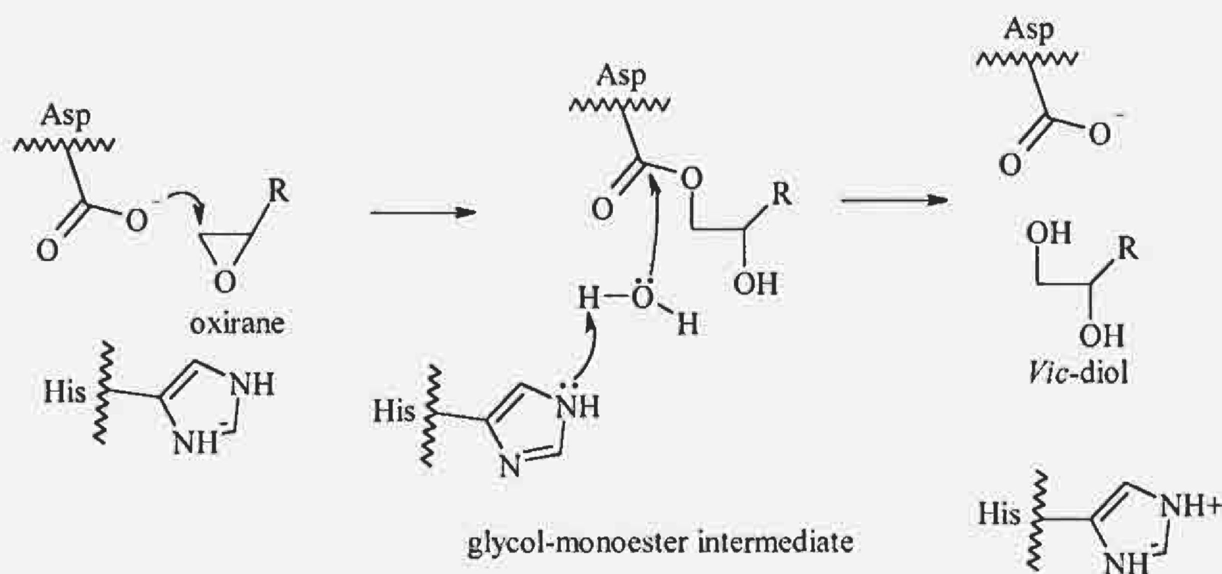
A study conducted by Jödicke *et al.* [81] concluded that a general decision on which of the two technologies was environmentally least harmful could not be made. This was especially true when the total impact of a work up of the reactions was considered. Therefore it can be said that each reaction has to be individually evaluated and compared to alternative methods considering the total environmental impact of each reaction.

5. Epoxide hydrolase enzymes

5.1 Mechanism of hydrolysis

Enzymes have an active site that is shaped in such a way that only a molecule with the correct shape can link into the enzyme. When the substrate is bound at the active site the molecule becomes orientated in a fixed position on the enzyme thereby increasing the probability of reaction at specific sites on the substrate [82]. At the active site of hydrolase enzymes are three residues - a serine, a histidine, and an aspartic acid, which are required for catalysis (known as the "catalytic triad"). Histidine acts as an acid-base catalyst in several steps of the reaction mechanism. In the initial step it removes a proton from serine to make serine a more powerful nucleophile and prepare it for covalent catalysis, the substrate then acetylates the serine side chain. The aspartate orients the histidine, and takes part in transition state stabilisation [77, 82]

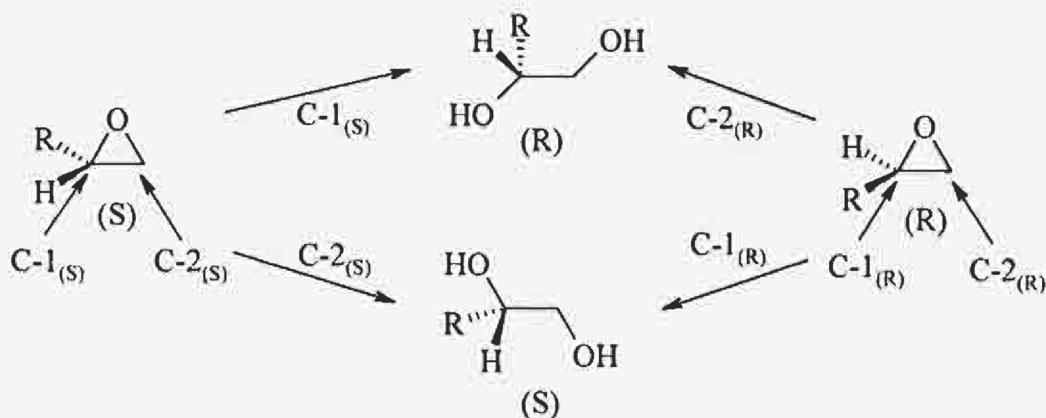
The general mechanism of EHs involves an initial attack of the oxirane by a carboxylate nucleophile (aspartic acid) forming a glycol-monoester-enzyme intermediate (Scheme 2-14). In the second step the intermediate is hydrolysed through the attack of OH^- from water, which is provided by the aid of a histidine residue, thereby releasing the *vic*-diol and liberating the enzyme [84].



Scheme 2-14 The catalytic mechanism of epoxide hydrolase [83, 84]

5.2 Stereoselectivity of epoxide hydrolase

Hydrolysis catalysed by EH can result in the preferential nucleophilic attack of either the benzylic or terminal carbon atom. Scheme 2-15 represents the different possibilities for the attack of a terminal epoxide. Attack of the (S)-enantiomer at the benzylic carbon atom ($C\text{-}1_{(S)}$) will lead to inversion of the original stereochemistry and thus the formation of the (R)-diol. Similarly, if the terminal carbon atom of the (R)-enantiomer ($C\text{-}2_{(R)}$) were preferentially attacked, it would lead to the preferential formation of the (R)-diol with retention of the original stereochemistry. For example the biohydrolysis of styrene oxide with *A. niger and B. sulferecens* (Scheme 2-8) [67]. Nucleophilic attack of the (R)-enantiomer (*A. niger*) at the least substituted carbon atom ($C\text{-}2_{(R)}$) leads to the formation of (R)-1,2-phenylethanediol therefore with retention of configuration. Nucleophilic attack at the benzylic carbon atom of the (S)-epoxide (*B. sulferecens*) however ($C\text{-}1_{(S)}$) also leads to the formation of the (R)-1,2-phenylethanediol thus with inversion of configuration.



Scheme 2-15 Regioselectivity of the oxirane ring opening [85, 30].

6. Conclusion

Pure enantiomers are of great importance in modern society, especially in the field of pharmaceutical drugs. In this chapter the consequences of chirality and the significance of enantiomers was described. An overview of the methods used to obtain pure enantiomers was also given with specific reference to methods of obtaining enantiopure epoxides. Known as "chiral building blocks", this class of substrate can be used during the synthesis of a variety of pharmaceutical substances and, if available in their enantiopure form, could be used to synthesize various pharmaceuticals in their enantiopure form.

The use of EH enzyme from micro-organisms to obtain enantiopure epoxides seems to be a very attractive method. The low cost of the biocatalyst as well as the abundant supply, create a potential for these reactions to replace reactions that involve the use of environmentally damaging heavy metal catalysts.

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Chapter 3

Synthesis of nitro substituted styrene oxides

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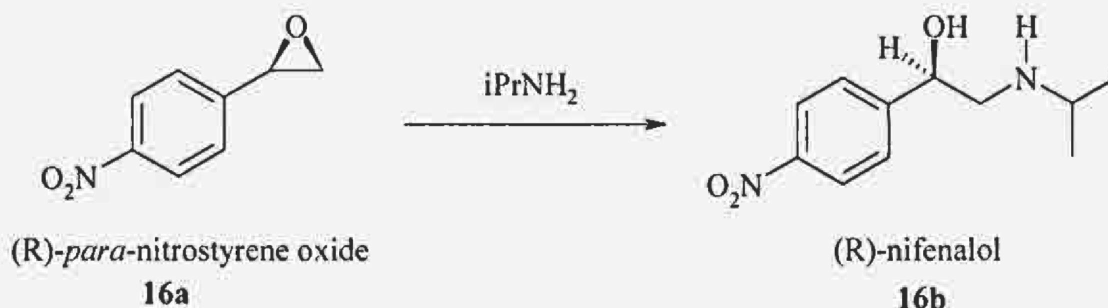
1. Introduction

As described in Chapter 2 (Scheme 2-2), terminal chiral epoxides have a multitude of uses during the organic synthesis of fine chemicals and medicinal drugs. Similarly, styrene oxides are very useful organic synhtons. One example is that of nifenalol® (Scheme 3-1). The synthesis involves the use of enantiomerically pure *para*-nitrostyrene oxide **(16a)** to yield (R)-nifenalol **(16b)**, a drug known to have β-blocking activity mainly attributed to the (R) enantiomer [1].



Scheme 3-1 Synthesis of (R)-nifenalol [1].

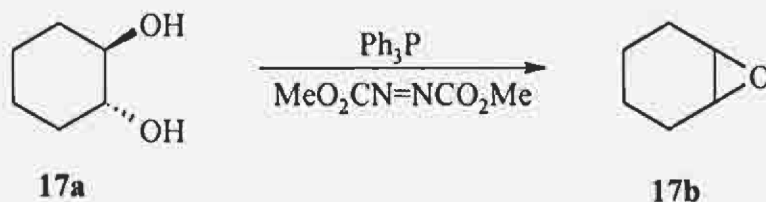
During the present study three epoxides were selected, i.e. *para*-nitrostyrene oxide **(pNSO)**, *meta*-nitrostyrene oxide **(mNSO)** and *ortho*-nitrostyrene oxide **(oNSO)** (Figure 3-1). If enantioselective hydrolysis of these epoxides could be achieved, it would not only present a route towards the pure enantiomers, but also illustrate the effect of the position of the nitro group upon enantioselective hydrolysis. Since none of these products were commercially available, they were all synthesised.

Figure 3-1 Styrene oxides selected for synthesis.

1.1 General synthesis of epoxides

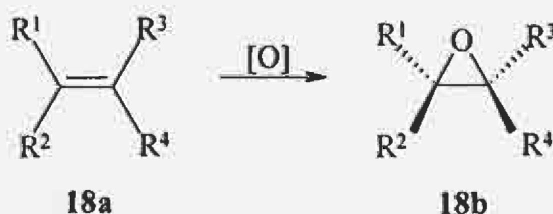
A variety of methods exist for the synthesis of racemic epoxides. Lewars [2] devides these methods into the following groups:

- Synthesis from non-heterocycles
    - By nucleophilic dispacement by oxygen of a β leaving group e.g. dehydration of 1,2-diols (Scheme 3-2).



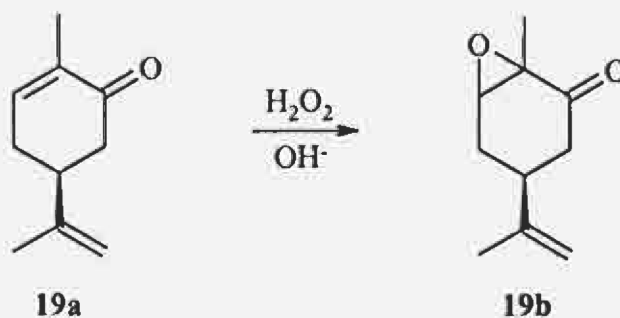
Scheme 3-2

- From [2+1] fragments
    - *By initial electrophilic attack (alkene epoxidation).* This is the most common route to epoxides and is illustrated in Scheme 3-3.



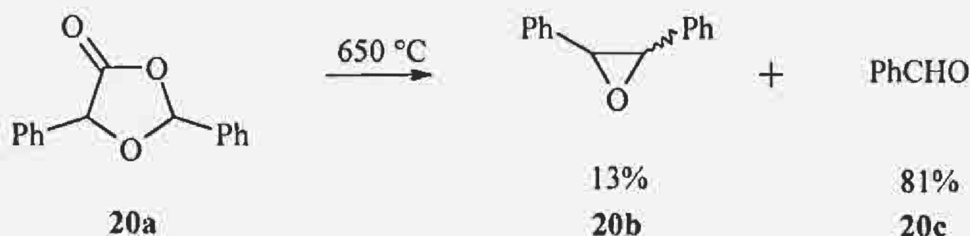
Scheme 3-3

- *By initial nucleophilic attack* e.g. epoxidation with hydrogenperoxide (Scheme 3-4).



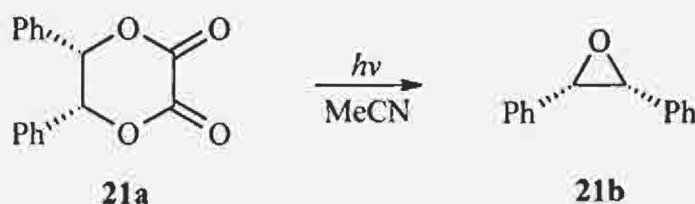
Scheme 3-4

- Synthesis from heterocycles
    - From five-membered heterocycles by extrusion, e.g. flash thermolysis of dioxazolones (Scheme 3-5).



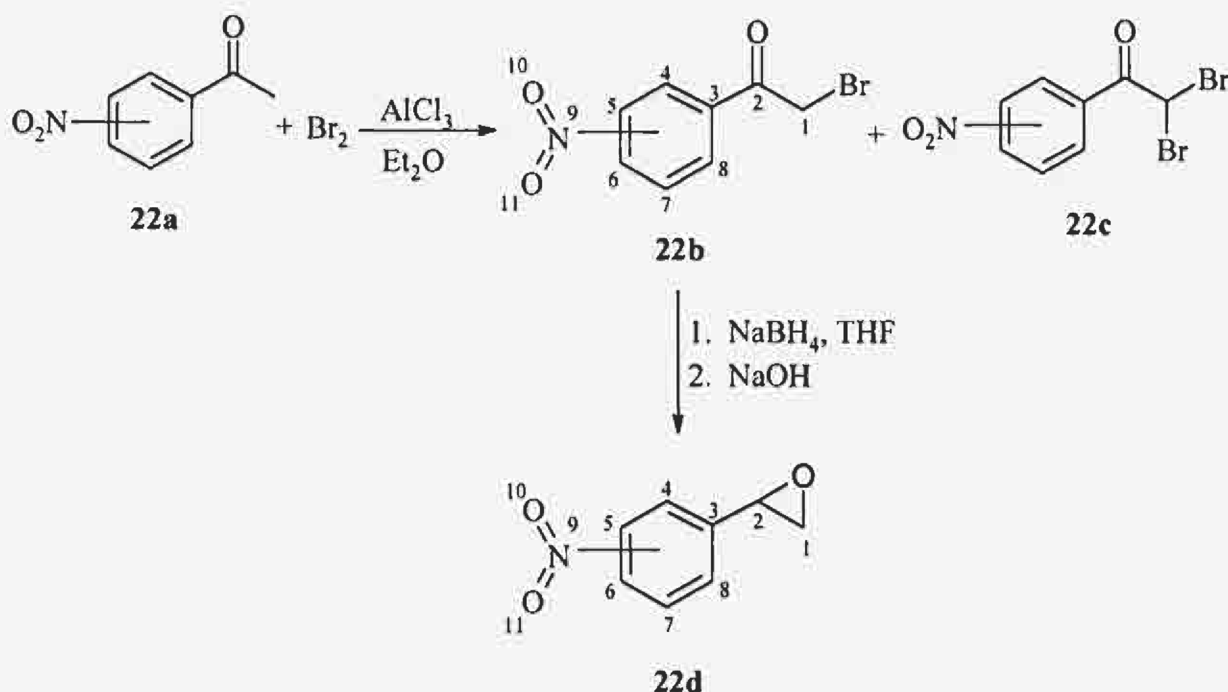
Scheme 3-5

- From six-membered heterocycles by rearrangement or extrusion e.g. photolysis of cyclic oxalates (Scheme 3-6).



Scheme 3-6

Scheme 3-7 illustrates a general method for the synthesis of nitro styrene oxides that was also used in this project. Starting with the corresponding nitro substituted acetophenone **(22a)**, bromination can be effected through the use of $AlCl_3$ as catalyst [3, 4]. This reaction leads to the formation of both the mono-bromide **(22b)** and di-bromide **(22c)** products. After isolation of the mono-bromide through chromatography, it can be epoxidised using $NaBH_4$ as catalyst [4].



Scheme 3-7 General method of synthesis of nitro substituted styrene oxides.

1.2 Chemistry of phenyl-substituted styrene oxides

Since studies conducted previously on the mechanism of epoxide hydrolase attribute it to a nucleophilic attack [5, 6, 7], this section will focus mainly on the chemical properties of phenyl-substituted styrene oxides that might influence nucleophilic attack.

The electronic properties of a substituent to an epoxide ring influence the position where preferred attack by a nucleophile will take place. For example in Figure 3-2, if the R group is electron withdrawing **(23a)**, nucleophilic attack at the most substituted or benzylic carbon atom will be promoted. If the R group is electron donating **(23b)**, nucleophilic attack at the least substituted carbon atom will be promoted.

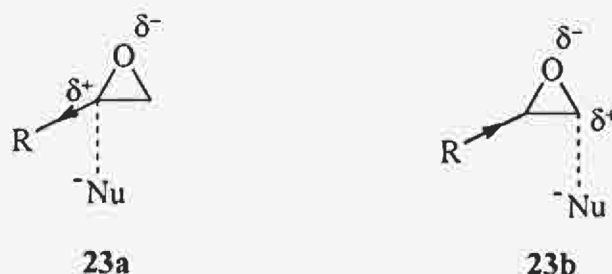


Figure 3-2 Effect of substitution on nucleophilic attack.

In the case of nitro styrene oxides, the nitro group exerts a very powerful electron withdrawing effect. This leads to the promotion of nucleophilic attack at the benzylic carbon atom. Figure 3-3 illustrates the effect of substitution at the *para*, *meta* and *ortho* position. In the case of **pNSO (24a)** and **oNSO (24c)** the electron withdrawing effect is the greatest because of a combination of the resonance and the inductive effect. Nucleophilic attack when the nitro group is in the ortho position may however be negatively influenced because of steric interference. When considering **mNSO (24b)** it can be seen that, even though an electron withdrawing effect still exists (only inductive), it is much less pronounced than in the case of **pNSO** and **oNSO.**

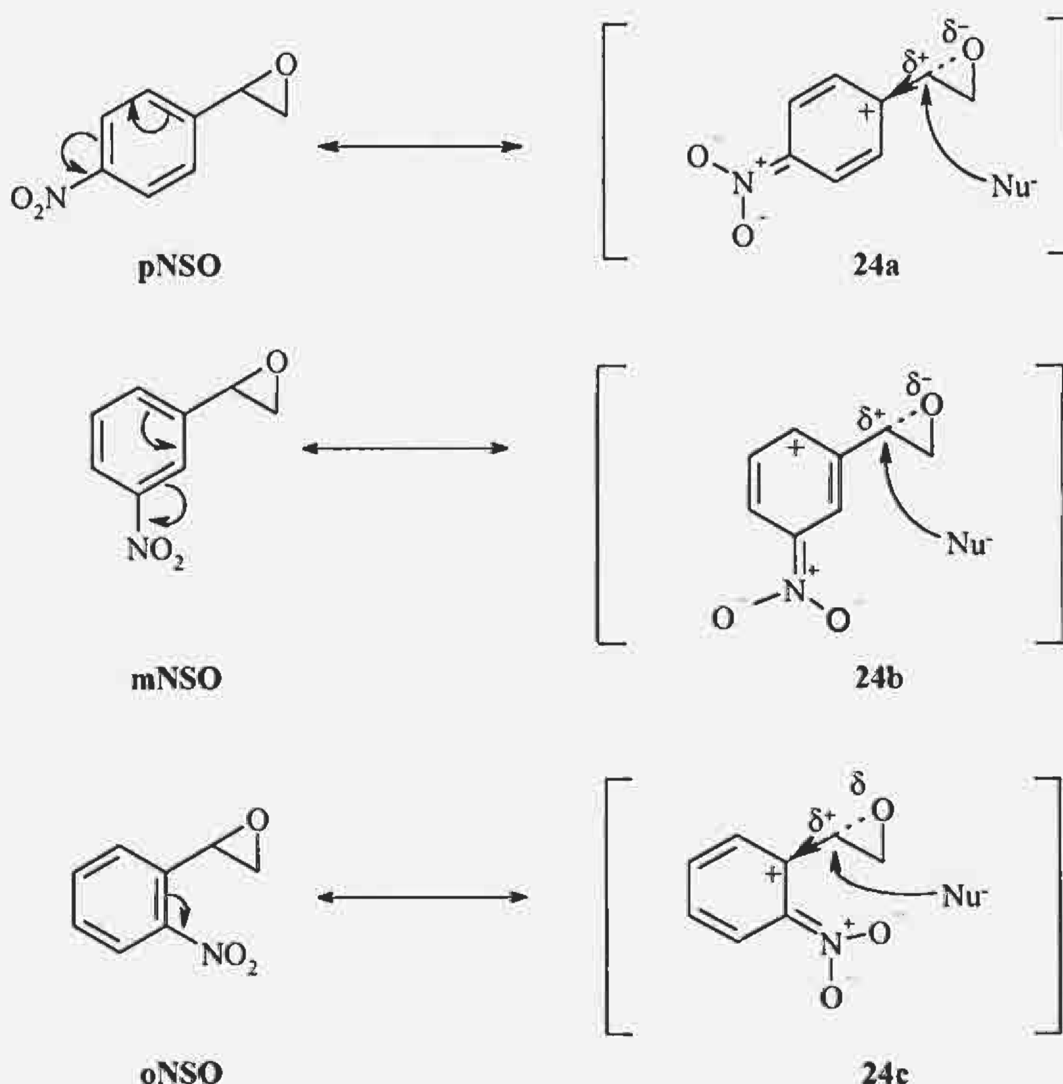


Figure 3-3 Effect of the position of the nitro group on transition state.

1.3 NMR and MS analysis of styrene oxides

During the NMR analysis of styrene oxides, the signals presented by the epoxide ring can be explained with Figure 3-4.

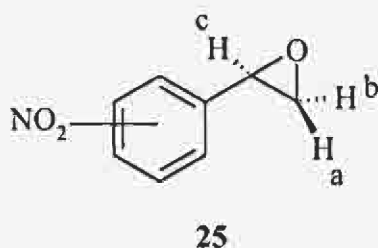


Figure 3-4 Phenyl-substituted styrene oxides.

H_a and H_b will present geminal spin-spin coupling. H_c will couple with H_a and H_b in different ways because H_a is *trans* and H_b is *cis* to H_c [8]. This also leads to the appearance of three different signals while only two protonated carbons exist. During mass spectrometry (MS) analysis phenyl-substituted styrene oxides tend to show only weak molecular ion peaks, because of rearrangement and fragmentation [2] (i.e. Appendix 1, spectrum 9).

2. Experimental

2.1 General method

All reactions were preformed under atmospheric pressure using air-dried glassware. The commercially available reagents were used as received without any further purification. The structures of the styrene oxides and of ortho-nitrophenacyl bromide were numbered as shown in **22d** and **22b** respectively (Scheme 3-2). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini-300 spectrometer in $CDCl_3$ at room temperature. ^{13}C NMR spectra were recorded at a frequency of 75,462 MHz while ^{1}H spectra were recorded at a frequency of 300,075 MHz. Chemical shifts are given in parts per million (ppm) downfield from tetramethylsilane. EI (electron impact) mass spectra were recorded on a VG70-70E mass spectrometer fitted with an Ion Tech B11N saddle field gun and a VG11-250J data system. All spectra are given in Appendix 1. Melting points were determined with a digital Büchi B-540 melting point apparatus.

2.2 Para-nitrostyrene oxide (pNSO)

To 2,50 g (10,2 mmol) of *p*-nitrophenacyl bromide (obtained from Aldrich) in 25 ml of methanol and 35 ml of THF at + 4 °C was gradually added 500 mg of sodium borohydride. The reaction was stirred at + 4 °C until completion. Hereafter 35 ml of 2 N NaOH was gradually added. After stirring at + 4 °C until completion, the reaction mixture was quenched with acetic acid (pH ≈ 6), extracted with 150 ml of ethyl acetate, washed with saturated sodium bicarbonate and water, and dried over sodium sulphate [4]. The product obtained after evaporation of the solvent was purified by flash chromatography using ethyl acetate and dichloromethane (1:1) as mobile phase and silica gel as stationary phase. Finally the product was recrystallised from ethanol. The synthesis yielded 1,5 g (52%) of light yellow crystals confirmed by NMR, MS and melting point analysis as being pure pNSO; mp 83,4 °C (79-81 °C [4]); $C_8H_7NO_3$; M^+ 165; m/z (EI, %) (Spectrum 1): 165 (40), 164 (22), 148 (90), 118 (94), 91 (62), 89 (100), 77 (24), 63 (84), 51 (49), 39 (59), 28 (61); δ_H (Spectrum 3): 2,82-2,65 (dd, 1H, H-2), 3,27-3,10 (dd, 1H, H-1a/H-1b), 4,00-3,86 (dd, 1H, H-1a/H-1b), 8,25-8,04 (d, 2H, H-4, 8), 7,49-7,33 (d, 2H, H-5, 7); δ_C (Spectrum 2): 51,30 (C-2), 51,47 (C-1), 145,36 (C-3), 148,00 (C-6), 126,25 (C-4, 8), 123,80 (C-5, 7); **DEPT** (Spectrum 4): CH_3 = 0, CH_2 = 1, CH = 3, Total protonated carbons = 4.

2.3 meta-Nitrostyrene oxide (mNSO)

The racemic epoxide was prepared in the same way as the para derivative, except that 2,50 g (10,2 mmol) *m*-nitrophenacyl bromide (obtained from Aldrich) was used. The synthesis yielded 1,52 g (89,8%) of a light yellow oil confirmed by NMR and MS analysis as being pure mNSO; $C_8H_7NO_3$; M^+ 165; m/z (EI, %) (Spectrum 5): 165 (39), 164 (29), 148 (73), 118 (73), 91 (76), 89 (100), 77 (48), 63 (85), 51 (64), 39 (74); δ_H (Spectrum 7): 2,84-2,69 (dd, 1H, H-2), 3,25-3,11 (dd, 1H, H-1a/H-1b), 3,99-3,88 (dd, 1H, H-1a/H-b), 7,65-7,55 (m, 1H, H-4/H-5), 7,55-7,43 (m, 1H, H-4/H-5), 8,19-8,05 (m, 2H, H-6, 8); δ_C (Spectrum 6): 51,25 (C-2), 51,30 (C-1), 120,62 (C-3/C-4/C-5/C-6, 8), 123,12 (C-3/C-4/C-5/C-6, 8), 129,62 (C-3/C-4/C-5/C-6, 8), 131,51 (C-3/C-4/C-5/C-6, 8); **DEPT** (Spectrum 8): CH_3 = 0, CH_2 = 1, CH = 5, Total protonated carbons = 6.

2.4 ortho-Nitrostyrene oxide (oNSO)

2.4.1 Synthesis of o-nitrophenacyl bromide

2,0 g (12,1 mmol) of *o*-nitroacetophenone was diluted in 40 ml of anhydrous ether. The solution was cooled in an ice bath and 500 mg of aluminium chloride was introduced. 2,0 g (12,5 mmol) of bromine was gradually added and, after completion of the reaction, both the ether and dissolved hydrogen were removed at once under reduced pressure [9]. The reaction yielded both the di-bromide, as well as the monobromide derivatives. The product was purified by flash chromatography using silica gel as stationary phase and benzene as mobile phase. Finally the product was recrystallised from methanol. The synthesis yielded 1,4 g (47%) of a pure white crystalline product, confirmed by NMR as being ortho-nitrophenacyl bromide; mp 55,3 °C (55-56 °C [10]); $C_8H_6BrNO_3$; M^+ 244; m/z (EI, %) (Spectrum 14): 244 (5), 236 (9), 228 (7), 213 (9), 191 (11), 170 (15), 149 (26), 137 (16), 129 (20), 109 (26), 97 (39), 95 (48), 91 (11), 71 (52), 69 (100), 57 (91), 55 (83), 43 (91), 41 (79), 29 (33); δ_H (Spectrum 16): 4,27 (s, 2H, H-1), 8,42-8,09 (d, 1H, H-4/H-5/H-6/H-7), 7,86-7,70 (t, 1H, H-4/H-5/H-6/H-7), 7,68-7,60 (t, 1H, H-4/H-5/H-6/H-7), 7,50-7,40 (d, 1H, H-4/H-5/H-6/H-7); δ_C (Spectrum 15): 33,62 (C-1), 194,39 (C-2), 131,31 (C-3/C-4, 8/C-5, 7/C-6), 134,74 (C-3/C-4, 8/C-5, 7/C-6), 129,22 (C-3/C-4, 8/C-5, 7/C-6), 124,48 (C-3/C-4, 8/C-5, 7/C-6); **DEPT:** CH_3 = 0, CH_2 = 1, CH = 4, Total protonated carbons = 5.

