Red yeast epoxide hydrolases: growth, activity and selectivity

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Abstract

Enantiopure epoxides are versatile compounds in the production of single enantiomer drugs, and are of high value as building blocks and intermediates in the preparation of more complex single enantiomer pharmaceuticals and agrochemicals.

Epoxide hydrolases, ubiquitous enzymes in nature, can be versatile tools in the biocatalytic production of these single enantiomer epoxides due to their capability of selectively hydrolysing one enantiomer of a wide range of these compounds, and thus rendering an enantiopure epoxide and diol. The value of epoxide hydrolases for the kinetic resolution of epoxide compounds are dependant on factors such as availability, ease of production, long term stability, activity and the displayed enantioselectivity.

The first objective of this study was to investigate and optimise the growth media and time for the production of two red yeasts, *Rhodotorula glutinis* and *Rhodosporidium toruloides*, and their epoxide hydrolysing enzymes. Maximum and minimum epoxide hydrolase (EH) activity for *R. glutinis* was respectively observed with the YMvit (0,26 $mM.min^{-1}$) and malt (0,17 $mM.min^{-1}$) media, while peak biomass production was observed from the YM medium (64,9 $mg.mL^{-1}$). For *R. toruloides*, the highest biomass was produced in the YM (130,8 $mg.mL^{-1}$) medium, with similar epoxide hydrolase activities (average c = 0,75 ± 0,01) displayed for the YM, YMvit and malt grown biocatalysts.

With varying the YM medium glucose concentration (0,5 - 2,0 %) the most biomass was produced for *R. glutinis* with the addition of 1,5 % glucose (60,0 ± 0,9 $mg.mL^{-1}$), with a slight drop in the biomass observed with the addition of 2,0 % glucose (56,0 ± 1,7 $mg.mL^{-1}$). No significant differences in epoxide hydrolase activity was observed for the lower glucose additive concentrations (0,5 – 1,5 %), while 2,0 % (m/v) rendered a biocatalyst with almost 20 % higher activity (0,29 $mM.min^{-1}$). For *R. toruloides* an increase in the glucose concentration lead to a significantly higher biomass production while the time needed to attain the stationary phase increased progressively from 40 to 96 hours. Almost equal activity was observed for the top three glucose concentrations (average c = 0,82 ± 0,01) at 36 hours growth time, but in all cases a decrease in the EH activity was observed during the stationary phase, with the most pronounced decrease for the 2,0 % (m/v) glucose concentration, that showed a drop in conversion of almost 62 % at 144 hours growth time.

The second objective was to synthesise *meta* and *para* nitro-, methyl- and methoxystyrene oxides and the successive production of their single enantiopure epoxides through *R. glutinis*

EH mediated kinetic resolution, and the determination of the absolute configuration of the pure residual enantiomers through VCD analysis. *R. glutinis* selectively hydrolysed the whole range of styrene oxide derivatives, with the highest activity displayed towards the *meta* substituted derivatives in the order of methyl > methoxy > nitro. *m*-Methylstyrene oxide reached a % *e.e.* of >98 within 60 minutes, with an exceptionally high yield of 42,5 %. The absolute configuration of the residual epoxide enantiomers of *m*-nitro, *m*-methyl and *m*-methoxystyrene oxides were determined to be of the (S)-configuration, indicating that *R. glutinis* EH preferentially hydrolyses the (R)-epoxides.

Thirdly, we attempted to increase the *R. glutinis* EH activity through the addition of hydroxypropyl-β-cyclodextrin (HPB) and to correlate the rate of chemical and *R. glutinis* EH mediated enzymatic hydrolysis, and the enzyme's enantioselectivity to the electronic properties of their substituents and the spatial arrangement of the substrates in relation to the EH catalytic triad of the EH active site.

An increase in the HPB concentration (0 - 20 % w/v) lead to a substantial increase in both the solubility as well as enzyme activity for *p*-NO_2 (*para*-nitrostyrene oxide) with a significant increase in the solubility of between 2,89 and 6,28 times for the substrate range with the addition of 5 % HPB in comparison to the buffer solution.

The acid induced chemical and *R. glutinis* EH mediated enzymatic reaction rate was correlated to both the Hammett constant as well as the Mulliken charge distributions. The Mulliken charge distribution over the protonated epoxides was correlated to the acid induced chemical hydrolysis rates, while the Mulliken charge distribution over the neutral epoxides could be correlated to the enzymatic reaction rates. An increase in the electron-donating properties of the styrene oxide substituent groups was correlated to an increase in both the chemical as well as the *R. glutinis* EH mediated hydrolysis reaction rates of the styrene oxide derivatives.

Docking of the possible conformers of the (R)- and (S)-enantiomers of these *meta* and *para* substituted styrene oxides into the EH binding site of the closely related *Aspergillus niger* displayed a closer and more preferential fit of the (R)-epoxides which is the faster reacting enantiomer for both *A. niger* and *R. glutinis* EHs.

The proven relationship between *R. glutinis* EH activity and selectivity and the electronic properties of substituent groups, as well as the relationship between spatial arrangement of the epoxide hydrolase binding site and the enantioselectivity of the enzyme, could open up the possibility to correctly predict both the enantioselectivity as well as the activity of *R. glutinis* EH,

and possibly other red yeasts, towards more complex epoxide substrates without the need of time consuming screenings.

Keywords: epoxide hydrolase, biocatalysis, kinetic resolution.

Uittreksel

Enantiosuiwer epoksiede is veelsydige verbindings vir die produksie van farmaseutiese middels as suiwer enantiomere. Die verbindings het hoë waarde as boustene en reagense vir die produksie van meer komplekse enantiomeries suiwer farmaseuties aktiewe bestanddele en landbouchemikalieë.

Epoksiedhidrolase (EH) is 'n volop ensiem in die natuur wat as 'n veelsydige hulpmiddel vir die biokatalitiese produksie van epoksiedverbindings as suiwer enantiomere kan optree. Die ensieme vertoon selektiewe hidrolise van een enantiomeer van 'n wye reeks van rasemiese epoksiede, en produseer dus in die proses die enantiosuiwer epoksied. Die waarde van EH vir die kinetiese resolusie van epoksiede is afhanklik van 'n verskeidenheid faktore, soos hul beskikbaarheid, die moeite waarmee hul geproduseer kan word, langtermynstabiliteit van die ensiem, aktiwiteit asook hul enantioselektiwiteit.

Die eerste doelstelling van hierdie projek was om die effek van die groeimedium en groeityd op die produksie van die twee rooi giste, *Rhodotorula glutinis* en *Rhodosporidium toruloides*, en hul epoksiedhidrolase-ensieme te ondersoek en te optimiseer. Maksimum en minimum EH-aktiwiteit vir *R. glutinis* is onderskeidelik in die YMvit- (0,26 $mM.min^{-1}$) en mout- (0,17 $mM.min^{-1}$) mediums verkry, terwyl die maksimum biomassa deur die YM-medium (64,9 $mg.mL^{-1}$) gelewer is. Vir *R. toruloides* is die hoogste biomassa (130,8 $mg.mL^{-1}$) in die YM medium geproduseer, terwyl vergelykbare aktiwiteit (gemiddelde omskakelingsfaktor = 0,75 ± 0,01) vertoon is vir biokataliste geproduseer in die YM-, YMvit- en moutmedia.

Met wisselende glukosekonsentrasies van die YM medium (0,5 -2,0 %) is die meeste biomassa deur *R. glutinis* tydens die byvoeging van 1,5 % glukose (60,0 ± 0,9 $mg.mL^{-1}$) geproduseer, terwyl 'n klein daling in die hoeveelheid biomassa met die byvoeging van 2,0 % glukose (56,0 ± 1,7 $mg.mL^{-1}$) waargeneem is. Geen beduidende verskil in die aktiwiteit van epoksiedhidrolase is by laer glukosekonsentrasies (0,5 – 1,5 %) opgemerk nie, terwyl 2,0 % (m/v) glukose 'n biokatalis met byna 20 % hoër aktiwiteit (0,29 $mM.min^{-1}$) gelewer het. Die biomassa asook die groeityd tot stasionêre fase van *R. toruloides* het skerp toegeneem (van 40 tot 96 uur) met 'n toename in glukose. Ongeveer dieselfde aktiwiteit is na 'n groeitydperk van 36 uur met die boonste drie glukosekonsentrasies (gemiddelde omskakelingsfaktor = 0,82 ± 0,01) verkry. 'n Afname in aktiwiteit tydens die stationêre fase is by alle glukosekonsentrasies waargeneem, met die mees uitgesproke afname (62 %) vir die konsentrasie van 2,0 % (m/v).

Die tweede doelstelling was om *meta*- en *para*-nitro-, metiel- and metoksistireenoksied te sintetiseer en die daaropvolgende produksie van die enantiosuiwer epoksiede deur kinetiese resolusie deur *R. glutinis* EH, en die bepaling van die absolute konfigurasie van die ongereageerde suiwer epoksiedenantiomere deur VCD-analise. *R. glutinis* EH het die hele reeks substrate selektief gehidroliseer, terwyl die hoogste aktiwiteit teenoor die *meta*-gesubstitueerde derivate in die volgorde van metiel > metoksi > nitro vertoon is. *m*-Metielstireenoksied het 'n enantiomeriese oormaat en opbrengs van onderskeidelik >98 en 42,5 % binne 'n reaksietyd van 60 minute bereik. Die absolute konfigurasie van die ongereageerde epoksiedenantiomere van *m*-nitro-, *m*-metiel- en *m*-metoksistireenoksied is as (S) bepaal wat die voorkeur van *R. glutinis* EH vir die (R)-enantiomere aantoon.

Die derde doelstelling was om die aktiwiteit van *R. glutinis* EH deur byvoeging van hidroksipropiel-β-siklodekstrien (HPB) te prodeer verbeter asook om die chemiese en *R. glutinis* EH-gemedieerde ensimatiese hidrolise en die ensiemenantioselektiwiteit te korrelleer met die elektroniese eienskappe van die substituentgroepe en die drie-dimensionele rangskikking van die substrate teenoor die katalitiese setel van EH.

'n Toename in HPB-konsentrasie (0 - 20 % m/v) het tot 'n aansienlike toename in beide die oplosbaarheid en die ensiemaktiwiteit teenoor *p*-nitrostireenoksied gelei. Met die byvoeging van slegs 5 % HPB is 'n toename in die oplosbaarheid van tussen 2,89 en 6,28 keer vir die substraatreeks in vergelyking met die skoon bufferoplossing waargeneem.

Die suurgekataliseerde chemiese en *R. glutinis* EH-gemedieerde ensimatiese reaksietempo's is met die Hammettkonstante asook die Mulliken-ladingverspreiding gekorreleer. Die Mulliken-ladingverspreiding oor die geprotoneerde epoksiede is met die tempo van suur geïnduseerde chemiese hidrolise gekorreleer terwyl die Mulliken-ladingverspreiding oor die neutrale epoksiede met die ensimatiese reaksietempo gekorreleer is.

'n Toename in elektronskenkende eienskappe van die substituent op die stireenoksiede het tot 'n toename in sowel die chemiese as die ensimatiese hidrolisetempo's gelei.

Modellering en passing van die moontlike konformere van die (R)- en (S)-enantiomere van die verbindings in die bindingsetel van EH van *Aspergillus niger* het duidelik die meer voordelige passing van die (R)-enantiomere in die setel getoon wat die vinniger reagerende enantiomeer vir beide *A. niger* en *R. glutinis* epoksiedhidrolase is.

Die verhouding tussen die aktiwiteit en selektiwiteit van *R. glutinis* epoksiedhidrolase en die elektroniese eienskappe van die substituente asook die verhouding van die ruimtelike

rangskikking van die aktiewe setel en die enantioselektiwiteit kan die voorspelling van sowel die aktiwiteit as die enantioselektiwiteit van die ensiem uit *R. glutinis*, maar moontlik ook vir ander rooi giste, vir meer komplekse stireenoksiedverbindings moontlik maak sonder dat tydrowende siftingseksperimente vooraf gedoen moet word.

Tref woorde: Epoksiedhidrolase, biokatalise, kinetiese resolusie

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Chapter 1

Introduction

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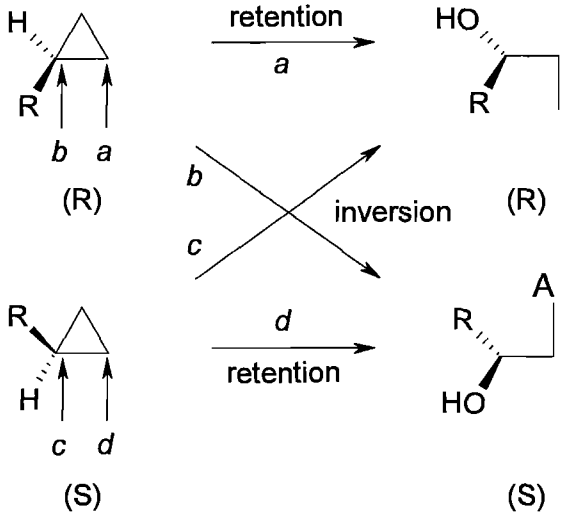
1 General Introduction

Two-thirds of prescription drugs are chiral, with the majority of new chiral drugs being single enantiomers (Halpern & Trost, 2004:5347). In a recent survey conducted of three top pharmaceutical companies by Carey *et al.* (2006:2338), 69 of the 128 drug candidate molecules were chiral drugs containing at least one stereogenic unit. Out of these 69 molecules, 67 are being developed as single enantiomers. When considering this and all the other examples of enantiopure building blocks for the preparation of pharmaceutical compounds, the need for the development of new and effective synthetic methods and the broadening of the scope of existing methods are quite obvious.

Today, there is an increasing interest in the application of enzymes and microorganisms as catalysts in organic chemistry, and specifically in the preparation of the enantiopure compounds. Biocatalysts are particularly versatile in comparison to the commonly used chemical catalysts. They not only catalyse specific reactions involving one or more structurally related compounds, but they also almost completely distinguish between isomers and regioisomers. This latter property is the main reason for the attractiveness of biocatalytic enzymes in the preparation of biologically and chemically useful optically active compounds (Duran, 2000).

One of the versatile enzymes currently widely researched is epoxide hydrolases (EH) from various organisms including mammals, plants, insects as well as microorganisms. EHs selectively hydrolyse racemic epoxides; thus rendering the unreacted enantiopure epoxide and enantiopure *vicinal* diol products (**Scheme 1-1**). These enzymes utilise an environmentally benign compound (water) as the only stoichiometric reagent (Finney, 1998:R73), are co-factor independent and relatively stable as pure enzyme preparations (Weijers & De Bont, 1999:199). The unreacted enantiopure epoxides obtained during EH mediated kinetic resolution are highly attractive molecules for organic synthesis due to their potential to be opened by reactions with halides, carbon, nitrogen and sulphur nucleophiles (Smit & Labuschagne, 2006:1146).



Scheme 1-1 Regio- and stereoselectivity of EHs (Smit & Labuschagne, 2006:1151).

The prevalence of EH enzymes in microorganisms give easy access to large amounts of these enzymes by fermentation (Archer et al., 1996:8819). The use of yeast EHs has the added advantage of easy removal of the cells from a reaction mixture through centrifugation (Weijers & De Bont, 1999:206). These organisms, including the basidiomycetous red yeasts *Rhodotorula glutinis* and *Rhodosporidium toruloides*, have been found to exhibit activity towards a wide range of aliphatic and aromatic epoxide substrates (Botes *et al.*, 1998:423; Choi *et al.*, 1999:9; Fantin *et al.*, 2001:2710; Kotik *et al.*, 2006:370; Yeates *et al.*, 2003:678).

2 Motivation

In order to increase the activity of already available EH biocatalysts and the range of possible substrates of these enzymes, the factors influencing their growth and mechanism of action needs to be better understood. This might lead to the production of more industrially viable enzymes, and the possible prediction and control of their specific activities and enantioselectivities.

3 Aim and Objectives

The aim of this study was to increase the understanding of the factors that influence red yeast EH activity. During this study there were focused on the residual epoxides, and not the formed diols, due to the ease in which the enantiopure diols can be synthesised through straightforward chemical hydrolysis of the enantiopure epoxides.

The first objective was to investigate and optimise the growth media for the production of both *R. glutinis* and *R. toruloides* and their epoxide hydrolysing enzymes, and secondly the synthesis

and absolute configuration determination of a range of styrene oxide type compounds to enable the absolute configuration determination of the unreacted epoxide and thus the enantioselectivity of the optimized *R. glutinis* EHs.

Thirdly, we attempted to correlate both the chemical and enzymatic hydrolysis, and enzymatic enantioselectivity of the *R. glutinis* EH enzyme towards the range of styrene oxide derivatives to the electronic properties of their substituents and the spatial arrangement of the substrates in relation to the EH catalytic triad.

4 Outline of this thesis

An overview on the implications of chirality and the most widely used methods for the synthesis of enantiopure compounds; including the use of the biocatalytic enzyme EHs in the synthesis of enantiopure epoxides is presented (Chapter 2).

The effect of different growth media (YM, YMvit and malt) and the addition of different glucose additive concentrations on the growth of two red yeasts, *R. glutinis* and *R. toruloides*, and their EH activity and selectivity are reported (Chapter 3).

The hydrolytic kinetic resolution of a range of synthesised substituted styrene oxide derivatives through the utilisation of *R. glutinis* (UOFS Y-0563) EH is presented. The resultant *meta* substituted enantiopure derivatives were also successfully synthesised on a semi-preparative scale and the absolute configuration determined through the utilisation of FTIR vibrational circular dichroism (Chapter 4).

The effect of hydroxypropyl-β-cyclodextrin (HPB) on the solubility as well as the *R. glutinis* EH enzymatic activity on a reference compound and the effect of HPB on the solubility on a range of styrene oxide derivatives were investigated. Furthermore, we attempted to correlate both the chemical and enzymatic hydrolysis of this range of styrene oxides to the electronic properties of their substituents and the spatial arrangement of the substrates in relation to the EH catalytic triad of the active site (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 and future research proposals (Chapter 6).

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Chapter 2

Optically Pure Compounds

Abstract

Due to the change of legislation regarding the production and use of chiral compounds in pharmaceutical substances, the synthesis and thus development of new effective synthetic methods for the production of enantiopure products have gained a lot of interest. Epoxides are one of a number of extremely versatile synthons for the production of more intricate pharmaceutical compounds in their enantiopure form, and are of interest to the synthetic chemist due to their possible regio- and stereoselective synthesis and control of the subsequent reactions.

This chapter provides an overview on the implications of chirality as well as the most widely used methods for the synthesis of enantiopure compounds, including the use of the biocatalytic enzyme epoxide hydrolases in the synthesis of enantiopure epoxides.

Keywords: chirality, enantiopure, epoxide and epoxide hydrolase

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1 Stereochemistry, chirality and chiral compounds

The concept of stereochemistry was discovered more than a hundred and fifty years ago when Louis Pasteur demonstrated that a polarized beam of light passing through solutions of tartar deposits rotates to the left, the right, or not at all. He also observed that the dissolved racemic tartaric acid crystallised into two different crystal shapes. It was subsequently shown that these crystals consisted of molecules that were mirror images of each other (Andersson, 2004:279).

In 1874 Van't Hoff and Le Bel independently gave the same explanation to Pasteur's discovery - Van't Hoff speculated that when a carbon molecule is occupied by the maximum possible number of dissimilar atoms, this molecule can occupy two distinctive spatial arrangements (Cornforth, 1976:121).

1.1 Definitions and nomenclature

Molecules with the same chemical formula, but different structures are referred to as isomers. Those isomers with identical structural formulas are referred to as stereoisomers (USAN, 2006). The chemistry of the tetravalent carbon (**Figure 2-1**) allows it to have a planar or three-dimensional centre, and can thereby generate stereoisomers (Cordato *et al.*, 2003:649). An organic compound may have more than one chiral centre and thus have n^2 stereoisomers (Daniels *et al.*, 1997:639), where n refers to the number of chiral centres.

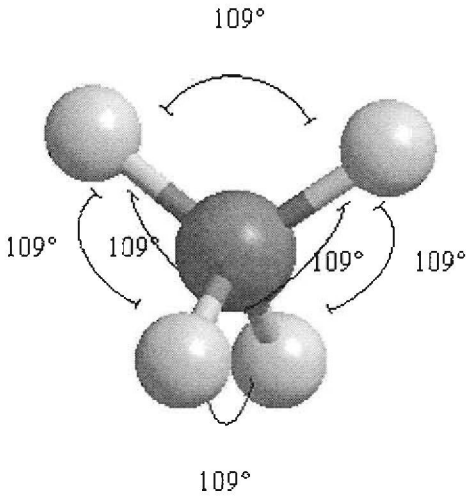


Figure 2-1 The tetravalent carbon atom.

Where stereochemistry refers to the three-dimensional arrangement of organic molecules in space, chirality refers to the properties of these molecules that cause them to be non-superimposable on their mirror images (Eliel & Wilen: 1994:1, 1194) by simple rotations or

translations (Petsko, 1992:1403). These non-superimposable stereoisomers are called enantiomers. The molecule structures are identical in an achiral environment, but vary in the spatial arrangement of atoms (Aitken, 1992:1). Due to their identical physical and chemical properties, enantiomers can not be separated by distillation, crystallisation, or chromatography on an achiral column (USAN, 2006), which leads to the necessity of a chiral environment for the separation of these compounds.

Diastereomers are the other form of stereoisomers. These compounds are also constituently the same as enantiomers, but incorporate all isomers that are not mirror-images of one another. Diastereomers have different physical and chemical properties that enable chemical separation by means of achiral chromatography and crystallisation (USAN, 2006), and other conventional methods.

Optical activity, as was shown by the initial studies of Pasteur, can be defined as the phenomenon where a beam of plane-polarized light is either bent or rotated to the left or the right when passing through a cell containing a sample of a single enantiomer. The degree and sign of the optical activity of an enantiomer is referred to as the specific or optical rotation, and is a function of the temperature, solvent and concentration used for the measurements (Buxton & Roberts, 1996:21). While enantiomer pairs of a specific compound have identical physical properties, they differ in terms of their optical rotation, which is equal in magnitude but opposite in direction (Daniels *et al.*, 1997:639). The composition of a mixture of two enantiomers may be characterized by its *optical purity*, which may in turn be determined from the ratio of the optical rotation of the mixture to that of the pure enantiomer (Jacques *et al.*, 1981:4).

While enantiomers can be described using various different methods, usually three main systems of nomenclature are used. The first is the previously mentioned optical rotation which designates the direction of the angle of optical rotation. The prefixes + or – and *dextro* or *levo* (*d/l*), is assigned (Daniels *et al.*, 1997:639). A distinct limitation of this method is that the organic solvent used to dissolve the compound, might alter the plane of polarized light (Cordato *et al.*, 2003:649). Another drawback is that the sign of optical rotation does not give any additional information on the spatial arrangement of a specific molecule.

The second method is the Cahn-Ingold-Prelog principle, where the *absolute configuration* of molecules is described by the stereodescriptors R and S, which are allocated by the sequence rule procedure (IUPAC, 1993). The different chemical groups are assigned a rank based on the sequence of groups around the chiral centre, in order of increasing atomic number (Caldwell & Weiner, 2001:S107).

The third is the Fischer convention where the molecule under investigation is chemically correlated to (+) glyceraldehyde (or another molecule of known configuration), which was arbitrarily assigned a "D" configuration (Cordato *et al.*, 2003:651), or (+)-glyceraldehydes (L configuration). The drawback of this method is that the assigned configuration is relative to other compounds, and examples do exist where the configuration has changed with the choice of reference compound. The D, L convention is mainly used for defining the absolute configuration of α aminoacids and sugars these days (Aboul-Enein & Basha, 1997:4).

The word racemate describes a mixture of equal amounts of a pair of enantiomers (Eliel & Wilen, 1994:153), and can thus be represented as (±) or dl-, (R/S), DL- or just *rac* (Cordato *et al.*, 2003:651).

1.2 Implications of chirality

1.2.1 Biological implications of chirality

In 1957 the drug Thalidomide was marketed for use against nausea and morning sickness for pregnant women, it was said that this drug was harmless and that a lethal dose could not be established. This drug was however found to be associated with congenital abnormality causing severe birth defects (Pannikar, 2003:9). It was later discovered that the (S)-(–)-enantiomer was responsible for the teratogenic effect, while the (R)-(+)-enantiomer showed none of these side-effects.