2.4.2 Synthesis of o-nitrostyrene oxide

The racemic epoxide was prepared in the same way as the *para* and *meta* derivatives, using 1,4 g (5,7 mmol) *o*-nitrophenacyl bromide. The synthesis yielded 540 mg (57%) of white

crystals confirmed by NMR, MS and melting point analysis as being pure oNSO; mp 63,4 °C (62-63,5 °C [4]); M^+ 165; m/z (EI, %) (Spectrum 9): 165 (14), 164 (6), 135 (30), 91 (63), 77 (93), 63 (84), 51 (54), 39 (59); δ_H (Spectrum 12): 2,79-2,47 (dd, 1H, H-2), 3,74-3,09 (dd, 1H, H-1a/H-1b), 4,61-4,21 (dd, 1H, H-1a/H-1b), 7,76-7,33 (m, 3H, H-4, 5, 6, 7), 8,26-7,97 (d, 1H, H-4, 5, 6, 7); δ_C (Spectrum 11): 50,48 (C-2), 50,54 (C-1), 124,687 (C-3/C-4, 8/C-5, 7/C-6), 127,044 (C-3/C-4, 8/C-5, 7/C-6), 128,587 (C-3/C-4, 8/C-5, 7/C-6), 134,300 (C-3/C-4, 8/C-5, 7/C-6); **DEPT:** CH_3 = 0, CH_2 = 1, CH = 5, Total protonated carbons = 6.

3. Conclusion

The results that were obtained through the use of NMR, MS and melting point analysis conclusively prove that all three the epoxides were obtained by the proposed method of synthesis. Even though the final yields obtained are low compared to alternative methods of synthesis that have been reported, for example Guss who reported a 88,5% overall yield for the synthesis of oNSO [11], the products were found to be chemically pure and could be used in further experiments without any further purification.

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Chapter 4

Screening of yeasts for the enantioselective hydrolysis of nitro substituted styrene oxides

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1. Introduction

The resolution of chiral epoxides into their pure enantiomers has become a very well researched subject in recent years. The main reason for this being the high value of the pure enantiomers of the epoxides, as well as their corresponding *vicinal* diols, as valuable building blocks during the organic synthesis of various pharmaceutical products and fine chemicals [1, 2, 3]. In addition to chemo-catalytic methods, the use of epoxide hydrolases (EHs) is a new, very actively emerging strategy for the access to enantiopure epoxides or vicinal diols [2]. Several biocatalytic methods utilising EHs for the production of enantiomerically pure epoxides have been evaluated as viable alternatives to other methods (Chapter 2, Section 4). Although EHs from mammalian origin are known to effect kinetic resolution of epoxides during the metabolism of xenobiotic compounds, their usefulness is limited due to their poor availability. The continued explorations into new biocatalytic methods lead to the discovery of highly enantioselective, abundantly available EHs, mainly originating from bacteria and filamentous fungi [4].

De Bont and Weijers [5, 6] found that the yeast strain *Rhodotorula glutinis* CIMW 147 displayed enantioselectivity during the hydrolysis of styrene oxide and various derivatives. Enantioselectivity was, without exception, preferential towards the hydrolysis of the epoxides (R)-enantiomer, leading to the formation of the corresponding (R)-diol in excess. Another example of the enantioselective hydrolysis exhibited by yeasts is that of 1,2-epoxyoctane. Hydrolysed by a strain of *R. toruloides*, this reaction also presented excellent enantioselectivity towards the hydrolysis of the (R)-epoxide and the formation of the (R)-diol [4, 7].

Various studies have been completed on styrene oxides and their derivatives, especially those with para-substituted phenyl rings, because of their high value in organic synthesis [8, 9, 10]. The aforementioned studies proved that substitution of the aromatic ring affects the reaction rate as well as the enantioselectivity to an extent that seems to depend upon the electronic properties of the substituents. Most of these studies however, were conducted using only EH from fungal or bacterial origin. The reported results from these studies were convincing factors when the decision was made to study the effect of nitro substitution to styrene oxide upon EH activity from yeast origin. Not only do the nitro derivatives have multiple uses for the organic synthesis of pharmaceutical products (antibacterial, antileukemic, antianginal and

antiarhythmatic drugs) [11, 12, 13], but these results would also deliver a better understanding of the influence of the position of nitro substitution on the enantioselectivity of the yeasts.

During the present study three nitro derivatives, *para*-nitrostyrene oxide **(pNSO)**, *meta*-nitrostyrene oxide **(mNSO)** and *ortho*-nitrostyrene oxide **(oNSO)** were chosen, and together with styrene oxide **(SO)**, were used to screen for enantioselective epoxide hydrolase (EH) activity within the yeast domain

2. Results and discussion

2.1 Screening

The results of the initial screening for EH activity are summarised in Table 4-1 (**SO** is not included because of the fact that it was not included in the initial screening, only 36 organisms with high enantioselective activity towards the nitro substituted styrene oxides were chosen and used to screen for the enantioselective hydrolysis of **SO**). EH activity was found to be widespread throughout the screened yeast domain, while the genera *Candida, Debaryomyces, Pichia, Rhodosporidium, Rhodotorula and Trichosporon* specifically were very successful in catalysing the hydrolysis of the substrates. When the enantioselectivity of the hydrolysis was analysed however, it was found that only a few of these reactions were enantioselective, but for the most part, were still localised in the aforementioned species. This corresponds well with previously reported results where strains from *Rhodosporidium sp.*, *Rhodotorula* sp. and *Trichosporon* sp. [4, 5] were shown to have enantioselective EH activity.

Twelve of the most promising yeast strains were chosen on the basis of their *ee* values for each substrate, namely 2 strains from *Debaryomyces* sp., 1 strain from *Rhodosporidium* sp., 3 strains from *Candida* sp., 2 strains from *Rhodotorula* sp. and 4 as yet unidentified strains. A complete list of the screened organisms together with the results from the time course experiments performed with the selected strains is given in Appendix 2. From this list it can be seen that only a few organisms were able to catalyse the hydrolysis of **oNSO**, presumably as a result of steric hindrance caused by the nitro group at the active site of the enzyme. This is confirmed by the fact that a much larger amount of organisms were able to catalyse the hydrolysis of **pNSO** and **mNSO**, where the nitro group is positioned further away from the epoxides ring.

Table 4-1 Distribution of epoxide hydrolase (EH) activity towards nitro substituted styrene oxides within the screened yeast domain.

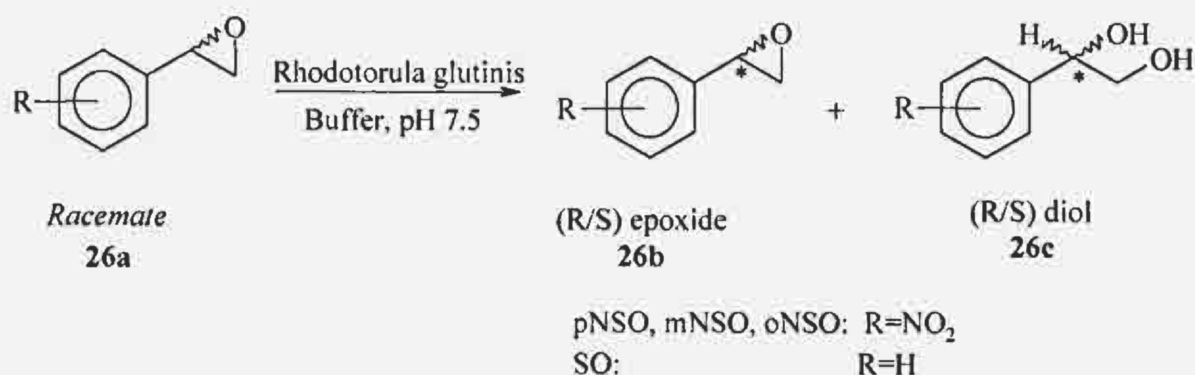
Genera	Number of species screened	Number of strains screened	Number with EH activity towards **pNSO**	Number with EH activity towards **mNSO**	Number with EH activity towards **oNSO**
Arthroascus	3	3			
Arxula	2	9	5	1	
Brettanomyces	4	5			
Bullera	1	2	1		
Bulleromyces	1	1			
Candida	30	52	19	16	
Chryseomonas (bacterium)	1	1	1	1	
Cryptococcus	1	1	1	1	
Cystofilobasidiella	1	1			
Cystofilobasidium	2	2			
Debaryomyces	1	27	17	3	
Dekkera	2	2			
Dipodascopsis	7	7			
Dipodascus	1	1			
Endomyces	2	2	1	1	
Endomycopsella	2	2			
Filobasidium	3	4		1	
Galctomyces	1	1			
Geotrichum	2	4	1	1	
Hormonema	1	1	1		
Hyphopichia	1	1			
Kluyveromyces	2	2	1		
Leucosporidium	1	1	1		
Lipomyces	2	2	1	1	
Myxozyma	2	2			
Pichia	7	22	6	6	

Genera	Number of species screened	Number of strains screened	Number with EH activity towards **pNSO**	Number with EH activity towards **mNSO**	Number with EH activity towards **oNSO**
Rhodosporidium	4	7	3	4	
Rhodotorula	14	44	20	23	2
Saccharomyces	2	1			
Saccharomycopsis	6	13			
Sporidiobolus	1	2	1		
Sporobolomyces	2	2		1	
Trichosporon	7	37	12	8	3
Wickerhamiella	1	1			
Wingea	1	1	1	1	
Yarrowia	1	7	2	1	
Unidentified		137	79	77	17
Total	132	410	177	148	22

2.2 Enantioselective hydrolysis

The 12 selected strains were used for time course reactions and the formation of the diols was analysed by chiral GC to reveal the optimal time for enantioselective hydrolysis for each of the strains. From these results one strain of *Rhodotorula glutinis* (UOFS Y-0653) was chosen because of its ability to enantioselectively hydrolyse **pNSO**, **mNSO**, **oNSO** as well as **SO** (Scheme 4-1). In addition the enantioselectivity of a strain of *Rhodosporidium toruloides* (UOFS Y-0471) for the hydrolysis of pNSO was investigated since the enantioselectivity of *Rhodotorula glutinis* for this substrate was very poor. The results of the GC analysis are shown in Figure 4-1. Unfortunately only the enantiomers of the epoxides could be analysed by GC. The use of several different fused cyclodextrin capillary columns (α-dex 120, β-dex 120, β-dex 225) was investigated and were found to be unable to sufficiently separate the produced diols into their enantiomers. This was true for all the produced diols except for 1-phenyl-1,2-ethane diol (produced by the hydrolysis of styrene oxide), which was analysed with a β-dex 120 column. The absolute configurations of the enantiomers were identified by adding a small amount of pure (S)-styrene oxide to the racemate (an example is given in Appendix 3, Chromatogram 1). Chiral GC analysis proved that, for styrene oxide, the enzyme enantioselectively hydrolysed the (R)-enantiomer of the epoxide to yield the (R)-enantiomer of the corresponding *vicinal* diol (an example is give in Appendix 3,

Chromatogram 2). To determine the absolute configuration of the residual epoxide and formed diol of **pNSO**, the directions of optical rotation were measured and compared to the values reported in the literature [8, 12]. This illustrated that the strain of *Rhodosporidium toruloides* hydrolysed the (R)-enantiomer of **pNSO** and, in contrast to the aforementioned reaction, produced the (S)-enantiomer of the corresponding diol. Optical rotations for **mNSO** and **oNSO** could not be found in the literature and were therefore not determined.



Scheme 4-1 Enantioselective hydrolysis of styrene oxide and its nitro derivatives.

The *ees*, the yields of the reactions, as well as the absolute configurations of the residual epoxides and the formed *vicinal* diols are reported in Table 4-2. From the absolute configurations of the residual epoxides and the formed *vicinal* diols, it can be seen that for **pNSO**, one yeast strain, *R. glutinis*, hydrolysed the (S)-enantiomer of the epoxide. Unfortunately the enantioselectivity of this reaction was so low that the absolute configuration of the formed diol could not be determined with certainty. *R. toruloides* however was found to hydrolyse the epoxide with a much greater enantioselectivity and this reaction could therefore be used in the determination of the absolute configuration by measuring the optical rotation of the products.

Reaction rates during the biocatalysis with *Rhodotorula glutinis* decreased in the order *meta>para>ortho* while unsubstituted styrene oxide's reaction rate was similar to that of mNSO (Figure 4-1 and Table 4-2). This shows that a strong electron withdrawing effect negatively influenced the reaction rate. These results are consistent with the results reported by Grogan *et al* [14]. Using the fungus *Beauveria densa* CMC 3240 they found that it did not catalyse the reaction with **pNSO** and attributed the phenomenon to the powerful electron withdrawing effect of the nitro group. This effect negatively influences attack at the benzylic carbon atom, the preferred site of attack for this enzyme. Since **oNSO** exhibited an even

lower reaction rate than **pNSO** other factors have to be implicated as well, such as restricted access to the terminal carbon atom because of steric interference and also because of steric hindrance of the substrate to fit into the catalytic site of the enzyme.

It would seem that the hydrolysis of **pNSO** catalysed by the EH enzyme found in *R. toruloides* is preferential towards nucleophilic attack at the benzylic carbon atom, which is similar to that exhibited by *Beauveria sulferescens* for styrene oxide and various other phenyl-substituted styrene oxides [15, 16]. In the case of **pNSO** however *B. sulfurescens* was shown to promote nucleophilic attack at the terminal carbon atom leading to the retention of configuration, but with poor enantioselectivity [16]. In contrast to this *R. toruloides* hydrolysed **pSNO** with inversion of configuration.

The fact that *R. glutinis* hydrolysed styrene oxide with retention of the original stereochemistry seems to indicate that attack is preferential towards the terminal carbon atom, as is seen with *Aspergillus niger* [15]. We could therefore assume that *R. glutinis* would lead to the preferential formation of the (R)-diol when catalysing the hydrolysis of pNSO. This would however have to be confirmed by optimising the enantioselectivity to such a degree that the optical rotation could be determined accurately. In the same sense, if the hypothesis concerning the preferred site of attack were true, it could be predicted that the hydrolysis of **mNSO** and **oNSO** with *R. glutinis* would be preferential towards attack at the terminal carbon atom. Once again this would have to be proven by a method such as x-ray crystallography.

The absolute configurations that were determined and the proposed regioselectivities are in perfect agreement with the electronic properties of the epoxide ring discussed in Chapter 3 (Section 1.2). For **pNSO** the benzylic carbon atom is the preferred site for nucleophilic attack, this effect is however less pronounced for **SO** and **mNSO** because of the absence of the resonance effect. Therefore the inability of *R. glutinis* to effectively hydrolyse **pNSO** could be a result of the reduced stability of an acid generated carbocation and point to an enzyme-catalysed hydronium-ion dependant hydrolysis with preferential attack at the terminal carbon atom as described by Grogan *et al.* [14].

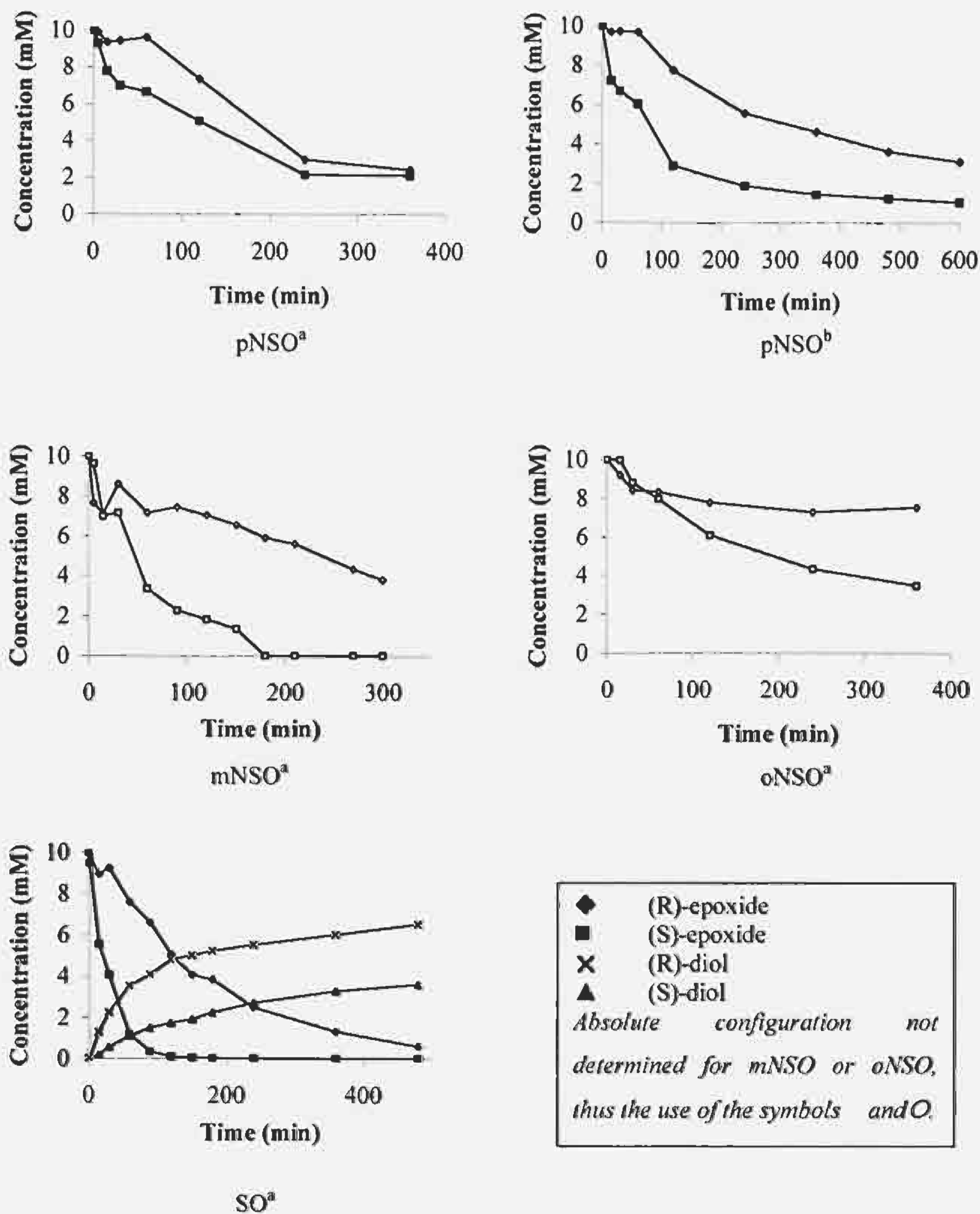


Figure 4-1 Enantioselective hydrolysis of chiral substrate and product formation over time during hydrolysis by *Rhodotorula glutinis*[a] and *Rhodosporidium toruloides*[b] (mM = 10^{-3} mol.dm^{-3}).

Table 4-2 Hydrolysis of various epoxides by *Rhodotorula glutinis* (UOFS Y-0653)[a] and *Rhodosporidium toruloides (UOFS Y-0471)*[b].

	Epoxide (residual)				Diol (product)			
	ee (%)	Yield (%)	Reaction time (h)*	Absolute configuration	*ee* (%)	Yield (%)	Reaction time (h)	Absolute configuration
pNSO[a]	18	13	1	(+) (S)	-	-	-	n.d.
pNSO[b]	55	35	6	(+) (S)	-	-	-	(+) (S)
mNSO[a]	>98	29	3	n.d.	-	-	-	n.d.
oNSO[a]	39	19	8	n.d.	-	-	-	n.d.
SO[a]	>98	19	3	(-) (S)	39	15	3	(-) (R)

n.d.: Not determined

*: Time where *ee* was highest

3. Experimental

3.1 General

All yeasts were obtained from the Yeast Culture Collection of the University of the Free State. Reactions were monitored and enantiomeric purities were analysed by GC (Hewlett-Packard 5890 series II equipped with FID) on fused silica cyclodextrin capillary columns (β-dex 225 (epoxides) and β-dex 120 (diols), obtained from Supelco) using N_2 as carrier gas. Optical rotations were measured with an Officine Galileo visual polarimeter at the sodium D-line.

3.2 Materials

pNSO, **mNSO** and **oNSO** were all synthesised as described in Chapter 3. **SO** was purchased from Sigma-Aldrich. Pure (S)-(-)-styrene oxide and (S)-(+)-1-phenyl-1,2-ethanediol were both obtained from Sigma-Aldrich.

3.3 Cultivation and preparation of yeast cells

Yeasts were grown in 250 ml shake-flask cultures containing 50 ml YM media (0,5% yeast extract, 2,0% malt extract, 0,5% peptone w/v) supplemented with 1,5% (w/v) glucose. 2,0% (v/v) of a vitamin solution was added through filter sterilisation to increase growth and decrease incubation time. The cells were centrifuged in falcon centrifuge tubes, the supernatant discarded, and the cells washed with phosphate buffer (50 mM, pH 7,5). After this the cells were resuspended in phosphate buffer containing 15% glycerol (1 ml of cells in 4 ml of buffer). 500 μl aliquots of the suspension were dispensed in aliquots of 1,5 ml into micro-centrifuge tubes and frozen. After several months of storage at below –15 °C the cells showed no significant loss of activity.

3.4 Screening for hydrolysis of styrene oxide and its nitro-derivatives.

The search for enantioselective yeast epoxide hydrolase enzymes with activity towards **SO** and its nitro derivatives, **pNSO**, **mNSO** and **oNSO**, was completed with a screening of 410 strains from more than 44 yeast genera. For the initial small-scale screening, hydrolase activity was identified by thin layer chromatography (TLC) using commercially available silica gel plates (Merck 5554 DC – Alufolien 60 F_{254}). Frozen cells were thawed (500 μl) after which the concerned nitro-derivative (10 μl of a 1 M DMSO stock solution) was added to give a final concentration of 20 mM. The reaction mixtures were incubated at 30 °C for 4 h with continuous shaking. Reactions were terminated by extracting the epoxide and formed

diol with 250 µl ethyl acetate. Compounds were visualised by spraying with vanillin/conc. H_2SO_4 (10 g/l). Only 36 organisms that exhibited enantioselective activity towards **pNSO, mNSO** or **oNSO** were used to determine enantioselective activity towards **SO.**

3.5 Procedure for time-course experiments.

Epoxides that were enantioselectively hydrolysed by certain yeast strains were identified by the chiral GC analysis of samples taken at appropriate time intervals. Frozen cells (500 µl) were thawed and substrate (10 µl of a 1 M DMSO stock solution) was added to give a final concentration of 20 mM. The reaction mixtures were incubated at 30 °C with continuous shaking. At different time intervals one micro-centrifuge tube of each reaction (poor substrate solubility prevented sample extraction from a single batch reaction) was removed and the epoxide and formed diol extracted with 250 µl of ethyl acetate. Chiral GC of the isolated products after biohydrolysis were done as follows:

Epoxides: pNSO, 150 °C, t_R (R) 41,0 min and t_R (S) 43,0 min,
mNSO, 150 °C, t_R (1) 26,9 min and t_R (2) 27,6 min*,
oNSO, 130 °C, t_R (1) 27,0 min and t_R (2) 27,6 min*,
SO, 90°C, t_R (S) 16,9 min and t_R (R) 17,5 min.

Vicinal diols: SO, 140°C, t_R (S) 37,0 min and t_R (R) 38,6 min.

* (1) and (2) represent the enantiomer to elute first and second respectively from the chiral GC column.

4. Conclusion

Enantioselectivity of one organism (*Rhodotorula glutinis* UOFS Y-0653) for all of the substrates **SO**, **pNSO**, **mNSO**, **oNSO** was demonstrated. To the best of our knowledge, this is the first time that enzymatic resolution of **mNSO** and **oNSO** is being reported. Another yeast strain (*Rhodosporidium toruloides* UOFS Y-0471) was found to have a higher enantioselectivity towards pNSO, but in contrast to the retention of stereochemistry observed with **SO** and *Rhodotorula glutinis*, proceeded with inversion of the original stereochemistry of the epoxide. Unfortunately the absolute configurations of the diols produced from the reaction of *R. glutinis* with **pNSO**, **mNSO** and **oNSO** could not be established and therefore it could not be determined whether these reactions proceeded with inversion or retention of the original stereochemistry.

The results clearly show that the yeast strains, *Rhodotorula glutinis* UOFS Y-0653 and *Rhodosporidium toruloides* UOFS Y-0471, can effectively be employed as biocatalysts for the enantioselective hydrolysis of *para-, meta-, and ortho*-nitrostyrene oxide as well as unsubstituted styrene oxide. The yields that were obtained during this study are comparatively low to those obtained by certain filamentous fungi and bacterial EHs [16], however yeasts have previously been shown to have the ability to hydrolyse very high concentrations of substrate without significant loss of activity [17] whereas EH from the fungus *A. niger* LCP521 is inhibited by the product when concentrations of styrene oxide higher than 2.5 g/L (approx. 21 mM) are used. This inhibition was overcome by removing the product and recycling the cells as well as increasing the fungus cell concentration [18], allowing the use of 10 g/L of substrate. The fact that yeasts have not shown this inhibition increases their potential for the preparative scale production of single enantiomers of epoxides as well as their corresponding vicinal diols. The simplicity with which these organisms can be grown and stored, as well their ability to hydrolyse a broad range of substrates further adds to this potential.

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Chapter 5

Optimisation of enantioselective reactions

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1. Introduction

Enzymes in general have three unique characteristics. Firstly, they are very efficient catalysts, possessing the ability to significantly accelerate a reaction. Secondly, most enzymes can be distinguished by a specifity of action, in other words an enzyme catalyses the conversion of a preferred substrate. The third characteristic is that the activity of many enzymes are regulated, meaning that they can change from a state of low activity to one of high activity and back [1].

All enzymes, except epimerases, exhibit at least some optical specifity [2]. In the case of epoxide hydrolases, enantioselective hydrolysis of epoxides is catalysed via the *trans* addition of water to the epoxide ring, resulting in the formation of the corresponding *vicinal* diol [3]. Many examples of enantioselective biocatalytic enzymes can be found in literature, for example the lipase enzyme found within *Candida rugosa* [4], the epoxide hydrolase enzyme found within *Bacillus megaterium* [5], *Aspergillus niger* [6] and *Syncephalastrum racemosum* [7], and the alcohol dehydrogenase enzyme found within *Candida parapsilosis* [8]. By varying certain parameters such as pH, temperature, co-solvent and substrate concentration these reactions were tuned to yield optimal enantioselectivity. Cleij *et al.* [9] reported another interesting phenomenon, using a two-liquid-phase reaction with a soluble EH extract from *Aspergillus niger* LCP 521 for the enantioselective resolution of *para*-bromo-α-methyl styrene oxide. In the biphasic system the E value of the reaction was increased 13-fold when compared to a regular batch reaction in aqueous phase.

In Chapter 4 results were presented on the screening of yeast strains that exhibit epoxide hydrolase (EH) activity towards certain phenyl-substituted styrene oxides namely **pNSO**, **mNSO**, **oNSO** and **SO**. From the results (Table 4-1) it was established that two yeast strains *Rhodotorula glutinis* (UOFS Y-0653) and *Rhodosporidium toruloides* (UOFS Y-0471) had significant activity for these four substrates. Due to the industrial and pharmaceutical importance of phenyl-substituted styrene oxides (Chapter 2), it was decided to try and improve upon these results. Therefore, in this chapter, the variation of various parameters and the effect thereof on the *ee*, yield and reaction rate is presented.

2. Results and discussion

2.1 Optimisation of pH

To determine the optimal pH value for the EH enzyme, *R. glutinis* UOFS Y-0653 was used to catalyse the hydrolysis of styrene oxide. A cell free extract was also prepared to determine the effect of mass-transfer upon the reaction and to determine whether the whole cells offered any protection to the enzyme from its surroundings.

The optimal pH for the hydrolysis reaction was determined by analysing the amount of formed diol by GC analysis (Figure 5-1). Within the whole cells (1-1) the enzyme exhibited high activity across a broad range of pH values, while the cell free extract (1-2) demonstrated a peak in activity at a pH value of 7,2.

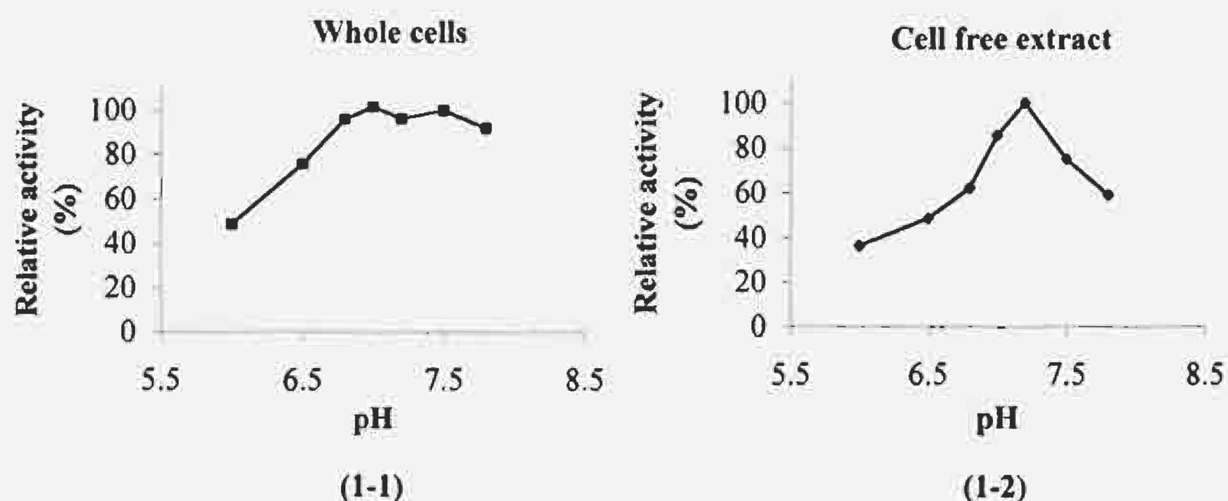


Figure 5-1 Formation of 1,2-phenylethane-diol at different pH values.

The ability of the whole cells to efficiently catalyse the reaction over a broad range of pH (6,5-7,5) values was probably due to the physical protection offered by the cell membrane against the immediate environment. A decrease in activity was observed at both slightly basic (pH 6,0) as well as slightly acidic (pH 8,0) conditions leading to the conclusion that a relatively neutral pH value of between 7,0 and 7,5 would be most advantageous for this reaction. This correlates well with previous work preformed using resting yeast cells at a pH value of 7,5 [10, 11]. The cell free extract showed a peak in selectivity at pH 7,2 which falls within the same range as was observed for the whole cells and correlates with the optimal pH range obtained by Botes with EH enzyme from *Rhodosporidium toruloides* CBS 0349 [12].

2.2 Optimisation of temperature

The optimal temperature for the hydrolysis reaction was determined for both whole cells as well as cell extract, once again using *R. glutinis* and **SO**. The results are shown in Figure 5-2. The whole cells demonstrated optimal activity over a broad range of temperature values (2-1), while the cell extract exhibited maximum activity at around 45 °C (2-2).

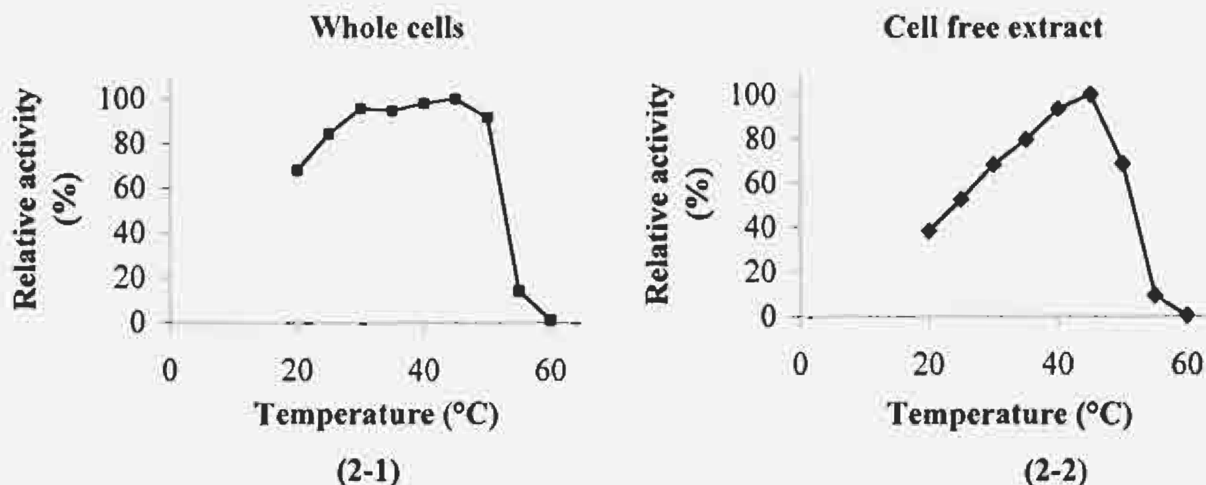


Figure 5-2 Formation of 1,2-phenylethane-diol at different temperatures.

Both the whole cells and the cell extract exhibited decreased relative activity at temperatures below 30 °C and higher than 50 °C (deactivation of enzyme). Nellaiah *et al.* [6] previously reported that an enzyme preparation from *Aspergillus niger* showed an increase in initial reaction rate up to 35 °C while losing nearly 60% of its original activity at 40 °C after 1 hour of incubation. The cell free extract of *R. glutinis* however showed an increase in activity at higher temperatures and can therefore be said to be reasonably stable at temperatures as high as 45 °C. The stability of the enzyme at high temperatures makes it a very useful catalyst for the hydrolysis of substrates with poor solubility in water, as is the case with nitro substituted styrene oxides.

2.3 Optimisation of substrate concentration

To determine the substrate concentration that would give the highest initial reaction rate, hydrolysis was preformed with increasing amounts of initial substrate concentration. The optimal substrate concentration of each reaction is dependant upon the substrate, and therefore, was determined for each of the four substrates using the biocatalyst best suited for hydrolysis of the substrate. Thus *R. glutinis* was used for the biocatalysis of **SO**, **mNSO** and **oNSO** and *R. toruloides* for the biocatalysis of **pNSO**. The decrease in the concentration of epoxide was measured over time by chiral GC analysis. By plotting the slope of the initial

reduction (Appendix 2, Section 3) in epoxide concentration (V) against the substrate concentration (S), the concentration leading to the highest initial reaction rate could be determined. The results are shown in Figure 5-3.

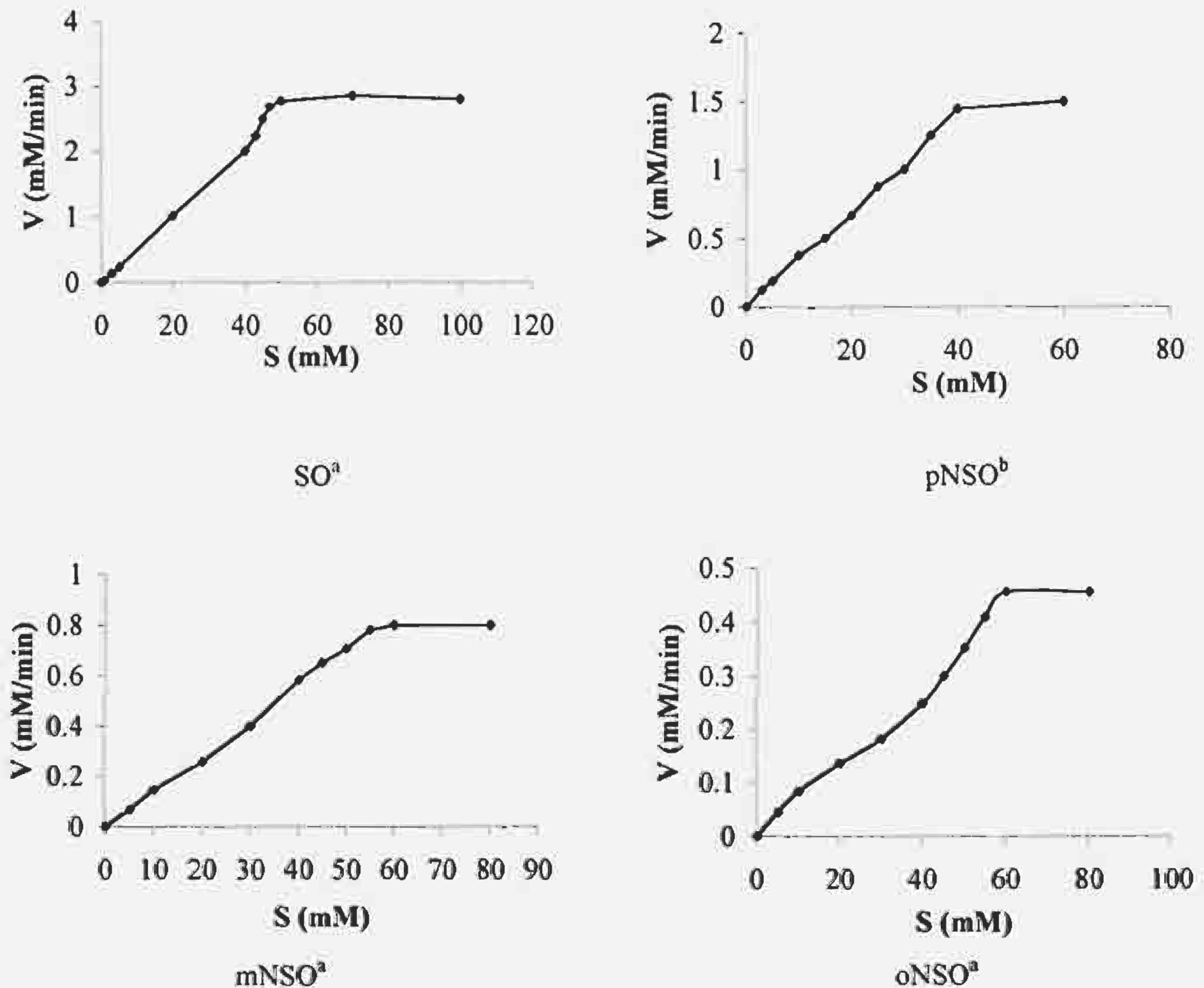


Figure 5-3 Hydrolysis of substrate over time at various initial substrate concentrations during hydrolysis by *Rhodotorula glutinis*[a] and *Rhodosporidium toruloides*[b].

For all four substrates, it was found that an increase in the initial substrate concentration lead to an increase in the initial reaction rate. This behaviour was observed up to a certain value for each substrate after which the initial reaction rate was no longer dependant upon an increase in the substrate concentration. The highest initial reaction rate (V_m) as well the corresponding substrate concentration (S_m) leading to this rate are summarised in Table 5-1.

Table 5-1 Maximum reactions rates and corresponding substrate concentrations for the hydrolysis of nitro-substituted styrene oxides with *R. glutinis*[a] and *R. toruloides*[b].

Epoxide	V_m (mM.min^{-1})	S_m (mM)
SO[a]	2.78	50
pNSO[b]	1.5	40
mNSO[a]	0.8	60
oNSO[a]	0.45	60

At the S_m concentration it can be assumed that either all the active enzyme-binding sites are saturated and therefore more epoxide molecules would have no effect, or the poor water solubility of the substrates does not allow a higher rate. During a study conducted by Nellaiah *et al.* [6] on the hydrolysis of **pNSO** with *Aspergillus niger*, a precipitate was observed in the aqueous reaction at concentrations as low as 8 mM even in the presence of DMSO. Similarly, throughout the current study, formation of a precipitate at concentrations of 10 mM and above was observed for both **pNSO** and **oNSO** in the presence of 2% DMSO. The results that were obtained during this study are however slightly different to those reported by Nellaiah *et al*. The sharp increase in initial reaction rate during hydrolysis with *A. niger* decreased at concentrations higher than 20 mM. This effect was attributed to the poor solubility of the substrate since no substrate inhibition was observed [6]. Hydrolysis of **pNSO** with *R. toruloides* and **mNSO**, **oNSO** and **SO** with *R. glutinis* however, proceeded with an increase in initial reaction rate up to a concentration of at least 40 mM.

2.4 Time course reactions

The hydrolysis of the epoxides at 45 °C with an initial substrate concentration of 60 mM were analysed with chiral GC over time. The results are shown in Figure 5-4 and summarised in Table 5-2.

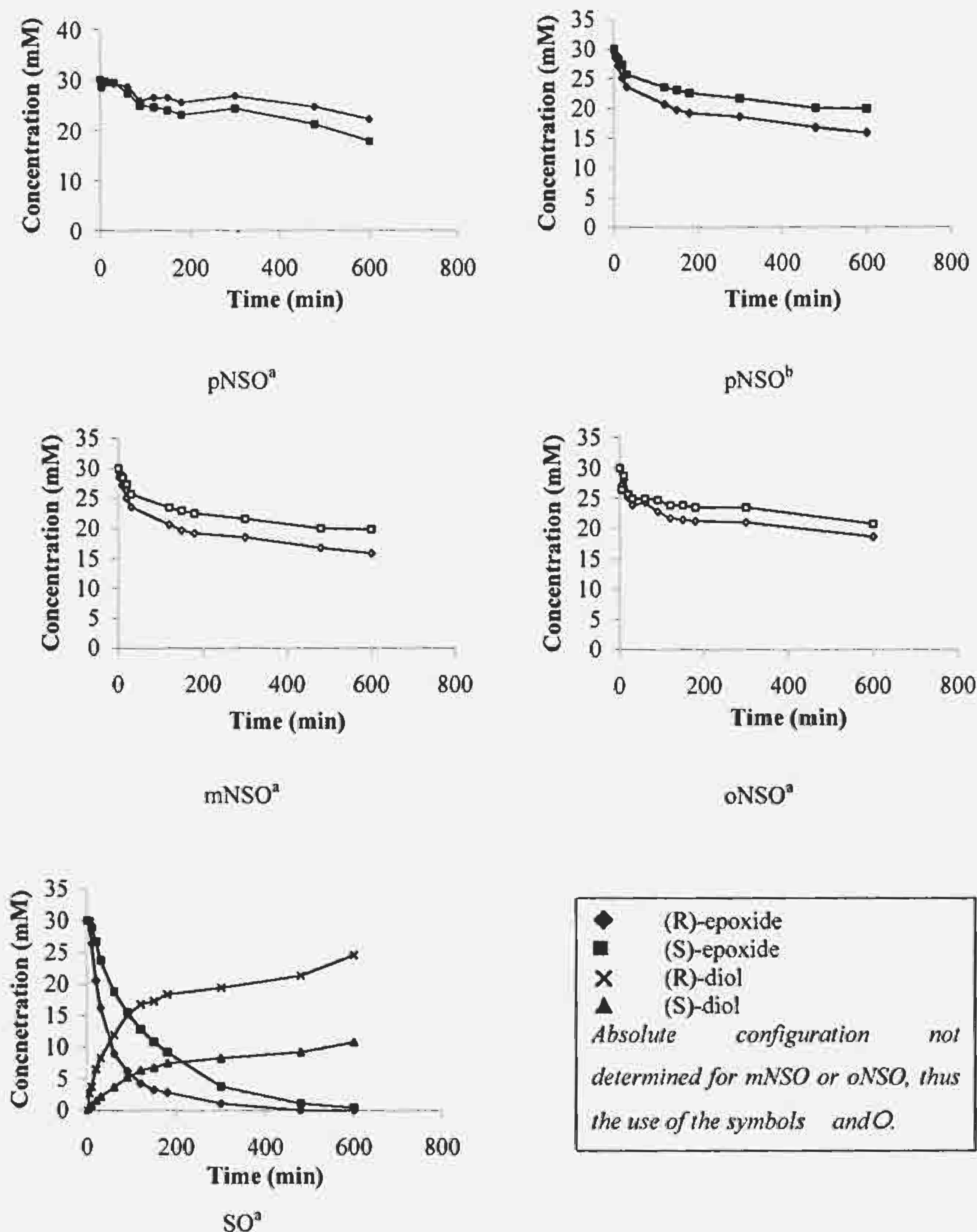


Figure 5-4 Enantioselective hydrolysis of chiral substrate and product formation over time during hydrolysis by *Rhodotorula glutinis*[a] and *Rhodosporidium toruloides*[b] at 45 °C.

Table 5-2 Hydrolysis of various epoxides by *Rhodotorula glutinis* (UOFS Y-0653)[a] and *Rhodosporidium toruloides (UOFS Y-0471)*[b] at 45 °C.

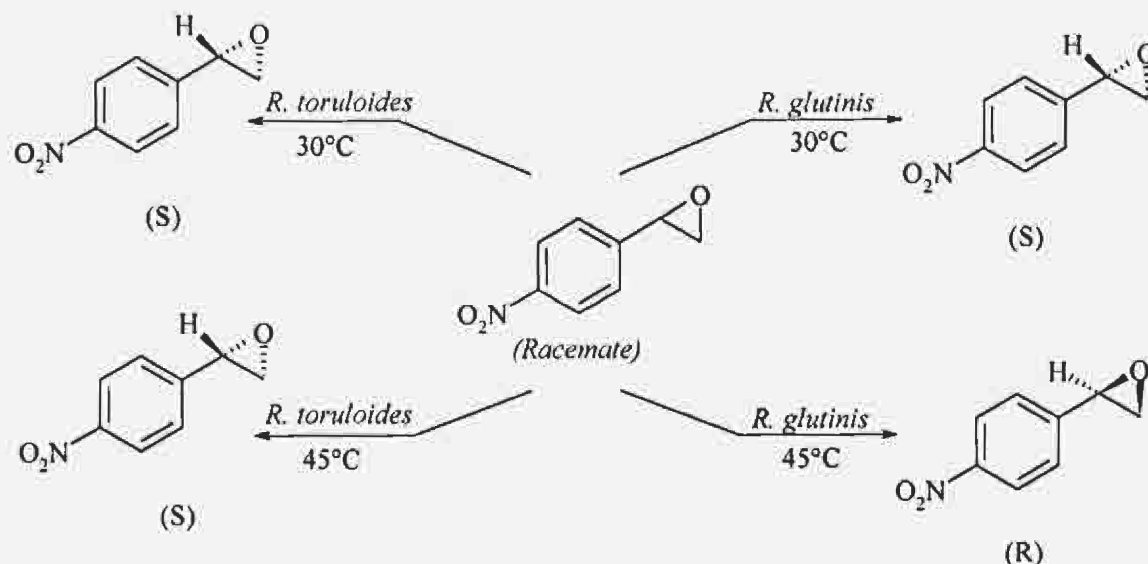
	Epoxide (residual)				Diol (product)			
	ee (%)	Yield (%)	Reaction time (h)*	Absolute configuration	*ee* (%)	Yield (%)	Reaction time (h)	Absolute configuration
pNSO[a]	10	7	10	(-) (R)	-	-	-	n.d.
pNSO[b]	11	7	10	(+) (S)	-	-	-	(+) (S)
mNSO[a]	11	7	10	n.d.	-	-	-	n.d.
oNSO[a]	6	4	5	n.d.	-	-	-	n.d.
SO[a]	>98	2	8	(-) (S)	20	40	8	(-) (R)

n.d. Not determined

*: Time where *ee* was highest

These results show that, in all cases, enantioselectivity decreased significantly when compared to the results found during the initial screening (Chapter 4), especially **mNSO** for which an *ee* of >98% was found previously. This could be as a result of the higher substrate concentration possibly because of inhibition of the enzyme, as seen with *B. sulferecens* and **SO** [13], or as a result of the increased temperature. Phillips [14] similarly reported a decrease in *E* value at high temperatures with *Thermoanaeobacter ethanolicus* and 2-pentanol.

Another interesting phenomenon was that, at these conditions, the preference of *R. glutinis* for the hydrolysis of either the (R)- or (S)-enantiomer of **pNSO** was reversed. In Chapter 4 it was found that both *R. toruloides* and *R. glutinis* enantioselectively hydrolysed the (R)-enantiomer of pNSO (Appendix 3, Chromatogram 8). In this case however (45 °C), *R. glutinis* preferentially catalysed hydrolysis of the (S)-enantiomer (Scheme 5-1) (Appendix 3, Chromatogram 9). *T. ethanolicus* also exhibited this temperature dependant reversal of enantiospecificity during the oxidation of 2-butanol [14].



Scheme 5-1 Biocatalytic hydrolysis of pNSO by *R. toruloides* and *R. glutinis* at different temperatures.

2.5 Effect of temperature on enantioselectivity

The question whether the loss of enantioselectivity was as a result of an increased temperature or an increased substrate concentration had to be solved next. Therefore the hydrolysis of **SO** was repeated at a lower substrate concentration (20 mM) and a lower temperature (15 °C) than those used previously (60 mM, 45 °C). The results of the chiral GC analysis are shown in Figure 5-5 and summarised in Table 5-3.

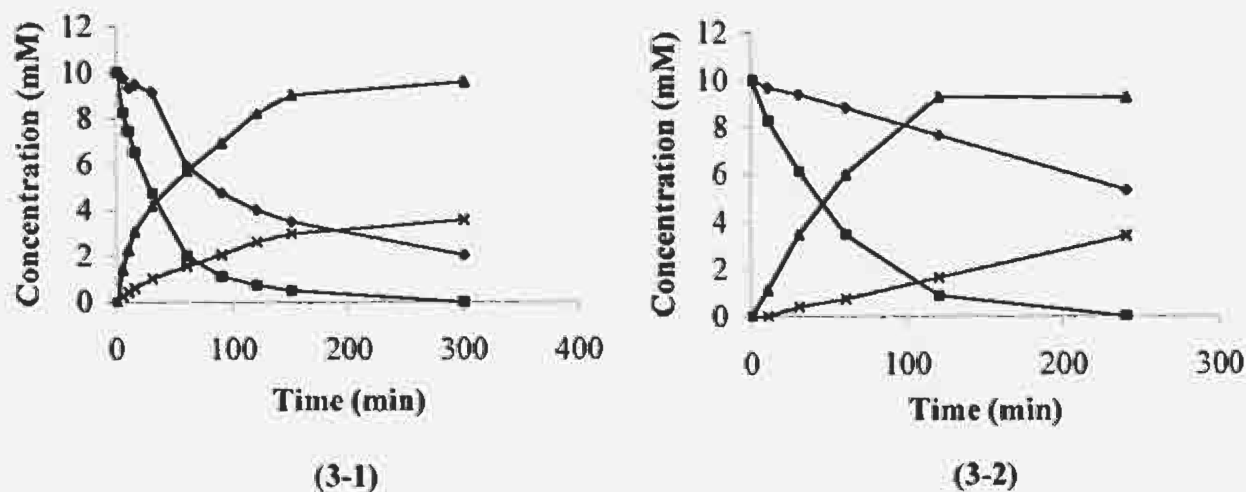


Figure 5-4 Hydrolysis of racemic styrene oxide (20 mM) at 45 °C (3-1) and 15 °C (3-2) with *R. glutinis*. (♦-(S)-epoxide, ■-(R)-epoxide, ▲-(R)-diol, ✕-(S)-diol).

Table 5-3 Hydrolysis of styrene oxide at 45 °C[a] and 15 °C[b] with *Rhodotorula glutinis* (UOFS Y-0653)

	Epoxide (residual)				Diol (product)			
	ee (%)	Yield (%)	Reaction time (h)	Absolute configuration	*ee* (%)	Yield (%)	Reaction time (h)	Absolute configuration
SO[a]	>98	10	5	(-) (S)	46	30	5	(-) (R)
SO[b]	>98	27	4	(-) (S)	46	30	4	(-) (R)

It can be seen that a decrease in the initial substrate concentration (3-1) at 45 °C had relatively little effect on the enantioselectivity of the reaction as the yield remained low, even though an *ee* of >98% was achieved after 5 hours. However at 15 °C (3-2), enantioselectivity increased dramatically, reaching an *ee* of >98% after 4 hours with a yield of 27%. Phillips obtained similar results during the oxidation of 2-pentanol [14], reporting an increased E value at low temperatures. Unexpectedly however the reaction time was not greatly influenced by the decrease in temperature.

Process parameters that have to be considered during enantioselective hydrolysis are pH, temperature, substrate concentration and solubility, ratio of catalyst to substrate (as these parameters influence reaction rates) and the enantioselectivity of the reaction. While it would be ideal to apply those conditions that yield highest activity to complete a reaction within the shortest time, in practice, as shown in this study, it is often not possible. This means that some compromise has to be found between the different parameters such as enantioselectivity, activity and reaction time to reach 98% *ee*, depending on the final process requirements.

3. Experimental

3.1 General

The strains of *Rhodotorula glutinis* (UOFS Y-0653) and *Rhodosporidium toruloides* (UOFS Y-0471) were obtained from the Yeast Culture Collection of the University of the Free State. Reactions were monitored and analysed by GC (Hewlett-Packard 6890 plus equipped with FID) on a HP4 non-polar column (non-chiral analysis) and a β-dex 225 (obtained from Supelco) fused silica cyclodextrin capillary column (chiral analysis) using H_2 as carrier gas. Phenyl-substituted styrene oxides were synthesised as described in Chapter 3. Racemic styrene oxide was obtained from Aldrich.

3.2 Cultivation and preparation of whole yeast cells

Whole cells were cultivated and stored as described in Chapter 4.

3.3 Preparation of yeast cell-free extract

Yeast cells were cultivated as before (Chapter 4) except that they were suspended in a phosphate buffer containing 5 mM EDTA and 150 mM KCl. The cells were broken with a Biospec Beadbeater® using zirconium beads and cell breakage was evaluated with a Nikon® light microscope. The remaining whole cells were centrifuged at 3 500 g and discarded. The cell extract containing the enzyme was centrifuged at 30 000 g and the supernatant discarded. The cell extract was resuspended in phosphate buffer and frozen.

3.4 Optimisation of pH

Frozen cells with different pH values were thawed (500 µl) after which styrene oxide (10 µl of a 1 M DMSO stock solution) was added to give a final concentration of 20 mM. The reaction mixtures were incubated at 30 °C for 30 min while continuously being shaken in a Memmert® shaking water bath. Extracting with 250 µl of ethyl acetate terminated the reactions. After this the ethyl acetate fraction was analysed with GC.

3.5 Optimisation of temperature

Frozen cells with a pH of 7,5 were thawed (500 µl) after which styrene oxide (10 µl of a 1M DMSO stock solution) was added to give a final concentration of 20 mM. The reaction mixtures were incubated at different temperatures for 30 min while continuously being shaken in a Memmert® shaking waterbath. Extracting with 250 µl of ethyl acetate terminated the reactions. After termination the ethyl acetate fraction was analysed with GC.

3.6 Determining the optimal substrate concentration

Frozen cells with a pH value of 7,5 were thawed (500 µl) after which a specified amount of the specified substrate (1 M DMSO stock solution) was added to give the various specified concentrations. The reaction mixtures were incubated at 45 °C and extracted with ethyl acetate (500 µl) at various time intervals while continuously being shaken. The ethyl acetate fractions were analysed with GC.

3.7 Time course reactions

Frozen cells were thawed and substrate (30 µl of a 1 M DMSO stock solution) was added to give a final concentration of 60 mM. The reaction mixtures were incubated at 45 °C with continuous shaking. At different time intervals one micro-centrifuge tube of each substrate was removed and the epoxide and formed diol extracted with 500 µl of ethyl acetate. Chiral GC analysis of the isolated products after biohydrolysis were done as follows:

Epoxides:

pNSO, 130 °C, t_R (R) 37,7 min and t_R (S) 38,7 min (Appendix 3, Chromatogram 5),
mNSO, 115 °C, t_R (1) 60,7 min and t_R (2) 61,9 min (Appendix 3, Chromatogram 6),
oNSO, 130 °C, t_R (1) 15,7 min and t_R (2) 16,3 min (Appendix 3, Chromatogram 7),
SO, 90 °C, t_R (R) 9,6 min and t_R (S) 10,1 min (Appendix 3, Chromatogram 3),

Vicinal diols:

1-Phenyl-2-ethanediol, 150 °C, (S) t_R 11,6 min and (R) t_R 12,0 min (Appendix 3, Chromatogram 4).

4. Conclusion

Enantioselective hydrolysis of terminal epoxides by yeast EH is influenced by various factors such as pH, temperature, co-solvent and initial substrate concentration. During this work it was shown that the pH value as well as the temperature of the surrounding area could influence the relative activity exhibited by the enzyme. High temperatures (30 °C-50 °C), substrate concentrations of between 40 and 60 mM and neutral pH values (6,5-7,5) lead to increased enzymatic activity. It was concluded that, for optimal enzymatic activity, these reactions had to be operated at 45 °C and at a pH of 7,2. The use of a cell free extract did not have any significant beneficial effects upon the activity of the enzyme and therefore, considering the cost and time involved in acquiring the extract, would not be necessary in further experiments.

Initially it seemed that the acceptability of the enzyme for an increased temperature could be advantageous, especially in the case of water insoluble epoxides, where the higher temperature could increase solubility. It was found however that an increase in temperature was responsible for a severe decrease in enantioselectivity, with the lower temperature (15 °C) yielding a much more selective reaction. Lower temperature influenced the reaction rate and thus reaction time. However, the hydrolysis rate of the fast reacting enantiomer was not influenced by the lower temperature, only that of the slow reacting enantiomer, which was hydrolysed at an even lower rate. Reaction time to reach 98% *ee* was thus not negatively influenced, while a better yield was obtained due to enhanced enantioselectivity.