While this tragic event lead to the revision of the U.S. Pure Food and Drug Act of 1906 (Eliel & Wilen, 1994:204), the possible difference in physiological effect between enantiomers had already been discovered 30 years earlier by Cushny during a study on the enantiomeric adrenalines (Easson & Stedman, 1933:203).

It is important to realise that when drugs are administered to the body, chiral interaction will probably occur. Nucleic acids, proteins and carbohydrates are single stereoisomers and pharmacological targets such as receptors, enzymes and ion channels are chirally distinctive, resulting in many of the processes essential for life being stereospecific (Andersson, 2004:280). The active enantiomer of a drug has a three-dimensional structure that can be aligned with the binding site (**Figure 2-2**). Even though the inactive enantiomer has exactly the same structure, the three-dimensional arrangement prevents the inactive enantiomer from interacting and thus having a biological effect. In some instances, the portion of the molecule containing the chiral centre may be in a region that does not play a role in the molecule's ability to interact with its target. In these instances the enantiomers may exhibit equal effects (McConathy & Owens, 2003:71).

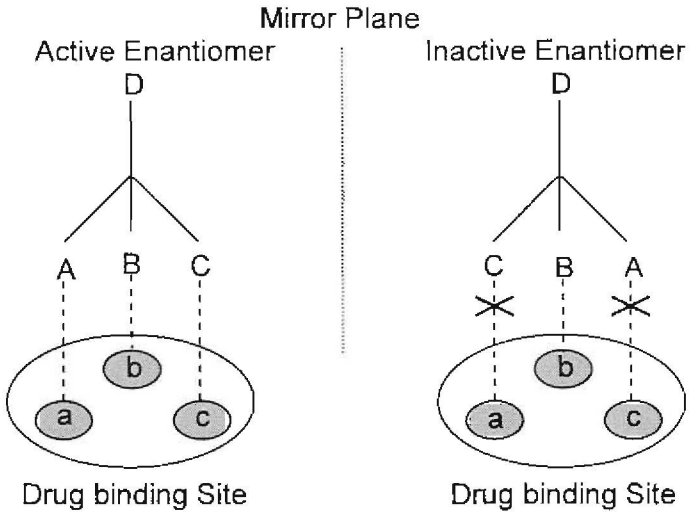
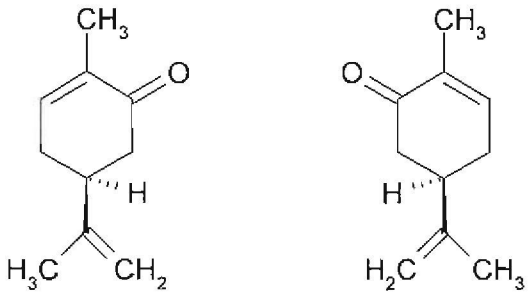


Figure 2-2 Schematical representation of receptor-drug interaction (McConathy & Owens, 2003:71).

Another simple example of chiral recognition is observed for carvone (**Scheme 2-1**). (–)-Carvone is a natural product with the smell of spearmint oil, while (+)-carvone smells like caraway seeds. The fact that our noses can detect a difference in smell between these two enantiomers indicates that smell is stereospecific, again confirming that our bodies (receptors) can discriminate between enantiomers (Cairns, 2003:84-85).



Scheme 2-1 Structures of (+)- and (–)-carvone (Cairns, 2003:84-85).

Stereoselective interactions in the human body may also cause variation in absorption, distribution, metabolism and elimination of the different enantiomers (Aboul-Enein & Basha, 1997:17). Due to these mentioned effects of stereoselective interactions, different enantiomers may exhibit a difference in potency (the one enantiomer may have a smaller or no effect), the enantiomers might have totally different effects (Ahuja, 1997:289), one of the enantiomers might cause adverse effects or one enantiomer might have antagonistic effects to the other

enantiomer (Andersson, 2004:280). Some examples of chiral pharmaceutical compounds and their pharmacological effect are presented in **Table 2-1**.

Table 2-1 Physiological activities of stereoisomers.

Compound	Stereoisomers Activities	Reference
Omeprazole	(S)-isomer has a lower total metabolic clearance than (R)-omeprazole, which leads to higher plasma levels and different clinical effects.	Äbelö *et al.*, 2000:972
Ifosfamide	The (S)- and (R)-isomer of this *chemotherapeutic agent have equal* therapeutic activity, but the (S)-enantiomer exhibits nephrotoxicity.	Aleska *et al.*, 2006:398
Metoprolol	All the β-locking activity resides in the (S)-enantiomer, whereas the (R)-enantiomer does not contribute to this effect.	Stoschitzky *et al.*, 2001:344
Promethazine	Both enantiomers have equal antihistaminic activity as well as equal adverse effects.	Aboul-Enein & Wainer, 1997:11
Propoxyphene	(d)-Propoxyphene is an analegesic agent, whereas (l)-propoxyfene is an effective antitussive.	Aboul-Enein & Wainer, 1997:14
Quinine and Quinidine	Quinidine, the (+)-enantiomer, is an antiarrhythmic agent, while, quinine is the (–)-enantiomer with anti-malarial activity.	Ahuja, 1997:289; Aboul-Enein & Wainer, 1997:14

In 1956 Pfeiffer postulated that the effective dosage of a drug is inversely proportional to the difference in the pharmacological effect of the individual optical isomers, and the more potent a drug is the more likely it is to show stereoselectivity (Burke & Kratochvil, 2002:20). Ariens stipulated that this generalisation only holds true in cases where the point of chirality is near a point of close approach of a ligand to a receptor (Mitscher, 2005:574). The ratio of the activity

of an active enantiomer (eutomer) to that of the less active enantiomer (distomer) is referred to as the eudismic ratio (Aboul-Enein & Basha, 1997:6) for a particular biological action. Thus, the larger the difference in activity between two enantiomers, the more probable the need will be to separate the two enantiomers.

A combination of factors including the possible difference in physiological effect between enantiomers, recent advances in chiral technology and the ability to synthesise enantiomerically pure compounds, together with the regulatory influences, have led the pharmaceutical industry to attempt, wherever relevant and possible, to develop new chemical compounds as single isomers. At the same time, there is an interest to replace already approved racemate drugs by their single enantiomers which is known as "chiral switching" (Tucker, 2000:1086).

Not only does this switching to single enantiomers have major potential advantages for the patient (Agranat & Caner, 1999:313), but it also has an economical impact on the pharmaceutical industry as a whole.

1.2.2 Economical implications of chiral switching

From the time of the publication of the U.S. Food and Drug Administrations` policy statement for the development of new stereoisomeric drugs in 1992 (FDA, 1992:1) and the subsequent publication of the European Unions regulatory document, "Investigation of chiral active substances" in 1994 (TGA, 1994:381), world-wide production and sales of chiral single enantiomer drugs continue to grow (Caner *et al.*, 2004:105).

Despite the fact that the number of new drugs derived from chiral switches is less than that predicted during the 1980s (Caldwell, 2001:S70), the economic impact of the industrial production of chiral drugs is substantial. In 1997, already more than 50 % of the 500 top selling drugs were sold as single-enantiomers. Sales have further increased by more than 20 % to a total world-wide distribution of single enantiomer drugs of 55 % in 2002 (**Figure 2-3**). World-wide sales of enantiomeric drugs exceeded US$100 billion for the first time in the year 2000, with chiral drugs then presenting close to one third of all world-wide sales (Burke & Henderson, 2002:573). According to TCI world-wide sales of chiral drugs are estimated to reach US$200 billion by 2008 (Stinson, 2001b).

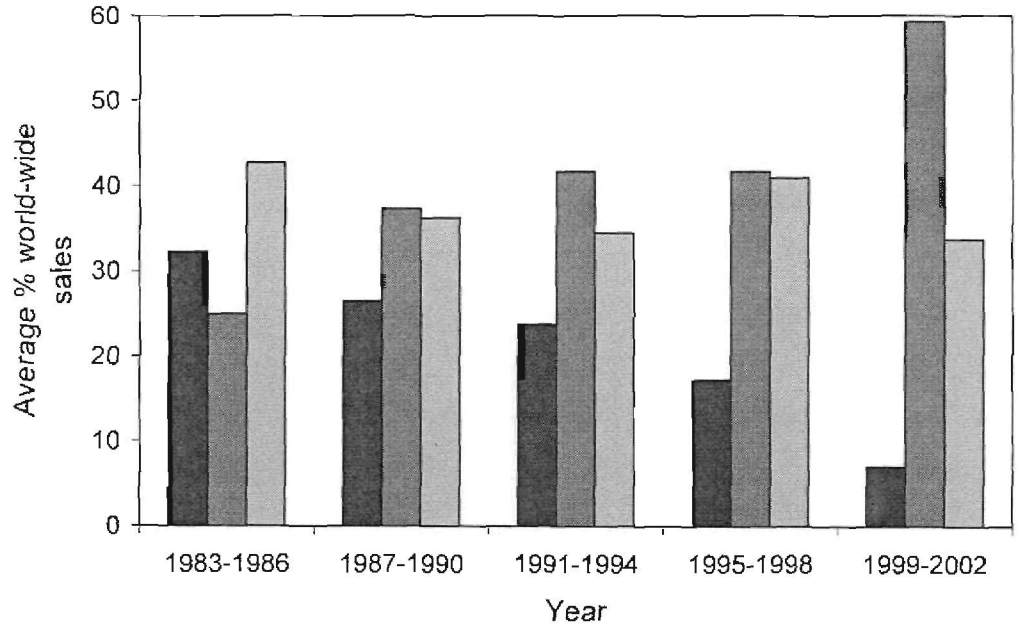


Figure 2-3 Average distribution of world-wide pharmaceutical sales in four year intervals (■, racemates; ▩, enantiomers; □, achiral) (Caner *et al.*, 2004:107).

When considering the number of highly successful chirally pure drugs on the market, the importance of stereochemistry to the pharmaceutical industry is apparent. The chiral switch process presents a strategy to prolong the profitable life of a successful pharmaceutical drug and may result in extended patent protection, thus providing an advantage against generic competition (Hutt & Valentová, 2003:15).

There are however financial risks associated with the development of single enantiomer drugs previously available as racemates. Apparently the research and development costs for the single enantiomer drug dilevalol (a drug used in the treatment of high blood pressure) were US$ 100 million (Hutt & Valentová, 2003:16). Dilevalol was withdrawn from the U.S. market and subsequently world-wide, after severe liver injury was indicated for patients on this therapy (Kessler, 1996). This indicates the need for thorough research to determine the necessity, advantages as well as possible disadvantages that a single enantiomer drug might deliver.

Thus, due to the high cost of development, an ideal candidate for a chiral switch must have a number of features, including (i) problems with the racemate in terms of efficacy or toxicology, (ii) the activity of interest resides in the one enantiomer, (iii) predictable therapeutic benefits, (iv) practical and commercially viable production chemistry and (v) possible patent protection for the single enantiomer (Caldwell, 2001:S69).

2 Obtaining optically pure compounds

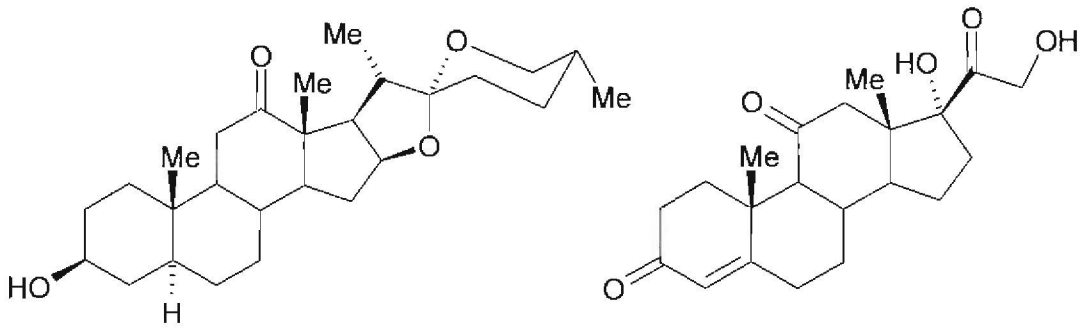
The organic chemist spends a lot of energy and time in seeking methods for the production of optically pure compounds. Currently, there are several different methods to achieve this goal (Buxton & Roberts, 1996:187).

2.1 Utilisation of materials from the chiral pool

The term "*chiral pool*" refers to fairly inexpensive, readily available, all natural asymmetric compounds. A wide variety of these chiral compounds are found in nature as components in plants and animals (i.e. sugars, steroids, carbohydrates, amino acids and lipids) (Stinson, 2001a) or alternatively as secondary metabolites produced by microorganisms for example penicillin from *Penicillium* fungi (Buxton & Roberts, 1996:188; Crosby, 1992:5).

All the stereogenic units of the product are directly derived from the chiral pool compound. The functional groups already present can be modified and/or the backbones rearranged. Thus, natural occurring compounds can be transformed into compounds of the same structurally related types, or more intricate molecules (Buxton & Roberts, 1996:189). If the compound from the chiral pool is used for the synthesis of alternative complex compounds, it is only practical if no reaction takes place on the stereogenic unit of value.

A good example is the preparation of cortisone from hecogenin (**Scheme 2-2**) which is obtained from the sisal plant. Nearly all the stereogenic centres in cortisone are already present in hecogenin, making the latter an extremely convenient starting material (Aitken & Gopal, 1992:71).



Hecogenin Cortisone

Scheme 2-2 The chiral precursor hecogenin for the synthesis of cortisone (Aitken & Gopal, 1992:71).

2.2 Asymmetric synthesis from prochiral substrates

According to Aitken (1992:5), asymmetric synthesis can be defined as a synthetic route in which an achiral unit in a group of substrate molecules is converted to a chiral unit, so that stereoisomers in unequal amounts are attained. Asymmetric synthesis transforms a known reaction into an enantioselective process by incorporating simple reagents and chiral auxiliaries or a catalysts (Bonini & Righi, 2002:4981).

Development of an asymmetric synthesis method is extremely time consuming, which makes it suitable if large quantities of a compound is essential, but unsuitable where only small amounts of a compound is required, for instance in the early stages of drug discovery (Andersson & Allenmark, 2002:12).

Generally, asymmetric synthesis can be divided into non-enzymatic and enzymatic catalysed reactions. Due to many available examples in asymmetric synthesis, only the main strategies and characteristics thereof will be discussed.

2.2.1 Non-enzymatic asymmetric synthesis

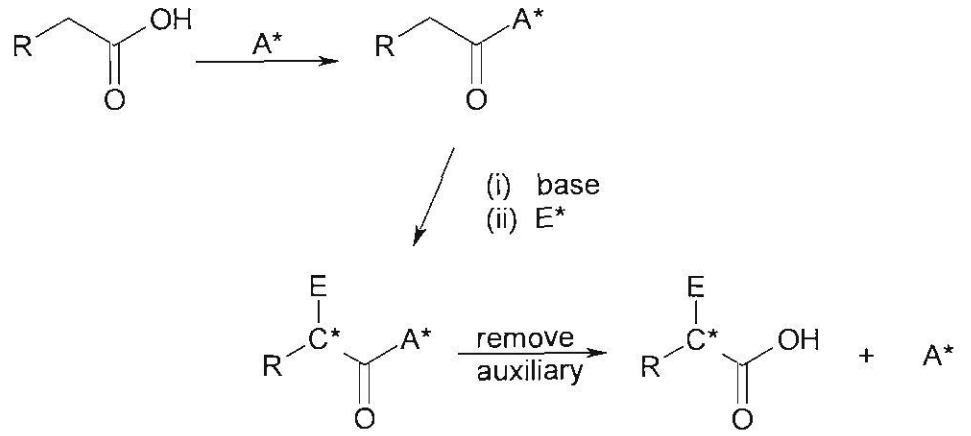
Aitken and Gopal (1992:72-77) distinguish between four different types of non-enzymatic asymmetric synthesis:

2.2.1.1 Substrate-controlled asymmetric synthesis

The synthesis is intramolecularly directed by a stereogenic unit present in the chiral substrate. A second stereogenic unit is formed after the reaction with an achiral reagent. The need for enantiomerically pure starting material is the main drawback of this method (Aitken & Gopal, 1992:74).

2.2.1.2 Auxiliary controlled asymmetric-synthesis

An auxiliary is a facilitator in the transformation of an achiral substrate into a chiral product (**Scheme 2-3**), without appearing in the final product. During the synthetic process a chiral auxiliary-achiral substrate intermediate is formed. The auxiliary directs the course of further reactions, due to steric hindrance or directing groups, by only making the preferred path of attack available to other chiral reagents for further reaction (Buxton & Roberts, 1996:203).



Scheme 2-3 Schematic representation of auxiliary-controlled asymmetric synthesis (Buxton & Roberts, 1996:203).

2.2.1.3 *Catalyst controlled asymmetric synthesis*

The processes in this category fall into three different groups (Eliel & Wilen, 1994:947) according to the catalyst used: catalysis by chiral transition metal complexes, chiral bases and chiral Lewis acids. The relative stereochemistry is predictably controlled by using an external element - a nonracemic catalyst. The catalyst converts an achiral substrate directly to a chiral product. By definition the unchanged catalyst can be recovered after the reaction and reused (Taylor & Jacobsen, 2004:5371, Aitken & Gopal, 1992:77).

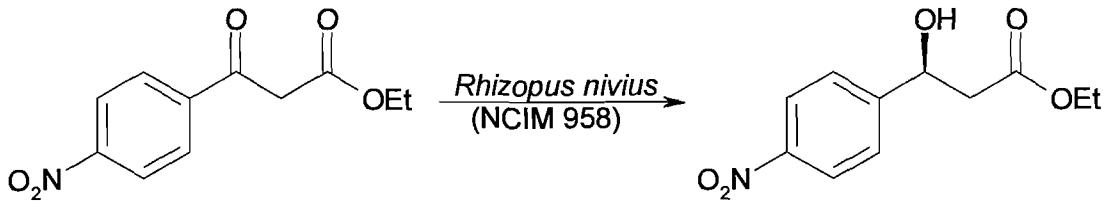
2.2.1.4 *Reagent controlled asymmetric synthesis*

In reagent controlled synthesis the asymmetry is derived from the enantiopure reagent and not from the starting material or the auxiliary (Kilenyi & Aitken, 1992:143). This is obviously an attractive procedure due to fact that no auxiliary has to be added or removed, but unfortunately the range of reagents available are limited (Aitken & Gopal,1992:75).

2.2.2 Enzymatic asymmetric synthesis

Due to higher selectivity under milder reaction conditions, an increasing number of stereoselective synthetic processes incorporate enzymatic steps (Halgăs, 1992:1). These enzymatic reactions include processes like oxidation, hydrogenation, reductive animation, transamination, reductive amination, ammonia addition, hydration and cyanohydrin formation (Crosby, 1992:37-52). The most important advantage of enzymatic catalysis is that many enzymes will accept and thus convert not only their natural, but also some "unnatural" substrates. As with reagent controlled synthesis, the choice of substrate is far wider, since it does not need to come from the chiral pool (Kilenyi & Aitken, 1992:143,181).

A very recent example of an enzyme catalysed synthesis is the asymmetric enzymatic reduction of 3-nitro-3-aryl-3-keto ester (1) (Salvi & Chattopadhyay, 2006:4914) to yield the corresponding (S)-keto acids (2), which are important building blocks in organic synthesis. For this reaction a *Rhizopus* species was utilised as catalyst (**Scheme 2-4**). The corresponding (S)-acid was recovered with a 79,9 % yield and >98 % *e.e.*



(1) (2)

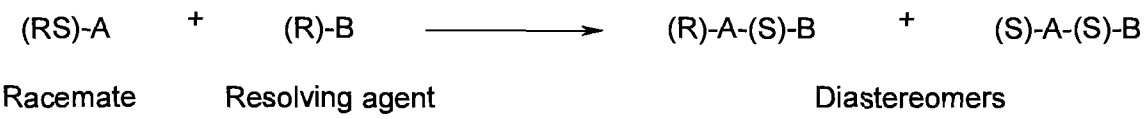
Scheme 2-4 Enzymatic asymmetric synthesis (Salvi & Chattopadhyay, 2006:4914).

2.3 Resolution of racemates

Numerous methods for the separation of enantiomers exist, of which some are used as analytical method and others employed for preparative scale separation (Maier *et al.*, 2001:9). The methods discussed in this section only include the industrially relevant preparative methods.

2.3.1 Classical resolution

Classical resolution (**Scheme 2-5**) is the most widely used method for obtaining enantiomerically pure compounds from racemates (Bruggink, 1997:81). Separation is obtained by converting the enantiomers into a mixture of diastereomers, usually salts, by reaction with a pure enantiomer of a second reagent.



Scheme 2-5 Formation of diastereomeric salts from a racemic mixture (Bruggink, 1997:81).

As previously mentioned; diastereomers differ in physical and chemical properties. Thus, the formed diastereomers can be separated by a range of methods such as fractional crystallization or chromatographic procedures (Bayley & Vaidya, 1995:70; Carey & Sundberg, 2000:88).

2.3.2 Chromatographic enantioseparation

Chromatography entails any separation process which is based on the partitioning of compounds between a flowing fluid and a solid adsorbent. Separation relies on differential migration of the compounds through the stationary phase (where the chiral selector is chemically bound, coated, or otherwise attached to the surface of a support material) under the influence of a mobile phase of any flowing solvent or carrier stream. There are a wide variety of chiral stationary phases (CSPs) (Bommarius & Riebel, 2004:233; Del Rio *et al*, 2005:S74) and chiral selectors available, in both (R) and (S) enantiopure forms, including proline, hydroxyproline, phenylalanine, valine, pipecolinic acid and cyclodextrins (Francotte & Junker-Buchheit, 1992:31).

Even though the outcome of a specific chromatographic resolution can not be predicted, and the optimisation procedure can be time consuming (Andersson & Allenmark, 2002:11), chiral chromatography has become increasingly important in the industrial production of enantiomers. Both enantiomers are usually attained in high optical purity, which makes this method particularly favourable for preliminary comparative biological testing where both enantiomers are obviously required (Francotte, 2001:380). Chromatography is usually also the method of choice where a kilogram or less of a racemate needs to be resolved (Miller, 1999a:316). The most commonly used chromatographic methods include gas chromatography, liquid chromatography, and more recently, supercritical fluid chromatography.

2.3.2.1 Gas chromatography

Enantioselective gas chromatography is particularly suited for chiral analysis when no sample derivatisation is required. However, there are some essential prerequisites for the use of this method: the analyte has to be volatile, thermally stabile and resolvable (Schurig, 2001:277).

Although gas chromatography, due to the difficulty in upscaling, is more widely used as an analytical method (Zhang *et al.*, 2005:572), but has been studied on a semi-preparative and preparative scale. On a semi-preparative scale, highly enriched enantiomers of *all-trans*-perhydrotriphenylene (PHTP) with purities of up to 99,6 % were obtained after a single separation step (Schürch *et al.*, 2001:175), while Juza *et al.*, (1997:127) has successfully separated the enantiomers of inhalation anaesthetics enflurane, isoflurane and desflurane on a preparative scale.

2.3.2.2 Liquid chromatography

a) High pressure liquid chromatography (HPLC)

According to Lao & Gan (2006:184) HPLC is currently the best option to obtain pure enantiomers in analytical and small-scale preparative separations. HPLC is also the most widespread chiral separation technique used in drug discovery due to the common availability and demonstrated success on analytical as well as preparative scales (Zhang *et al.*, 2005:572).

Miller *et al.* (1999b:213-220) demonstrated preparative separation for a number of pharmaceutical intermediates and final compounds on a 150mg to 15g scale per single injection, while obtaining yields of between 87 and 98 %, with % e.e. of 98 to 99,5 for both enantiomers.

Even though HPLC is a highly successful resolution method, it does have disadvantages such as sample dilution and extremely high consumption of organic solvents (Toribio *et al.*, 2003:156).

b) Counter current chromatography (CCC)

Counter current chromatography is a support-free liquid-liquid chromatographic method. The liquid that contains the chiral selector is kept stationary, while a second liquid is pumped through it, and the chromatographic separation process occurs between the two phases. Although this is a powerful preparative technique because of the high capacity, low cost of stationary phases and low solvent consumption (10 times less than HPLC), they show poor efficiency and longer separation times in comparison with other chromatographic methods. Another major difficulty when implementing this method is the availability effective chiral selectors that remain highly selective in a specific solvent, or combination of solvents, while the solvent retains the capacity to elute the chiral isomers of interest (Kim *et al*, 2004:119; Foucault 2001:366).