In future work it will be determined whether even lower temperatures will lead to further increased *ee* values. Since a decrease in temperature did not greatly influence the reaction time, it is hoped that lower temperatures will yield even more viable biocatalytic reactions.

Another interesting result is that at a temperature of 45 °C, the preference of *Rhodotorula glutinis* for either the (R)- or (S)- enantiomer of the epoxide was reversed. In Chapter 4, during the screening of the organisms, it was found that *R. glutinis* preferentially hydrolysed the (R)-enantiomer of **pNSO**. At 45 °C though, the enzyme preferentially hydrolysed the (S)-enantiomer (Scheme 5-1). This is in agreement with the results obtained by Phillips [14], where the preference for either (R)- or (S)-enantiomer of 2-pentanol was reversed with *T. ethanolicus*. If the enantioselectivity of the hydrolysis with *R. glutinis* at 45 °C could be optimised, it would yield a biocatalytic route to both of the enantiomers of **pNSO**.

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Chapter 6

Conclusions

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3. Potential of these reactions for the production of enantiopure epoxides 92
4. Future work 92
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1. Biocatalytic resolution of phenyl-substituted styrene oxides

Epoxides are versatile intermediates during the organic synthesis of fine chemicals and drugs and due to this a large amount of research has been conducted on the reactions catalysed by epoxide hydrolase [1, 2, 3].

In this study the ability of two yeast strains, *Rhodotorula glutinis* (UOFS Y-0653) and *Rhodosporidium toruloides* (UOFS Y-0471), to enantioselectively hydrolyse four terminal epoxides namely styrene oxide **(SO)**, *para*-nitrostyrene oxide **(pNSO)**, *meta*-nitrostyrene oxide **(mNSO)** and *ortho*-nitrostyrene oxide **(oNSO)** was demonstrated (Chapter 4). The nitro group substituted to the phenyl ring, because of its high reactivity, could be used in a broad range of synthetic applications.

In the cases of **mNSO** and **SO** *ee* values of >98% with relatively high yields, were found. This makes these reactions ideal for industrial production of the pure epoxide enantiomers. To the best of our knowledge this was the first time that the enantioselective hydrolysis of **mNSO** and **oNSO** were reported. In addition to this, the results clearly show the consequences of nitro substitution to the phenyl ring of styrene oxide and the effect of the position of the substitution.

2. Optimisation of biocatalytic reactions

The activity of the epoxide hydrolase enzyme found within these yeast strains was optimised to determine the conditions needed for the most favourable reactions to take place (Chapter 5). It was found that different pH values, initial substrate concentrations and especially temperatures all greatly affected the resolution of the substrates.

Increases in the initial substrate concentration seemed to increase the initial reaction rate up to a value far exceeding the solubility of the epoxide. This led us to believe that there may be another method of solubilisation involved possibly originating from the yeast.

Variation in temperature not only had an effect upon the enantioselectivity of the reactions, but also upon the preference of the enzyme to catalyse the hydrolysis of either the (R)-enantiomer or the (S)-enantiomer (Scheme 5-1).

3. Potential of these reactions for the production of enantiopure epoxides

- Various different routes can be applied in the preparation of enantiopure intermediates for the synthesis of drugs. Microbial and enzyme-catalysed reactions however carry the advantage that they can be preformed at room temperature and atmospheric pressure, minimising problems usually experienced during chemical processes (e.g. isomerisation, racemisation and rearrangement).

- Biocatalytic reactions generally follow a green chemistry approach, in other words they are preformed in aqueous solution. This makes them much less harsh on the environment and potentially less hazardous to scientists handling them. Additionally they lessen the need for the use of environmentally benign heavy-metal catalysts.

- Costs for the production of enantiopure products can be cut drastically through the use of enantiospecific biocatalysts. Obtaining yeast cells, especially whole cells, is extremely simple and the cost minimal. Furthermore the source of the biocatalysts is not depleteable and can be restocked at any time with ease.

- In the case of South Africa, the production of low cost chiral drug intermediates holds obvious advantages. By investigating synthetic routes from chiral intermediates, such as epoxides, to commonly used chiral drugs (e.g. propranolol), a variety of generic medicines can be produced. This would decrease the cost of essential medicine and improve health care within our borders and in the rest of Africa. An added advantage of biocatalytic resolution reactions is that they are simple and yet very effective.

4. Future work

Even though this work addressed various factors influencing the biocatalytic resolution of nitro substituted styrene oxides, it is foreseen that the following issues will have to be dealt with in the near future:

- Since very little data concerning the physical properties of **mNSO** and **oNSO** is available, the absolute configuration of the residual epoxides and formed diols could as yet not be determined. X-ray three-dimensional structure determination (x-ray crystallography) could possibly be employed to elucidate the absolute configuration.

- During the analysis of the enantioselective hydrolysis of **pNSO**, **mNSO** and **oNSO**, we were unable to detect the formed *vicinal* diols with chiral gas chromatography. A method of analysis, possibly through the use of chiral high-pressure liquid chromatography (HPLC) will have to be evaluated.

- Since the results showed that high temperatures negatively influence enantioselectivity, it is believed that lower temperatures (even as low as 5 °C) could be employed to increase enantioselectivity. If this were to be the case these reactions would be ideal for scale-up in bioreactors.

- The development and optimisation of continuous processes, whether they are continuous or continuous batch processes, could hold an assortment of advantages for the South African industry as well the industry worldwide.

- Current developments in separation technology are increasing this potential even further. The use of membranes and membrane bioreactors for the continuous extraction and production of enantiopure epoxides and diols is an actively researched and developing area [4, 5, 6] and should be developed for these specific reactions.

- The prospective use of new technologies such as zeolite membranes [7], submerged membranes [8], liquid crystals [9] and two phase liquid reactors could be investigated as possible substitutes for the conventional methods of biocatalytic resolution such as large batch reactors.

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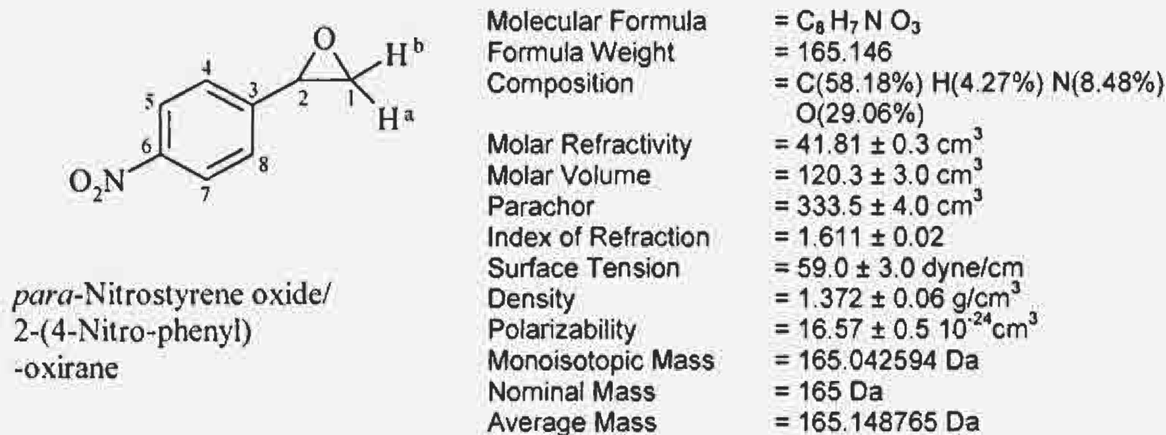
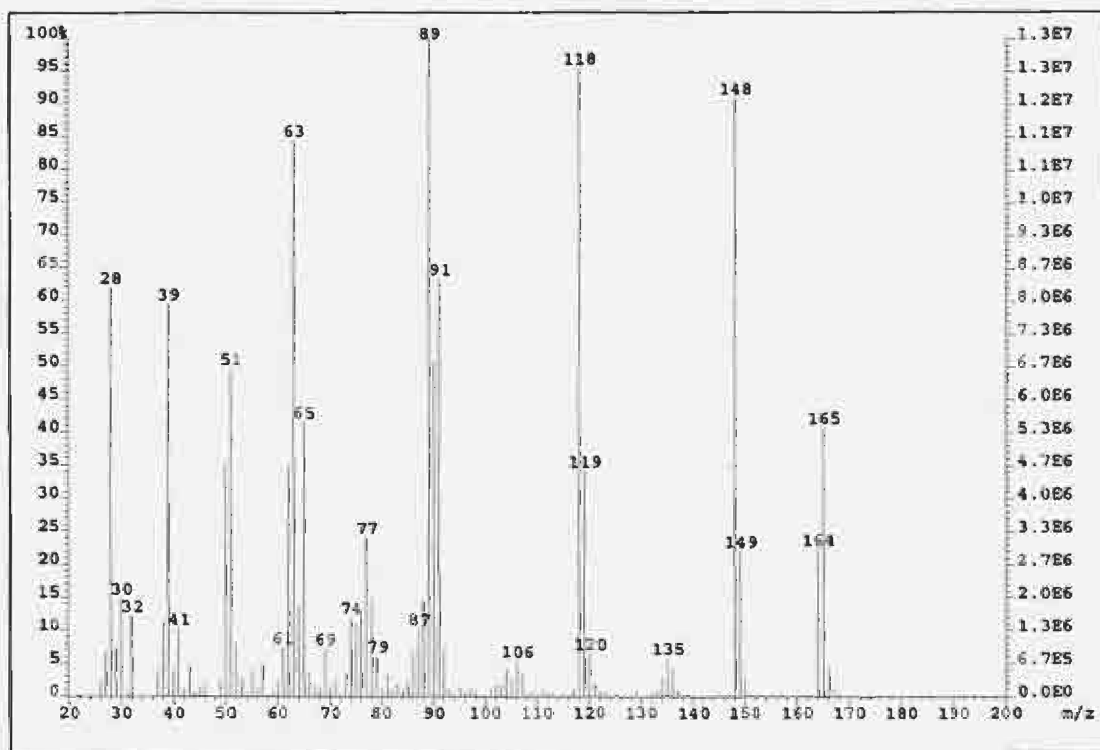
Appendix 1

Structures and properties (calculated from structure with ACD chemsketch®), ^{1}H, ^{13}C and DEPT NMR spectra and MS spectra of para-nitrostyrene oxide (pNSO), meta-nitro styrene oxide (mNSO), ortho-nitrophenacyl bromide (oNPhBr) and ortho-nitrostyrene oxide (oNSO).

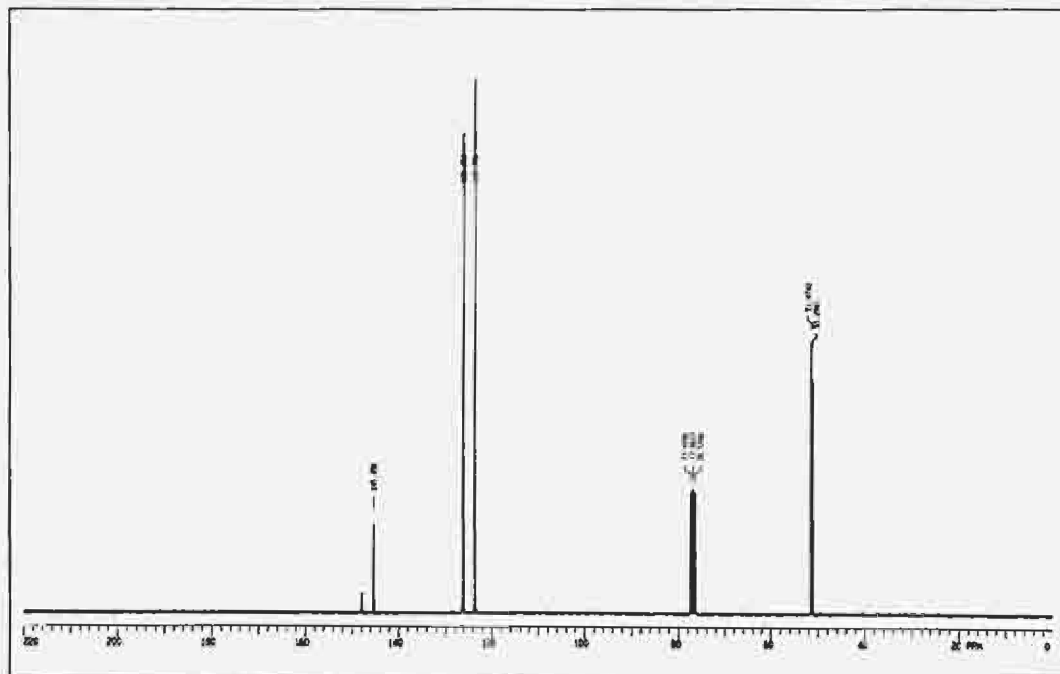


Property	Value
Molecular Formula	= $C_8H_7NO_3$
Formula Weight	= 165.146
Composition	= C(58.18%) H(4.27%) N(8.48%) O(29.06%)
Molar Refractivity	= 41.81 ± 0.3 cm^3
Molar Volume	= 120.3 ± 3.0 cm^3
Parachor	= 333.5 ± 4.0 cm^3
Index of Refraction	= 1.611 ± 0.02
Surface Tension	= 59.0 ± 3.0 dyne/cm
Density	= 1.372 ± 0.06 g/cm^3
Polarizability	= 16.57 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass	= 165.042594 Da
Nominal Mass	= 165 Da
Average Mass	= 165.148765 Da

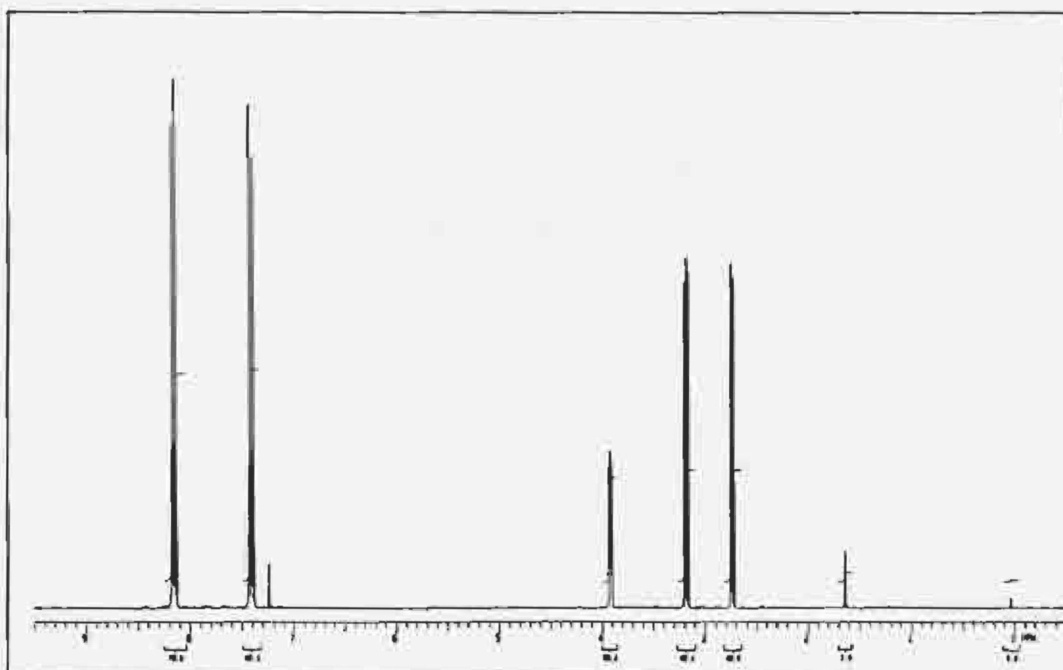
Figure 1 Structure and properties of pNSO



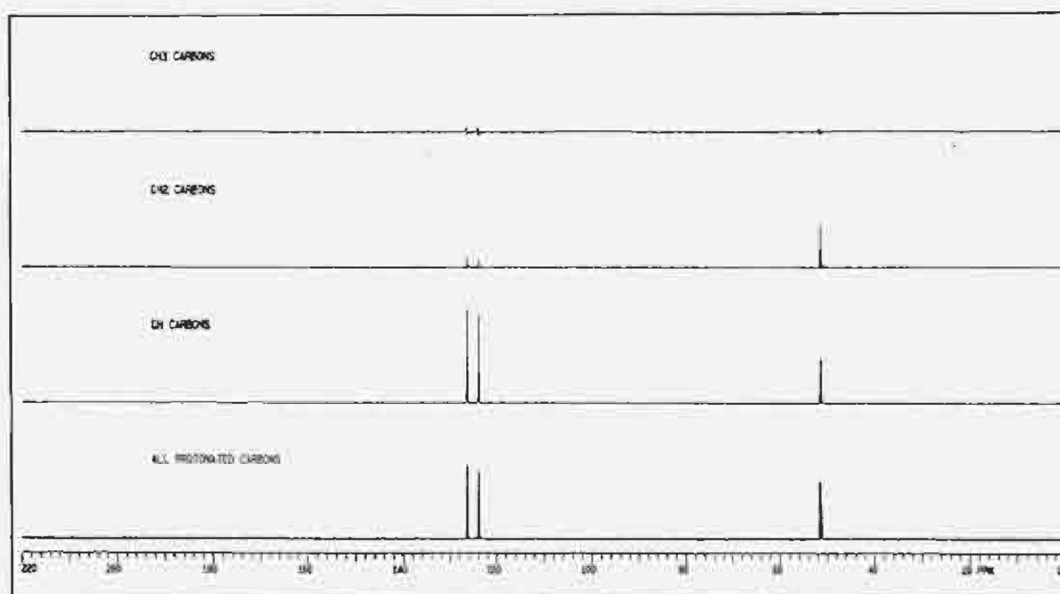
Spectrum 1: MS spectrum of pNSO

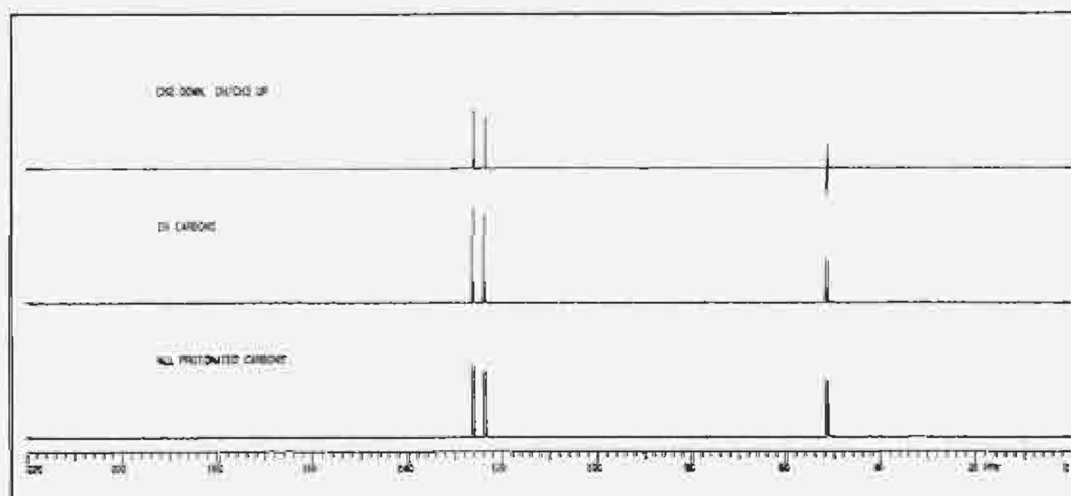


Spectrum 2: ^{13}C NMR spectrum of pNSO

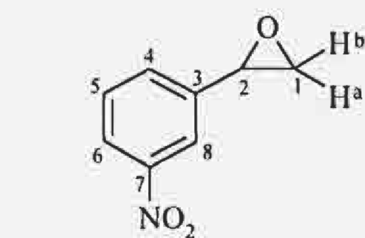


Spectrum 3: ^{1}H NMR spectrum of pNSO





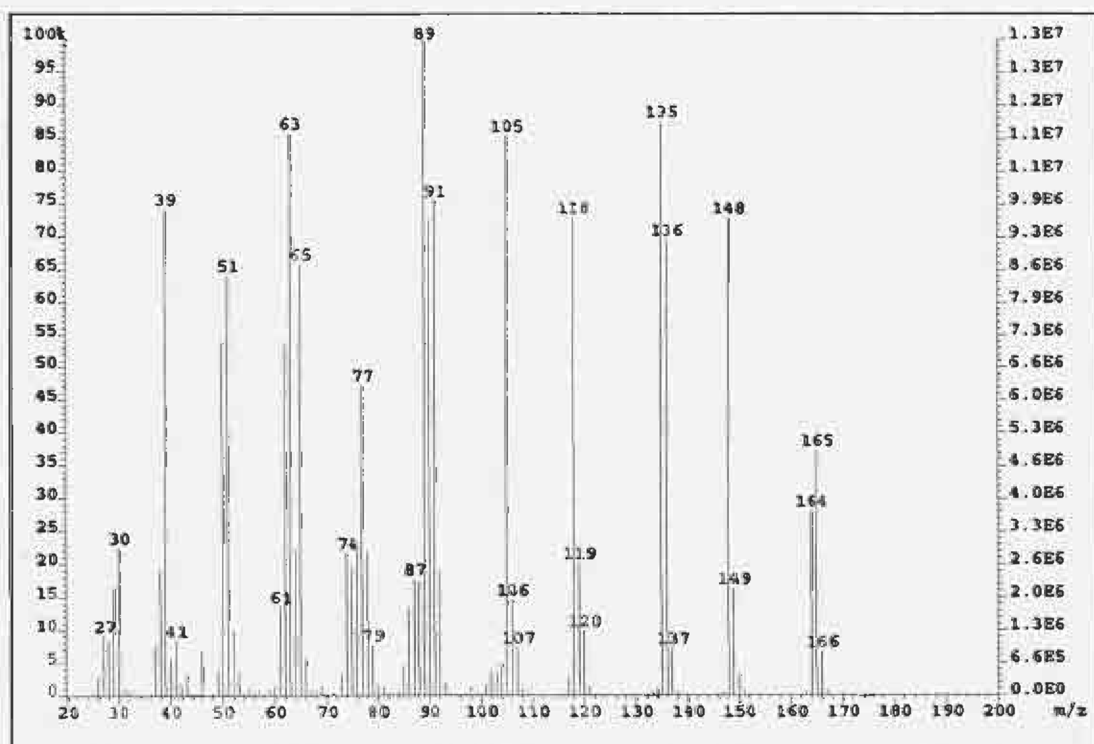
Spectrum 4: DEPT spectrum of pNSO



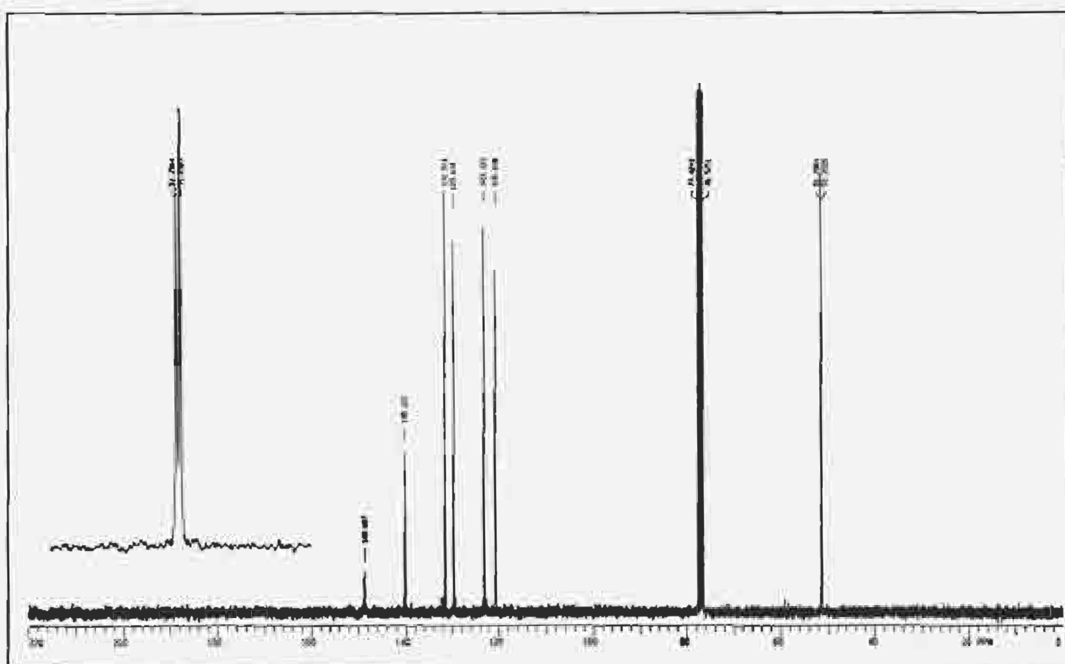
meta-Nitrostyrene oxide/
2-(3-Nitro-phenyl)
-oxirane

Molecular Formula	= $C_8H_7NO_3$
Formula Weight	= 165.146
Composition	= C(58.18%) H(4.27%) N(8.48%) O(29.06%)
Molar Refractivity	= 41.81 ± 0.3 cm^3
Molar Volume	= 120.3 ± 3.0 cm^3
Parachor	= 333.5 ± 4.0 cm^3
Index of Refraction	= 1.611 ± 0.02
Surface Tension	= 59.0 ± 3.0 dyne/cm
Density	= 1.372 ± 0.06 g/cm^3
Polarizability	= 16.57 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass	= 165.042594 Da
Nominal Mass	= 165 Da
Average Mass	= 165.148765 Da

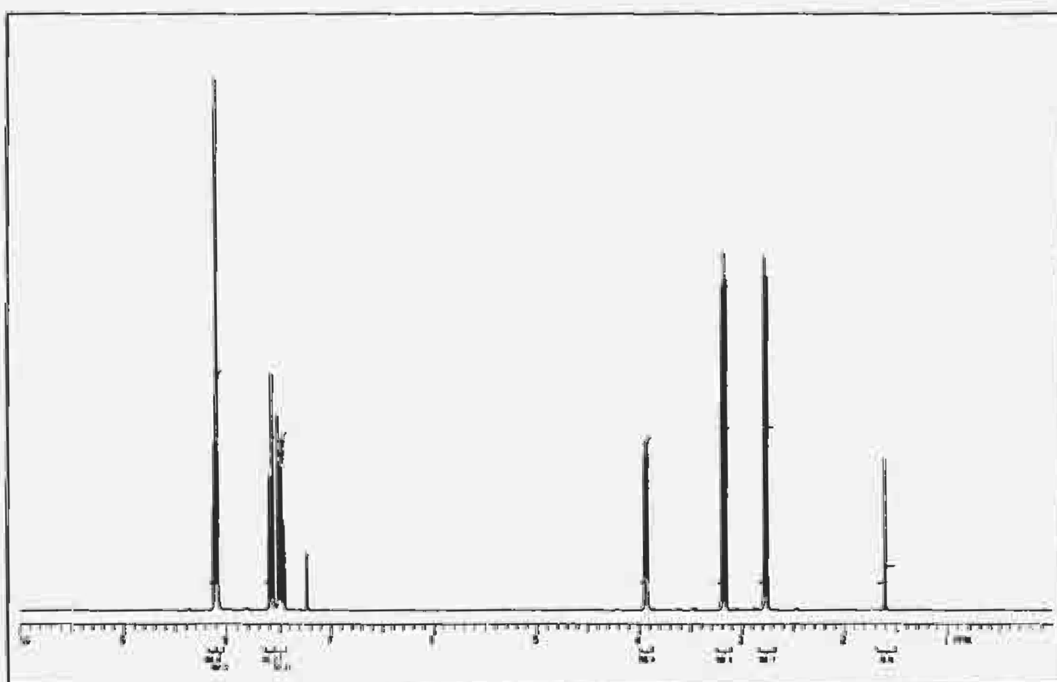
Figure 2 Structure and properties of mNSO



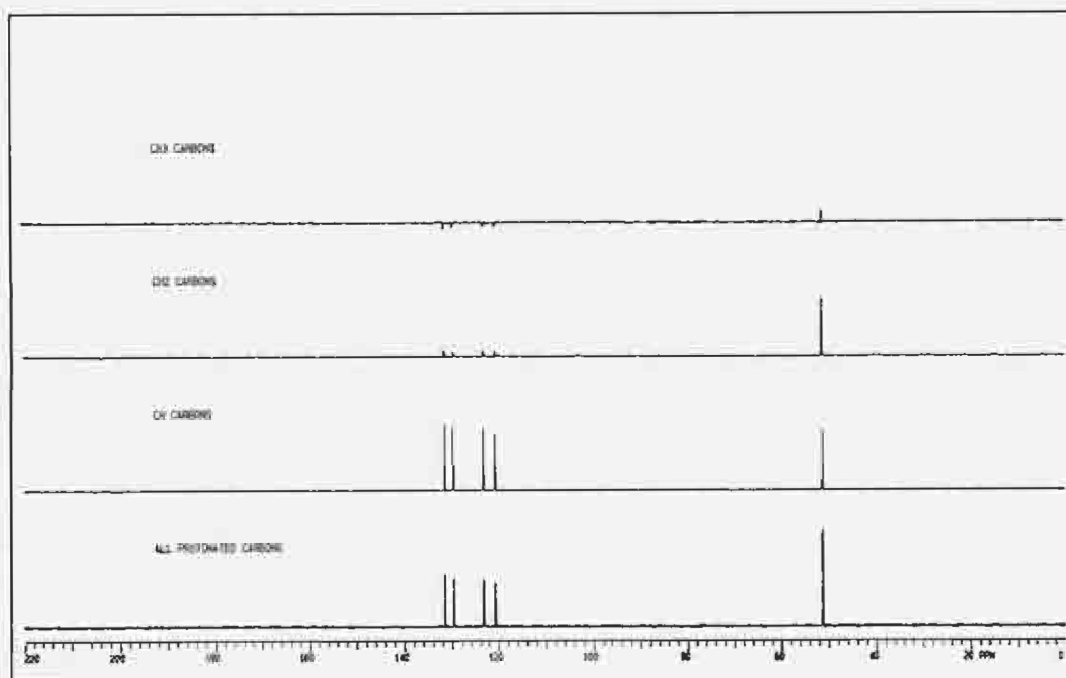
Spectrum 5: MS spectrum of mNSO

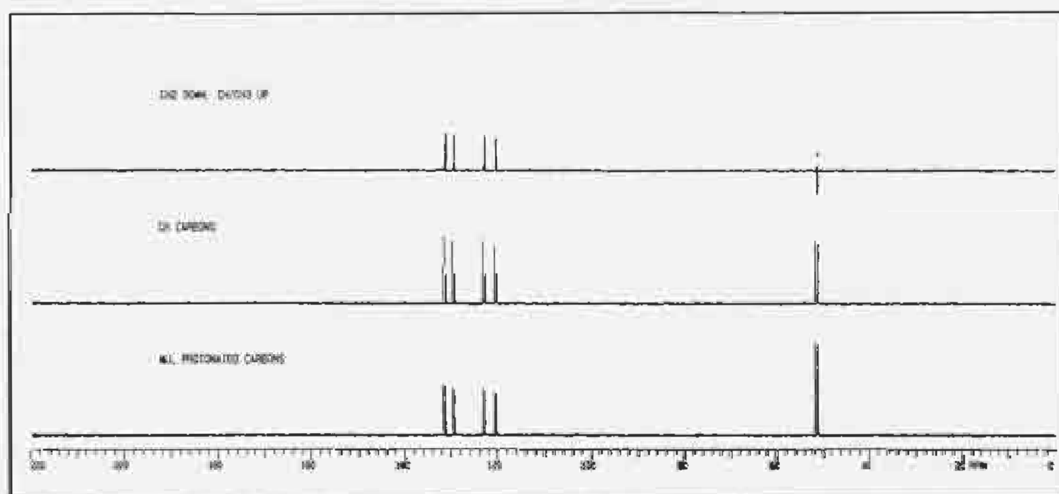


Spectrum 6: ^{13}C NMR spectrum of mNSO

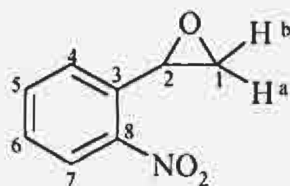


Spectrum 7: ^{1}H NMR spectrum of mNSO





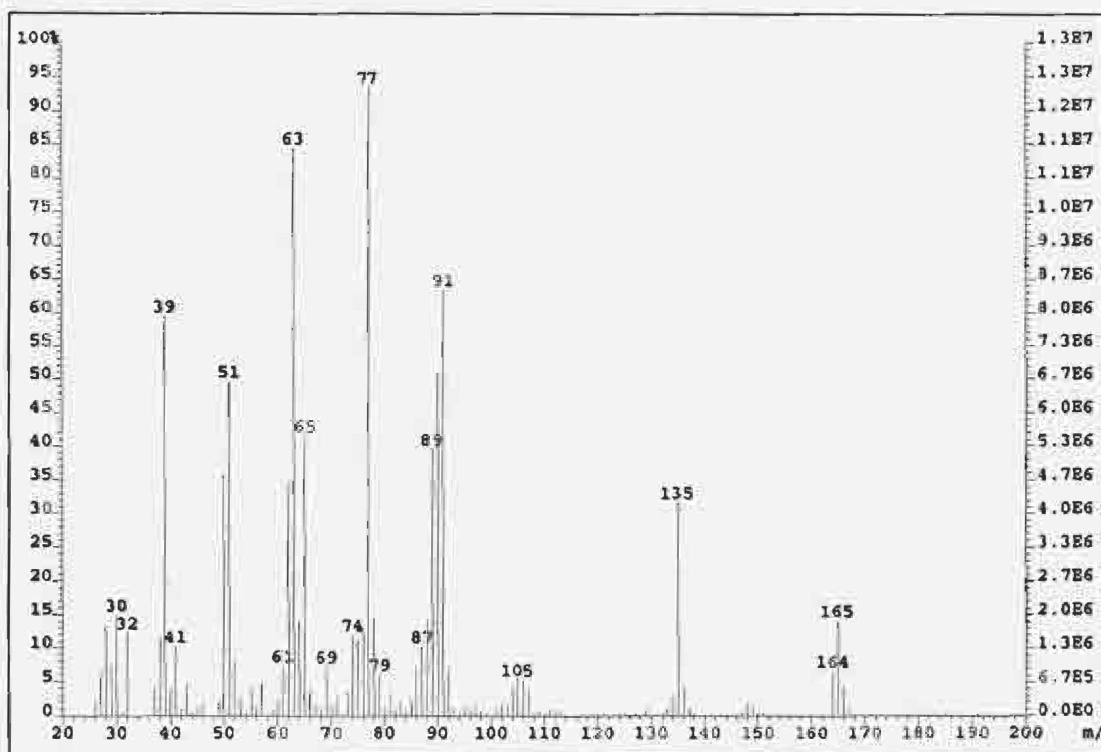
Spectrum 8: DEPT spectrum of mNSO



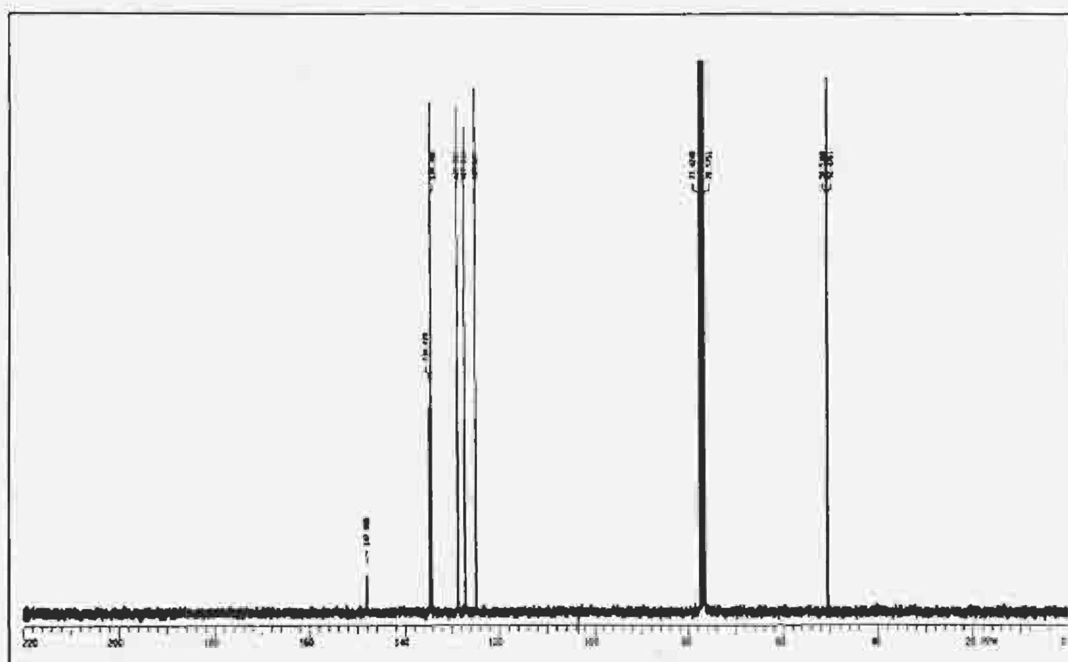
ortho-Nitrostyrene oxide/
2-(2-Nitro-phenyl)
-oxirane

Molecular Formula = $C_8H_7NO_3$
Formula Weight = 165.146
Composition = C(58.18%) H(4.27%) N(8.48%) O(29.06%)
Molar Refractivity = 41.81 ± 0.3 cm^3
Molar Volume = 120.3 ± 3.0 cm^3
Parachor = 333.5 ± 4.0 cm^3
Index of Refraction = 1.611 ± 0.02
Surface Tension = 59.0 ± 3.0 dyne/cm
Density = 1.372 ± 0.06 g/cm^3
Polarizability = 16.57 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 165.042594 Da
Nominal Mass = 165 Da
Average Mass = 165.148765 Da

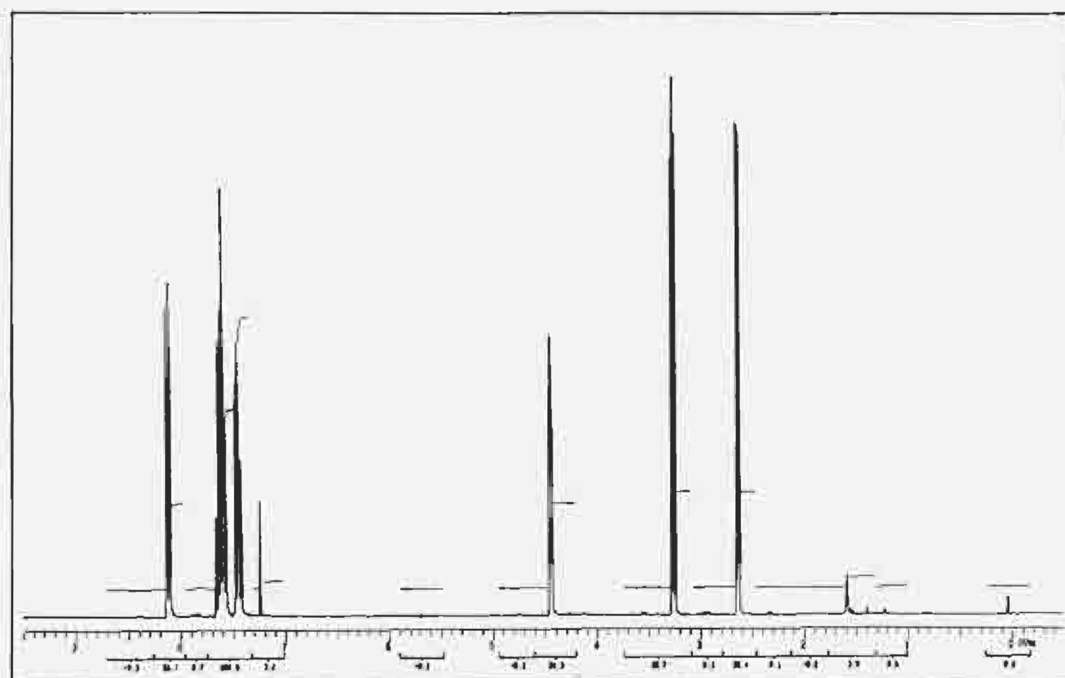
Figure 3 Structure and properties of oNSO



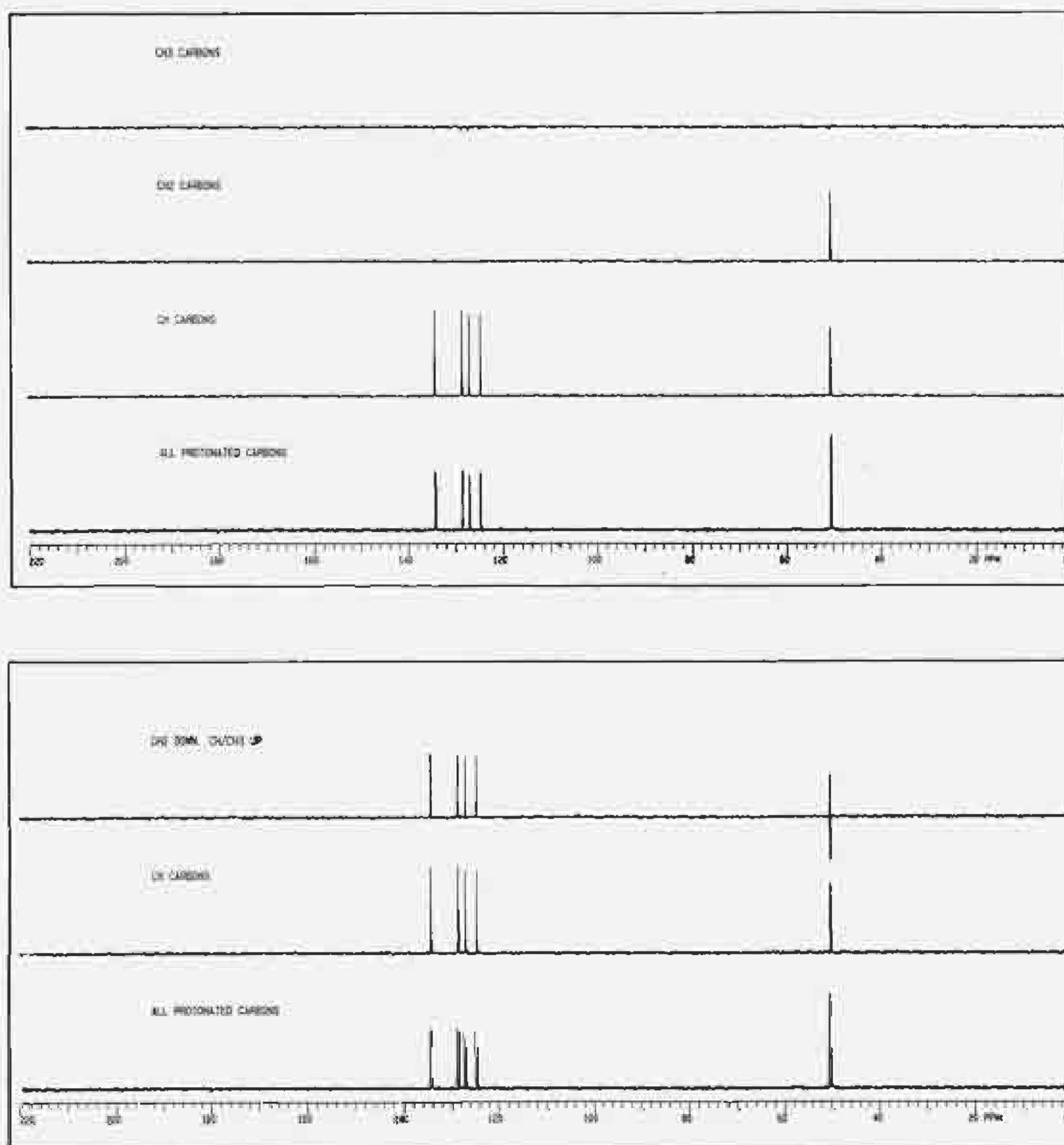
Spectrum 9: MS spectrum of oNSO



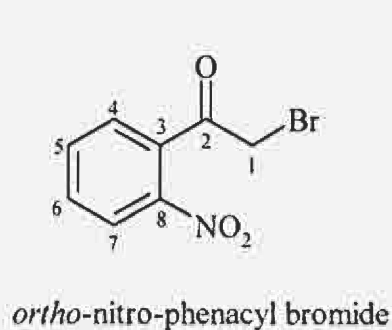
Spectrum 11: ^{13}C NMR spectrum of oNSO



Spectrum 12: ^{1}H NMR spectrum of oNSO



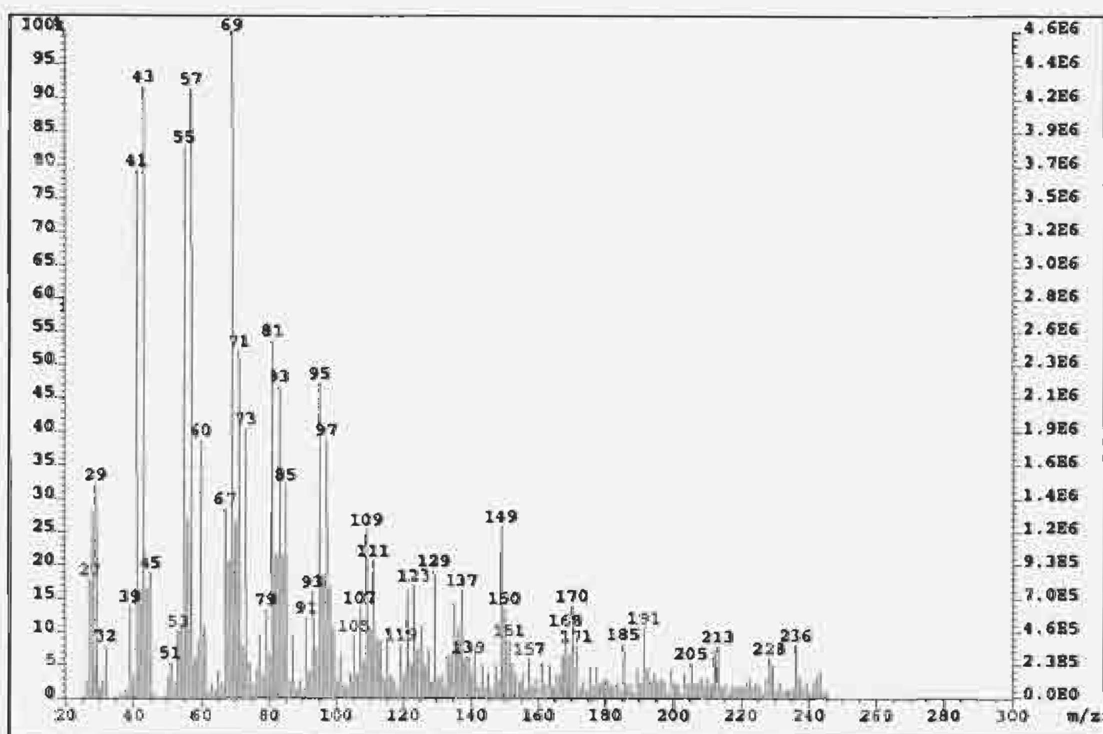
Spectrum 13: DEPT spectrum of oNSO



ortho-nitro-phenacyl bromide

Property	Value
Molecular Formula	= $C_8 H_6 Br N O_3$
Formula Weight	= 244.042
Composition	= C(39.37%) H(2.48%) Br(32.74%) N(5.74%) O(19.67%)
Molar Refractivity	= 50.55 ± 0.3 cm^3
Molar Volume	= 145.9 ± 3.0 cm^3
Parachor	= 400.0 ± 4.0 cm^3
Index of Refraction	= 1.609 ± 0.02
Surface Tension	= 56.4 ± 3.0 dyne/cm
Density	= 1.671 ± 0.06 g/cm^3
Polarizability	= 20.04 ± 0.5 10^{-24}cm^3
Monoisotopic Mass	= 242.953107 Da
Nominal Mass	= 243 Da
Average Mass	= 244.044318 Da

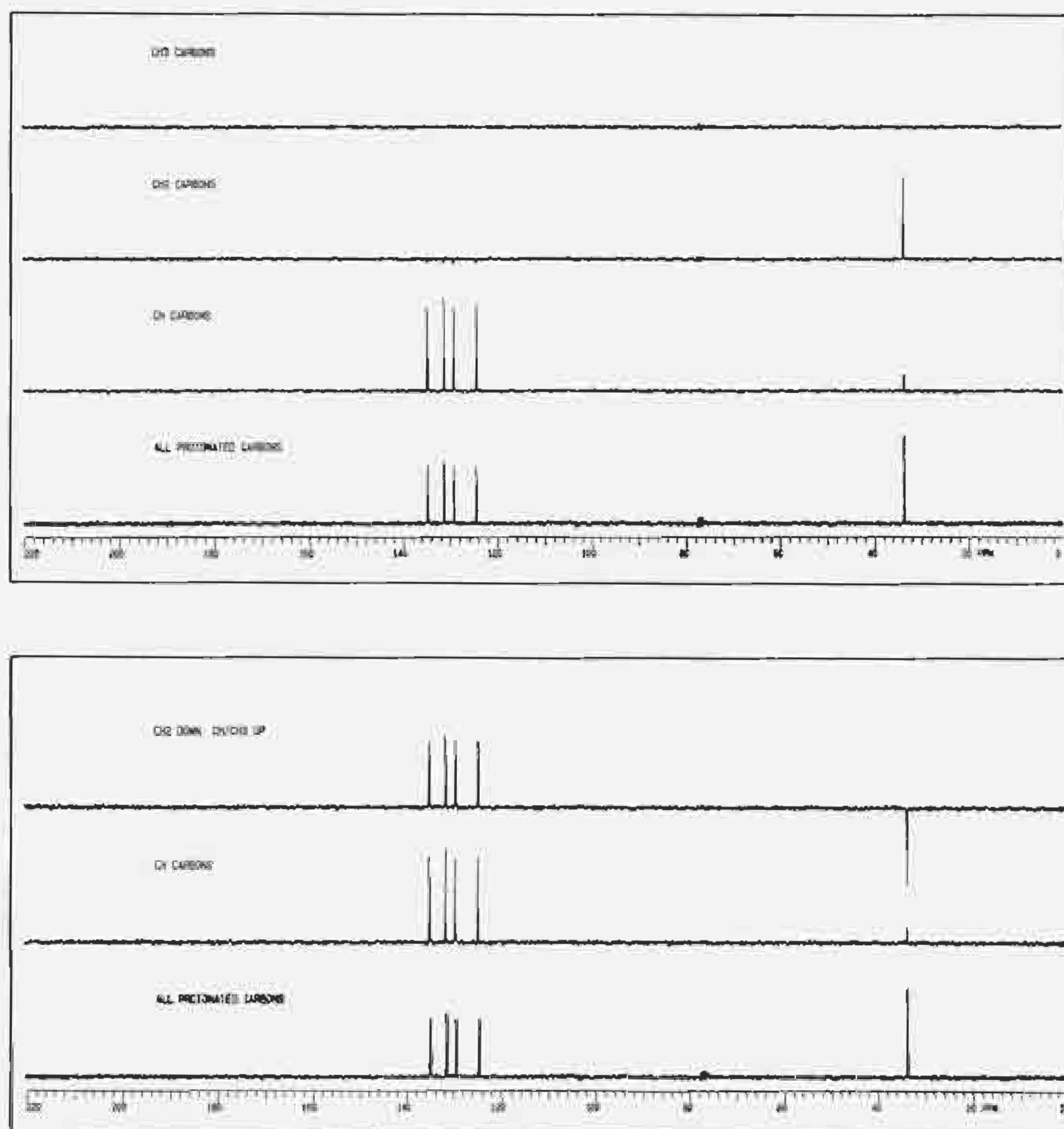
Figure 4 Structure and properties of oNPhBr



Spectrum 14: MS spectrum of oNPhBr

Spectrum 15: ^{13}C NMR spectrum of oNPhBr

Spectrum 16: ^{1}H NMR spectrum of oNPhBr



Spectrum 17: DEPT spectrum of oNPhBr

Appendix 2

1. Screening

This section contains a complete list of screened yeast strains (Table 1), including species, strain and identification number. Strains were considered as having EH activity if formation of the *vic* diol was observed. A strain was considered to be enantioselective (✓, ×, ★ or •) if the calculated e.e was above 30 % and very enantioselective (✓✓, etc.) if the e.e. was above 70 %. As mentioned in Chapter 4, only 36 organisms that exhibited enantioselective activity towards the nitro substituted styrene oxides were used during the screening of SO.

Table 1 List of screened yeast strains (pNSO (✓), mNSO (×), oNSO (★), SO (•)).

	Species	Culture collection number	EH activity	Enantio-selective
1	*Arthroascus fermentans*	CBS 7830		
2	*Arthroascus javanensis*	CBS 2555		
3	*Arthroascus schoenii*	CBS 7223		
4	*Arxula adeninivorans*	CSIR Y-0677	✓	
5	*Arxula adeninivorans*	CSIR Y-1117		
6	*Arxula adeninivorans*	CSIR Y-1118		
7	*Arxula adeninivorans*	CSIR Y-1136	✓	
8	*Arxula adeninivorans*	CSIR Y-1138		
9	*Arxula adeninivorans*	CSIR Y-1147	✓	
10	*Arxula adeninivorans*	CSIR Y-1148	✓	
11	*Arxula adeninivorans*	CSIR Y-1149	✓, ×	
12	*Arxula terrestris*	CBS 7376		
13	*Brettanomyces anomalus*	CBS 0077		
14	*Brettanomyces bruxellensis*	UOFS Y-0937		
15	*Brettanomyces naardenensis*	UOFS Y-1227		
16	*Brettanomyces naardenensis*	CSIR Y-0876		
17	*Brettanomyces* sp.	UOFS Y-0553		
18	*Bullera dendrophila*	CBS 6074	✓	
19	*Bullera dendrophila*	UOFS Y-1231		
20	*Bulleromyces albus*	UOFS Y-0230		

	Species	Culture collection number	EH activity	Enantio-selective
21	*Candida albicans*	UOFS Y-0536	✓, ×	✓
22	*Candida apris*	CBS 2674		
23	*Candida bombicola*	CBS 6009		
24	*Candida cantarellii*	CBS 4878		
25	*Candida castellii*	CBS 4332		
26	*Candida ernobii*	CBS 1737		
27	*Candida ernobii*	CSIR Y-0533		
28	*Candida famata*	UOFS Y-0058	✓, ×	✓
29	*Candida famata*	UOFS Y-0203	×	
30	*Candida guillermondii*	UOFS Y-0796	✓, ×	
31	*Candida guillermondii*	UOFS Y-0053	✓, ×, •	✓
32	*Candida guillermondii*	UOFS Y-0054	✓, ×, •	✓✓
33	*Candida guillermondii*	UOFS Y-0057	✓, ×	
34	*Candida haemulonii*	CBS 5149		
35	*Candida haemulonii*	UOFS Y-1291		
36	*Candida humilis*	CBS 5658	✓	
37	*Candida kruisii*	UOFS Y-0177		
38	*Candida magnoliae*	CBS 0166		
39	*Candida magnoliae*	CBS 0166 T		
40	*Candida magnoliae*	UOFS Y-0451	✓, ×	
41	*Candida magnoliae*	UOFS Y-1761	✓, ×	
42	*Candida magnoliae*	UOFS Y-1040	✓, ×, •	✓, •
43	*Candida magnoliae*	UOFS Y-1041	✓, ×	
45	*Candida magnoliae*	UOFS Y-0799	✓, ×, •	✓✓
46	*Candida magnoliae*	UOFS Y-0535	✓, ×	✓
47	*Candida magnoliae*	UOFS Y-1297	✓, ×, •	✓, ×, •
48	*Candida maris*	CBS 5151		
49	*Candida milleri*	CBS 2664		
50	*Candida nitratophilia*	CBS 2027		
51	*Candida norvegica*	CBS 2669		

	Species	Culture collection number	EH activity	Enantio-selective
52	*Candida norvegica*	CBS 2670		
53	*Candida norvegica*	CBS 4035		
54	*Candida norvegica*	CBS 4049		
55	*Candida norvegica*	CBS 4148		
56	*Candida norvegica*	CBS 4737		
57	*Candida norvegica*	CSIR Y-0532		
58	*Candida parapsilosis*	UOFS Y-0206		
59	*Candida pignaliae*	CBS 6071		
60	*Candida pinus*	CBS 0970		
61	*Candida rugosa*	UOFS Y-0537	✓, ×	✓
62	*Candida rugosa*	UOFS Y-0538	✓	
63	*Candida salmanticensis*	CBS 5121		
64	*Candida sonorensis*	CBS 6792		
65	*Candida sorbophila*	CBS 6739		
66	*Candida* sp.	CBS 1784		
67	*Candida* sp.	CSIR Y-0756		
68	*Candida tenuis*	UOFS Y-1328	✓	✓
69	*Candida tropicalis*	UOFS Y-0210		
70	*Candida tropicalis*	UOFS Y-0534	✓, ×	
71	*Candida vanderwaltii*	CBS 5524	✓	
72	*Candida vartiovaarae*	CBS 4289		
73	*Candida wickerhamii*	CBS 6395		
74	*Chryseomonas luteola (bacterium)*	UOFS Y-1621	✓, ×	
75	*Cryptococcus albidus*	UOFS Y-2127	✓, ×	
76	*Cystofilobasidiella infirmo-miniatum*	CBS 2205		
77	*Cystofilobasidium capitaum*	IGC 4309		
78	*Cystofilobasidium infirmo-miniatum*	CBS 2205		
79	*Debaryomyces hansenii*	UOFS Y-1991		
80	*Debaryomyces hansenii*	UOFS Y-1991		
81	*Debaryomyces hansenii*	TVN 133		