Although all these disadvantages do exist, CCC does have some major advantages including the fact that it is free from the tedious column packing procedures, no loss of compound due to adsorption onto packing material, and the recovery of samples and reagents are possible without contamination or decomposition (Kim *et al*, 2004:119). Ma *et al.*, (1995:79) demonstrated the analytical and preparative (up to 2 g scale) resolution of (±)-DNB-leucine using pH-zone refining CCC.

c) Simulated moving bed (SMB)

The simulated moving bed process is based on the continuous counter-current movement of a stationary and a mobile phase (Schulte, 2001:401). Compared to other chromatographic processes, the SMB requires less stationary as well as mobile phases with which a more cost-effective large-scale separation can be accomplished (Miller *et al.*, 1999a:316).

The separation of pharmaceuticals has been conducted with great effectiveness, and high productivity. On a pilot-scale, racemic DOLE, a cholesterol reducing agent (Nagamatsu *et al*, 1999:63) was separated with a productivity of 0,268 kg enantiomer per kilogram CSP per day, with a % e.e. of 99,4 in the raffinate. Miller *et al.* (2003: 279) also reported the separation of a pharmaceutical racemate with a maximum average production of 24 kg chirally pure product per day, at an enantiomeric purity of 99 %.

This technology can make chromatographic resolution a feasible process at the metric ton scale, which is required for the pharmaceutical manufacturing industry (Miller *et al.*, 1999a:316; Guiochon, 2002:153), but has distinct disadvantages such as high expense and large solvent consumption (Mc Cormick, 2006).

2.3.2.3 Supercritical fluid chromatography (SFC)

The separation of enantiomers on CSPs has been one of the most successful applications of supercritical fluid chromatography. SFC is the term used to describe the use of mobile phases at temperatures and pressures above, or just below the supercritical point (Williams & Sander, 1997:150). Carbon dioxide with added methanol or acetonitrile is the most commonly used mobile phase in SFC for both chiral and achiral separations (Smith, 1999:93).

SFC has gained ground as a powerful alternative to HPLC for the purification and resolution of enantiomers (Olson *et al.*, 2002:69) due to the use of carbon dioxide instead of large volumes of solvent, as well as the availability of highly-automated systems (Chester *et al.*, 1994:106R). On an analytical scale, the higher speed and faster re-equilibration, in comparison to other chromatographic processes, make method development dramatically faster. These advantages become more compelling as the scale of chromatography increases due to the lower operating costs, but hardware complexity and high initial capital cost still limits the use of SFC in laboratories (Maftouh *et al.*, 2005:80).

A good example of the advantage of this method was given by Toribio *et* al., (2003:161) who separated the enantiomers of ricobendazole on a semi-preparative scale. With a 15 mg sample

loading the first eluted enantiomer was obtained with a purity of >99,9 % (37 mg/h) and the second eluted enantiomer with a purity of 95 % (36,5 mg/h).

2.3.3 Membrane facilitated enantioseparation

Enantioseparation methods utilizing membrane technology can be divided into two major categories: (i) supported liquid membranes: where the chiral selectors are located in the retentate phase, or inside the porous solid membrane layer (Hadik *et al.*, 2005:223), and (ii) dense membranes which is prepared with a chiral polymer that can invoke enantiospecific interactions during sorption and/or diffusion of the racemate (van der Ent *et al.*, 2001:208).

Although membrane technology is only an emerging technique in chiral resolution (Maier *et al.*, 2001:11), there is a vast range of literature available on this subject. Kim *et al.* (2003:273) separated racemic tryptophan with 98 % *e.e.* on a polymeric dense-membrane. Through the use of a supported liquid membrane Nakamura *et al.* (1998:57) also succeeded in separating the enantiomers of tryptophan. Bovine serum albumin was immobilized into the membrane which produced a separation factor of 12.

Enantiomer resolution via membrane processes has a number of attractive features. The separation is usually performed at ambient temperatures, membrane processes can be easily up scaled, and membranes can be tailor-made (Strathmann, 1986:3).

The major problems encountered with membrane facilitated chiral resolution include:

- added resistance to mass transfer due to the membrane,
- membranes are subject to fouling,
- increases in process cost due to the limited life of a membrane (Gabelman & Hwang, 1999:63), and
- loss of impregnated chiral selectors from the membrane (Krieg *et al*, 2000:184).

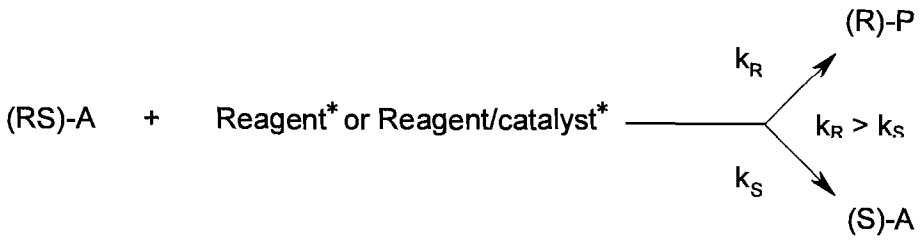
2.3.4 Crystallisation

Crystallisation techniques provide a powerful aid in the manufacture of chiral compounds (Wood, 1997:149). Racemic solutions can crystallise in one of two forms, either as a conglomerate, or a racemic compound. In a conglomerate, an individual crystal only contains one enantiomer, where as a racemic compound's crystals contain an equivalent amount of both enantiomers (Crosby, 1992:24). For this method of enantioseparation to be of use, a racemate, or a reversible derivative thereof, has to crystallise as a conglomerate (Eliel & Wilen, 1994:300).

The individually crystallised enantiomers can be separated by hand, but this method is obviously tedious. Another method is the circulation of a supersaturated solution of the racemate through two crystallisation chambers containing seed crystals of the individual enantiomers (Buxton & Roberts, 1996:195), resulting in localised crystallisation.

2.3.5 Kinetic resolution

Kinetic resolution involves a reaction of a racemate, in which one of the enantiomers forms a product at a faster rate. The difference in the reaction rates is due to the difference in the activation energy that the different enantiomers need to reach their respective transition states. The enantiomers react either with a chiral reagent, or an achiral reagent in combination with a chiral catalyst (**Scheme 2-6**). Ideally only one enantiomer should react, and thus be converted to the product, while the other enantiomer remains unchanged (Aitken & Gopal, 1992:77; Eliel & Wilen, 1994:395, 1201, Gayet & Andersson, 2005:4805).



Scheme 2-6 Principles of kinetic resolution (*, chiral) (Aitken & Gopal, 1992:77).

The term enantiomeric excess, gives an indication of the excess of the predominant enantiomer expressed as a percentage (van Eikeren, 1996:21). A *complete kinetic resolution* (Atkinson, 1995:24) is accomplished if only one enantiomer reacts while the other enantiomer is left in its enantiopure form. The enantiomeric excess (*e.e.*) (**Equation 2-1**) is calculated from the concentration of the individual enantiomers ([A] and [B]) (Crosby, 1992:28).

$$e.e. = \frac{[A]-[B]}{[A]+[B]} \qquad \textbf{(2-1)}$$

The efficiency of a resolution is stated by the enantiomeric ratio, E (**Equation 2-2**) (Wong & Whitesides, 1994:11), and serves as a measure of the enantioselectivity at a certain degree of conversion (c) (**Equation 2-3**). The enantiomeric ratio is also connected to the enantiomeric excess of the recovered reactant ($e.e._R$) as well as the recovered product ($e.e._P$) (Crosby, 1992:28; Bommarius & Riebel, 2004:31).

$$E = \frac{\ln[(1-c)(1-e.e._R)]}{\ln[(1-c)(1+e.e._R)]} = \frac{\ln[1-c(1+e.e._P)]}{\ln[1-c(1-e.e._P)]} \qquad \textbf{(2-2)}$$

The conversion factor represents the decrease of the individual substrates from the initial individual enantiomer concentrations ($[A_0]$ and $[B_0]$) up to the enantiomer concentrations after a certain reaction time (Chen *et al.*, 1982:7294).

$$c = 1 - \frac{([A]+[B])}{([A_0]+[B_0])} \qquad \textbf{(2-3)}$$

The enantiomeric ratio can also be determined directly (**Equation 2-4**) (Rakels *et al.*, 1993:1052) using both the calculated *e.e.* values while excluding the conversion factor. This method renders more accurate results in cases of very high or very low conversions (Faber, 2000:41).

$$E = \frac{\ln\left(\left(1-ee_R\right) \Big/ \left(1+\left(ee_R / ee_P\right)\right)\right)}{\ln\left(\left(1+ee_R\right) \Big/ \left(1+\left(ee_R / ee_P\right)\right)\right)} \qquad \textbf{(2-4)}$$

By definition, is the maximum yield of one product during kinetic resolution 50 %. This results in an inherently wasteful process for producing optically active compounds. If the reaction is however carried out under conditions where the enantiomers of the substrate can interconvert (racemisation), the entire substrate can in principle be converted to the enantiopure product (Aitken & Gopal, 1992:77). This process of interconvertion is also known as dynamic kinetic resolution (DKR) (see section 2.3.6).

According to Bommarius & Riebel (2004:32), the FDA's threshold for the use of a biocatalyst in the preparation of a pharmaceutical compound, is that the biocatalytic process should either render an E-value of 100, or a compound with 99 % *e.e.*

2.3.5.1 Stoichiometric and chemical-catalytic kinetic resolution

Kinetic resolution was first observed in 1899 by Marckwald and McKenzie who discovered the chemical kinetic resolution of (±)-mandelic acid with (–)-menthol which yielded a pair of diastereomeric esters (Nógrádi, 1995:15).

In order for kinetic resolution to compete with the more conventional methods for the synthesis of enantiopure compounds, the difference in the reaction rate between the enantiomers has to be very high (Nógrádi, 1995:17). Although this was initially, with a few exceptions, only accomplished with enzymes, chemical kinetic resolution has expanded and been widely employed as a tool for the production of easily functionalisable molecules with high enantiomeric purity (Robinson *et al.*, 2003:1407, Visser & Hoveda, 1995:4386).

2.3.5.2 Enzymatic kinetic resolution

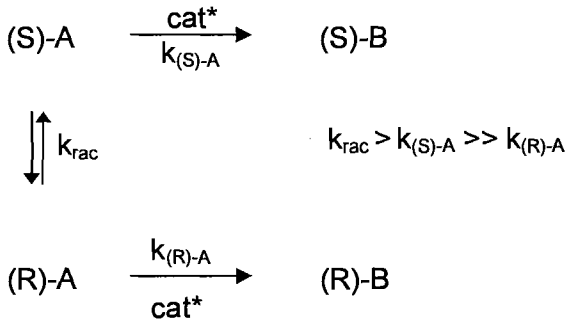
Due to the highly specific selectivities displayed by enzymes, biocatalysts can accomplish reactions often impossible for chemically synthesised catalysts (Faber, 2000:5). During enzymatic kinetic resolution, the addition, change, or removal of a functional group is enzymatically catalysed on only one of the enantiomers (Wong & Whitesides, 1994:9).

A vast quantity of literature is available on a wide variety of enzymatic kinetic resolutions by numerous enzymes for a very broad spectrum of substrates. Few of the more widely applicable enzymes used for the kinetic resolution of organic compounds include:

- lipases (Paizs *et al.*, 2003:1943),
- esterases (Koul *et al.*, 2005:2576),
- proteases (Bianchi *et al.*, 1988:105),
- epoxide hydrolases (Monfort *et al.*, 2004:602),
- nitrilases (Kaul *et al.*, 2004:209), and
- acylases (Chenault *et al.*, 1989:6354).

2.3.6 Dynamic kinetic resolution

As mentioned previously, classical kinetic resolution has the distinct disadvantage of having a maximum theoretical yield of 50 %. DKR (*asymmetric synthesis*) (**Scheme 2-7**) combines the resolution step of kinetic resolution with an *in situ* racemisation of the chirally labile substrate (Gihani & Williams, 1999:11).



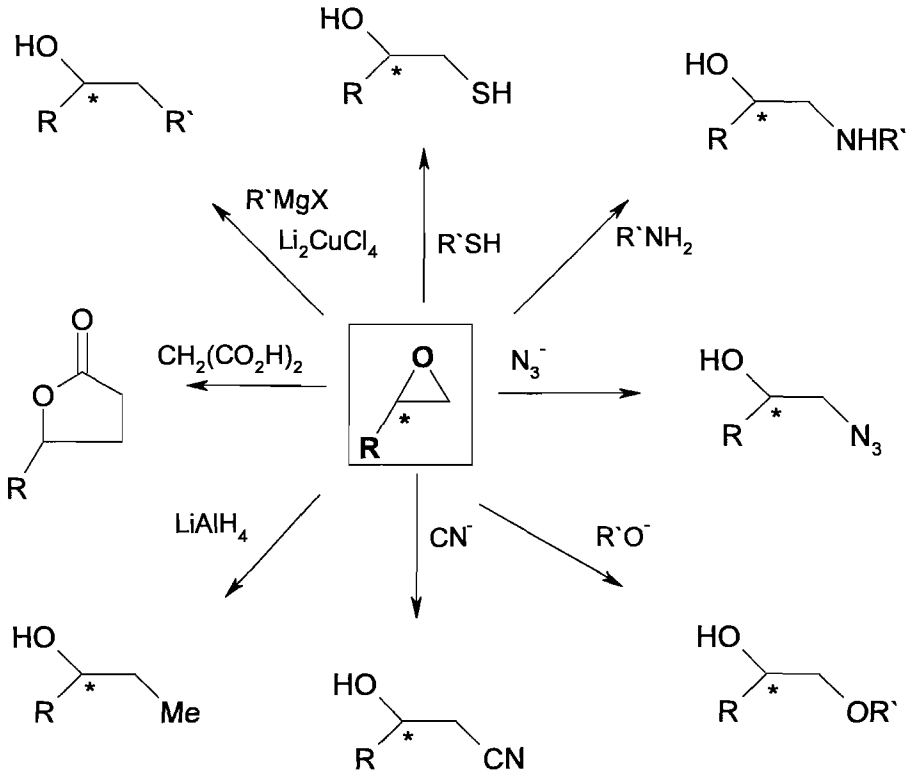
Scheme 2-7 Schematic representation of dynamic kinetic resolution (Ward, 1995:1475).

Thus, under conditions where the tempo of racemisation (k_{rac}) is faster than the enzymatic conversion of (S)-(A) to (S)-B ($k_{(S)-A}$), which in turn, is much faster than the formation of (R)-B, a theoretical yield of 100 % can be accomplished for (S)-B. If the reaction generates a new chiral centre, an additional stereo-control should be considered in order to obtain a single enantiomer (Sugimura, 2006:233).

3 Epoxides

3.1 Introduction

The epoxide functional group and vicinal diols are extensively employed high-value intermediates for the synthesis of enantiomerically pure bioactive compounds (Faber, 2000:135). Epoxides are extremely versatile and can form a wide variety of bifunctional compounds after ring-opening (**Scheme 2-8**). According to Sharpless, the principal reason for the synthetic interest in, and importance of the epoxide moiety, is the existence of the possible regio- and stereoselective synthesis and control of the subsequent reactions (Rossiter *et* al, 1981:464).



Scheme 2-8 Epoxide reactions with nucleophiles (Archelas & Furstoss, 1997:492).

Several simple epoxides, for instance ethylene oxide and racemic propylene oxide, have a long history as bulk chemicals (De Bont, 1993:1331), while other epoxides are very important chiral building blocks in organic synthesis and can be used as key intermediates (**Table 2-2**) in the preparation of more intricate enantiopure bioactive compounds.

Not only are epoxides key building blocks in the synthesis of pharmaceutical compounds, but are also found in biological end-products such as the gypsy moth pheromone (+)-dispalure (Besse & Veschambre, 1994:8886), epithilone A, an antitumor agent (Finney, 1998:R74) and (R)-(+)-methyl palmoxirate which is a potent hypoglycemic agent (Ruano *et al*, 1994:534).

Table 2-2 Examples of chiral epoxides as intermediates in organic pharmaceutical synthesis.

Therapeutic group	Compound	Reference
Antibiotics	Naturally occurring optically active erythromycin is used in the treatment of respiratory tract and middle ear infection.	Besse & Veschambre, 1994:8886
	Ferensimycin B for the elimination of gram + bacteria which is a naturally occurring optically pure substance.	Evans *et al.*, 1991:7618; Kusakabe *et al*, 1982:1119
Antivirals	A-792611 used in the treatment of HIV-1	Engstrom *et al*, 1996:5370
	Oxetanosine natural nucleoside derivate against HIV-1 and HIV-2	Nair *et al, 2004:*10261
	BMS-200475 analogue against Hepatitis B virus	Ruediger *et al.*, 2004:739
Steroids:	Vitamin D analogue for the treatment of osteoporosis and the prevention of bone fractures.	Ono *et al*, 2006:536
	(S,R)-Trifluoromethyl alcohol	Lee *et al.*, 2006:654
B-Blockers	(S)-propranolol and (S)-practolol	Leftheris & Goodman, 1990:216.
Leukotriene		Bellamy *et al*, 1992:355

3.2 Obtaining optically pure epoxides and diols

Due to the increased awareness of the economic importance and physiological implications of single enantiomer drugs, and thus the need for suitable methods of obtaining these compounds, research on the production of optically pure epoxides has exploded in the past years, Subsequently, quite a number of comprehensive review papers have been published on this subject (Archelas & Furstoss, 1997; De Bont, 1993; Goswami *et al,* 1999; Leak *et al*, 1992).

To give another overview of all the possible methods for the synthesis of chiral epoxides would fall beyond the scope of this literature study. Therefore only the most industrially competitive catalytic methods will be discussed.

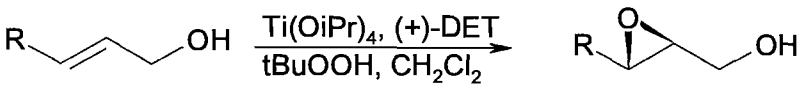
3.2.1 Chemical Synthesis

While a number of chemical asymmetric methods for the synthesis of optically pure epoxides do exist, e.g. from chiral precursors, the use of phase transfer catalysts, and the use of chiral reagents like oxaziridines and borates, they are mostly overshadowed (Besse & Veschambre, 1994, 8898) by the methods developed by Sharpless and Jacobsen.

3.2.1.1 Sharpless asymmetric epoxidation

A few of the advantages of catalytic epoxidation include a decrease in the volume of utilised reagent, minimization of the waste generation, as well as access to enantioselective reactions for which there is no non-catalytic alternative (Finney, 1998:R74).

In terms of simplicity, reliability, and the ease of access to reagents, all other chemical synthetic routes to enantiopure epoxides are dominated by one of the most important developments in chemical enantioselective synthesis (Eliel & Wilen, 1994:948). In 1980 Katsuki and Sharpless (1980:5974) reported the metal titanium catalysed asymmetric epoxidation of primary allylic alcohols, when used in combination with (+)-or (–)-diethyl tartrate and *tert*-butyl hydroperoxide (**Scheme 2-9**). After a minor alteration of the reagents, Martin *et al.* (1981:6237) reported the successful asymmetric catalysis of secondary allylic alcohols utilising DIPT, instead of DET as catalyst.



Scheme 2-9 Sharpless epoxidation of allylic alcohols (Katsuki & Sharpless, 1980:5975).

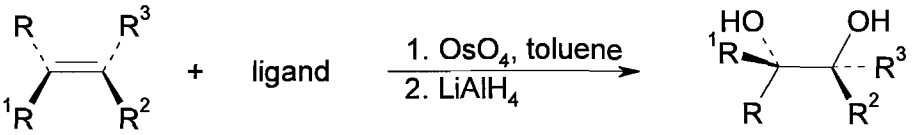
Another major advantage of this epoxidation reaction is that the stereochemical outcome can always be predicted: the use of (+)-diethyl tartrate give rise to epoxide oxygen addition from the top, and from the bottom when (–)-diethyl tartrate is used (Katsuki & Sharpless, 1980:5975). A wide variety of allylic alcohols have been shown to be suitable substrates for this reaction (Gao *et al.*, 1987:5767). With a small number of exceptions, the (E)-allylic alcohols tend to be the most suitable substrates for high enantioselelectivity, whereas the properties of the substituent groups on (Z)-allylic alcohols are inclined to have a large influence on the selectivity of the reaction (Bonini & Righi, 1992:4983).

Although this method has had wide industrial and academic implications (Klunder *et al.*, 1986:3710), it has definite limitations and drawbacks (Besse & Veschambre, 1994:8893):

- The reaction is very sensitive to pre-existing chirality,
- (Z)-allylic alcohols are relatively poor substrates (Martin *et al*, 1981:6237-6238),
- Very poor yields are realized for moderately water soluble epoxyalcohol products (Katsuki & Sharpless, 1980:5978), and
- This reaction is limited to the use of allylic alcohols as substrates (Armstrong *et al*, 2002:8610).

3.2.1.2 *Sharpless asymmetric dihydroxylation*

Hentges and Sharpless (1980:4263) reported the first asymmetric induction of olefins into *cis*-vicinal diols (**Scheme 2-10**) through the reaction of an alkene compound with a stoichiometric amount of osmium tetroxide in pyridine, following with reductive hydrolysis. Through the addition of a ligand Jacobsen and co-workers succeeded in delivering improved reaction rates and useful levels of asymmetric induction (Jacobsen *et al.*, 1988:1968).

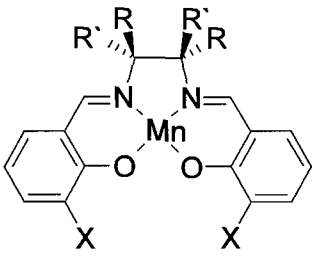


Scheme 2-10 Sharpless dihydroxylation (Hentges & Sharpless, 1980:4264).

Subsequently new ligands were developed to synthesise a wider range of epoxides (Sharpless *et al.*, 1991:4586; Sharpless *et al.*, 1992:2769). With the addition of the aromatic ether ligand group and the phthalazine group, all the different substituted epoxides (except for the *cis*-di- and tetra-substituted oxiranes) are good substrates with enantioselectivities of up to 99 %. Even though this method has a wide applicability, it does have the distinct disadvantage of the incorporation of osmium which is exceptionally expensive and toxic (Jacobsen *et al.*, 1988:1968).

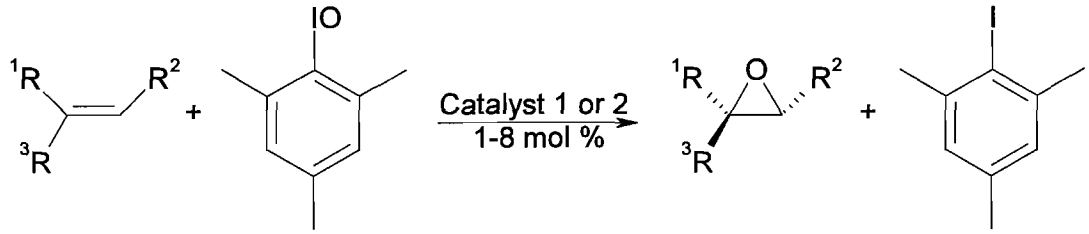
3.2.1.3 *Jacobsen epoxidation*

The enantioselective epoxidation of alkyl- and aryl- substituted olefins with manganese complexes of chiral Schiff bases was reported for the first time in 1990 (Zhang *et al.*, 1990:2801-2802) by Jacobsen and co-workers. Jacobsen epoxidation delivered the highest enantioselectivity up to that point for a non-enzymatic catalyst for the asymmetric catalysis of Z- and tri-substituted alkenes (**Scheme 2-11**).



(S,S)-1: R = Ph, R` = H, X = H
(R,R)-1: R = H, R` = Ph, X = H
(R,R)-2: R = H, R` = Ph, X = ^{t}Bu

Mn(III)salen complex catalyst

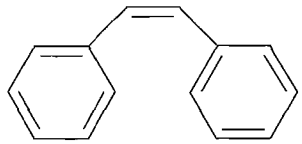
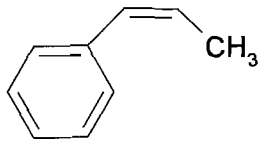
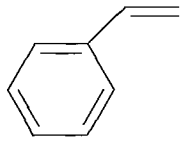
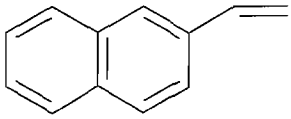
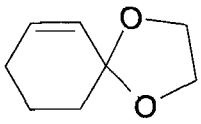
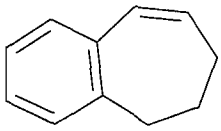
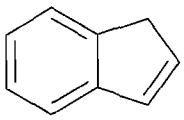
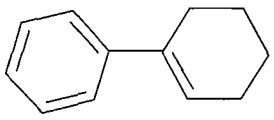


Scheme 2-11 Jacobsen asymmetric synthesis (Zhang *et al.*, 1990:2801).

The efficiency and ease of this method was later increased by (i) an easier synthetic route of the manganese catalyst, (ii) the use of commercial bleach as the stoichiometric oxidant instead of iodosylarenes (Zhang & Jacobsen, 1991:2296) and (iii) the immobilization of the catalyst onto a support (Pini *et al.*, 1999: 3883, Silva *et al.*, 2005:2096), which increases the ease of product and catalyst separation and recovery (Piaggio *et al.*, 1998:1167). The fourth improvement was the replacement of the substituent groups on the Mn(III)salen complex that lead to higher and wider selectivity (Palucki *et al.*, 1994:9333; Wang *et al.*, 2006:249).

The Jacobsen manganese catalyst is currently the most efficient chemical catalyst available for the enantioselective epoxidation of unfunctionalised olefins (Canali & Sherrington, 1998:86). A number of the compounds that have been successfully converted to optical pure epoxides with high enantioselectivity and yield are summarized in **Table 2-3**.

Table 2-3 Substrates for asymmetric epoxidation with the Jacobsen Mn-salen complex.

Substrate	% e.e.	% Yield	Product configuration	Reference
	78	86	*trans*	Piaggio *et al.*, 1998:1168
	62	97	(1R,2S)	Minutolo *et al.*, 1996:2296
	26	86	(R)	
	67	72	(+)	Zhang *et al.*, 1990:2802
	93	52	(–)	
	90	72	(5S,6R)	Pietikäinien. 2000:418
	72	n.a.	(1S,2R)	Zhang *et al.*, 2006:357
	84	n.a.	(S,S)	

3.2.2 Biological synthesis

Two basic methods exist for the biological synthesis of enantiopure epoxides, of which the first is the formation of an epoxide ring from a precursor, and secondly, the resolution of a racemic compound already bearing an epoxide ring (Archelas & Furstoss, 1999:159).

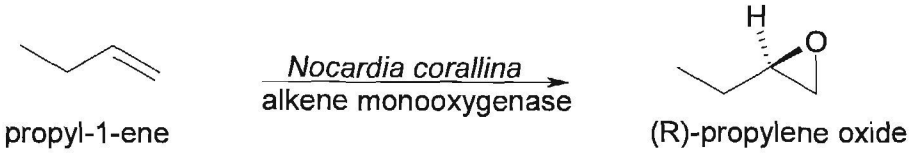
3.2.2.1 Asymmetric epoxidation

Biological epoxidation is classed as an oxygenation reaction which potentially catalyses the regio and stereospecific incorporation of one or more oxygen atoms into a molecule which are very difficult to carry out by chemical methods (Besse & Veschambre, 1994:8901). This can either be accomplished through the direct epoxidation of alkenes utilising the biocatalytic potential of a number of monooxygenases and chloroperoxidase, or through an indirect method where both haloperoxidases and halohydrin epoxidases are utilised in a two-step biocatalytic approach (Leak *et al.*, 1992:258).

a) Monooxygenase (MO)

The reaction mechanism of the monooxygenase enzyme group differs depending on the sub-type of enzyme, but their mode of oxygen-activation is the same (Faber, 2000:227). MO use molecular oxygen to insert one oxygen atom into the substrate while the second oxygen is reduced with the electros from NADH to NADPH to yield water (Li *et al., 2002:136).* These enzymes are found in most living organisms including bacteria, yeast, insects, plants and mammals. They are employed in asymmetric reactions as purified enzymes (cytochrome P450) or as whole-cell microorganisms (Besse & Veschambre, 1994:8902).

An example is *Nocardia corallina* B-276 which possesses a multi-component alkene monooxygenase which catalyses the epoxidation of terminal (**Scheme 2-12**) and sub-terminal alkenes. Propylene was successfully epoxidised producing the (R)-propylene oxide with a 85 % *e.e.*



Scheme 2-12 Reactivity of monooxygenase (Gallagher *et al.*, 1997:639).

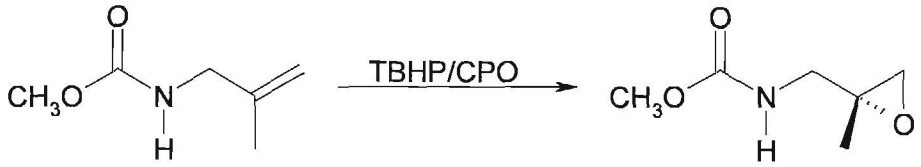
Direct epoxidation by MO generally yields terminal epoxides of (R) configuration (with the exception of microsomal MO), with an average yield and high enantioselectivity (Besse & Veschambre, 1994:8912).

b) Haloperoxidases and halohydrin epoxidases

An indirect method for the synthesis of non-racemic epoxides from olefins is through the synthesis of halohydrins with haloperoxidase enzymes that catalyse halogenation and the subsequent selective cyclisation into epoxides (Archelas & Furstoss, 1997:503) by halohydrin

epoxidase enzymes (Geigert *et al.*, 1983:369). Haloperoxidases are subdivided into groups according to the range of halide ions they can utilise. Iodoperoxidases can only employ iodide ions, bromoperoxidase both iodide and bromide ions, and chloroperoxidase can utilise chloride as well as iodide and bromide ions (Zhang *et al.*, 1999:87).

However, chloroperoxidase from C. *fumago* has the ability to yield epoxides directly from olefins in the absence of halide ions (Archelas & Furstoss, 1997:503; Allain *et al*, 1993:4415) if hydrogen peroxide is present. Chloroperoxidase has been shown to catalyse the asymmetric synthesis of a wide variety of olefins including 1,1-disubstituted alkenes (Dexter *et al.*, 1995:6413) and 1,2-disubstituted alkenes (Allain *et al*, 1993:4415). The selective conversion of olefins with chloroperoxidase (**Scheme 2-13**) is extremely dependant on the length of the carbon chain, increasing up to a maximum carbon length for a specific enzyme followed by a decline in *e.e.* beyond that point (Hager *et al.*, 1998:98, Lakner *et al.*, 1997:444).



94% e.e.
82% yield

Scheme 2-13 Chloroperoxidase mediated epoxidation (Hager *et al.*, 1998:98).

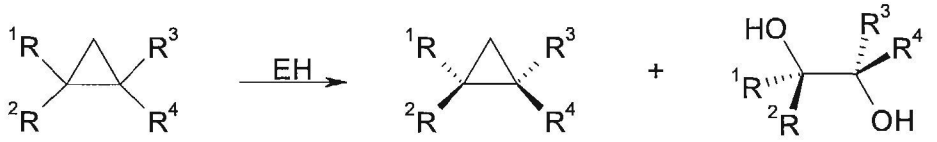
The enantiopure epoxides synthesised through the use of chloroperoxidases have a wide applicability for the further synthesis of bioactive targets such as (R)-phenylethanolamine, (R)- or (S)-ibuprofen, (S)-ketoprofen, and (R)-(–)-mevalonolactone (Hager *et al.*, 1998:98).

3.2.2.2 Enzymatic kinetic resolution

Enzymatic resolution of epoxide enantiomers can be accomplished through the selective addition of another functional group, or through the difference in the rate of metabolism (Besse & Veschambre, 1994:8917). One of the biggest advantages of standard kinetic resolution (metabolism) as opposed to asymmetric epoxidation is that the quality of the product, thus the enantiomeric excess, can be increased by sacrificing on the yield (Anthonsen *et al.*, 1996:2633). Although epoxide hydrolase (EH) and lipase aren't the only two enzymes available for the kinetic resolution of epoxides, they are the most widely used.

a) Epoxide hydrolases

Epoxide hydrolases refer to a collection of enzymes that catalyse the addition of a water molecule to an epoxide ring (**Scheme 2-14**), to yield a corresponding diol (Archelas & Furstoss, 1998:108).



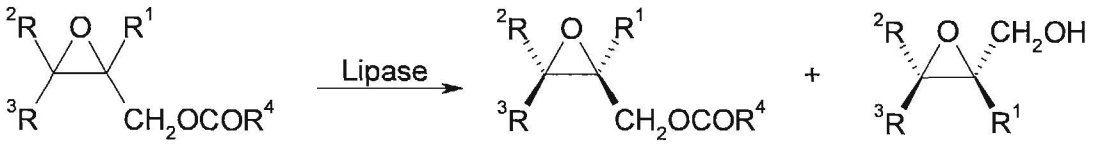
Scheme 2-14 Theoretical representation of epoxide hydrolase catalysed kinetic resolution (Archelas & Furstoss, 1998:108).

Due to their widespread use and their industrial applicability they will be discussed separately in Section 4.

b) Lipases

According to Brockerhoff, advantages of lipases include their acceptance of a wide range of structures with acceptable enantioselectivities and that they are not deactivated by the reaction with the epoxide moiety (Ladner & Whitesides, 1984:7250). Generally lipases also react at the same prochiral centre irrespective of the substrate (Schoffers *et al.*, 1996:3772).

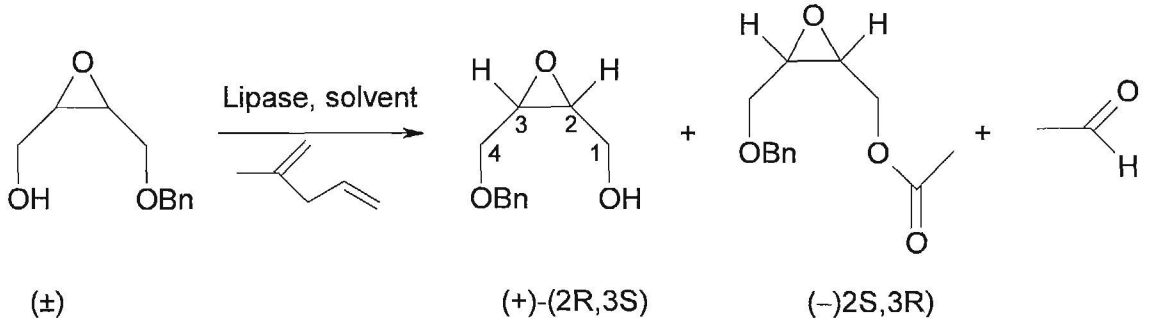
Lipases selectively hydrolyse racemic glycidyl esters (**Scheme 2-15**) to produce enantiomerically pure or enriched epoxyalcohols. The enantioselectivity of lipases is dependant on the acid component structure (R^4), the longer the n-alkyl group, the higher the enantioselectivity (Ladner & Whitesides, 1984:7250).



	Residual ester % e.e.
$R^1 = R^2 = R^3 = H$, $R^4 = CH_3$	58
$R^1 = R^3 = CH3$, $R^2 = H$, R^4 = n-propyl	75
$R^1 = R^2 = R^3 = H$, R^4 = n-pentyl	95

Scheme 2-15 Lipase catalysed hydrolysis of glycidyl esters (Ladner & Whitesides, 1984:7250).

Lipases are not only limited to hydrolytic reactions, but have also been implemented for enantioselective alcoholysis, transesterification (Fukusaki & Satoda, 1997:262) and acylation (**Scheme 2-16**) of epoxyalcohols (Faigl *et al.*, 2005:3841).



Scheme 2-16 Enantioselective lipase-catalysed acylation of *cis*-4-benzyloxy-2,3-epoxybutanol (Faigl *et al.*, 2005:3841).

4 Epoxide hydrolases

Epoxide hydrolase (EH) enzymes are being implemented in an increasing number of biotransformations. This can be attributed to a number of reasons:

- Epoxide hydrolases frequently exhibit remarkable chemio-, regio-, and stereoselectivity on a wide range of substrates (Xu *et al.*, 2004b:1217),
- Water-insoluble substrates can be handled due to the maintained catalytic activity of epoxide hydrolase in the presence of (Lotter *et al.*, 2004a:1191), or in pure organic solvents (Karboune *et al.*, 2006:320),
- These hydrolytic enzymes are co-factor independent, they can be purified and used as an enzymatic powder (Archer, 1997:15617),
- Substrates (racemic epoxides) are relatively cheap and readily available (Archelas & Furstoss, 1998:112), and
- Both possible hydrolases products (optically pure epoxide and optically pure diol) can be used as synthons in asymmetrical synthesis (Swaving & de Bont, 1998:19).

4.1 Occurrence of epoxides hydrolases

Epoxide hydrolases are ubiquitous in nature and have been found in most mammals (Archer, 1997:15620), insects (Debernard *et al.*, 1998:409), plants (Pinot *et al.*, 1997:103) and microbes (Orru & Faber, 1999:16). The preference and acceptance of natural and unnatural substrates differ according to the occurrence and physiological role that the enzyme plays within different organisms.

4.1.1 Mammalian epoxide hydrolases

To a large extent, the metabolism of lipophilic compounds has a detoxifying function. The first line of chemical defence against xenobiotic-derived epoxides is the liver enzyme epoxide hydrolases which converts lipophilic compounds, which can not be eliminated from the body unchanged, to water-soluble readily excreted metabolites and conjugates (Walker & Oesch, 1983:351; Argiriadi *et al.*, 1999:10637).

Two major epoxide hydrolase enzymes can be found in mammals; microsomal (mEH) and soluble epoxide hydrolase (sEH). These enzymes differ in terms of their location, primary physiological role, as well as their range of substrate and product specificity and enantioselectivities (Weijers & de Bont, 1999:201-202). The most notable difference in substrate specificity is styrene oxide, which is readily hydrolysed by mEH, but is not a substrate for sEH (Jefcoate, 1983: 52).

mEH are located in the endoplasmic reticulum (Arand *et al.*, 1994:251) of mammalian liver microsomes where they are primarily involved in the enzymatic detoxification of xenobiotics (Bellucci *et al.*, 1989a:5978) by catalysing the *trans* addition of water to epoxide substrates (Fretland & Omiecinski, 2000: 49). mEH exhibits low substrate specificity, with often remarkable stereoselectivity (Bellucci *et al.*, 1980:299), which makes it extremely well suited for the detoxification of a wide range of exogenous compounds (Bellucci *et al.*, 1989b:968). This detoxification process is particularly important due to the capability of many epoxides to irreversibly bind to nucleophilic sites present in tissue macromolecules and which could initiate toxic or carcinogenic conditions (Wang *et al.*, 1982:5469).

The broad range of substrates which are stereoselectively catalysed, or where products are stereoselectively formed, includes certain racemic anhydrosugars (Barili *et al.*, 1987:2889; Barili *et al.*, 1989:1561), styrene oxide (Oesch *et al.*, 1971:691) and *cis* 1,2-epoxdes (Bellucci *et al.*, 1993:1157; Bellucci *et al.*, 1996:200), poly-aromatic and *meso* epoxides (Bellucci *et al.*, 1989b:969;Jerina *et al.*, 1970:1059).

sEH, also called cytosolic epoxide hydrolase (cEH) (Arand *et al.*, 1994:251) are situated in both the cytosol and peroxisomal matrix of liver cells (Chang 1991:273) where they catalyse the *trans* addition of water to epoxide compounds (Gill & Hammock, 1979:969). These enzymes play a physiological role in the hydration of potentially toxic and mutagenic xenobiotics, but also in the regio- and stereo- selective (Zeldin, *et al.*, 1995:443) hydrolysis of endogenous *cis*- and *trans*-epoxymethylstearates (Gill & Hammock, 1979:969) and other epoxy fatty acids (Halarnkar *et al.*, 1989:227,232). Product and substrate stereo- and enantioselectivity towards cis-dialkyl

substituted oxiranes was also demonstrated, even though the selectivities were proven to be highly dependant on the substituent groups (Chiappe & Palese, 1999:11591). sEH also selectively hydrolyses *trans* epoxides, thereby complementing the activity of microsomal epoxides, for which *cis* epoxides are better substrates (Meijer and DePierre, 1988:207; Chiappe *et al.*, 2004:245).

Although mammalian epoxide hydrolases exhibit high enantioselectivity towards a wide variety of epoxides, it can only be exploited on small scale kinetic resolutions due to the lack of availability (Bellucci *et al.*, 1993:1158).

4.1.2 Insect epoxide hydrolases

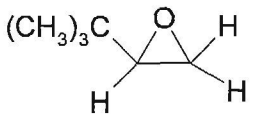
Insect epoxide hydrolases are involved in the degradation of juvenile hormone (JH) (Khalil, 2006: 669), as well as epoxides acquired through the diet (Otea & Hammock, 1986:319). The JH plays multiple roles, including the regulation of metamorphosis in larval insects (Debernard *et al.*, 1998:409) and in the reproduction of adults (Anspaugh *et al.*, 2005:533). Anspaugh and Roe (2005:533) also recently found the involvement of these enzymes in xenobiotic metabolism.

Although substrate specific JH epoxide hydrolase with stereo- and regiospecificity has been found (Linderman *et al.*, 1995:10845, Graham & Prestwich, 1994: 2956; Roe, 2005:146), large scale production is still fairly difficult, hindering their catalytic applications (Weijers & de Bont, 1999: 203).

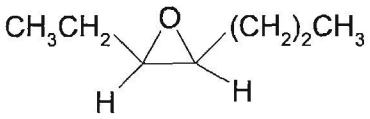
4.1.3 Plant epoxide hydrolases

Only a small portion of enzymes used in biotransformation is obtained from plant sources (Faber, 2000:25). In principle; plant epoxide hydrolases are useful for the synthesis of enantiopure epoxy fatty acids and dihydroxy fatty acids due to their stereochemical features and their relatively high activities (Weijers & de Bont, 1999:203). This specific activity was reported for the hydrolysis of cis-9,10-epoxystearate (Bleé & Schuber, 1992:714), 9,10-epoxy-12(Z)-octadecenoic and cis-12,13-epoxyocta-9(Z)-decenoic acids by soybean epoxide hydrolase (Bleé & Schuber, 1995:230).

Pinot *et al.*, (1997:103) discovered a hydrolytic activity in both the microsomal and soluble fractions of the seedlings of *Vicia sativa* using 9,10-epoxystearic acid as substrate. Subsequently Chiappe *et al*, (2001:128) also demonstrated the asymmetric hydrolysis of a variety of mono- and disubstituted epoxides (**Scheme 2-17**) with high selectivity utilizing the soluble enzyme fraction for the hydrolysis process.



(a)



(b)

Substrate	Hydrolysis (%)	Residual Epoxide		Formed diol	
		% e.e.	A.C.	% e.e.	A.C.
2-*tert*-butyloxirane	10	8	S	>98	R
2-ethyl-3-propyloxirane	30	n.d.	n.d.	80	(3R,4R)

A.C.: Absolute Configuration; n.d.: not determined

Scheme 2-17 *Vicia sativa* plant EH mediated kinetic resolution (Chiappe *et al.*, 2001:128).

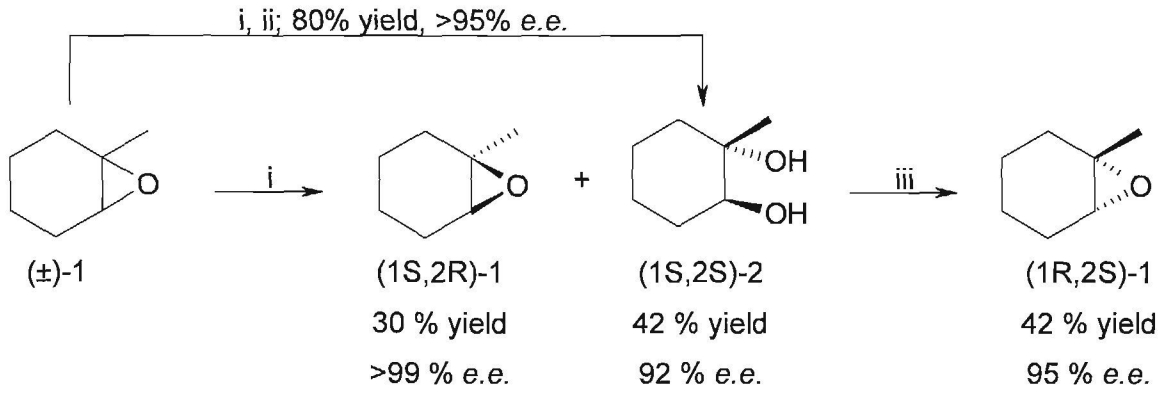
Recently another source (mung beans) for two novel epoxide hydrolase enzymes was discovered: both of these enantioselectively catalysed the hydrolysis of styrene oxides. Interestingly, the enantiospecificity of these enzymes for *p*-nitrostyrene oxide was complementary with a regioselectivity of more than 90 %. Thus, the combined use allows for a cheap and practical biocatalytic pathway to the (R)-diols of racemic epoxides (Xu *et al.*, 2006:1737, 1738).

4.1.4 Microbial epoxide hydrolases

4.1.4.1 Bacterial epoxide hydrolases

Enzymatic epoxide hydrolases represent an important step in carbon catabolism, but also facilitates the detoxification reactions essential for the survival of the cell (Osprian *et al.*, 1997:65). Bacterial epoxide hydrolases can be divided into (i) essentially produced enzymes and (ii) enzymes involved in the metabolism of specific epoxides (Weijers & de Bont, 1999: 203).

The essential enzymes are primarily used for the selective hydrolysis of mono and 2,2-disubstituted epoxides (Osprian *et al.*, 1997:67, Helström *et al.*, 2001:169, Gong & Xu, 2005:253). Even though lower selectivity is observed towards 2,3-di- and tri-substituted epoxides (Mischitz *et al.*, 1996:2044), Archer *et al.* (1996:8820) reported a whole cell chemo-enzymatic procedure to obtain both enantiomers of 1-methyl-1,2-epoxycyclohexane (**1**) in high enantiopurity (**Scheme 2-18**) through cyclisation of methylcyclohexane-1,2-diol (**2**).



Scheme 2-18 Chemo-enzymatic resolution and deracemisation of 1-methyl-1,2-epoxycyclohexane. (i) *Corynebacterium* EH, (ii) 0,02M $HClO_4$, (iii) $MeSO_2$/NaH (Archer *et al.*, 1996:8820).

Metabolism related EH usually exhibits narrow substrate specificity, with high specific activity limited to the compounds structurally related to the growth substrate (Weijers & de Bont, 1999: 204). Van der Werf *et al.*, (1998:5053) demonstrated the induction of a novel type of metabolism related epoxide hydrolase, by growing *Rhodococcus erythropolis* on monoterpenes (e.g. (+)-Limonene, (–)-Limonene and (+)-Limonene-1,2-epoxide). The specific activity (hydrolysis of (+)-limonene-1,2-epoxide) increased up to 53 times in contrast to the activity of succinate grown cells.

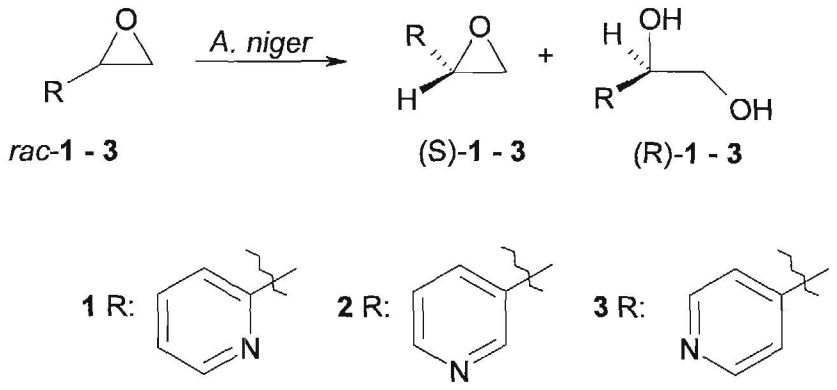
4.1.4.2 Fungal epoxide hydrolase

Fungal epoxide hydrolases exhibit a relatively broad substrate specificity and high enantioselectivity (Weijers & de Bont, 1999:206), which seems to be complementary to that of bacterial EH (Moussou *et al.* 1998:1566).

Although a large number of fungi have been tested and used for the kinetic resolution of epoxides in screenings (Grogan *et al.*, 1996:239; *Moussou et al.*, 1998:1565; Kotik *et al.*, 2005:370), the most widely applicable fungi, looking at activity ranges and selectivity, seem to come from the *Aspergillus* species (Smit, M.S, 2004:123).