	Species	Culture collection number	EH activity	Enantio-selective
82	*Debaryomyces hansenii*	TVN 159		
83	*Debaryomyces hansenii*	UOFS Y-1937	✓, ×	✓✓
84	*Debaryomyces hansenii*	UOFS Y-0492	✓	✓
85	*Debaryomyces hansenii*	UOFS Y-0539	✓, ×	
86	*Debaryomyces hansenii*	UOFS Y-0542	✓	
87	*Debaryomyces hansenii*	UOFS Y-0543	✓	
88	*Debaryomyces hansenii*	UOFS Y-0544	✓	✓✓
89	*Debaryomyces hansenii*	UOFS Y-0545	✓	
90	*Debaryomyces hansenii*	UOFS Y-0554	✓, ×	
91	*Debaryomyces hansenii*	UOFS Y-0600		
92	*Debaryomyces hansenii*	UOFS Y-0601		
93	*Debaryomyces hansenii*	UOFS Y-0603	✓	✓
94	*Debaryomyces hansenii*	UOFS Y-0604	✓	
95	*Debaryomyces hansenii*	UOFS Y-0605	✓	
96	*Debaryomyces hansenii*	UOFS Y-0606		
97	*Debaryomyces hansenii*	UOFS Y-0607		
98	*Debaryomyces hansenii*	UOFS Y-0608	✓	✓
99	*Debaryomyces hansenii*	UOFS Y-0609		
100	*Debaryomyces hansenii*	UOFS Y-0610	✓	
101	*Debaryomyces hansenii*	UOFS Y-0611		
102	*Debaryomyces hansenii*	UOFS Y-0612		
103	*Debaryomyces hansenii*	UOFS Y-0613	✓	✓✓
104	*Debaryomyces hansenii*	UOFS Y-0614	✓	
105	*Debaryomyces hansenii*	UOFS Y-0615		
106	*Dekkera anomala*	UOFS Y-1062		
107	*Dekkera bruxellensis*	CSIR Y-0562		
108	*Dipodascopsis aggregarus*	CBS 175.53 T		
109	*Dipodascopsis albidus*	CBS 766.85 T		
110	*Dipodascopsis ambrosiae*	CBS 749.85 T		
111	*Dipodascopsis armillariae*	CBS 817.71		

	Species	Culture collection number	EH activity	Enantio-selective
112	*Dipodascopsis capitatum*	CBS 572.82		
113	*Dipodascopsis geniculatus*	CBS 184.80 T		
114	*Dipodascopsis magnusii*	CBS 108.12		
115	*Dipodascus ovetensis*	CBS 192.55 T		
116	*Endomyces fibuliger*	UOFS Y-0665		
117	*Endomyces fibuligera*	CSIR Y-0269	✓, ×	×
118	*Endomycopsella crataegensis*	CSIR Y-0204		
119	*Endomycopsella* sp.	CSIR Y-0212		
120	*Filobasidium capsuligenum*	CBS 4736	×	
121	*Filobasidium capsuligenum*	UOFS Y-0475		
122	*Filobasidium floriforme*	CBS 6240		
123	*Filobasidium uniguttulatum*	CBS 2770		
124	*Galactomyces reesii*	CSIR Y-0585		
125	*Geotrichum* sp.	UOFS Y-0111		
126	*Geotrichum* sp.	VDW 0153		
127	*Geotrichum terrestre*	UOFS Y-2162	✓, ×	
128	*Geotrichum terrestre*	CSIR Y-0803		
129	*Hormonema* sp.	UOFS Y-0067	✓, ×	
130	*Hyphopichia "buitorii"*	CSIR Y-0608		
131	*Kluyveromyces maxianus var*	CSIR Y-0878		
132	*Kluyveromyces yarrowia*	CBS 6070	✓	
133	*Leucosporidium scottii*	CBS 0614		
134	*Lipomyces* sp.	UOFS Y-2159	✓, ×	
135	*Lipomyces tetrasporus*	UOFS Y-2158		
136	*Malay 36163*	TVN 292	✓, ×	
137	*Myxozyma melibiosi*	UOFS Y-1761		
138	*Myxozyma monticola*	EP 14		
139	*Pichia angusta*	CBS 7031		
140	*Pichia fabianii*	CBS 5482		
141	*Pichia fabianii*	CBS 5641		

	Species	Culture collection number	EH activity	Enantio-selective
142	*Pichia fabianii*	CBS 6212		
143	*Pichia fabianii*	CBS 6550		
144	*Pichia fabianii*	UOFS Y-0052		
145	*Pichia fabianii*	UOFS Y-0056	×	
146	*Pichia fabianii*	UOFS Y-0110		
147	*Pichia fabianii*	UOFS Y-0152 T		
148	*Pichia guillermondii*	UOFS Y-0209		
149	*Pichia guillermondii*	UOFS Y-1033	✓, ×	
150	*Pichia guillermondii*	TVN 157	✓	✓
151	*Pichia guilliermondii*	UOFS Y-01028	×	
152	*Pichia guilliermondii*	UOFS Y-1030	✓	
153	*Pichia guilliermondii*	UOFS Y-0209		
154	*Pichia haplophila*	UOFS Y-0903	✓, ×	
155	*Pichia haplophila*	UOFS Y-2136	✓, ×	
156	*Pichia haplophila*	UOFS Y-2161	✓, ×	×
157	*Pichia holstii*	UOFS Y-0140		
158	*Pichia jadinii*	CBS 0841		
159	*Pichia jadinii*	UOFS Y-0520		
160	*Pichia subpelliculosa*	UOFS Y-0136		
161	*Rhodosporidium lusitaniae*	UOFS Y-1619	×	
162	*Rhodosporidium paludigenum*	UOFS Y-0482	×	
163	*Rhodosporidium paludigenum*	CBS 6566		
164	*Rhodosporidium paludigenum*	UOFS Y-0481	✓	
165	*Rhodosporidium sphaerocarpum*	UOFS Y-0480		
166	*Rhodosporidium toruloides*	UOFS Y-0471	✓, ×, •	•
167	*Rhodosporidium toruloides*	UOFS Y-0472	✓, ×	✓✓
168	*Rhodotorula acuta*	UOFS Y-0493		
169	*Rhodotorula araucariae*	UOFS Y-0473	✓, ×	
170	*Rhodotorula aurantiaca*	TVN 142	✓	
171	*Rhodotorula aurantiaca*	UOFS Y-2049	×	

	Species	Culture collection number	EH activity	Enantio-selective
172	*Rhodotorula ferulica*	CBS 7416	×	
173	*Rhodotorula foliorum*	CBS 5234		
174	*Rhodotorula glutinis*	UOFS Y-0653	✓, ×, ★, ●	××, ●●
175	*Rhodotorula glutinis*	TVN 149	✓, ×	
176	*Rhodotorula glutinis*	UOFS Y-0123	✓, ×, ●	✓, ●
177	*Rhodotorula glutinis*	UOFS Y-0459	×, ●	×, ●●
178	*Rhodotorula glutinis*	UOFS Y-0489	✓, ×	
179	*Rhodotorula glutinis*	UOFS Y-0519		
180	*Rhodotorula graminis*	CBS 2826	✓, ×	
181	*Rhodotorula lactosa*	CBS 5826	✓	
182	*Rhodotorula lactosa*	CBS 5827	×	
183	*Rhodotorula minuta*	UOFS Y-1616	✓	
184	*Rhodotorula minuta*	UOFS Y-1626	✓, ×	
185	*Rhodotorula minuta*	UOFS Y-0835	✓, ×	
186	*Rhodotorula minuta*	CBS 2221	✓	
187	*Rhodotorula minuta*	UOFS Y-0129		
188	*Rhodotorula minuta*	UOFS Y-0143	✓	
189	*Rhodotorula minuta*	UOFS Y-0125	×	×
190	*Rhodotorula minuta*	UOFS Y-0125	✓	
191	*Rhodotorula minuta*	UOFS Y-0126	✓	
192	*Rhodotorula minuta*	UOFS Y-0138	✓, ×	
193	*Rhodotorula mucilagina*	UOFS Y-0137	×	
194	*Rhodotorula mucilaginosa*	CBS 0017		
195	*Rhodotorula mucilaginosa*	CBS 5804		
196	*Rhodotorula mucilaginosa*	CBS 5951		
197	*Rhodotorula mucilaginosa*	UOFS Y-0137		
198	*Rhodotorula mucilaginosa*	UOFS Y-0124		
199	*Rhodotorula mucilaginosa*	UOFS Y-0133	×	
200	*Rhodotorula mucilaginosa*	UOFS Y-0226	✓, ×	
201	*Rhodotorula mucilaginosa*	UOFS Y-0478		

	Species	Culture collection number	EH activity	Enantio-selective
202	*Rhodotorula philyta*	UOFS Y-0134		
203	*Rhodotorula philyta*	UOFS Y-0134	×	
204	*Rhodotorula rubra*	UOFS Y-0112	×	
205	*Rhodotorula* sp.	UOFS Y-0448		
206	*Rhodotorula* sp.	UOFS Y-0139		
207	*Rhodotorula* sp.	UOFS Y-0142	✓, ×, ★	
208	*Rhodotorula* sp.	UOFS Y-2042		
209	*Rhodotorula* sp.	UOFS Y-2043	✓, ×	
210	*Rhodotorula* sp.	UOFS Y-2045	✓, ×	
211	*Rhodotorula* sp.	UOFS Y-2046	✓	
212	*Saccharomyces exiguus*	CBS 0835		
213	*Saccharomycopsis capsularis*	CSIR Y-0447		
214	*Saccharomycopsis capsularis*	CSIR Y-0651	×	
215	*Saccharomycopsis capsularis*	CSIR Y-0652		
216	*Saccharomycopsis capsularis*	CSIR Y-0653		
217	*Saccharomycopsis capsularis*	CSIR Y-0654		
218	*Saccharomycopsis capsularis*	CSIR Y-0655		
219	*Saccharomycopsis crataegensis*	CBS 6447		
220	*Saccharomycopsis malanga*	CBS 6267		
221	*Saccharomycopsis malanga*	CSIR Y-0557		
222	*Saccharomycopsis malanga*	CSIR Y-0557		
223	*Saccharomycopsis selenospora*	CBS 2562		
224	*Saccharomycopsis synnaedendra*	CBS 6161		
225	*Saccharomycopsis vini*	CBS 4110		
226	*Sporidiobolus johnsonii*	CSIR Y-0011		
227	*Sporidiobolus johnsonii*	UOFS Y-0856	✓	
228	*Sporobolomyces roseus*	UOFS Y-0452		
229	*Sporobolomyces tsugae*	CBS 7096	×	
230	*Trichosporon beigelii*	UOFS Y-1580	✓, ×	
231	*Trichosporon beigelii*	UOFS Y-0061		

	Species	Culture collection number	EH activity	Enantio-selective
232	*Trichosporon beigelii*	UOFS Y-0062		
233	*Trichosporon beigelii*	UOFS Y-0063	✓	
234	*Trichosporon beigelii*	UOFS Y-0102		
235	*Trichosporon beigelii*	UOFS Y-0103		
236	*Trichosporon beigelii*	UOFS Y-0113	✓, ×	
237	*Trichosporon cutaneum*	UOFS Y-0989		
238	*Trichosporon delbrueckii*	TVN 126		
239	*Trichosporon delbrueckii*	TVN 140		
240	*Trichosporon delbrueckii*	TVN 145		
241	*Trichosporon delbrueckii*	UOFS Y-1939		
242	*Trichosporon mucoides*	UOFS Y-0116		
243	*Trichosporon mucoides*	UOFS Y-2041		
244	*Trichosporon ovoides*	UOFS Y-0106	✓, ×	
245	*Trichosporon pullulans*	CBS 2535	×, ★	
246	*Trichosporon pullulans*	UOFS Y-1617		
247	*Trichosporon* sp.	CBS 2485		
248	*Trichosporon* sp.	CBS 2488	✓	
249	*Trichosporon* sp.	UOFS Y-0861	✓	
250	*Trichosporon* sp.	UOFS Y-1615		
251	*Trichosporon* sp.	CBS 5597		
252	*Trichosporon* sp.	CBS 6858		
253	*Trichosporon* sp.	CBS 8152	✓	
254	*Trichosporon* sp.	UOFS Y-0118		
255	*Trichosporon* sp.	UOFS Y-0119	✓, ×	
256	*Trichosporon* sp.	UOFS Y-0451		
257	*Trichosporon* sp.	UOFS Y-0546	★	
258	*Trichosporon* sp.	UOFS Y-0547		
259	*Trichosporon* sp.	UOFS Y-0548		
260	*Trichosporon* sp.	UOFS Y-0552		
261	*Trichosporon* sp.	UOFS Y-0616	✓	

	Species	Culture collection number	EH activity	Enantio-selective
262	*Trichosporon* sp.	UOFS Y-2113		
263	*Trichosporon* sp.	UOFS Y-0449	✓, ×, ★	
264	*Trichosporon* sp.	UOFS Y-0533	✓, ×	
265	*Trichosporon* sp.	UOFS Y-0450	✓, ×	
266	*Trichosporon* sp.	UOFS Y-0119		
267	Unidentified	Car 001	✓, ×	
268	Unidentified	Car 002	✓, ×	
269	Unidentified	Car 003	✓, ×, ★	
270	Unidentified	Car 005		
271	Unidentified	Car 006	✓, ×	
272	Unidentified	Car 009		
273	Unidentified	Car 011	✓, ×	
274	Unidentified	Car 012		
275	Unidentified	Car 013	✓	
276	Unidentified	Car 016	✓, ×, ★	
277	Unidentified	Car 019	✓, ×	
278	Unidentified	Car 020	✓, ×, ★	
279	Unidentified	Car 022	✓, ×	
280	Unidentified	Car 026		
281	Unidentified	Car 031		
282	Unidentified	Car 032		
283	Unidentified	Car 033		
284	Unidentified	Car 036		
285	Unidentified	Car 038	✓, ×	
286	Unidentified	Car 039		
287	Unidentified	Car 040	✓, ×	✓
288	Unidentified	Car 043	✓, ×	
289	Unidentified	Car 044		
290	Unidentified	Car 046	✓, ×	✓, ×
291	Unidentified	Car 050	✓, ×, ★	

	Species	Culture collection number	EH activity	Enantio-selective
292	Unidentified	Car 052	✓, ×	✓
293	Unidentified	Car 054	✓, ×	✓
294	Unidentified	Car 055		
295	Unidentified	Car 057		
296	Unidentified	Car 058		
297	Unidentified	Car 059	✓, ×, ★	
298	Unidentified	Car 060	✓, ×	
299	Unidentified	Car 061	✓, ×, ★	
300	Unidentified	Car 062	✓, ×	✓
301	Unidentified	Car 066	✓, ×, ★	
302	Unidentified	Car 067	✓, ×, ★	
303	Unidentified	Car 069	✓, ×	
304	Unidentified	Car 070	✓, ×	
305	Unidentified	Car 075	✓, ×, ★	
306	Unidentified	Car 076	✓, ×	
307	Unidentified	Car 077	✓, ×	
308	Unidentified	Car 078	✓, ×	
309	Unidentified	Car 089		
310	Unidentified	Car 091		
311	Unidentified	Car 092	✓, ×	
312	Unidentified	Car 093	✓, ×	
313	Unidentified	Car 094	✓, ×	
314	Unidentified	Car 095		
315	Unidentified	Car 096		
316	Unidentified	Car 097		
317	Unidentified	Car 098		
318	Unidentified	Car 099	✓, ×	
319	Unidentified	Car 100	✓, ×	
320	Unidentified	Car 102	✓, ×, ★	
321	Unidentified	Car 103	✓, ×	

	Species	Culture collection number	EH activity	Enantio-selective
322	Unidentified	Car 104		
323	Unidentified	Car 108	✓, ×	✓
324	Unidentified	Car 113		
325	Unidentified	Car 114		
326	Unidentified	Car 116		
327	Unidentified	Car 118	✓, ×, ★	
328	Unidentified	Car 120	✓, ×	
329	Unidentified	Car 121	✓, ×, ★	
330	Unidentified	Car 125	×	
331	Unidentified	Car 126	✓, ×	
332	Unidentified	Car 131	✓, ×, ★	
333	Unidentified	Car 134	✓, ×, ★	
334	Unidentified	Car 137	✓, ×	
335	Unidentified	Car 138	✓, ×	
336	Unidentified	Car 139	✓, ×	
337	Unidentified	Car 141	✓, ×	
338	Unidentified	Car 142	✓, ×	
339	Unidentified	Car 200	✓, ×	✓
340	Unidentified	Car 204	✓, ×, ★	
341	Unidentified	Car 205	✓, ×	
342	Unidentified	Car 206	×	
343	Unidentified	Car 207	✓, ×	
344	Unidentified	Car 210	✓, ×	
345	Unidentified	Car 220	×	
346	Unidentified	Car 223	✓, ×	
347	Unidentified	Car 224	✓, ×	
348	Unidentified	Car 225	✓, ×	
349	Unidentified	Car 226	✓, ×	
350	Unidentified	Car 400	✓, ×	
351	Unidentified	IGC4524		

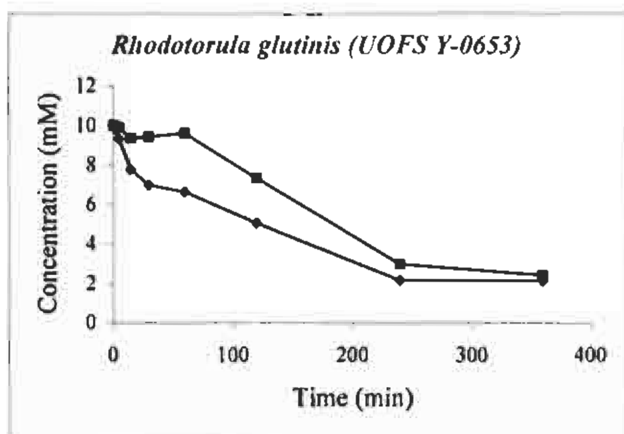
	Species	Culture collection number	EH activity	Enantio-selective
352	Unidentified	UOFS Y-2114	✓, ×	
353	Unidentified	TVN 292		
354	Unidentified	UOFS Y-1938	×	
355	Unidentified	UOFS Y-0505	✓, ×	
356	Unidentified	UOFS Y-0506		
357	Unidentified	UOFS Y-0507		
358	Unidentified	UOFS Y-0508	✓, ×	
359	Unidentified	UOFS Y-0509	✓, ×	
360	Unidentified	UOFS Y-0510		
361	Unidentified	UOFS Y-0511		
362	Unidentified	UOFS Y-0512		
363	Unidentified	UOFS Y-0513	✓, ×, ●	×, ●●
364	Unidentified	UOFS Y-0514	✓, ×	
365	Unidentified	UOFS Y-0515	★	
366	Unidentified	UOFS Y-0516	★	
367	Unidentified	UOFS Y-0517	✓, ×	
368	Unidentified	UOFS Y-0518	✓, ×, ●	×
369	Unidentified	UOFS Y-0560	✓	
370	Unidentified	UOFS Y-0561		
371	Unidentified	UOFS Y-2121	✓, ×	
372	Unidentified	VDW 69		
373	Unidentified	ZZ 003	✓, ×	
374	Unidentified	Y 12	✓	
375	Unidentified	Y 13		
376	Unidentified	Y 11	✓, ×	
377	Unidentified	Y 10		
378	Unidentified	Y 15		
379	Unidentified	Y 09		
380	Unidentified	Y 14	✓, ×	
381	Unidentified	TVN 219		

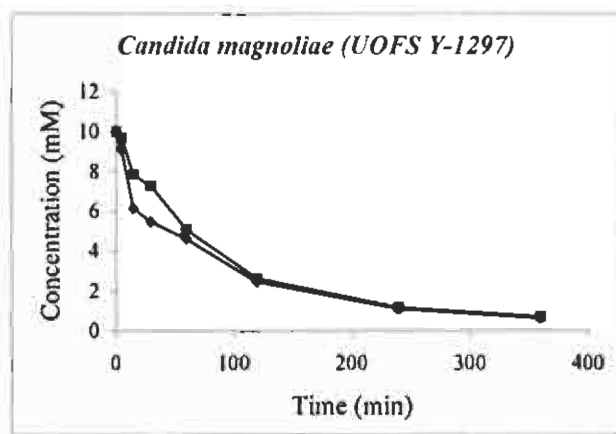
	Species	Culture collection number	EH activity	Enantio-selective
382	Unidentified	TVN 221		
383	Unidentified	TVN 222	✓, ×	
384	Unidentified	TVN 223	✓	
385	Unidentified	TVN 224		
386	Unidentified	TVN 225		
387	Unidentified	TVN 226		
388	Unidentified	TVN 227	✓, ×	
389	Unidentified	TVN 228		
390	Unidentified	TVN 229		
391	Unidentified	TVN 230	✓, ×	
392	Unidentified	TVN 231		
393	Unidentified	TVN 232		
394	Unidentified	TVN 233		
395	Unidentified	TVN 234		
396	Unidentified	TVN 235	✓	✓
397	Unidentified	TVN 115		
398	Unidentified	TVN 116	✓	
399	Unidentified	TVN 117		
400	Unidentified	TVN 118		
401	Unidentified	TVN 119		
402	Unidentified	TVN 120		
403	*Wickerhamiella domercqiae*	CBS 4733		
404	*Wingea robertsiae*	UOFS Y-0891	✓, ×	
405	*Yarrowia lipolytica*	UOFS Y-1138		
406	*Yarrowia lipolytica*	CSIR Y-0087		
407	*Yarrowia lipolytica*	UOFS Y-2160		
408	*Yarrowia lipolytica*	CSIR Y-0513	✓	
409	*Yarrowia lipolytica*	CSIR Y-0516		
410	*Yarrowia lipolytica*	CSIR Y-0518	✓, ×	

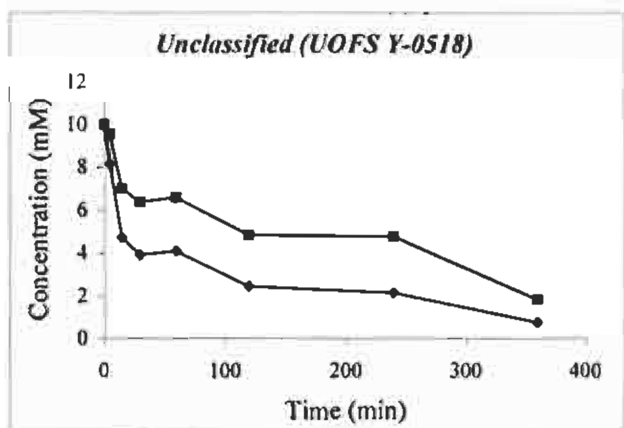
2. Time course reactions

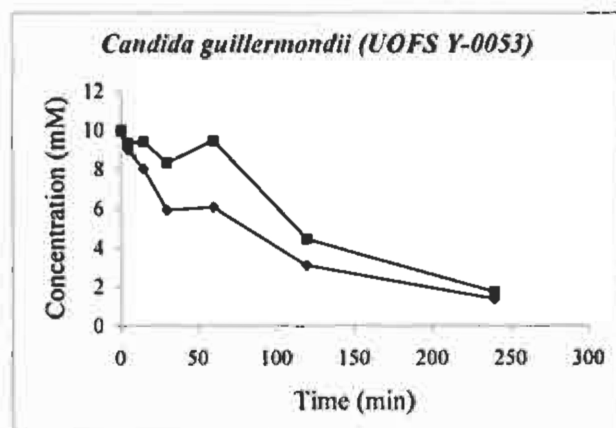
Time course reactions for the twelve most promising yeast strains (Chapter 4) for each substrate are presented in the following section. In each case (♦) represents the epoxide enantiomer that was eluated first and (■) represents the enantiomer that was eluated second from the chiral GC column. (mM = 10^{-3} mol.dm^{-3})

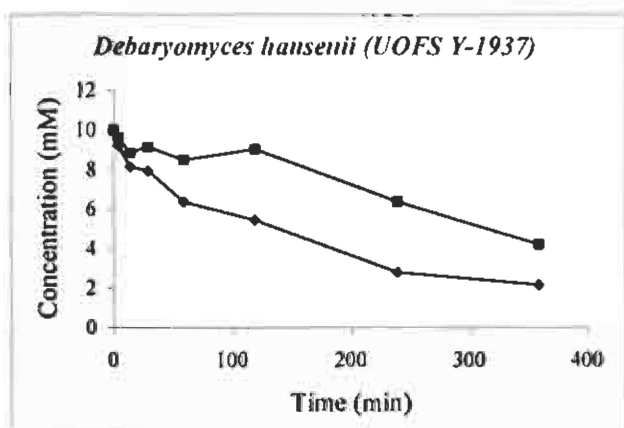
2.1 Para-nitrostyrene oxide

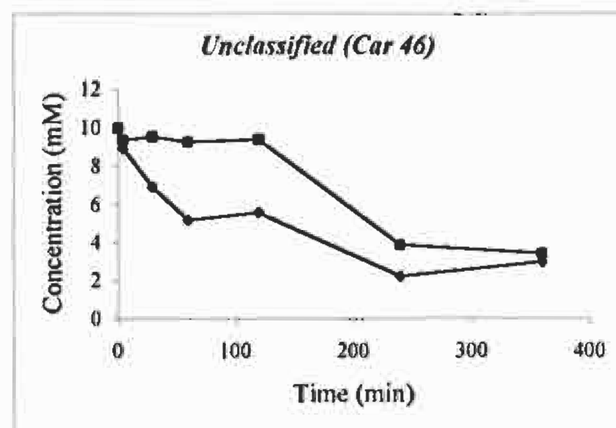


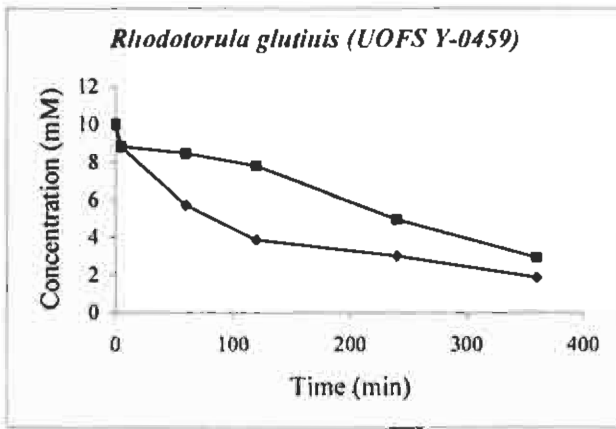


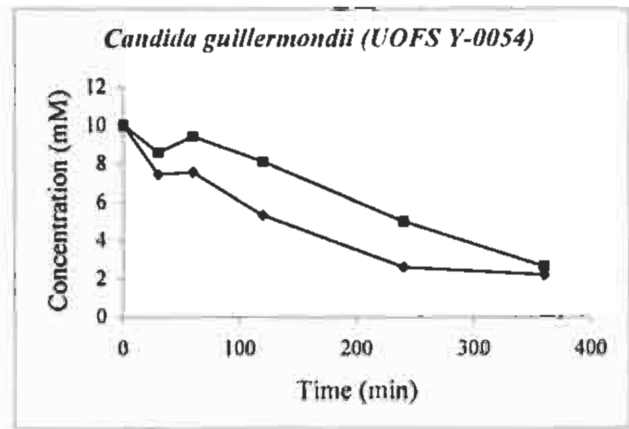


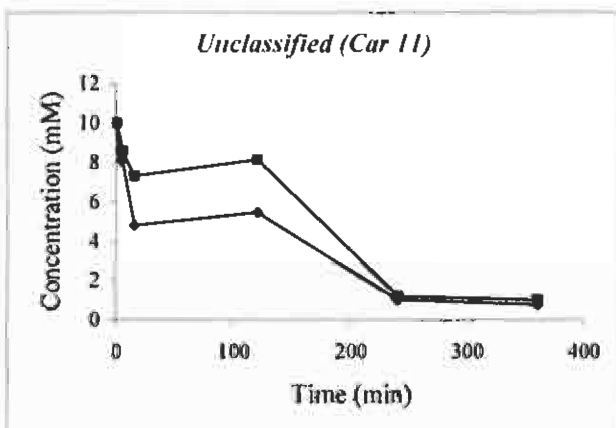


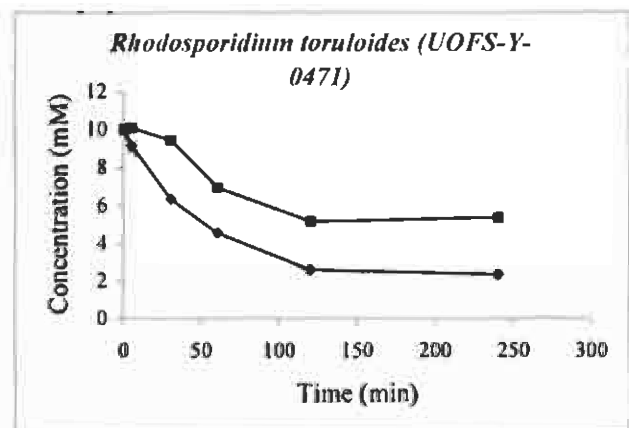


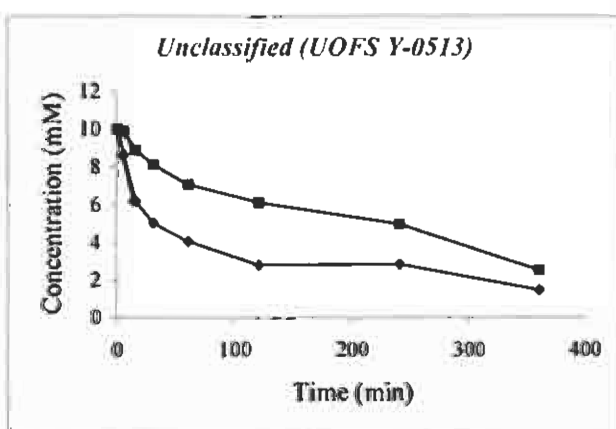


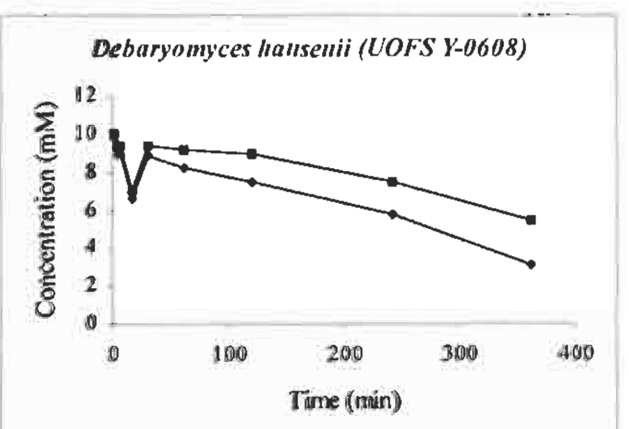

Rhodotorula glutinis (UOFS Y-0459)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)