Aspergillus niger strains exhibit broad substrate specificity with substrate structure dependant absolute configuration and yield. High enzyme activity and enantioselectivity has been demonstrated for aryl- (Choi *et al.*, 1998:226) and glycidyl (Kotik *et al.*, 2005:372) terminally substituted epoxides, as well as for styrene oxide (Liu *et al.*, 2006a:279) and styrene oxide type epoxide substrates (Morisseau *et al.*, 1999:390, Pedragosa-Moreau *et al.*, 1996:4594).

The kinetic resolution of pyridyloxirane derivatives (**Scheme 2-19**), poor substrates for heavy-metal-based catalysts, was accomplished on a 10 $g.L^{-1}$ preparative scale. The (S)-pyridyloxirane compounds were all synthesised with an optical purity of > 98 % *e.e.* (Genzel *et al.*, 2001:539).



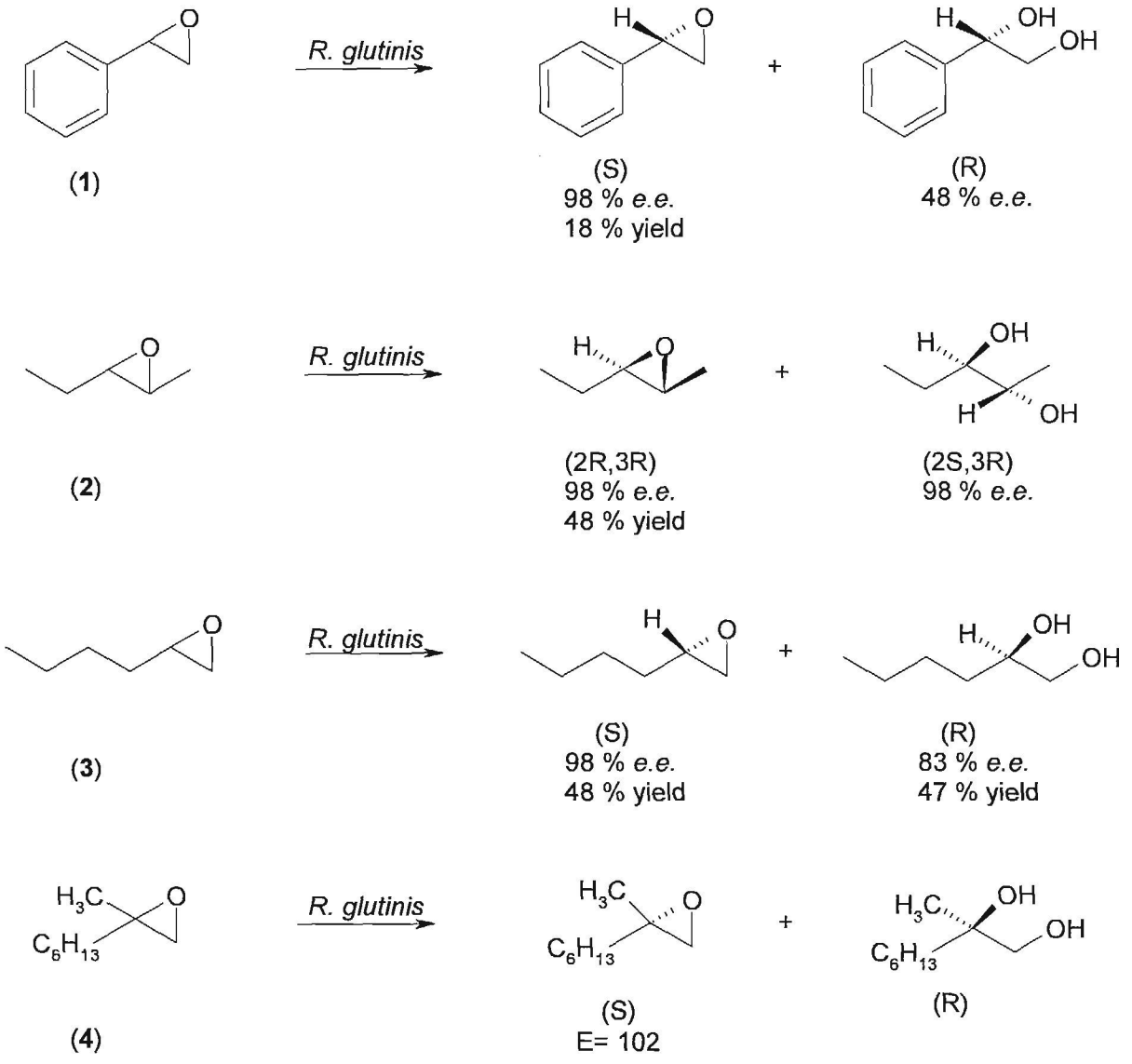
Scheme 2-19 Kinetic resolution of pyridyloxirane compounds (Genzel *et al.*, 2001:539).

4.1.4.3 *Yeast epoxide hydrolases*

The use of yeast epoxide hydrolases is of extreme interest due to the ease with which these organisms are cultivated and the easy removal of the cells from a reaction mixture through centrifugation (Weijers & De Bont, 1999:206). After Weijers (1997:639) reported EH activity in yeasts, a wide variety of yeast strains have been extensively screened against a broad range of substrates (**Scheme 2-20**) with most of the pioneering work probably done by Botes and co-workers (Botes *et al.*, 1998:421; Weijers *et al.*, 1998:467; Botes *et al.*, 1999:3327).

Rhodotorula glutinis was the first yeast where EH activity was discovered (Weijers, 1997:639). Subsequent studies showed enantioselective activty of this epoxide hydrolase enzyme towards terminal epoxides (**1; Scheme 2-20**) with low regioselectivity to (R)-diols with retention of configuration, and methyl substituted epoxides (**2**) with inversion of configuration at the most hindered carbon (Weijers & De Bont, 1999:206). Although high activity and enantioselectivity was obtained with both aliphatic and aromatic substituted epoxides, it was quite obvious that the enzyme prefers aromatic substrates.

Since the first discovery, this yeast has been extensively researched and used for its stereoselective activity. Later studies revealed biocatalytic usable enantioselectivities towards unbranched aliphatic mono substituted (**3**) (Weijers *et al.*, 1998:469), 2,2-disubstituted (**4**) (Botes *et al.*, 1999:3330) 1,2-epoxides, and other styrene oxide type epoxides (Yeates *et al.*, 2003:678).



Scheme 2-20 Example of substrates for yeast EHs (see references in text).

Other yeasts with enantioselective EH activities towards a number of different substrates include *Rhodosporidium toruloides* (Krieg *et al.*, 2001:38), *Rhodotorula araucariae* and *Rhodotorula rubra* (Botes *et al.*, 1998:423) *Trichosporon loubierii* (Xu *et al.*, 2004a:156), and also *Cryptococcus laurentii* and *Cryptococcus podzolicus* (Botes *et al.*, 2005:27).

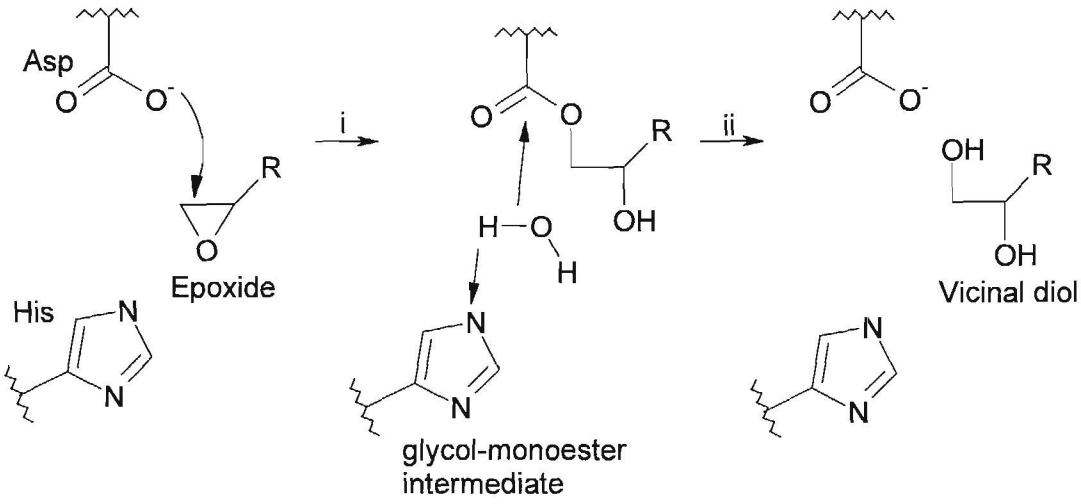
4.2 Mechanism of epoxide hydrolases

Epoxides can be opened via direct attack of the nucleophile on the epoxide ring or via an intermediate in which there is a covalent link between the enzyme and the substrate. Most epoxide hydrolases belong to the α/β-hydrolase fold family of enzymes which also includes many lipases, esterases and dehalogenases (De Vries & Janssen, 2003:114). All the enzymes

in this family have a nucleophile-His-acid triad involved in a two step catalytic mechanism participating in the formation of a covalent intermediate (Holmquist, 2000:212).

The most extensive mechanistic studies have been done on enzymes of mammalian origin, but in the light of qualitative similarities among the epoxide hydrolases of different species, it is widely accepted that most epoxide hydrolases function in a similar manner (Finney, 1998:R75).

In the first step of the catalytic process (**Scheme 2-21**), the epoxide is bound to the active site of the enzyme. The substrate epoxide is polarized by two tyrosine residues via hydrogen bonding with the epoxide oxygen. At the same time the nucleophilic carboxylic acid of the Aspartame, attacks the epoxide from behind. This nucleophilic attack can occur at any of the two carbon centres, and is not limited to only one of the two, due to the incomplete regioselectivity observed for enzymes. This implies that the absolute configuration may be retained or inverted, depending on the substituent arrangement on the attacked carbon atom (i). An ester bond intermediate is formed between the enzyme carboxylic acid and one alcohol functionality of the diol due to the opening of the epoxide ring. The formed intermediate is known as the hydroxyl alkyl-enzyme- or glycol-monoester intermediate. Subsequently, the histidine moves away from the nucleophilic acid allowing a water molecule to be activated by the acid-histidine pair. This water molecule then attacks the carbonyl of the ester (ii), to release the diol product as well as the original enzyme (Finney, 1998:R75, Morisseau & Hammock, 2005:315-317; Orru & Faber, 1999:16).



Scheme 2-21 General simplified method of EH activity (Orru & Faber, 1999:16).

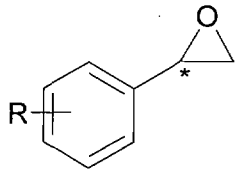
4.3 Microbial EH synthesis of enantiomerically pure styrene oxides

The usefulness of epoxide hydrolases as biocatalysts for the production of enantiopure epoxides and diols, are dependant on factors like availability, stability, and activity of the enzyme, as well as the enantioselectivity and regioselectivity of the individual enzymatic reactions (Kotik *et al,* 2005:372).

Although microbial epoxide hydrolases were only discovered in the late 1990`s, an immense amount of research has been conducted on these enzymes. An important group of epoxides under investigation is the styrene oxide type oxiranes due to their possible use in the synthesis of more complex pharmaceutical organic structures.

In order to obtain a better understanding of the scope of this research a summary of biocatalysts used to obtain enantiopure epoxides from styrene oxide derivatives is given in **Table 2-4**. When considering the different epoxide substrates, it can be seen that there are a number of possible biocatalysts capable of stereospecifically hydrolysing most of the epoxides. The residual epoxide, in other words, the enantiopure obtained epoxide, as well as the obtained diol can be regulated by using different species of organisms as biocatalyst sources.

Table 2-4 Yeast EH utilised for the biocatalytic resolution of styrene oxides.



Species	Residual Epoxide		Formed diol		E	References
	% e.e.	% Yield	% e.e.	% Yield		
Styrene oxide						
Rhodotorula glutinis	(S) >98	18	(R) 48			Weijers, 1997:642
Sphingomonas sp. HXN-200	(S) >99					Liu, *et al.*, 2006b:50
Streptomyces fradiae Tu27	(R) 70		(S) 23		4	Zocher *et al.*, 2000: 290
Streptomyces aranae Tu 495	(R) 52		(S) 38		3	
Streptomyces antibioticus	(R) 99		(S) 72		31	
Agrobacterium radiobacter 1219F	(S)		(R)		91	Rui *et al.*, 2005:2998
Aspergillus niger	(S) 100	32				Choi *et al.*, 1998:227
Aspergillus niger CGMCC	(S) 75	15	(R) 52	36		Jin *et al.*, 2003:410
Beauveria densa CMC3240	(R) >95	18	(R) 78	34		Grogan *et al.*, 1997:256
Aspergillus niger SQ-6	(S)		(R) >99			Liu *et al.*, 2006a:276
Solanum tuberosum	(R)96	47	(R) >99	47		Manoj *et al.*, 2001:698
Beauveria bassiana	(R)		(R)	28		Moussou *et al.*, 2000:419
Aspergillus niger	(S) 96	23	(R) 51	54		Pedragosa-Moreau *et al.*, 1993:5533
Beauveria sulfurescens	(R) 98	19	(R) 83	47		
Yarrowia lipolytica	(R) 52	22				Fantin *et al*, 2001:2710
Rhodoturula glutinis	(S) 98	36				Lee *et al.*, 2004:630

Species	Residual Epoxide		Formed diol		E	References
	% e.e.	% Yield	% e.e.	% Yield		
Styrene oxide (*continue*)						
Solanum tuberosum	(R) 94		(R) 95		30	Monterde *et al.*, 2004:2803
Rhodoturula sp. Y-0448	(S) 65	33	(R) 67	28	6,16	Lotter *et al.*, 2004b:1197
***o*-CH_3-Styrene oxide**						
Beauveria densa CMC3240	(R) 12	24	(R) 5	36		Grogan *et al.*, 1997:256
***m*-CH_3-Styrene oxide**						
Beauveria densa CMC3240	(R) 50	19	(R) 67	44		Grogan *et al.*, 1997:256
***o*-NO_2-Styrene oxide**						
Aspergillus niger CGMCC	(S) 98	34	(R) >99	38		Jin *et al.*, 2003:410
***m*-NO_2-Styrene oxide**						
Aspergillus niger CGMCC	(S) 57	40	(R) 79	33		Jin *et al.*, 2003:410
***p*-NO_2-Styrene oxide**						
Aspergillus niger CGMCC	(S) 97	47	(R) 93	46		Jin *et al.*, 2003:410
Aspergillus niger	(S) 100					Nellaiah *et al.*, 1996:76
Aspergillus niger	(S) 96	38	(R) 66	49		Pedragosa-Moreau *et al.*, 1997:9709
Aspergillus niger	(S) 98		(R) 92		85	Petri *et al.*, 2005:222
***p*-CH_3-Styrene oxide**						
Beauveria densa CMC3240	(R) > 95	21	(R) 61	48		Grogan *et al.*, 1997:256
Aspergillus niger CGMCC	(S) 70	45	(R) 63	43		Jin *et al.*, 2003:410
***p*-I-Styrene oxide**						
Aspergillus niger CGMCC	(S) 97	25	(R) 72	39		Jin *et al.*, 2003:410

Species	Residual Epoxide		Formed diol		E	References
	% e.e.	% Yield	% e.e.	% Yield		
***p*-F-Styrene oxide**						
Beauveria densa CMC3240	(R) > 95	18	(R) 77	36		Grogan *et al.*, 1997:256
Aspergillus niger CGMCC	(S) 41	24	(R) 74	33		Jin *et al.*, 2003:410
***o*-Cl-Styrene oxide**						
Aspergillus niger CGMCC	(S) 47	17	(R) 84	37		Jin *et al.*, 2003:410
Solanum tuberosum	(R) 31		(R) 30		2.5	Monterde *et al.*, 2004:2803
***m*-Cl-Styrene oxide**						
Aspergillus niger CGMCC	(S) 28	47	(R) 74	40		Jin *et al.*, 2003:410
Solanum tuberosum	(R) 61		(R) 94		6	Monterde *et al.*, 2004:2803
***p*-Cl-Styrene oxide**						
Beauveria densa CMC3240	(R) >95	17	(R) 82	36		Grogan *et al.*, 1997:256
Aspergillus niger CGMCC	(S) 95	32	(R) 85	46		Jin *et al.*, 2003:410
Solanum tuberosum	(R) >99		(R) 88		70	Monterde *et al.*, 2004:2803
***o*-Br-Styrene oxide**						
Aspergillus niger CGMCC	(S) >99	22	(R) 80	46		Jin *et al.*, 2003:410
***m*-Br-Styrene oxide**						
Aspergillus niger CGMCC	(S) 35	43	(R) 70	43		Jin *et al.*, 2003:410
***p*-Br-Styrene oxide**						
Beauveria densa CMC3240	(R) >95	19	(R) 87	41		Grogan *et al.*, 1997:256
Aspergillus niger CGMCC	(S) >99	30	(R) 86	43		Jin *et al.*, 2003:410

Species	Residual Epoxide		Formed diol		E	References
	% e.e.	% Yield	% e.e.	% Yield		
***o*-CF_3-Styrene oxide**						
Aspergllus niger	(S)		(R) 77		5	Deregenaucourt *et al.*, 2006:1166
Aspergillus niger CGMCC	(S) 98	34	(R) 82	31		Jin *et al.*, 2003:410
***m*-CF_3-Styrene oxide**						
Aspergillus niger	(S)		(R) 13.2		10	Deregenaucourt *et al.*, 2006:1166
***p*-CF_3-Styrene oxide**						
Aspergillus niger	(S)		(R) 84.3		50	Deregenaucourt *et al.*, 2006:1166
***p*-OCF_3-Styrene oxide**						
Aspergillus niger	(S)		(R) 94.5		30	Deregenaucourt *et al.*, 2006:1166
***p*-SCF_3-Styrene oxide**						
Aspergillus niger	(S)		(R) 85		160	Deregenaucourt *et al.*, 2006:1166
***p*-CH_2CH_3-Styrene oxide**						
Aspergillus niger CGMCC	(S) 63	29	(R) 70	41		Jin *et al.*, 2003:410

5 Why biocatalysis?

As can be seen, many chemical and biological approaches are available for the synthesis of enantiomerically pure epoxides and diols. The synthetic route of choice for a preparative purpose obviously will depend on the compound under investigation, as well as the expertise available in a research group or company (De Bont, 1993:1337).

Having said this, the use of biocatalysts does have a number of advantages over chemical catalysts, including:

- Enzymatic catalysis can be carried out at ambient temperatures and atmospheric pressure (Patel *et al.*, 2003:258),
- Possible elimination of the use of toxic and expensive heavy-metal reactants or catalysts (Manoj *et al.*, 2001:695),
- High chemoselectivity, regioselectivity and more specifically stereoselectivity (Schulze & Wubbolts, 1999:609),
- Enzymes can be utilised in crude or isolated form or as whole-cells,
- The use of enzymes excludes the tedious blocking and unblocking steps common in enantioselective and regioselective organic syntheses (Schulze & Wubbolts, 1999:609),
- Whole-cells have added advantages such as the simple and inexpensive preparation, and protection of the enzyme from environmental factors which makes them more stable in the long run (Ishige *et al.*, 2005:174),
- Enzymes demonstrate high substrate specificity, with remarkable broad substrate tolerance, and high stability in a range of temperatures, organic solvents and media (Ader *et al.*, 1992:1165),
- Screening a range of enzymes and mutants, the ones that are most suited for the conversion of a specific substrate, can be identified (De Vries & Janssen, 2003:415), and
- Synthetic routes to products that might have been extremely difficult, or even impossible to produce through chemical methods (Genzel *et al.*, 2002:217).

These advantages, and the development of a wide variety of biocatalyst optimisation techniques, such as immobilisation (Genari, Passos & Passos, 2003:2783) and the use of metabolic flux analysis, together with the characterisation of metabolic networks, for the identification of targets for genetic engineering (Stafford & Stephanopoulos, 2001:336), have lead to the increased industrial application of enzymes for both single- (Haberland *et al.*,

2002:458, Kaptein *et al.*, 1998:14), and chemo-enzymatic methods (Van Langen, 2003: 828) to obtain the required compound.

6 Conclusion

In this chapter a broad overview was given on the different motivations for the production of enantiopure compounds, different synthetic methods for the production of these compounds, especially epoxides, with more emphasis on the possible biological routes, including the use of epoxide hydrolases for the kinetic resolution of racemic epoxide.

The need for enantioseparation or the synthesis of enantiopure compounds due to the possible difference in biological effect that these compounds might exhibit is clearly demonstrated through the numerous examples available in literature. Both effective chemical and biological synthetic routes, as well as physical separation methods exist for this purpose, differing in effectiveness (yield as well as enantiomeric purity), possible disadvantages (including the use of large volumes of organic solvents, the use high pressure and temperature systems, which utilises the already decreased source of fossil fuels).

The effective chemical synthetic routes for the synthesis of enantiopure epoxides all include the use of expensive, and hazardous heavy-metal catalysts, and have a limited applicability in the range of possible substrates.

The use of epoxide hydrolases from micro-organisms and other sources for the kinetic resolution of chiral racemic epoxides seems to be an elegant method. The enzyme is ubiquitous in nature, thus, nature provides an abundant supply, it is cheap, can be easily and safely discarded, and the different epoxide hydrolases from different sources shows activity towards a wide range of diverse epoxides.

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Chapter 3

An activity growth study of two epoxide hydrolysing biocatalysts – *Rhodotorula glutinis* and *Rhodosporidium toruloides*

Abstract

The effect of different growth media (YM, YMvit and malt) as well as the addition of different initial glucose concentrations (0,5, 1,0, 1,5 and 2,0 % (w/v)) on the growth (time and biomass) as well as on the activity and selectivity of two highly similar epoxide hydrolase enzymes from *R. glutinis* (UOFS Y-0563) and *R. toruloides* (CBS-0349) were investigated. For both yeasts the malt medium lead to the production of less biomass in comparison to the YM and YMvit media. The medium used had almost no effect on the enzyme activity or selectivity for *R. toruloides* EH, but the contrary was found for *R. glutinis* EH. The two EH enzymes were also not equally affected by the addition of glucose. An increasing concentration of glucose lead to glucose mediated catabolic repression for *R. toruloides* EH, but this was not observed for *R. glutinis* EH in the glucose concentration range tested.

Keywords: epoxide hydrolases, growth, vitamin and glucose

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1 Introduction

Enantiopure epoxides are versatile chiral synthons in organic synthesis. These compounds are used as valuable building blocks and reactive intermediates (Faber, 2000:135) in the preparation of more complex enantiopure bioactive compounds such as pharmaceuticals and agrochemicals. The high reactivity of epoxides is due to the unstable three-membered ring structure and the electronic polarization present in these compounds (Choi *et al.*, 1999:7).

Due to the versatility of these epoxide compounds they have received widespread attention as can be seen from the number of papers that have been written in recent years extensively reviewing their chemical and biological synthetic pathways (Archelas & Furstoss, 1997; De Bont, 1993; Goswami *et al.*, 1999; Leak *et al.*, 1992). One of the most promising methods for the production of enantiomerically pure epoxides is the use of the widely available co-factor independent epoxide hydrolase (EH) enzymes. These enzymes enantiospecifically hydrolyse racemic epoxides to their corresponding *vicinal* diols (Archer, 1997:15617).

While EHs can be obtained from most living organisms, microorganisms are the most suitable source, due to the ease of cultivation of large amounts of biomass, thus easily producing large quantities of enzymes (Kronenburg, *et al.*, 1999:519). Since EH production is influenced by several factors, including carbon and nitrogen sources (Morisseau *et al.*, 1998:508), optimisation of the production and enhancement of its activity may have great economic significance.

The EHs from two red yeasts, *Rhodotorula glutinis* and *Rhodosporidium toruloides,* have been shown to be potentially versatile and user friendly biocatalysts (Smit, 2004:123) for the kinetic resolution of both styrene oxide type (Yeates *et al.*, 2003:680) and aliphatic epoxides (Botes *et al.*, 1998:425). Despite the reported difference in activity, the nucleotide and amino acid sequence similarity between these two basidiomycetous yeast EHs were found to be between 96 and a 100 % (Visser *et al.*, 2002:1690; Smit & Labuschagne, 2006:1154).

According to the guidelines of green chemistry, synthetic routes need to be improved by a reduction in solvent use and emission, as well as by the use of renewable resources. Green chemistry, i.e. biocatalysis, does not only aim to protect the environment, but also reduces costs involved in the synthetic process by decreasing the costs for waste water treatment, decreasing energy use, as well as organic carbon resources (Held *et al.*, 2000:1337). To further reduce the cost of large-scale production and commercialisation of enzymes, less expensive energy sources, thus growth media, are needed (Khanna, 2005:2173).

In this study the effect of using only a single malt energy source growth medium in comparison to the more widely used YM medium (with and without vitamin solution) on the growth time and biomass production, as well as the EH activity and selectivity was investigated. In addition, the effect of the initial glucose supplement concentration was also investigated in both *R. glutinis* and *R. toruloides* to determine not only the effect of energy source and supplements on a single organism, but also to compare the effect of these growth conditions on two almost identical EH enzymes.

2 Experimental procedure

2.1 General

The strains of *R. toruloides* (CBS-0349) and *R. glutinis* (UOFS Y-0563) were respectively obtained from the *Centraalbureau voor Schimmel cultures, Fungal and Yeast Collection*, The Netherlands, and the Yeast culture collection of the University of the Free State (South Africa). Racemic styrene oxide (SO) and 1,2-epoxyoctane (EO) were obtained from Fluka and Aldrich, South Africa respectively. The vitamin solution was obtained from the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, South Africa.

Reactions were monitored and enantiomeric purities analysed by chiral gas chromatography (ThermoFinnigan® Focus GC equipped with a FID and a Thermo Electron AS 3000 Series auto sampler) on a fused silica cyclodextrin Chirasil-DEX-CB Varian® capillary column, using H_2 as carrier gas.

2.2 Cultivation and preparation of yeast cells

2.2.1 Effect of growth media on biomass production and enzyme activity

Both microorganisms were grown at 27 °C in a light protected Labcon® rotary platform shaking incubator. All media solutions were prepared using single distilled water and autoclaved for 20 minutes at 121 °C. The media solutions were left at room temperature for 24 hours to cool down before inoculation. YM media pre-inoculum cultures (0,5 % yeast extract, 2,0 % malt extract, 0,5 % peptone (w/v)) supplemented with 1,5 % (w/v) glucose was grown for 24 hours in 250 mL shake flasks after which 5 mL of the 50 mL pre-inoculum was transferred under sterile conditions 1 L shake flasks containing 200 mL growth media.

Three growth media were selected for this study: YM media, YM media supplemented with 2,0 % (v/v) vitamin solution, and a third growth medium (YMvit), and a single carbon source medium, containing only 4,0 % malt extract (w/v). The vitamin solution was added to the YM medium after cooling, using 0,22 µm sterile Whatman® syringe filters. The vitamin solution comprised of: 20,0 $mg.mL^{-1}$ biotin, 200,0 $mg.mL^{-1}$ calcium pantothenate, 0,2 $mg.mL^{-1}$ folic acid,

1000,0 mg.mL^{-1} inositol, 40,0 mg.mL^{-1} niacin, 20,0 mg.mL^{-1} *p*-amino benzoic acid, 40,0 mg.mL^{-1} pyridoxine HCl, 20,0 mg.mL^{-1} riboflavin and 40,0 mg.mL^{-1} thiamine HCl.

The flask cultures were incubated for a maximum of 144 hours during which time samples were taken every 4 hours to determine the amount of biomass present in the growth media. Cells for the enzyme activity assay were harvested every 12 hours.

Cells were harvested by centrifugation (3500×*g*, 5 min) and washed with phosphate buffer (KH_2PO_4/K_2HPO_4, 50 mM, pH 7,3). Botes *et al.* (1999:3335) observed no noteworthy decrease in enzyme activity after several months of storage, therefore the washed cells were resuspended (25 %, w/v) in phosphate buffer containing 1,0 % (v/v) glycerol (pH 7,3), frozen and stored below −18°C in 50 mL centrifuge tubes until hydrolytic reactions were conducted within three weeks.

2.2.2 Effect of glucose concentration on enzyme activity and biomass

For this study all growth and harvesting procedures were followed as was explained in Section 2.2.1, including the preparation of the pre-inoculum. The glucose concentration of the YM inoculum medium was varied between 0,5 and 2,0 % (w/v). The flask cultures were left to grow for 144 hours. Samples were taken at 4 hours intervals to determine the amount of biomass present, and cells were harvested at 12 hours intervals for hydrolytic reactions.

2.3 Determination of biomass

In order to evaluate the growth and determine the formed biomass, 500 µL samples were taken from the culture flasks at specific time intervals. All samples were taken under sterile conditions in a vertical laminar flow cabinet.

The culture flasks were lightly swirled to homogenously distribute yeast cells in the culture media before the samples were taken. Samples were transferred into 1,5 mL weighed Eppendorf tubes and centrifuged at 9000 rpm for 3 minutes. After centrifugation, the supernatant was extracted and discarded. Distilled water was added to the cell pellet to eliminate all growth media; it was vortexed until all cells were resuspended, and centrifuged for a second time after which the supernatant was again removed and discarded. All supernatant that could not be removed through extraction with a pipette was absorbed by applying a small piece of absorbent cotton paper towling onto the cell pellet. The mass of the cell pellets were determined through subtracting the weight of the Eppendorf tube from the weight of the tube with the cell pellet.

2.4 Determination of enzyme activity

The enzyme activity of the yeast strains was determined by following the asymmetric hydrolysis of a specific epoxide substrate for each yeast strain. The enzyme activity of the *Rhodosporidium* sp. was determined through the asymmetric hydrolysis of EO and the hydrolysis of SO for the *Rhodotorula* sp. Samples were taken in duplicate at selected times to ensure reliability of the data. For the repeated data points the average as well as the error bars are plotted on the graphs.

The previously harvested and frozen cell suspensions were thawed and vortexed to ensure homogeneous distribution of yeast cells. 3 mL cell suspensions were placed in McCartney bottles with metal screw caps. The cell suspensions were incubated at 15 °C for 5 minutes to ensure that the reaction temperature was stable. Substrates were added with a Hamilton syringe to a final concentration of 20 mM. The reaction vessels were vigorously vortexed for one minute before being placed back into the incubator at 15 °C. Reaction vessels were vortexed with regular intervals during the reaction time as well as just before sampling.

Samples (500 µL for *R. glutinis* and 250 µL for *R. toruloides*) were taken from the reaction mixture at specific points during the reaction time, placed in 1,5 mL Eppendorf micro-centrifuge tubes and submerged into liquid nitrogen to stop the hydrolytic reaction. The residual epoxide was extracted through the addition 250 µL ethyl acetate. The ethyl acetate was added to the frozen samples, and vortexed until completely defrosted. The reaction mixtures were vortexed for another 3 minutes, after which it was centrifuged for 3 minutes at 10 000 rpm. The ethyl acetate was extracted and dried over anhydrous Na_2SO_4 before analysis and quantification of the residual epoxide on chiral GC.

2.5 Analysis

Chiral GC analysis of the isolated substrates (**Appendix 1**) were conducted as follows: The analysis of SO was done non-isothermally with a initial temperature of 130 °C which was kept constant for 0,5 min, followed by a decrease in temperature at a rate of 10 °C.min^{-1} to 110 °C, after which the temperature was kept constant at 110 °C. The retention times (tR) were 5,28 and 5,54 minutes for the (R) and (S) enantiomers respectively. EO was analysed isothermally at 50 °C, tR (R) 55,26 min and tR (S) 56,65 min The inlet and detector temperatures were 170 °C and 190 °C respectively. DMF and DMSO were used as internal standards. Split flow was set at 50 and 20 mL.min^{-1} for SO and EO respectively with split ratios of 33 and 20.

3 Results and Discussion

In the past, vitamin solutions consisting of a mixture of components including biotin, calcium pantothenate, folic acid, inositol, niacin, *p*-amino benzoic acid, pyridoxine HCl, riboflavin and thiamine HCl have been added to the cultivation medium for quite a number of microorganisms to enhance the production of enzymes. Examples where these additives have been used include the production of lipases (Dalmau *et al.*, 2000:658), protopectinases (Ferreyra *et* al., 2002:499) and EHs (Le Roux *et al.*, 2005:129). The vitamin solution however consists of a number of expensive ingredients and will thus increase the large scale production cost of these enzymes, and hence the cost of the enantiopure chemicals. It is thus ideal to grow these microorganisms in the cheapest possible medium that still deliver biocatalysts in bulk, with the highest possible activity in the shortest possible time-frame.

Malt extract is an effective slow releasing source of carbon (Vahidi *et al.*, 2004:608) and nitrogen (Jackson & Bothast 1990:3436). In a previous study conducted by Milanova *et al.*, (1996:91), it was demonstrated that a very high malt extract powder concentration (4,0 % w/v) in combination with 1,0 % (w/v) glucose and nutrient broth (0,8 % w/v) could be used as an effective growth medium for the fungus *Cunninghamella elegans*, utilised the biocatalytic hydroxylation of triptophenolide to triptoquinone. No real difference was observed between the hydroxylation activity of the biocatalyst in this medium in comparison to the more commonly used YM-type media. Therefore, it was decided to investigate the effect of a single malt medium on both the enzyme activity and the biomass production.

It has previously been demonstrated that EH activity fluctuates substantially through out the growth curve of some microorganisms (Grogan *et al.*, 1997:255; Grogan *et al.*, 1996:242), therefore it was considered important to not only determine the EH activity at a single point during the growth period, but to investigate the enzyme activity throughout the complete growth time, even a number of hours into the stationary phase, not only to determine the optimum point of enzyme activity and thus harvesting, but also to compare the fluctuation in enzyme activity between the two biocatalysts.

3.1 Effect of growth media

In the first series of experiments the effect of three different growth media on the amount of produced biomass ($mg.mL^{-1}$ wet cell mass) as well as the EH activity for the *basidiomycetous* red yeasts *R. glutinis* and *R. toruloides*, was determined. As described in the experimental procedure, samples were taken from the culture flasks at specific time intervals during the growth period, to determine the biomass and EH activity of the cells.

It was previously reported that *R. glutinis* exhibited high activity towards SO (Yeates *et al.*, 2003:678) and *R. toruloides* towards EO (Botes *et al.*, 1998:421). These two different substrates were thus implemented to monitor the effect of different growth media and growth times on the EH mediated kinetic resolution (activity) of these two yeasts, and not to compare the specific activity of the yeasts towards a specific substrate.

The three growth media tested included YM media (YM), YM media with 2 % (v/v) added vitamin solution (YMvit) and a third single carbon source medium consisting of only 4 % (w/v) malt extract (malt).

3.1.1 Rhodotorula glutinis

Batch growth profiles for *R. glutinis* are shown in **Figure 3-1**. The data presented are the means of triplicates with average deviations of less than 5 %. Only every second measurement are presented on the graphs to eliminate any possible confusion due to the large number of data points, but the 3rd order polynomial trendlines are from all the collected data. From non-linear regression it was observed that the exponential growth phase only lasted the initial 4 hour growth time after inoculation for the YM culture and up to 8 hours for both the YMvit and malt grown cultures, after which the linear growth phase was entered. Although the exponential phase for the YM culture was shorter, the initial growth rate can clearly be seen to be substantially higher. For the YM culture linear growth was approximately 40 hours, 36 hours for the YMvit culture, and 24 hours for the malt medium culture, with respective linear growth rates ($mg_{wcw}.mL^{-1}.h^{-1}$) of 0,93, 0,95, and 0,62 after which stationary phase was reached at 84, 64, and 48 hours respectively.

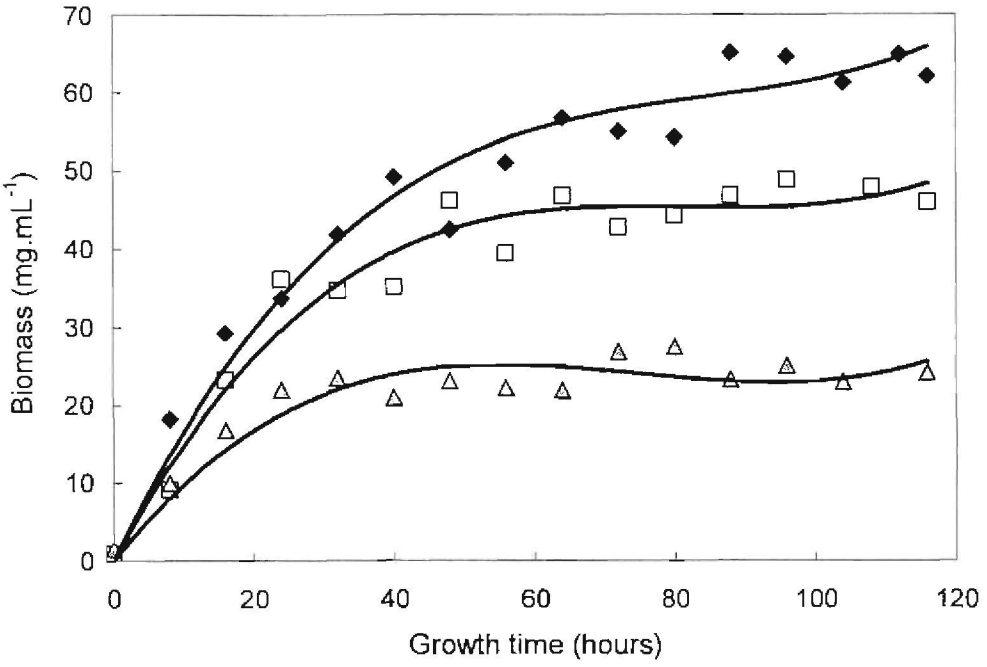

Figure 3-1 Effect of different growth media (YM (◆), YMvit (□) and malt (△)) on *R. glutinis* biomass production.

The maximum produced biomass varied significantly with a decrease of biomass observed for the vitamin supplemented growth media, and the single malt media. Maximum wet biomass weights at onset of stationary growth was 64,9 ± 1,3 (YM), 46,8 ± 0,2 (YMvit) and 23,1 ± 0,9 (malt) $mg.mL^{-1}$. A decrease in biomass production with the addition of vitamin solution has also been reported previously by Ferreyra *et al.* (2002:499) for *Geotrichum klebahnii.*

In order to compare the performance of *R. glutinis* EH during the growth time for the different growth media identical amounts of resting cells of *R. glutinis* was utilised for the kinetic resolution of SO. The change in the concentration and % *e.e.* of SO was followed over time as is shown in **Figure 3-2**. In all cases a linear decrease in both the concentrations of (R)-SO and (S)-SO were observed, but with a substantially higher decrease rate for (R)-SO; thus the (R)-enantiomer is preferentially hydrolysed by *R. glutinis* EH. This preferential hydrolysis leads to an increase in the % *e.e.* over time. These results are on accordance with previous work conducted by Yeates *et al.* (2003:678).

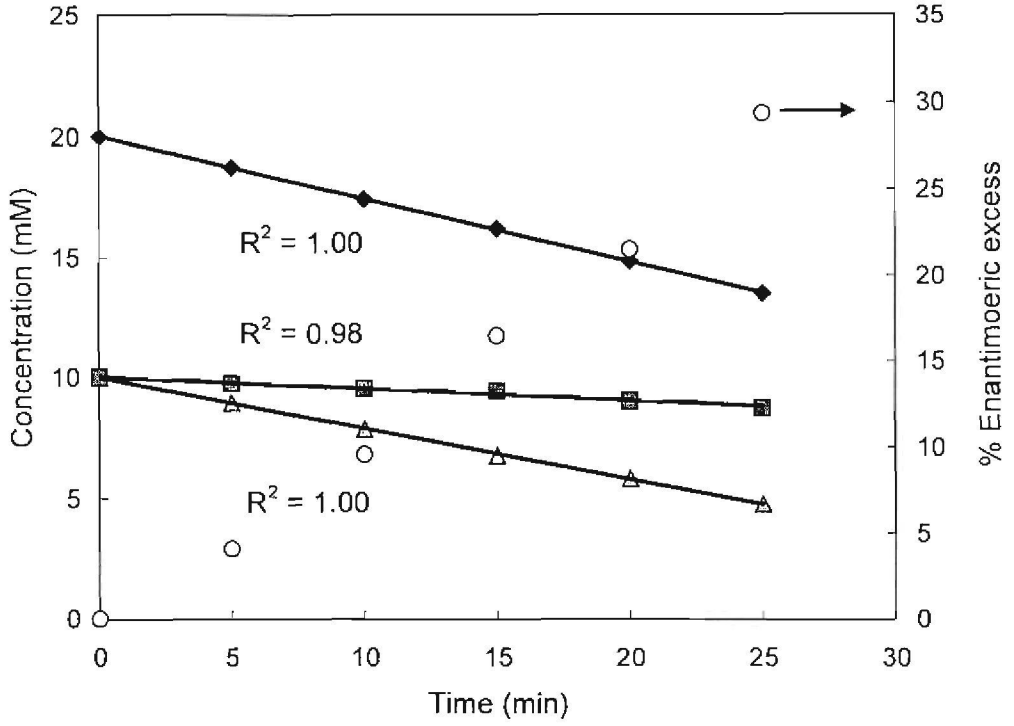

Figure 3-2 Time course reaction of SO hydrolysis by *R. glutinis* EH at 96 hours of growth time in malt medium (40 $g.L^{-1}$) indicating the decrease in total epoxide (◆), R- (△) and S-epoxide (▩) concentrations, represented on the left-hand axis, and the resultant % enantiomeric excess (·) on the right-hand axis.

The reaction rates (decrease in epoxide concentration (mM) over time (minutes) for the kinetic resolution of SO by *R. glutinis* EH were determined, over 25 minutes, from the total epoxide concentration, while the % *e.e.* was calculated from the concentrations (mM) of the SO stereoisomers at 25 minutes according to the following equation: % *e.e.* = ([S] − [R])|/([S] + [R])] × 100, where [S] and [R] represent the concentrations of the two stereoisomers. These reactions were conducted with the biomass samples taken at 12 hourly intervals throughout the growth time.

A significant increase in EH activity was observed in both YM and YMvit cultures between 12 and 24 hours growth time (**Figure 3-3**). This increase in EH activity has also been observed for the fungus *Aspergillus niger* (Morisseau *et al.*, 1998:806). For both YM and YMvit media the optimum activity and selectivity is reached at early stationary phase (approximately 84 and 60 hours respectively), with the malt media exhibiting an increase in EH activity even after the reach of the stationary phase. No real decrease in enzyme activity is observed over the monitored time during the stationary phase. Even 60 hours into stationary phase optimum activity is still observed for both the YM and YMvit grown biocatalyst.

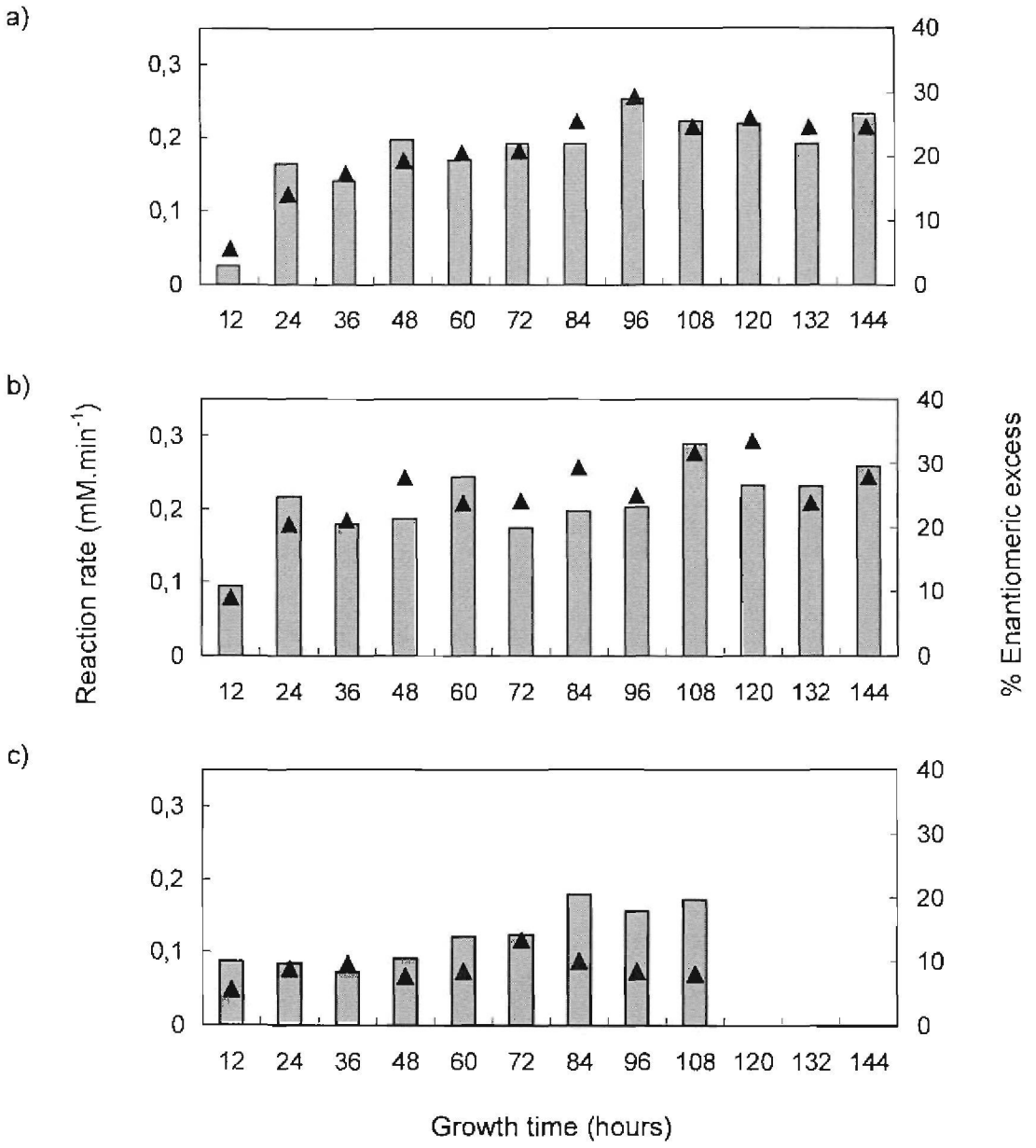

Figure 3-3 *R. glutinis* EH activity over the growth period under the influence of different growth media. Reaction rate (□) is plotted on left-hand axis, and % enantiomeric excess (▲) on the right-hand axis for the (a) YM, b) YMvit, and c) malt grown *biocatalyst.*

The malt medium rendered the lowest amount of biomass and an EH enzyme with substantially lower activity, while the YM and YMvit grown biocatalyst EHs displayed a smaller difference in EH activity and selectivity. The YM media produced almost 30 % more biomass than the YMvit medium. This implies that the amount of biocatalyst produced in a specific medium is not necessarily related to the quality of the biocatalyst.

These results also indicate that the vitamin solution has a negative effect on the total biomass production and only a small effect on EH activity for *R. glutinis*.

3.1.2 *Rhodosporidium toruloides*

Batch growth profiles for *R. toruloides* are shown in **Figure 3-4**. The data points shown are means of triplicates with average deviations of less than 5 %. As was previously explained for the growth curves of *R. glutinis*, the data points shown are again only every second measurement, but the trendlines (polynomial 4th order) are representative of all the collected data points. No explicit initial exponential phases could be detected in any of the growth media, but an initial lag phase with a limited increase in biomass was observed for the malt grown cells which were followed by a linear growth phase. Contrary to the growth profiles of *R. glutinis*, the growth of *R. toruloides* in the YM and YMvit media were characterized by not only one linear growth phase, but by two linear growth phases separated by a short lag phase (between 16 and 24 hours) where the biomass production slowed down almost completely. This has also been observed during the growth of the bacteria *Rhodococcus ruber* DSM 44540 and was said to be due to the depletion of the primary carbon source (glucose) and subsequent switch to the utilization of the secondary carbon sources (yeast extract and peptone) (Mayer *et al.*, 2002:166).

Although the difference in *R. toruloides* biomass production between the three growth media is characterised by a slight decrease in biomass production with the addition of the vitamin solution and a further decrease when grown in a single malt media, the difference is not as pronounced as was seen for *R. glutinis*. The stationary phase is respectively reached after 88, 108 and 64 hours with average wet biomass weights of 130,1 ± 4,8 (YM), 120,5 ± 1,1 (YMvit) and 75,7 ± 1,6 (malt) mg.mL^{-1} in the stationary phase.