Candida guillermondii (UOFS Y-0054)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

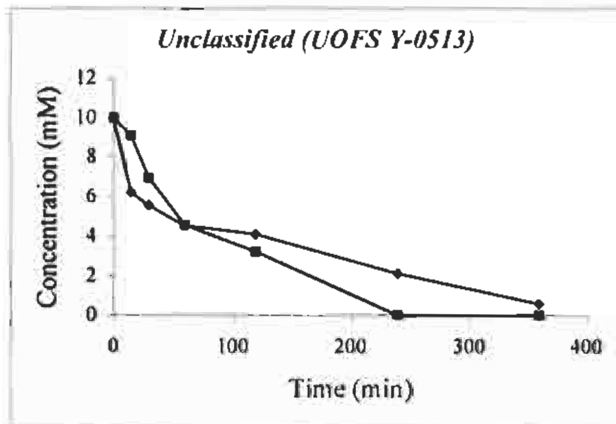

Unclassified (Car 11)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

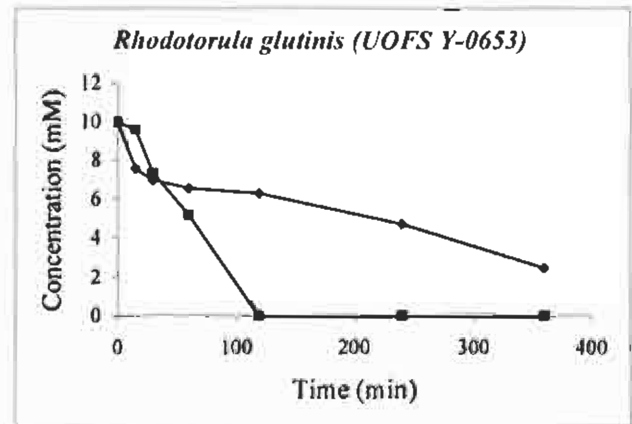

Rhodosporidium toruloides (UOFS-Y-0471)
Concentration (mM)
12
10
8
6
4
2
0
0
50
100
150
200
250
300
Time (min)

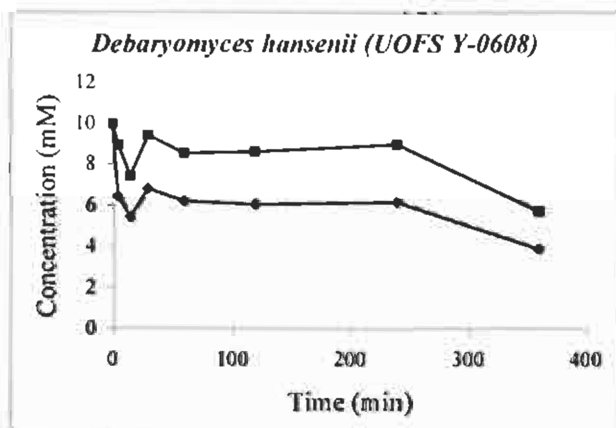

Unclassified (UOFS Y-0513)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

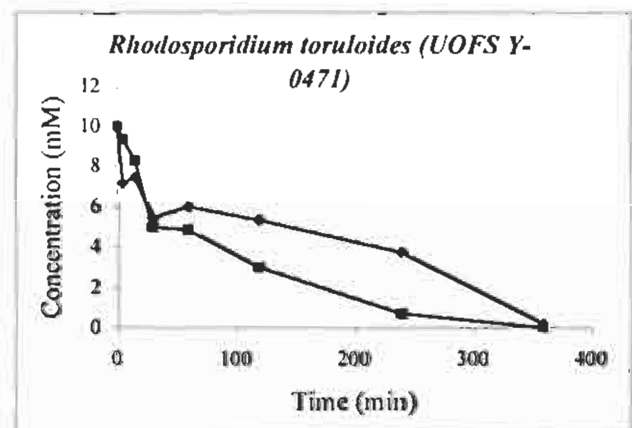

Debaryomyces hansenii (UOFS Y-0608)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

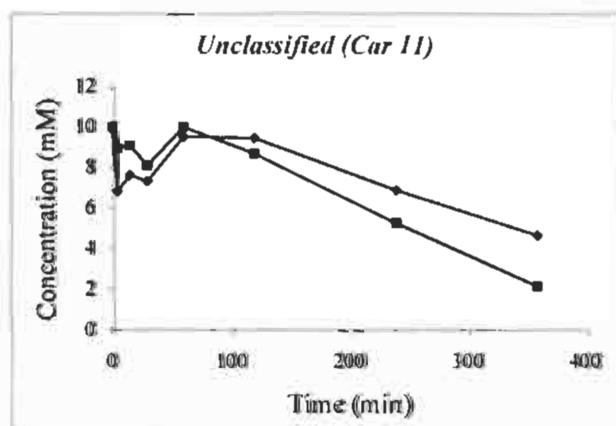
2.2 Meta-nitrostyrene oxide

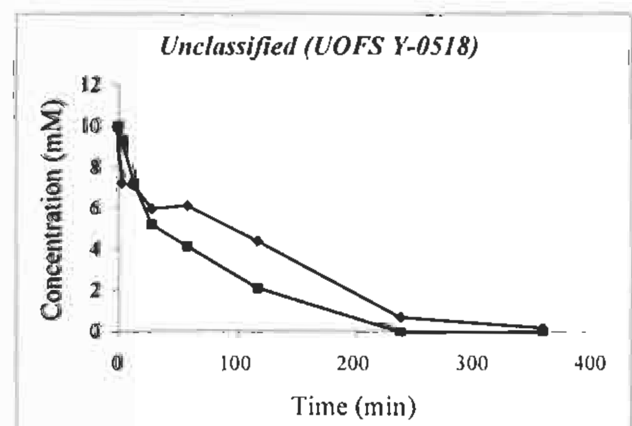


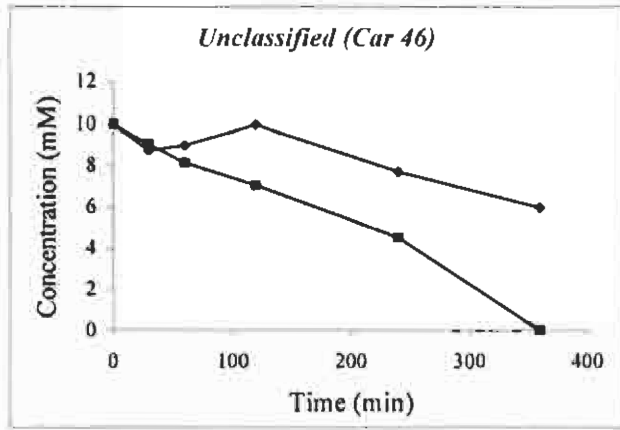


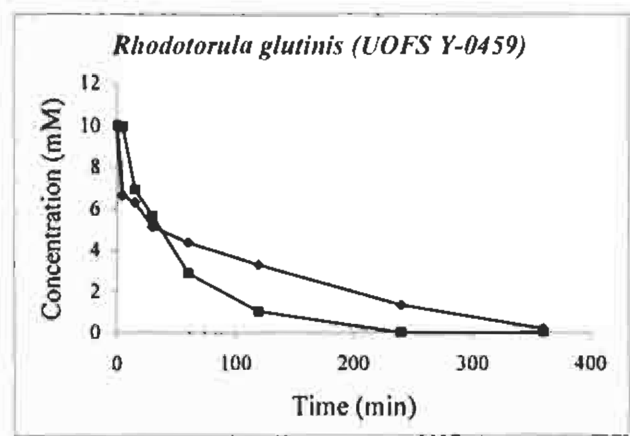


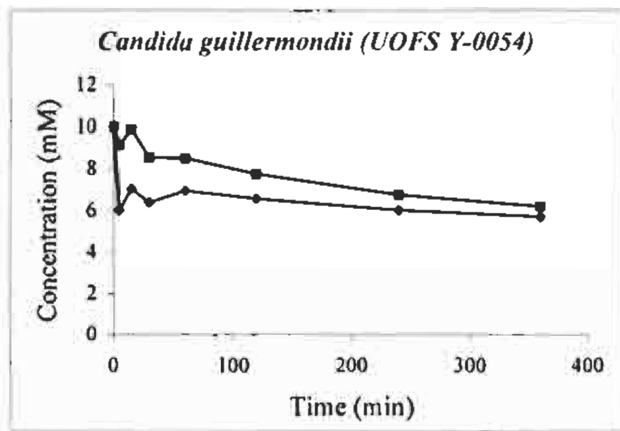


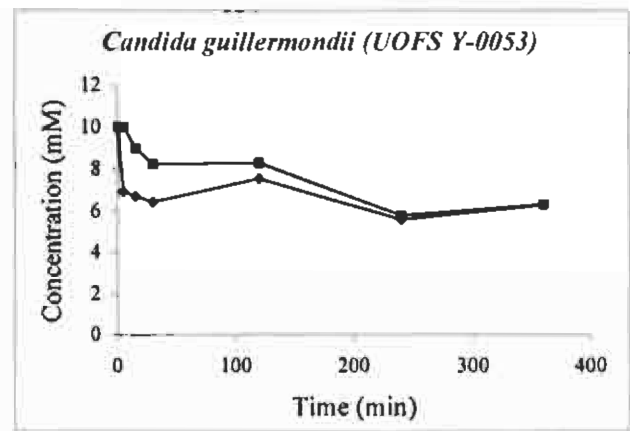


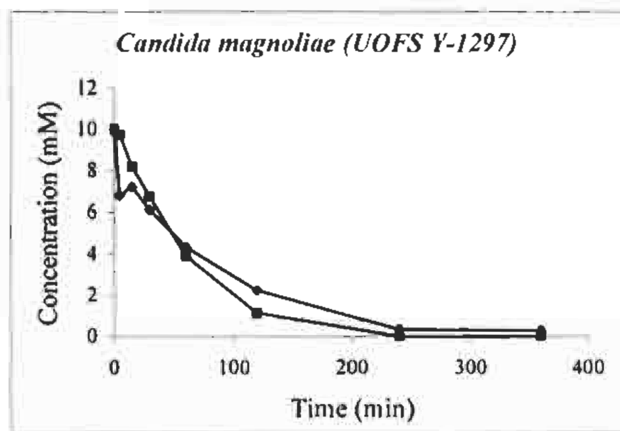


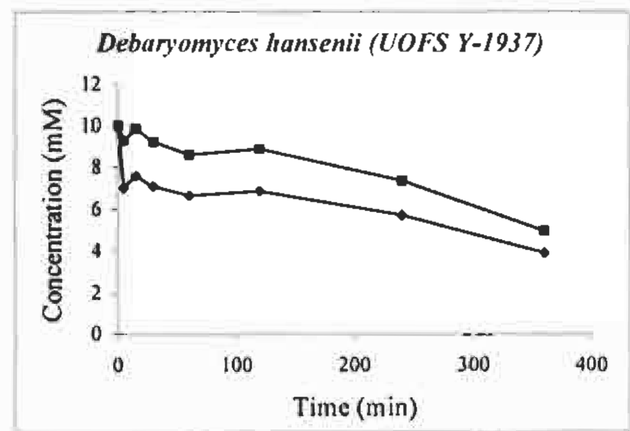

Unclassified (Car 46)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)


Rhodotorula glutinis (UOFS Y-0459)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

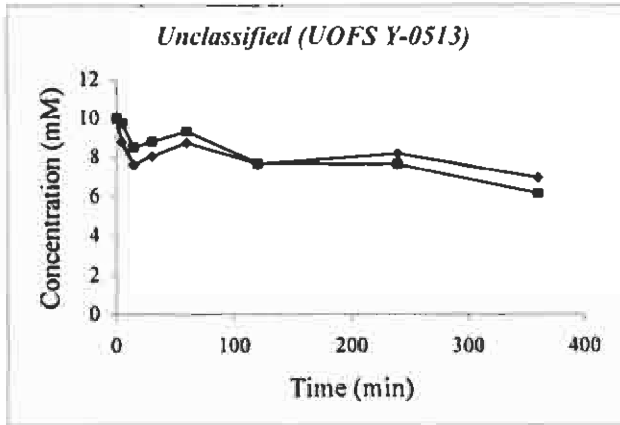

Candida guillermondii (UOFS Y-0054)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

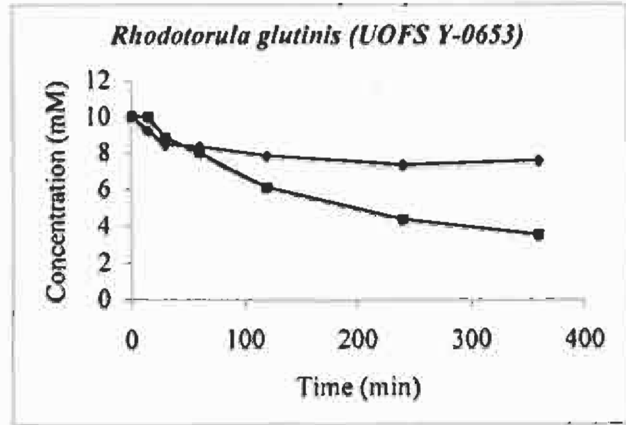

Candida guillermondii (UOFS Y-0053)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

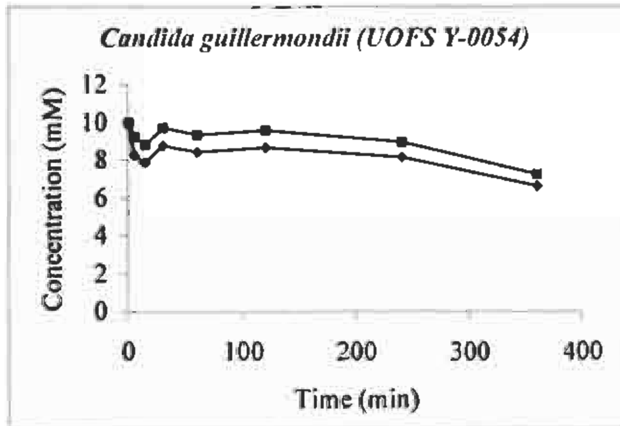

Candida magnoliae (UOFS Y-1297)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

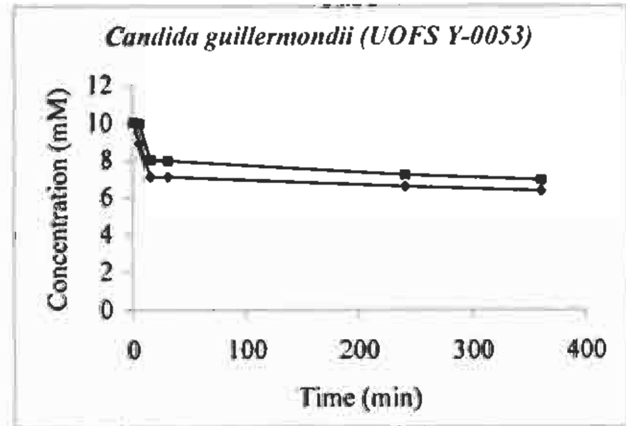

Debaryomyces hansenii (UOFS Y-1937)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

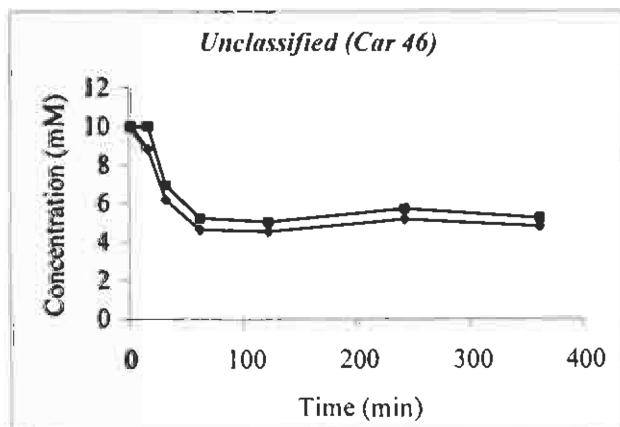
2.3 Ortho-nitrostyrene oxide

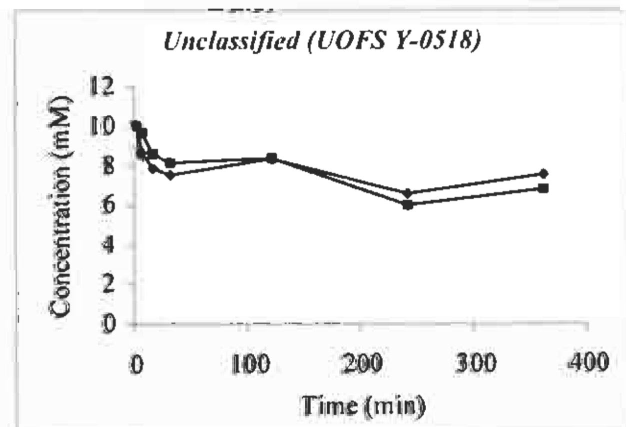


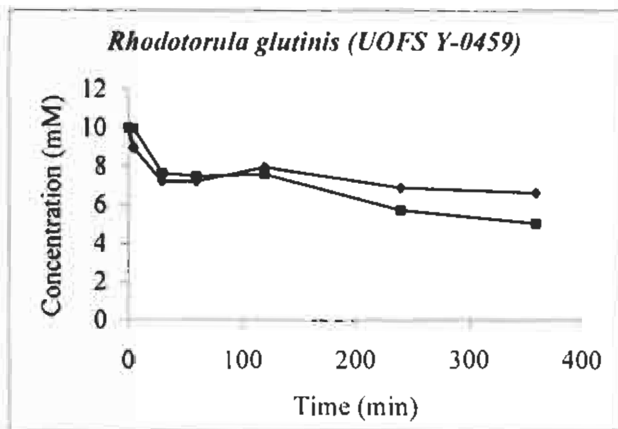


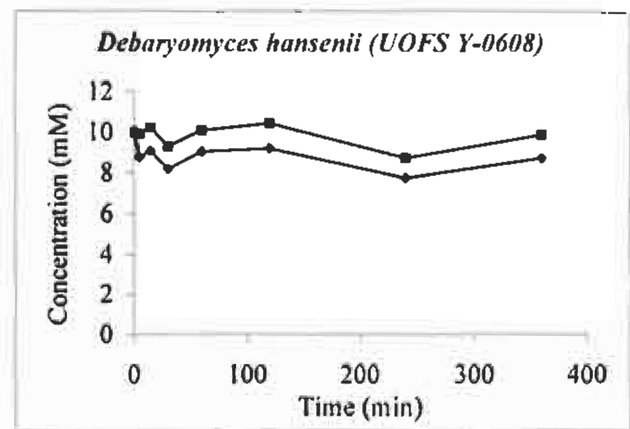


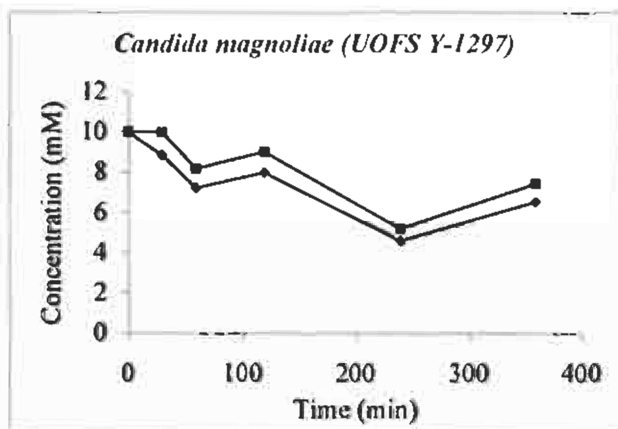


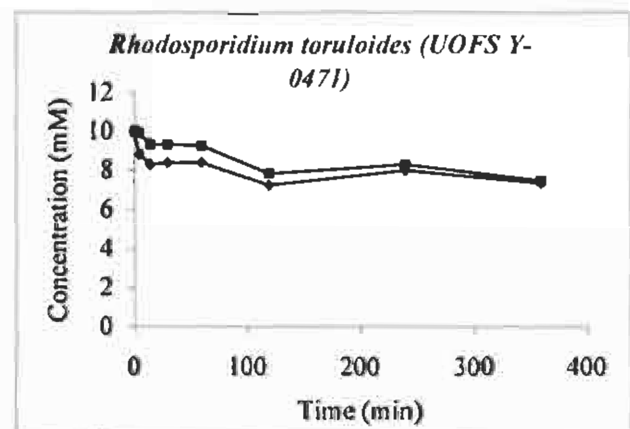


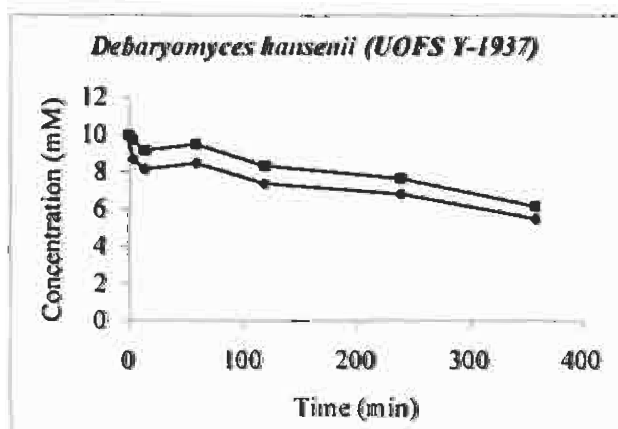


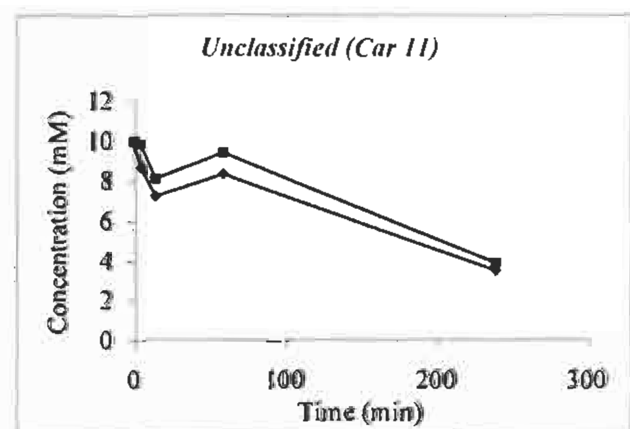

Rhodotorula glutinis (UOFS Y-0459)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)


Debaryomyces hansenii (UOFS Y-0608)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

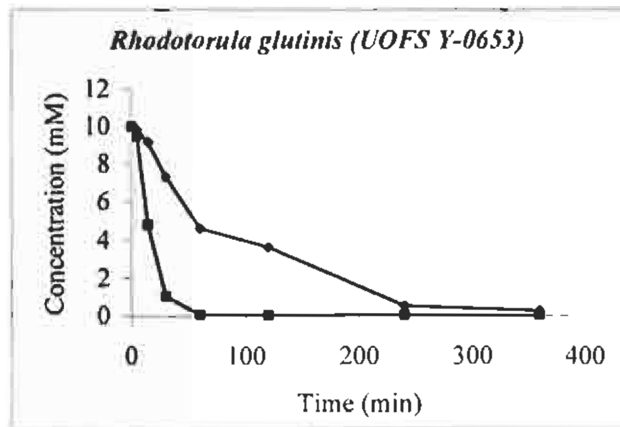

Candida magnoliae (UOFS Y-1297)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

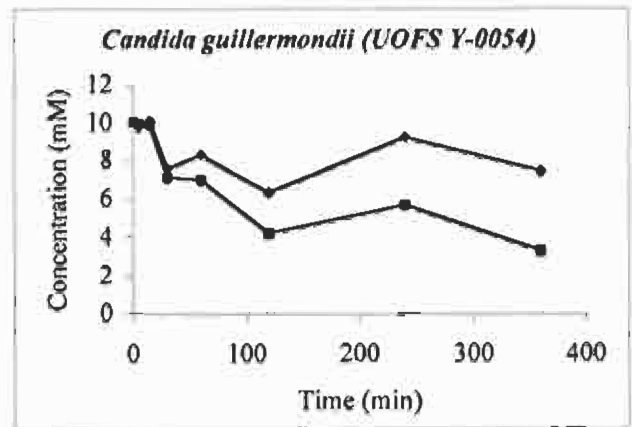

Rhodosporidium toruloides (UOFS Y-0471)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

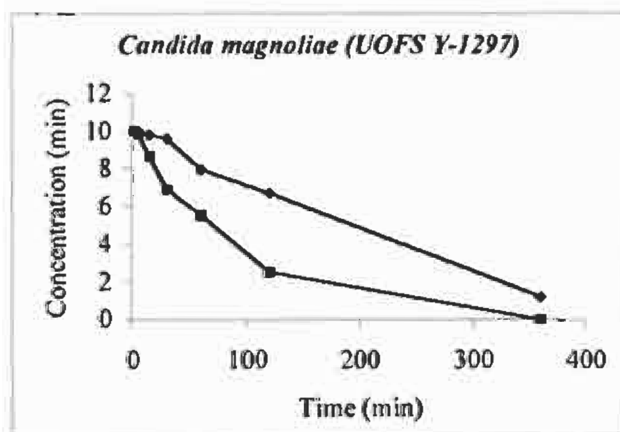

Debaryomyces hansenii (UOFS Y-1937)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

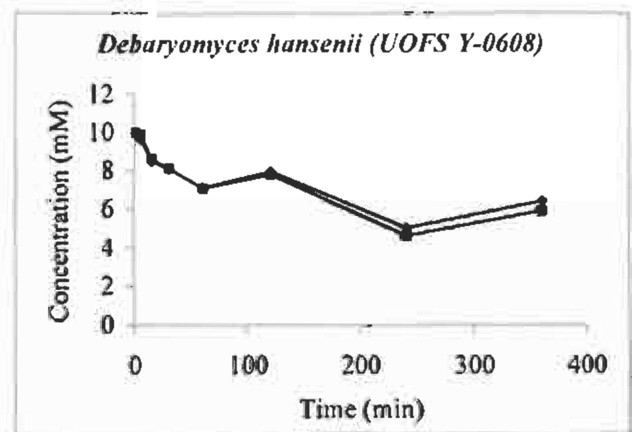

Unclassified (Car 11)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
Time (min)