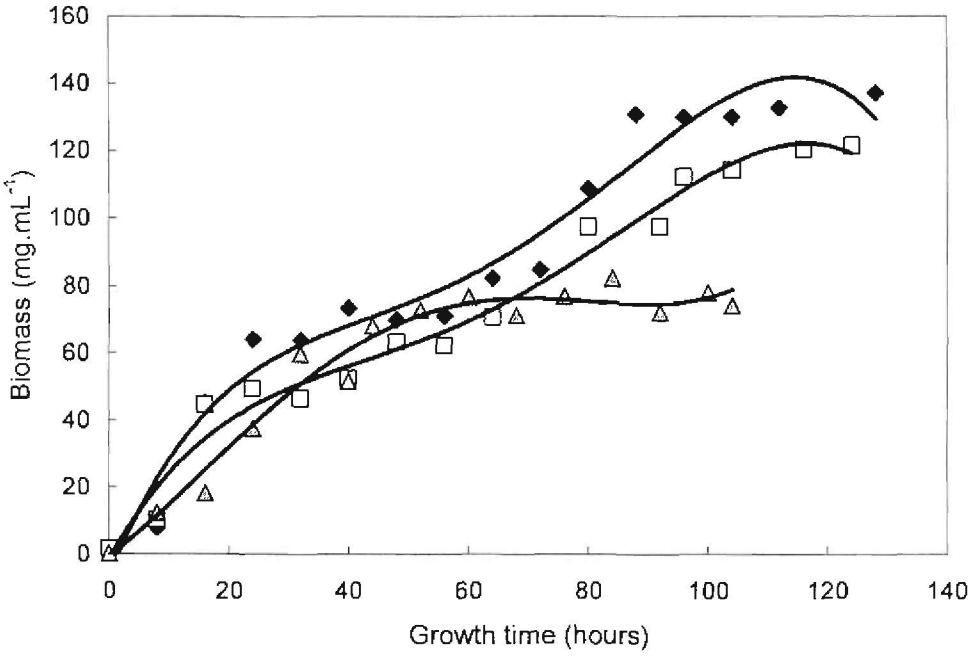

Figure 3-4 Effect of different growth media (YM (◆), YMvit (□), malt (△)) on the biomass production of *R. toruloides*.

In order to compare the performance of *R. toruloides* EH at different intervals during the growth time for the different growth media, identical amounts of resting cells were utilised for the kinetic resolution of the substrate EO. The change in the EO concentration and % *e.e.* over time was followed as is shown in **Figure 3-5**. In all cases a decrease in the concentration of (R)-EO and (S)-EO was observed, but with a higher rate of decrease for (R)-EO; thus, (R)-EO is preferentially hydrolysed. This preferential hydrolysis leads to an increase in the % *e.e.* over time. These results are in accordance with previous work conducted by Botes *et al.* (1999:3329).

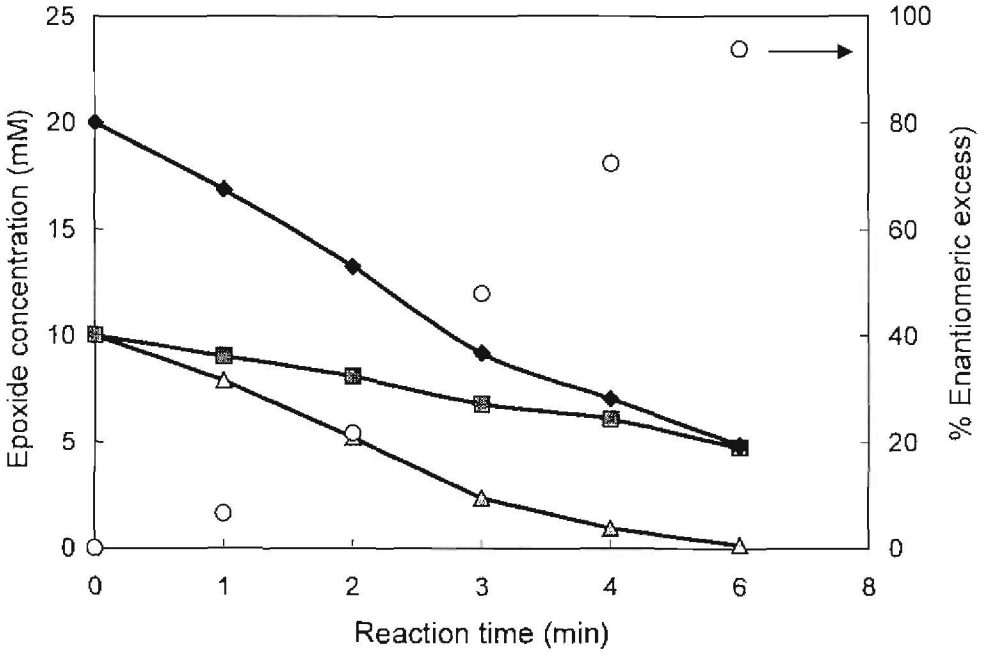

Figure 3-5 Time course reaction of 1,2-epoxyoctane hydrolysis by *R. toruloides* EH at 144 hours growth time in YMvit medium, indicating the decrease in total epoxide (◆), R- (△) and S-epoxide (■) concentrations, represented on the left-hand axis, and the resultant % enantiomeric excess (•) on the right-hand axis.

Although time-course reactions were conducted for each growth point in the different media, the conversion after 6 minutes was used for comparative purposes instead of the reaction rate due to the extremely fast reaction, and subsequent short linear reaction times resulting in an increased error. The conversion was calculated according to the equation: $c = 1 - [([S] + [R])/([S_0] + [R_0])]$ reported by Chen *et al.* (1982:7294), where [S] and [R] are the stereoisomer concentrations (mM) after a 6 min hydrolysis reaction and $[S_0]$ and $[R_0]$ represent the initial enantiomer concentrations (10 mM respectively for both (R) and (S)-EO). The % enantiomeric excess was calculated as previously described, but at a reaction time of 6 minutes.

A slight initial increase in enzyme activity as well as selectivity was observed between 12 and 36 hours of growth time (**Figure 3-6**) for *R. toruloides*, in all three media. The enzyme activity (conversion) from the YM and YMvit grown biocatalysts stabilised and reached a maximum between 36 and 48 hours of growth time.

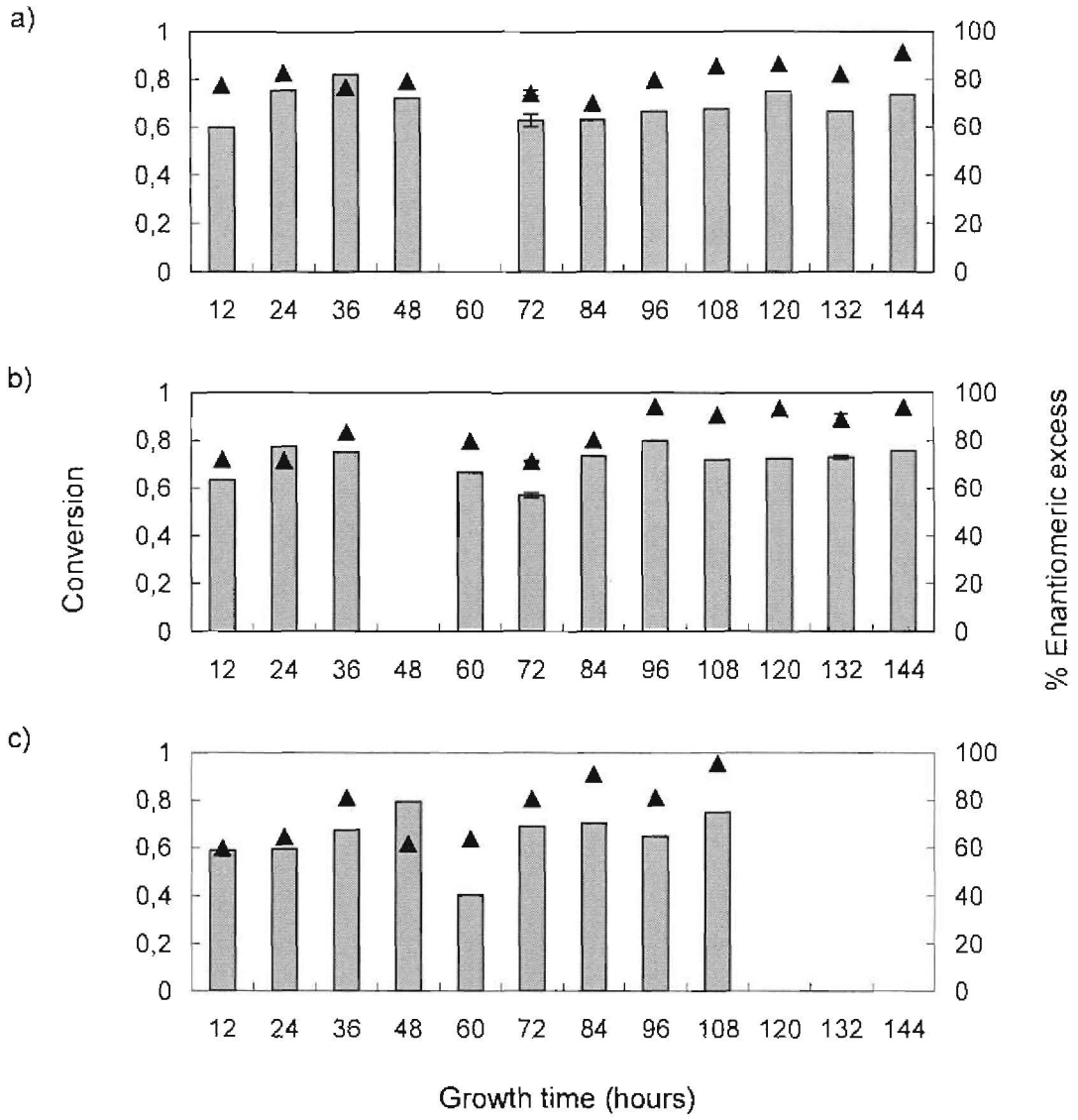

Figure 3-6 *R. toruloides* EH activity over the growth period under the influence of different growth media. Reaction rate (▭) is plotted on left-hand axis, and % enantiomeric excess (▲) on the right-hand axis for (a) YM, b) YMvit, and c) malt grown biocatalyst.

It seems that for both the YM and YMvit media the optimum activity is reached immediately after the depletion of the glucose supplement which is thought to be during the lag phase EH activity in malt medium reached an optimum at around 48 hours, thus in the early stationary phase.

EH activity and selectivity of *R. toruloides* stays relatively constant over the whole growth period (from 36 to 144 hours) for both the YMvit and malt cultures, with a slight decrease in activity, but increase in selectivity exhibited by the YM grown biocatalyst. Overall, it seems as if there is no statistically significant difference in the enzyme activity and selectivity expressed in all three

growth media, even though the malt medium again delivered a substantially lower amount of biomass in comparison to the other two media.

3.1.3 Summary

When plotting the produced biomass, activity and selectivity as values relative to the maximum (**Figure 3-7**), for both *R. glutinis* and *R. toruloides*, the relative effect of the different growth media can clearly be seen. All values are presented as a percentage of the maximum value attained in all three growth media for each biocatalyst which was at 144 hours for both YM and YMvit (*R. glutinis* and *R. toruloides*) and 108 hours for the malt grown biocatalysts. These specific times were chosen because there was no significant decrease in activity and selectivity in comparison to other growth times. For *R. glutinis*; the wet biomass reached a maximum concentration of 64,9 $mg.mL^{-1}$ with a maximum reaction rate of 0,26 $mMmin^{-1}$ (activity), and % *e.e* of 28 (selectivity). *R. toruloides* reached a maximum wet biomass concentration of 130,1 $mg.mL^{-1}$, and a conversion of 0,75 (activity), and 95 % *e.e*.

A growth media that produce enzymes with high activity and selectivity does not necessarily render the most biomass, thus, all these factors, including the cost of production, has to be weighed against each other in order to choose the best growth medium. For both *R. glutinis* as well as *R. toruloides* the highest biocatalyst production was delivered with the plain YM media, and the lowest with the single malt media.

The activity of the EH enzymes of the two biocatalysts were not equally affected by the available energy sources. While the activity and selectivity for the *R. toruloides* EH was similar in all three growth media, a pronounced drop could be observed for the malt grown *R. glutinis* EH.

The YMvit medium delivered an enzyme with almost 10 % higher activity (reaction rate and % enantiomeric excess) than the YM medium, and a substantially less active and selective enzyme was observed in the malt medium. For both *R. toruloides* and *R. glutinis*, the highest biomass concentration was achieved with the YM medium, and the lowest for the malt medium. As was previously reported (Morisseau *et al.*, 1998:808) these results clearly demonstrate that the growth medium does not only affect the biomass production, but also the expressed EH activity (reaction rate or conversion) and selectivity (% enantiomeric excess).

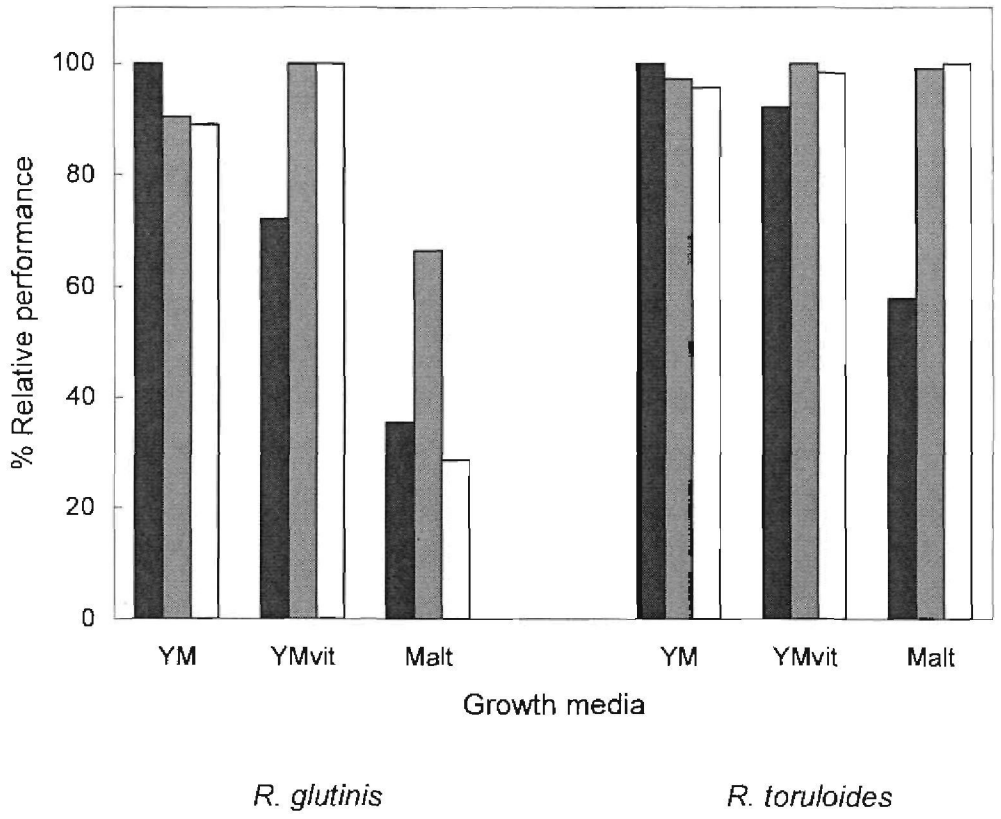

Figure 3-7 Relative effect of growth media on biomass production (■), EH activity (▨), and enantiomeric purity of the residual epoxide (□) for *R. glutinis* and *R. toruloides*.

In order to get a more clear representation of the effect of the growth media, the results presented in **Figure 3-7** are plotted in **Figure 3-8** in terms of an effectivity factor, thus, the maximum amount of substrate (mg) that can be hydrolysed from the amount of biocatalyst attained from one 200 mL growth medium in the given reaction time (25 min for *R. glutinis* (SO) and 6 minutes for *R. toruloides* (EO)) in a 20 mM reaction at the specific growth times mentioned for the data in **Figure 3-7**. For *R. glutinis* the maximum amount of substrate that could be hydrolysed under the specified reaction conditions with the attained cells was 36,48 mg, and 197,47 mg. for *R. toruloides.*

For both *R. glutinis* and *R. toruloides* a higher amount of substrate can be hydrolysed from the biomass attained from the YM medium (almost 20 % more in comparison to the YMvit medium for *R. glutinis*), with the effectivity of the malt media significantly lower for both biocatalysts. A slight increase in enantioselectivity (enantiomeric purity of the residual epoxide) is observed for the YMvit medium compared to the YM medium for both *R. glutinis* and *R. toruloides* EH, but in the case of *R. toruloides* this increase is not statistically significant.

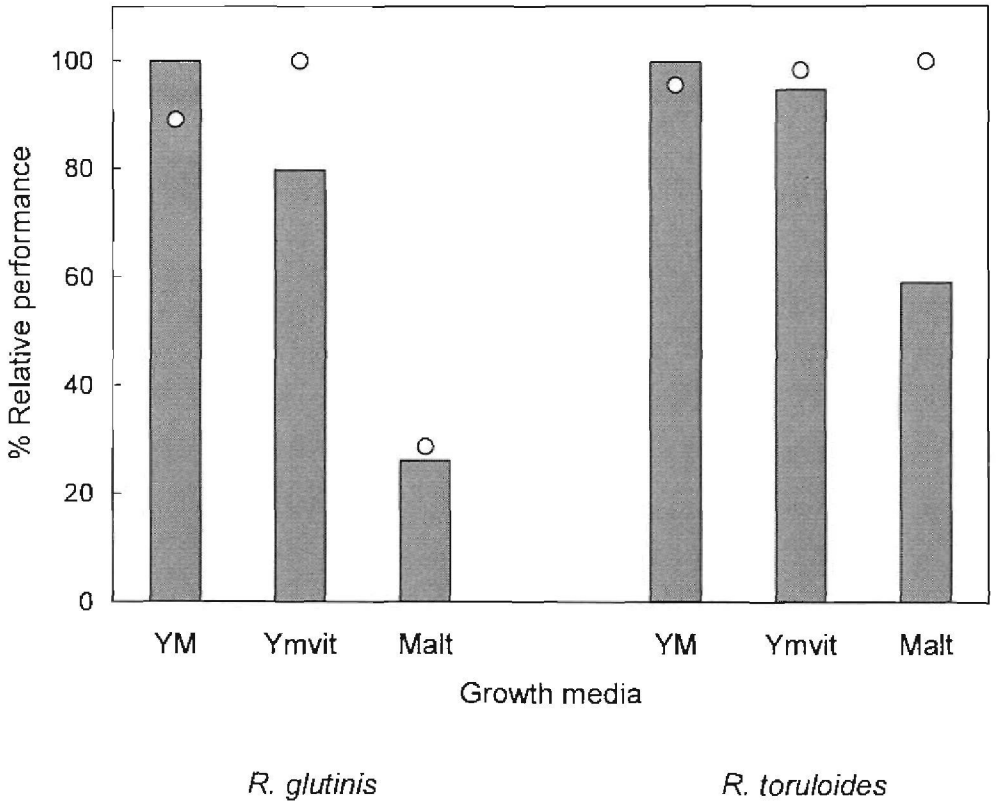

Figure 3-8 Relative effect of growth media on the amount of substrate hydrolysed (▩), and enantiomeric purity (•) of the residual epoxide for the biomass obtained from 200 mL growth medium.

According to these results, the YM medium was the medium of choice for both yeast strains, even though slightly lower enzyme selectivity was observed for *R. glutinis* in this medium. The YM medium also has the added advantage of being cheaper than the YMvit media due to the exclusion of the vitamin solution.

3.2 Effect of initial glucose concentration

The phenomenon of catabolite regression, i.e. the inhibiting effect of glucose on enzyme synthesis, has been widely reported on unrelated enzymes (Mayer *et al.*, 2002:167) such as nitrile hydratase (Cantarella *et al.*, 2002:408), α-amylase (Haseltine *et al.*, 1996:947) as well as EHs (Pan & Xu, 2003:530).

In order to quantify this effect, as well as compare the effect of initial glucose concentration on both *R. glutinis* and *R. toruloides*, flask cultures containing YM medium supplemented with different initial glucose concentrations were inoculated with both yeasts, and the biomass and

enzyme activity and selectivity measured at different stages during the growth time. The same substrates and experimental methods previously described were used.

3.2.1 *Rhodotorula glutinis*

Batch growth profiles for *R. glutinis* under the influence of different initial glucose concentrations are shown in **Figure 3-9**. No definite overall progressive increase was observed in the time needed to reach the stationary phase for the different glucose concentrations. Stationary phase was reached within 72 to 76 hours for all growth media.

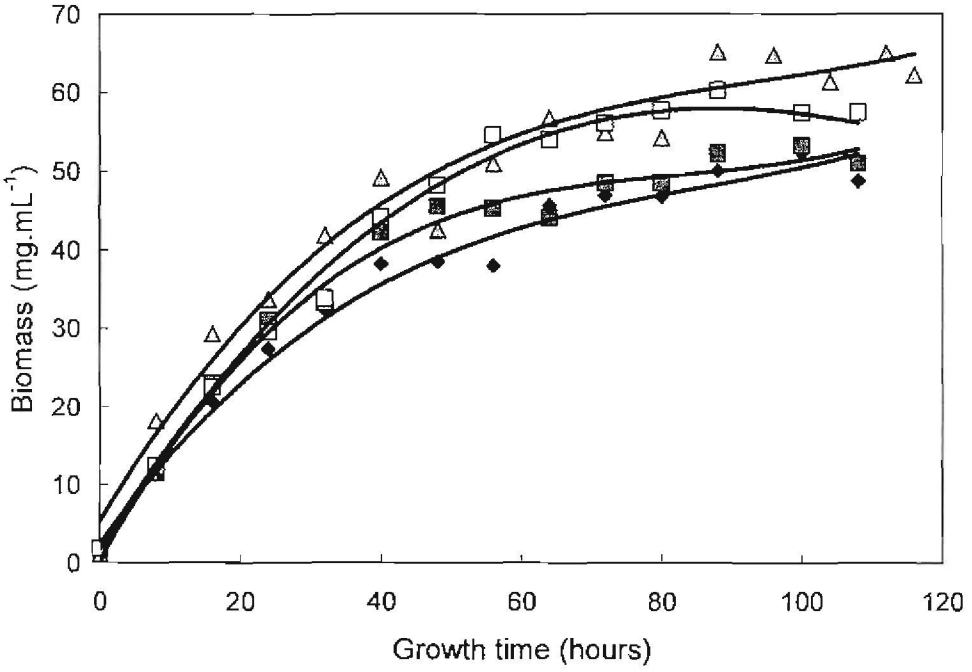

Figure 3-9 Effect of varying glucose concentrations (0,5 (◆); 0,1 (■); 1,5 (△) and 2,0 (□) % (w/v)) on the biomass production of *R. glutinis*.

No significant difference in the maximum wet biomass weight between 0,5 and 1,0 % (w/v) glucose (48,6 ± 1,1 and 48,4 ± 0,3 mg.mL^{-1} respectively) could be observed. It was expected that an increase in the glucose concentration would progressively deliver in increase in the produced biomass. From the data it can be seen that the 1,5 % (w/v) glucose YM medium did produce a higher biomass concentration (60,0 ± 0,9 mg.mL^{-1}) in comparison to the lower concentrations (5 and 1,0 % (w/v)), but that the 2,0 % (w/v) supplemented media however yielded a slightly lower (56,0 ± 1,7 mg.mL^{-1}) biomass. This drop in biomass production with the highest glucose concentration has been observed by Cantarella *et al.* (2002:409) for the growth of *Brevibacterium imperialis* where there was a diminished biomass production after a critical point of glucose present on the growth medium.

The same EH activity trends were observed for *R. glutinis* with varying glucose concentrations as was previously observed with the different growth media. EH activity (**Figure 3-10**) initially increases between 12 and 24 hours, after which it stabilises until early stationary phase (72 and 80 hours) followed by a further increase in activity. No significant decrease in enzyme activity was observed from early stationary phase up to 144 hours.

Similar to the biomass results, no meaningful trend was found for the effect of the different glucose concentrations on EH activity during the first 72 growth hours. During the stationary phase the activity stabilised with no significant difference between EH activity for the lower glucose concentrations, while the highest concentration (2,0 % (w/v)) rendered a biocatalyst with an almost 20 % higher activity.