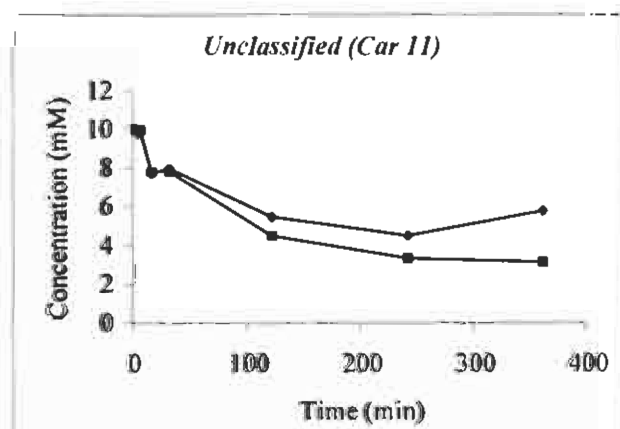
2.4 Styrene oxide

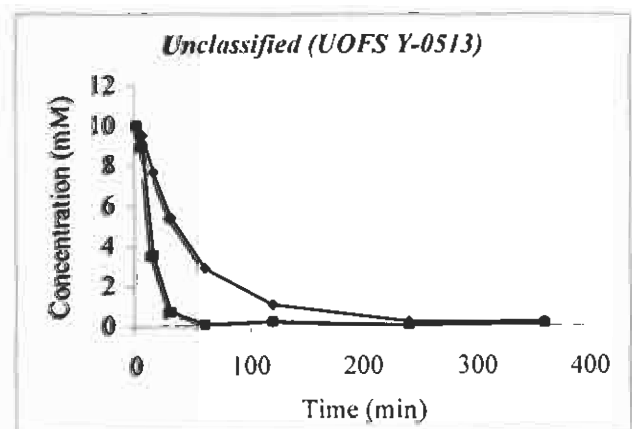


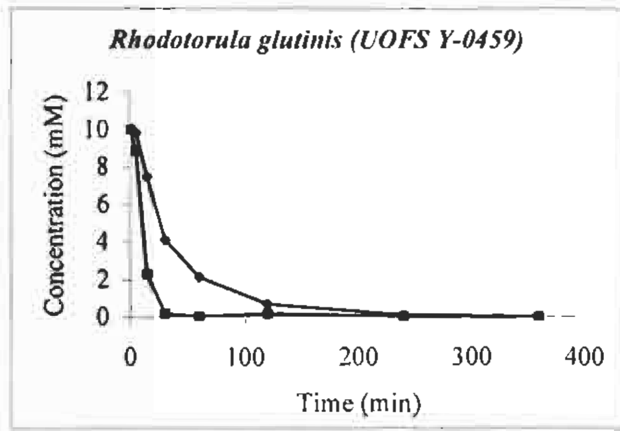


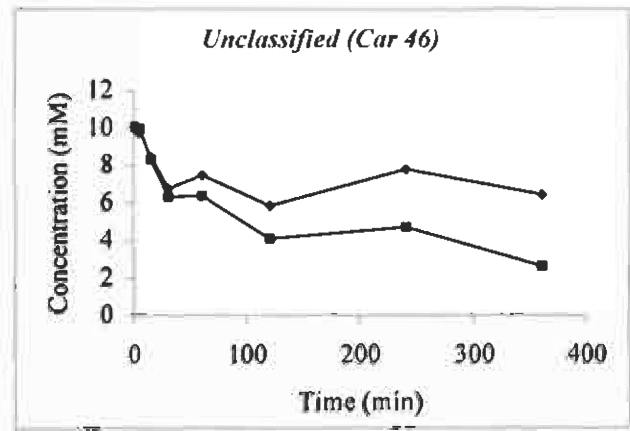


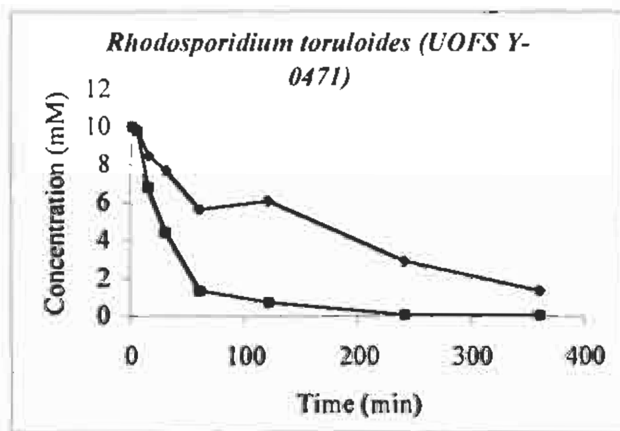


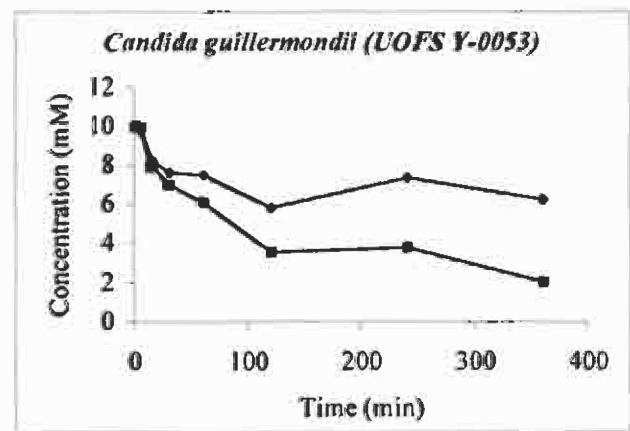


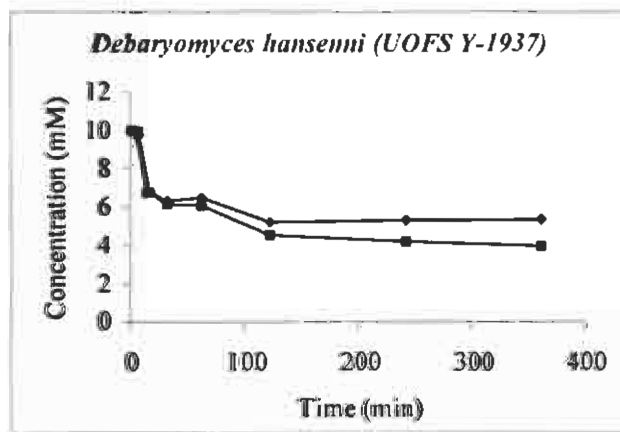


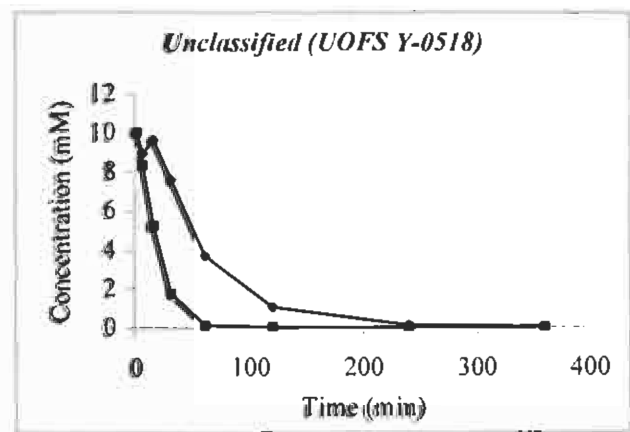

Rhodotorula glutinis (UOFS Y-0459)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)


Unclassified (Car 46)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)


Rhodosporidium toruloides (UOFS Y-0471)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)


Candida guillermondii (UOFS Y-0053)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

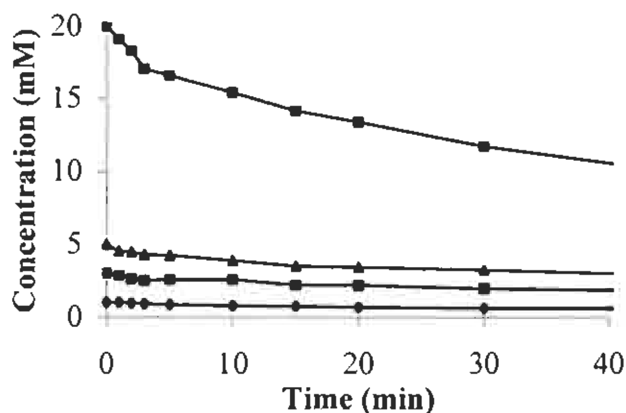

Debaryomyces hansenni (UOFS Y-1937)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

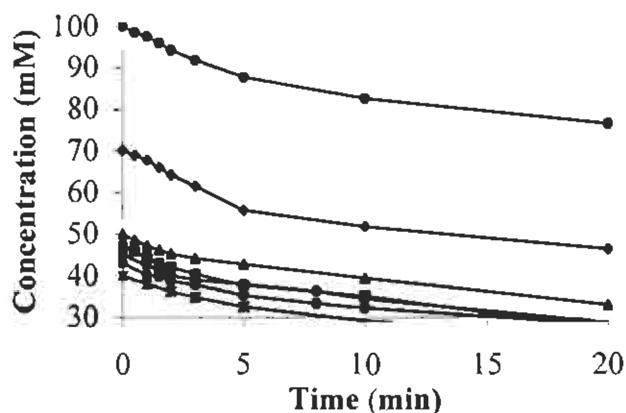

Unclassified (UOFS Y-0518)
Concentration (mM)
12
10
8
6
4
2
0
0
100
200
300
400
Time (min)

3. Optimisation

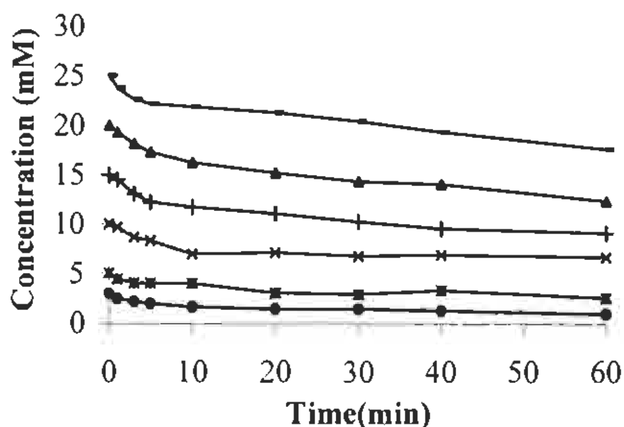
This section contains time course reactions obtained at increasing initial concentrations (Chapter 5). *R. glutinis* UOFS Y-0653 was used to catalyse the hydrolysis of **SO**, **mNSO** and **oNSO**, while *R. toruloides* (UOFS Y-0471) was used to catalyse the hydrolysis of **pNSO**. Low initial concentrations are shown on the left and higher concentrations on the right.

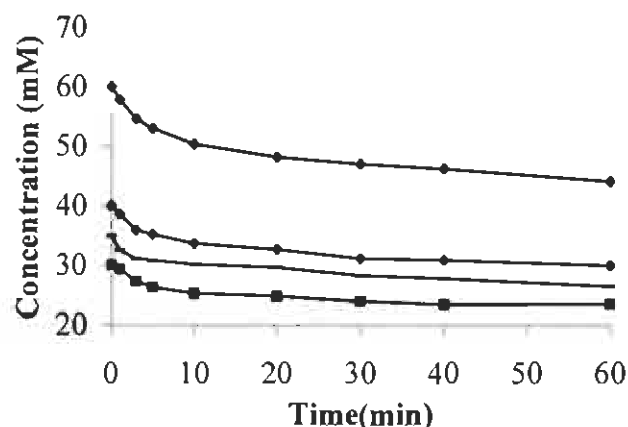
SO



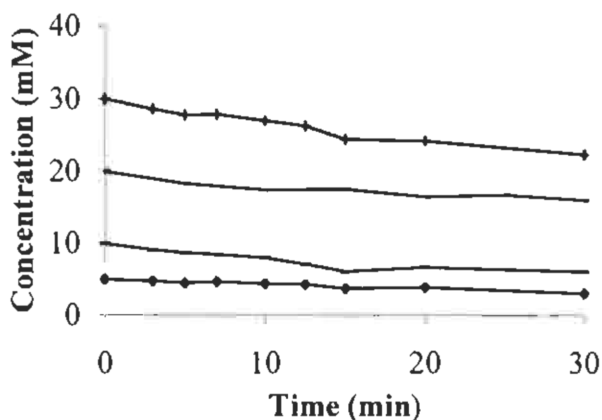


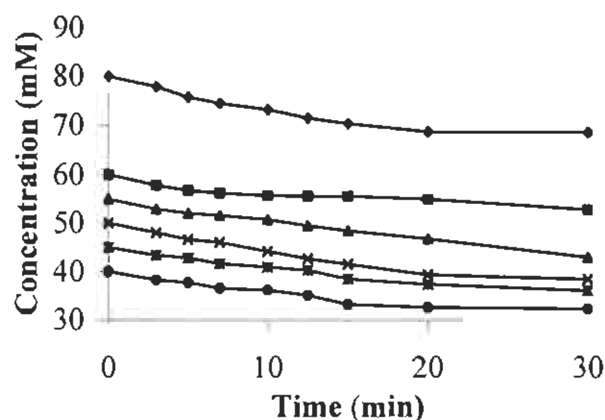
pNSO



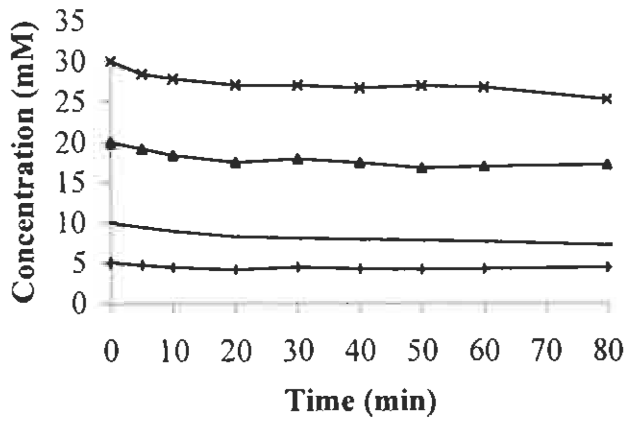


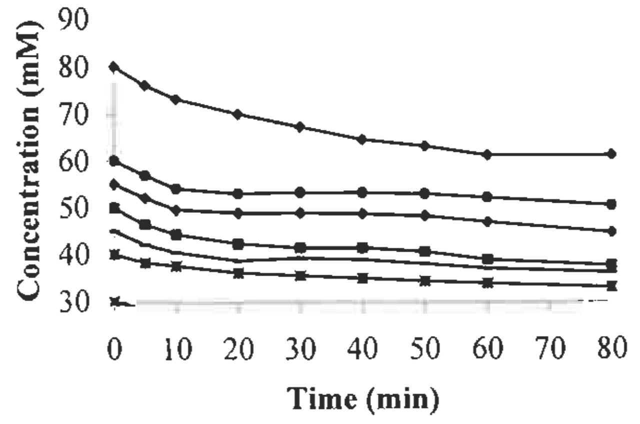
mNSO





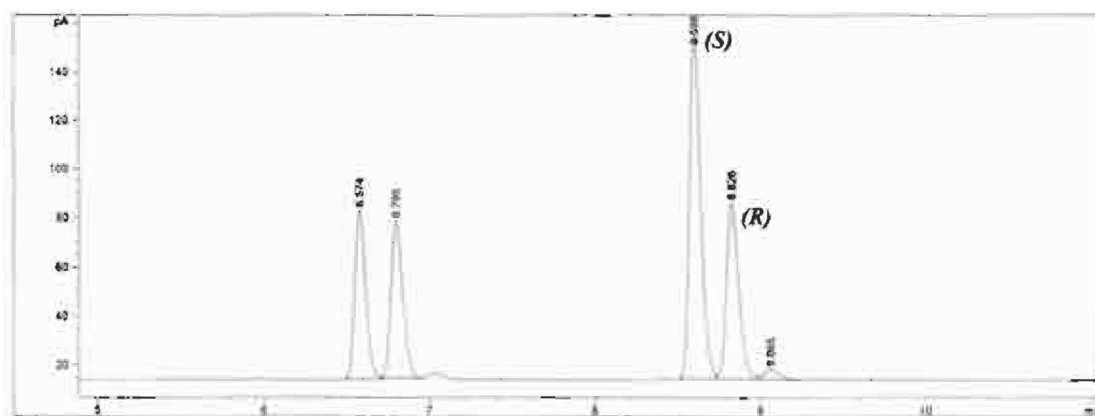
oNSO



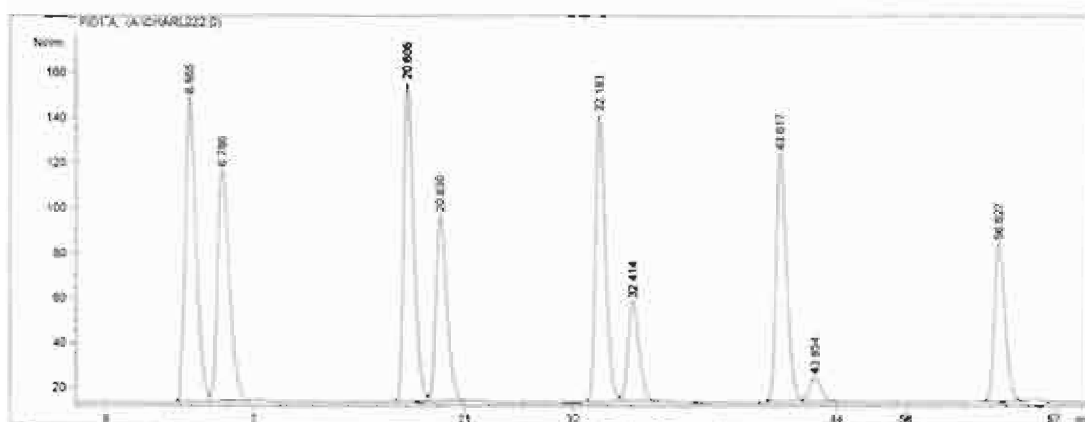


Appendix 3

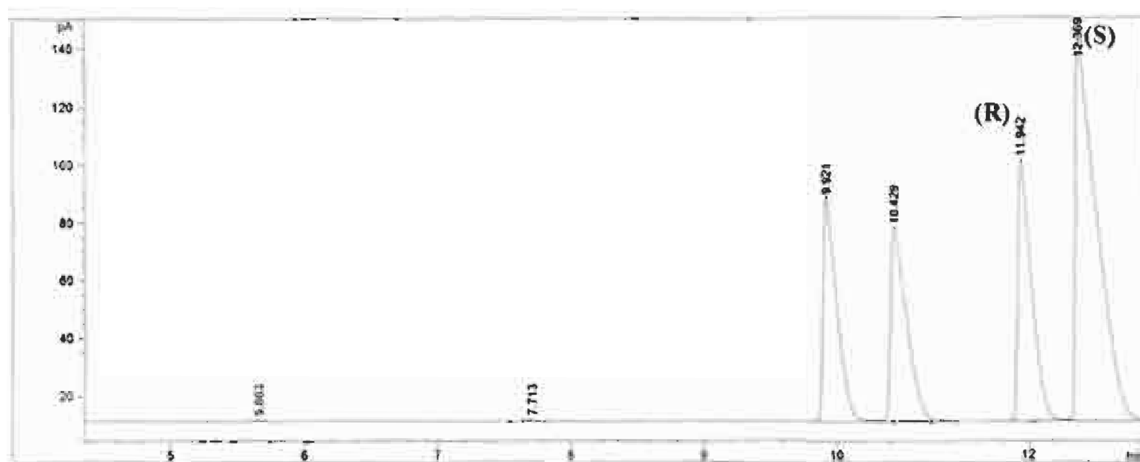
All the results shown were obtained using a HP 6890 GC, equipped with FID. In all cases H_2 was used as mobile phase.



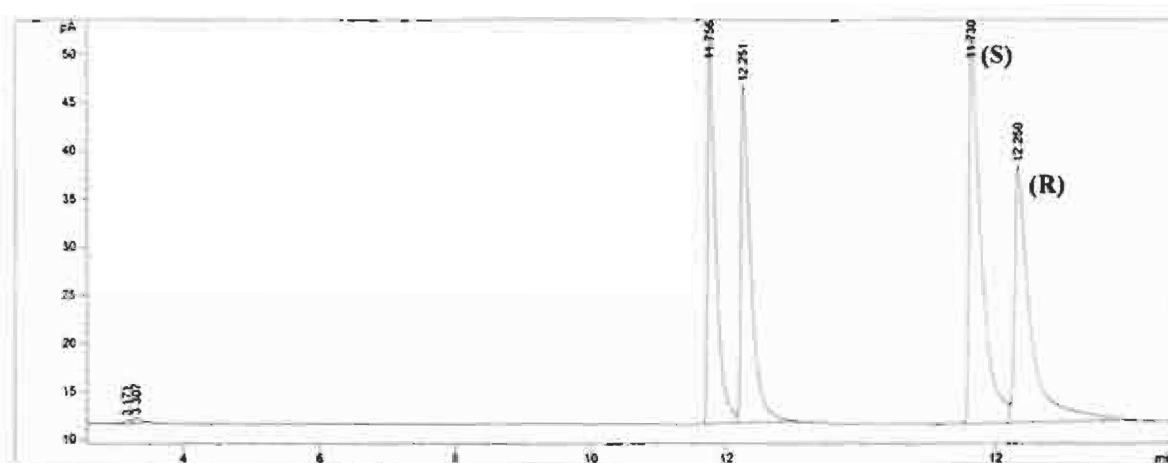
Chromatogram 1: Racemic **SO** and a racemic sample spiked with (S)-**SO** to reveal the elution order on a β-dex 225 column (90 °C).



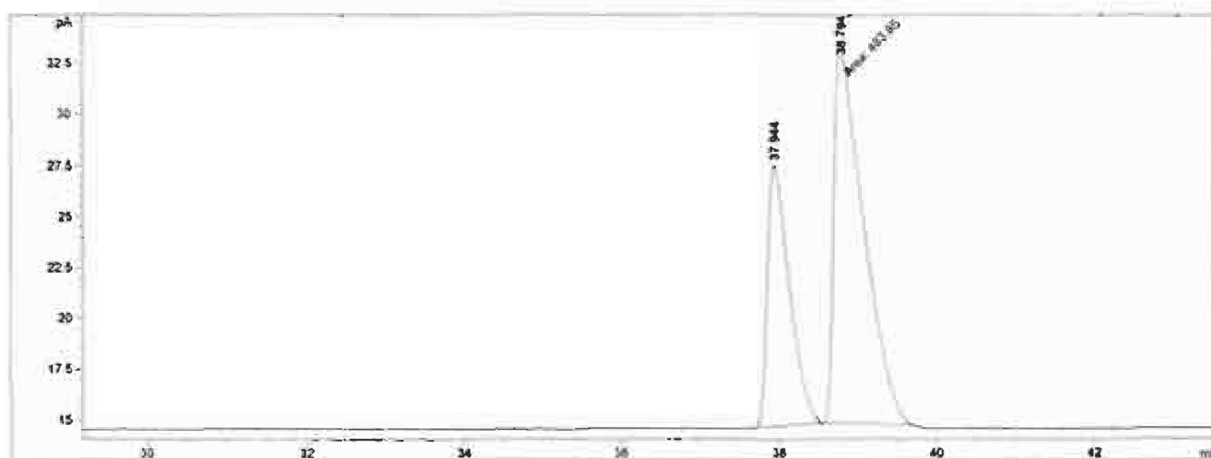
Chromatogram 2: Chiral GC analysis (β-dex 225) at different time intervals of the hydrolysis of **SO** catalysed by epoxides hydrolase (90 °C).



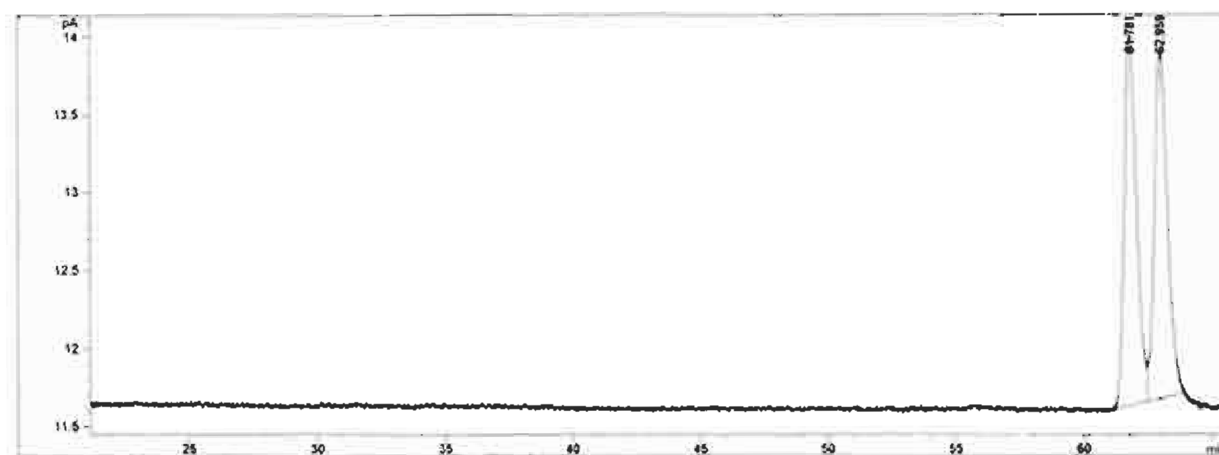
Chromatogram 3: Racemic **SO** and a racemic sample spiked with (S)-**SO** to reveal the elution order on a β-dex 120 column (90 °C).



Chromatogram 4: Racemic 1-phenyl-1,2-ethanediol and a racemic sample spiked with (S)-(+)-1-phenyl1,2-ethanediol to reveal the elution order on a β-dex 120 column (150 °C).

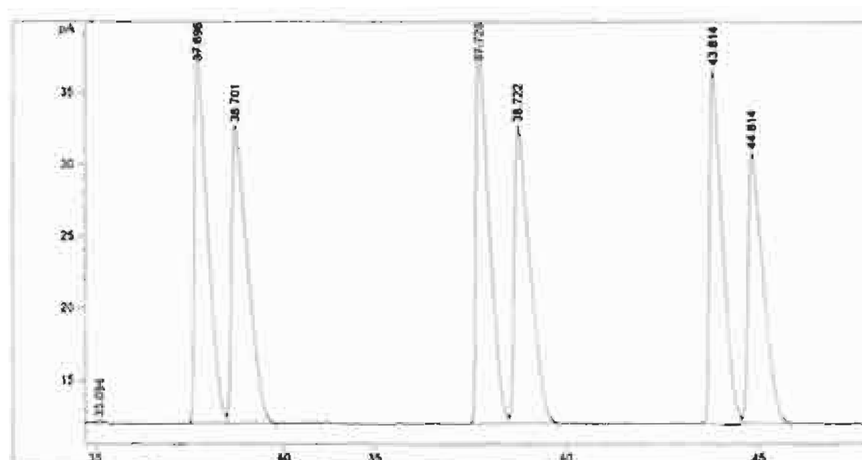


Chromatogram 5: Chiral analysis of the enantiomers of **pNSO** (β-dex 120, 130 °C).

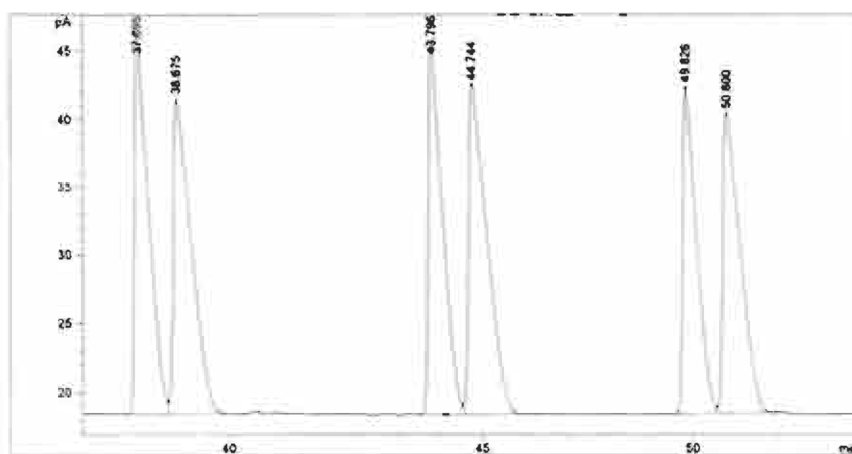


Chromatogram 6: Chiral analysis of the enantiomers of **mNSO** (β-dex 120, 115 °C)

Chromatogram 7: Chiral analysis of the enantiomers of **oNSO** (β-dex 120, 130 °C)



Chromatogram 8: Chiral GC analysis (β-dex 120, 90 °C) at different time intervals of the hydrolysis of **pNSO** catalysed by *R. glutinis* at 45 °C.



Chromatogram 9: Chiral GC analysis (β-dex 120, 90 °C) at different time intervals of the hydrolysis of **pNSO** catalysed by *R. glutinis* at 30 °C.