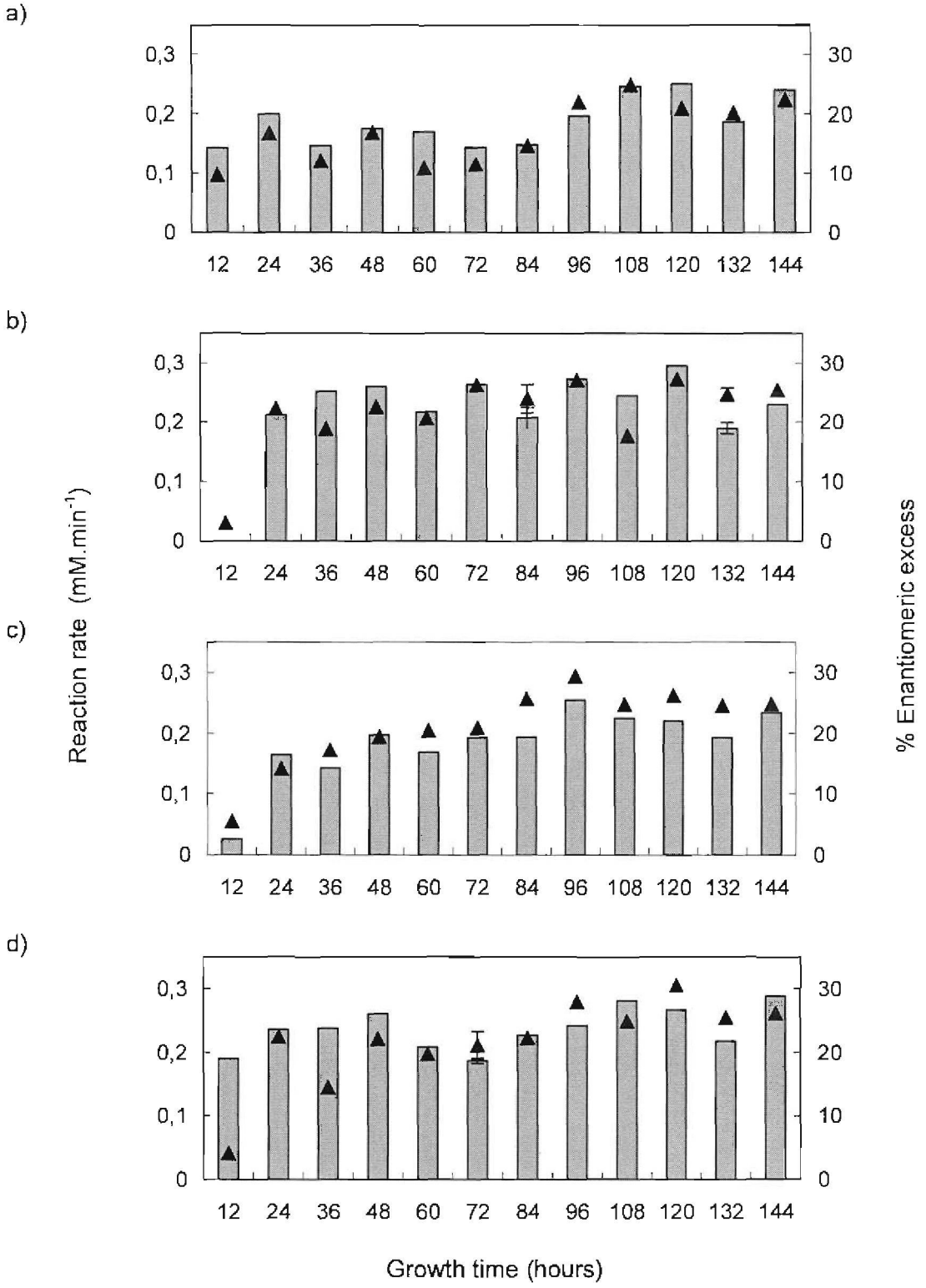

Figure 3-10 *R. glutinis* EH activity over the growth period as a function of the initial glucose concentration. Reaction rate (□) is plotted on left-hand axis, and % enantiomeric excess (▲) on the right-hand axis for (a) 0,5 b) 1,0 c) 1,5 and d) 2,0 % (w/v) glucose.

3.2.2 *Rhodosporidium toruloides*

Both the growth and EH activity of *R. toruloides* are more explicitly influenced by the increased initial glucose concentration. As was previously observed with the different growth media, an intermediate lag phase occurred at all glucose concentrations except for the lowest (0,5 % (w/v)) which is associated with depletion in the glucose concentration for *R. toruloides* (**Figure 3-11**). As was seen in **Figure 3-4** the single energy source malt medium also did not exhibit a lag phase as is observed for the 0,5 % (w/v) glucose additive concentration. Thus it is thought, that the initial glucose concentration is too low to be used as a single energy source, which leads to the simultaneous utilisation of both the glucose and the other components present in the YM media which explains why no intermediate lag phase is observed.

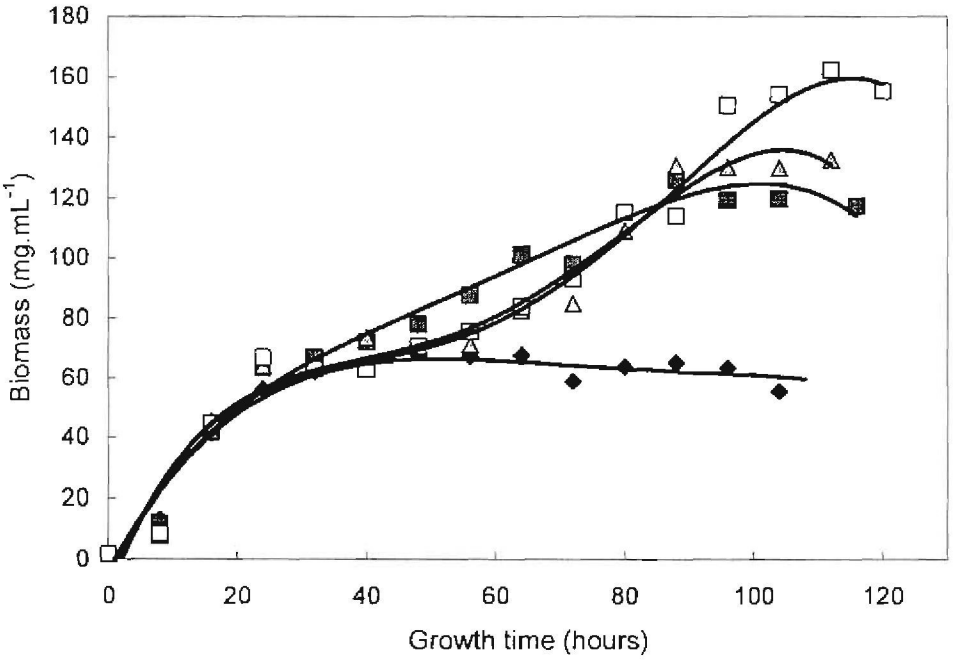

Figure 3-11 Effect of varying glucose concentrations on the biomass production of *R. toruloides* (0,5 (◆); 1,0 (▩); 1,5 (△) and 2,0 (□) % (w/v)).

The time needed to attain the stationary phase increased progressively with an increase in glucose concentration from 40 to 96 hours with maximum average produced wet biomass weights of 64,3 ± 2,9, 127,2 ± 0,4, 130,8 ± 4,8 and 150,5 ± 3,5 $mg.mL^{-1}$ wet biomass respectively for 0,5, 1,0, 1,5 and 2,0 % (w/v) initial glucose concentrations at the stationary phase.

An increase in EH activity and selectivity was observed for all initial glucose concentrations (**Figure 3-12**) between 12 and 36 hours of growth time for *R. toruloides*. Optimum EH activity

was reached between 24 and 36 hours in all cases, which coincides with the lag phase observed for the higher glucose concentrations. Contrary to what was found with *R. glutinis*, a decrease in enzyme activity was observed in all cases, between 36 and 144 hours. The highest activity and selectivity was observed for 1,0 % (w/v) initial glucose concentration between 24 and 72 hours, with an average enantiomeric excess of >98 %. The most pronounced decrease in EH activity was observed for the 2,0 % (w/v) glucose concentration which can be related to glucose mediated catabolic repression. Thus, it can be concluded that an increase in glucose concentration leads to the production of a higher concentration biomass, but the production of a less stable enzyme. The activity and selectivity was equally affected by the glucose concentration, and thus an enzyme with low activity also rendered low selectivity.

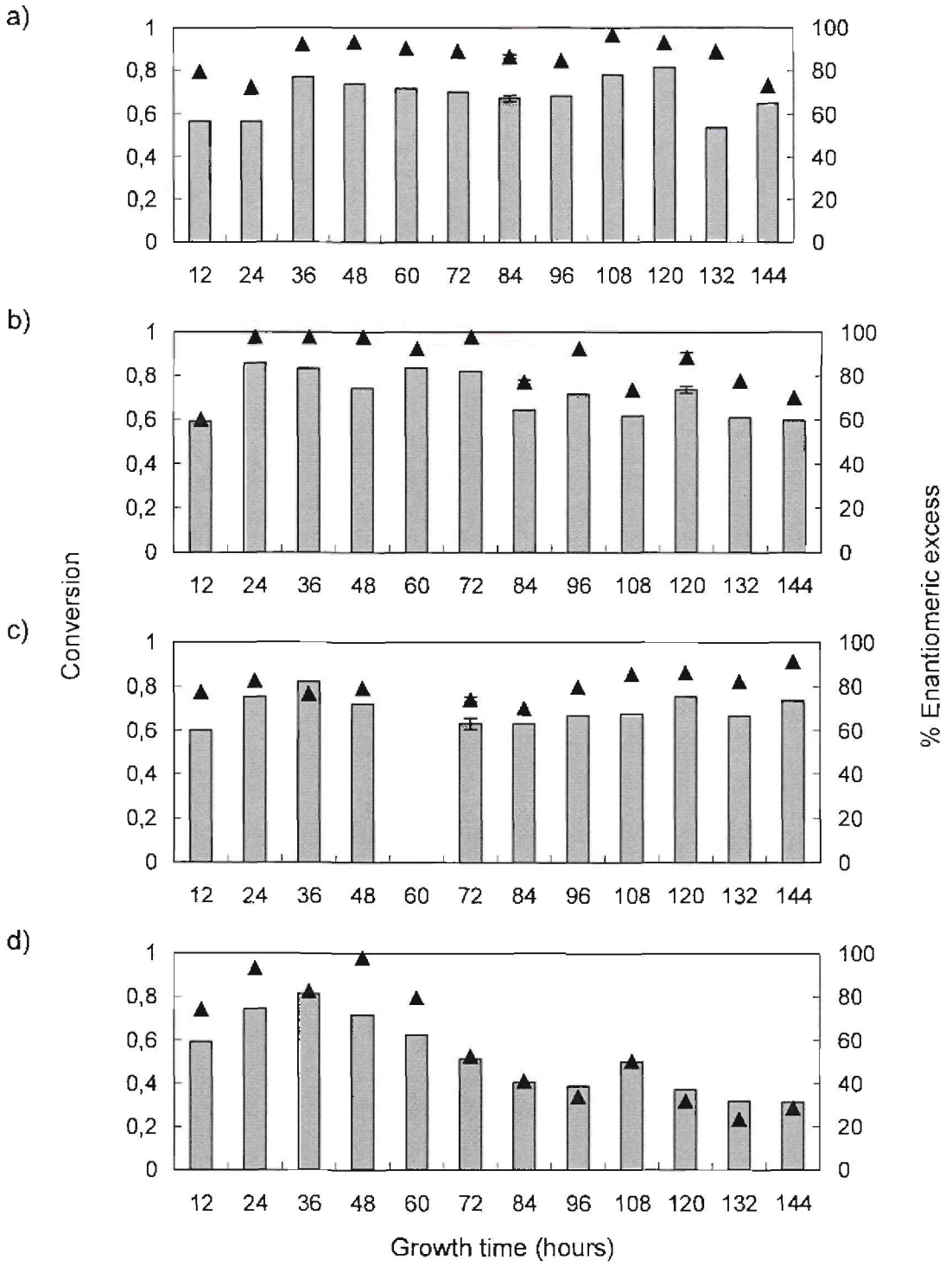

Figure 3-12 *R. toruloides* EH activity over the growth period as a function of the initial glucose concentration. Reaction rate (▨) is plotted on the left-hand axis, and % enantiomeric excess (▲) on the right-hand axis for (a) 0,5 b) 1,0 and c) 1,5 and d) 2,0 % (w/v) glucose.

3.2.3 Summary

Figure 3-13 illustrates the relative activity, selectivity (enantiopurity of the residual epoxide) as well as maximum average biomass for both *R.* and *R. toruloides* at 36 and 144 hours growth time. All values are presented as a percentage of the maximum value attained for each biocatalyst. For *R. glutinis* a maximum wet biomass concentration of 60 $mg.mL^{-1}$, reaction rate of 0,29 $mM.min^{-1}$ (activity), and % *e.e.* of 26 (selectivity) was achieved. *R. toruloides* reached a maximum biomass concentration of 150,5 $mg.mL^{-1}$, conversion of 0,83 (activity), and >98 % *e.e.* Activity as well as selectivity is still at a maximum at 144 hours for *R. glutinis*. *R. toruloides* exhibited maximum activity between 24 and 36 hours of growth, and has already decreased by 144 hours growth time, thus, plotting the 36 and 144 hour results, the resultant effect of the glucose concentrations can be clearly observed.

An increase in biomass, activity and selectivity was observed for *R. glutinis* from 36 to 144 hours (**Figure 3-13a**), with no statistically significant difference in activity between the lower three glucose concentrations, while a 2,0 % (w/v) glucose concentration did lead to an enzyme with almost 20 % higher activity and selectivity at 144 hour growth time. This increase in activity with an increase in the glucose concentration has also been demonstrated for the fungus *Alternaria alternate* (Morisseau *et al.*, 1999:2394). No catabolic repression could be observed for *R. glutinis* in the range of glucose concentrations tested. Thus, for a fast reaction with high selectivity, but compromising slightly on biomass yield, 2,0 % (w/v) glucose would be the additive concentration of choice, while 1,5 % (w/v) glucose additive would lead to a somewhat slower reaction, but a higher amount of biomass. Irrespective of the added glucose concentration, an increase in growth time had a positive effect on both enzyme activity as well as selectivity.

R. toruloides (**Figure 3-13b**) showed an almost equal biomass production as well as activity at 36 hours growth time, with lower selectivity for the top two glucose concentrations tested. Between 36 and 144 hours all glucose additive concentrations show a decrease in activity as well as selectivity, except for the 1,5 % additive concentration which only exhibited a slight decrease in activity. EH activity and selectivity was either progressively higher or equal for 0,5, 1,0 and 1,5 %, and drastically lower for the 2,0 % glucose additive concentrations (w/v) at 144 hours. This decrease in activity for the 2,0 % (w/v) glucose additive concentration would most likely be due to the effect of glucose mediated catabolic repression on *R. toruloides* EHs.

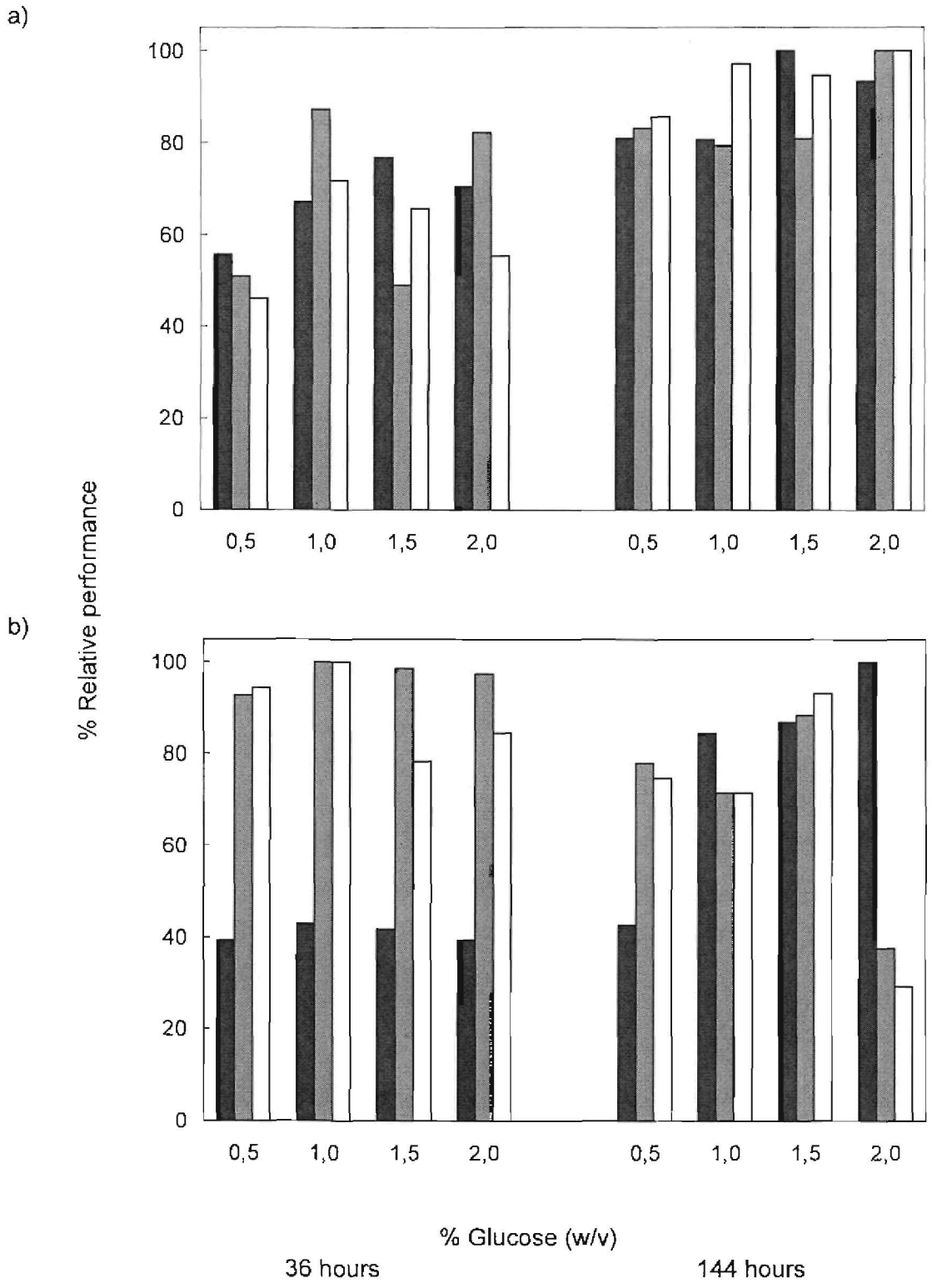

Figure 3-13 Relative effect of growth media on biomass production (■), EH activity (▨), and enantiomeric purity of the residual epoxide (□) for a) *R. glutinis* and b) *R. toruloides*.

For highest activity and selectivity, *R. toruloides* should be harvested around 36 hours growth time, but at a high loss of possible biomass, and in that case the lowest concentration glucose

can be added. If more biomass is required, 1,5 % (m/v) glucose would be the better choice for harvesting after stationary phase when the maximum biomass is produced.

4 Conclusion

In the course of this study the effect of growth media as well as additives (vitamin solution and glucose) on the growth and epoxide hydrolase characteristics of two related *basidiomycetous* red yeasts, *R. glutinis* and *R. toruloides*, was studied

The different growth media, YM, YMvit, and malt, had a definite effect on the amount of biomass produced as well as the activity and selectivity of the expressed EH for *R. glutinis*, with the highest activity and selectivity expressed for the YMvit grown EH and the highest biomass produced in the YM medium. *R. toruloides* also presented with the highest biomass produced in the YM medium, but with almost equal activity and selectivity for all three media tested.

An increase in glucose concentration lead to an increase in biomass, activity as well as selectivity for *R. glutinis*. *R. toruloides* presented with a decrease in activity as well as selectivity for the 2,0 % (w/v) glucose additive concentration which is attributed to glucose mediated catabolic repression of epoxide hydrolases.

It was clearly demonstrated that not only the growth, but also EH properties (activity and selectivity) of these two basidiomycetous red yeasts are not equally affected by the nutrient source and additives, demonstrating that all the growth parameters of related organisms (growth time, media and additives) have to be individually optimised, and that a general nutrient medium for all yeasts might not deliver the optimum growth and epoxide hydrolase activity for the individual organisms.

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Chapter 4

Synthesis and VCD analysis of enantiomerically pure styrene oxide derivatives

Abstract

The hydrolytic kinetic resolution of a range of synthesised styrene oxide derivatives through the utilisation of *R. glutinis* (UOFS Y-0563) epoxide hydrolase is presented. The residual *meta* substituted enantiopure derivatives were also successfully produced on a semi-preparative scale and the absolute configuration determined through FTIR vibrational circular dichroism. For all substrates, *R. glutinis* epoxide hydrolases preferentially hydrolysed the (R)-epoxide, thus rendering the unreacted enantiopure (S)-epoxides.

Keywords: epoxide hydrolases, styrene oxide, absolute configuration and VCD

Table of Contents

1 Introduction

The value of terminal epoxides as synthetic intermediates in the synthesis of optically active drugs emphasise the need to obtain these compounds with high enantiomeric purity (Moussou *et al.*, 1998a:1539). Various chemical and biological methods have been developed for the preparation of these derivatives (Archelas & Furstoss, 1999:159, Capriati *et al.*, 2002:2445). From stereochemical investigations it has been concluded that epoxide hydrolase (EH) enzymes are of great interest in the kinetic resolution of racemic epoxides (Weijers & De Bont, 1999:199, Yeates *et al.*, 2007:221) and probably one of the most promising methods for industrial environmentally friendly synthesis of these enantiopure synthons (Moussou *et al.*, 2000:414).

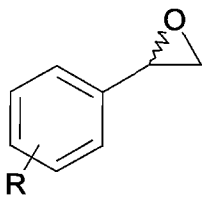
Chirality is a fundamental property of the "building blocks of life", such as amino acids and sugars, which are found in all living organisms. As a consequence, metabolic and regulatory processes mediated by biological systems are sensitive to stereochemistry and different physiological effects can often be observed when comparing the activities of a pair of enantiomers (Maier *et al.*, 2001:4). This difference in interaction of chiral compounds with biological systems (Kuppens, 2004:269) is the reason for the interest of enzymes in organic synthesis – the capability to differentiate between enantiomers, and thus preferentially react with one of them.

As was previously mentioned, enzymes do not only show selectivity for a specific substrate, but also to a single enantiomer (enantioselectivity) of that specific racemic pair (Wong, 1989:1146). Thus, enzymatic resolution of chiral compounds can be accomplished through either the selective addition of another functional group, or through a difference in the rate of metabolism (Besse & Veschambre, 1994:8917) between the individual enantiomers by the enzymes. In order to describe these interactions between chiral entities it is important to distinguish between the enantiomers. The absolute configuration is such a descriptive method that gives the specific enantiomers` arrangement in space. Determination of the absolute configuration is a long-standing problem to molecular stereochemistry (Freedman *et al.*, 2003:743).

The most common method for the determination of the absolute configuration of chiral compounds is the use of x-ray diffraction on enantiopure crystallised compounds. Although x-ray diffraction is a highly reliable method, there are quite a number of factors that have to be taken into consideration (Boyle, 2006). The requirement of a single crystal of sufficient size and quality slows down the analysis of some molecules, or in some cases even totally excludes it entirely (Freedman *et al.*, 2003:743).

Vibrational circular dichroism (VCD) offers a practical alternative to x-ray analysis. In fact, according to Barron (1994:402), VCD or Raman optical activity (ROA) analysis, in combination with *ab initio* computation is arguably the best method for determining the absolute configuration of molecules consisting of upto 50 atoms (excluding hydrogen). Given that VCD spectra (the difference in the IR absorbance, A, of a specific molecule for left versus right circulatory polarized radiation during a vibrational transition) are typically measured in the solution state (Nafie, 2004:4, Bose & Polavarapu, 1990:207), the tedious process of growing a crystal is avoidable. The absolute configuration is subsequently derived by comparing the solution-phase VCD spectrum to the results of an *ab initio* quantum chemistry calculation (Solladié-Cavallo *et al.*, 2001:2606).

Herein, the EH mediated kinetic resolution of different *meta* and *para* substituted styrene oxide derivatives (**Figure 4-1**), as well as the assignment of the absolute configuration of the *meta*-substituted derivatives, is reported.



1. R = H
2. R = *meta*-NO_2
3. R = *para*-NO_2
4. R = *meta*-CH_3
5. R = *para*-CH_3
6. R = *meta*-OCH_3
7. R = *para*-OCH_3

Figure 4-1 Substituted styrene oxides for kinetic resolution by *R. glutinis* EHs.

2 Experimental

2.1 General

Rhodotorula glutinis (UOFS Y-0563) was obtained form the yeast culture collection of the University of the Free State (South Africa), and the reagents were from Aldrich (3-chloroperbenzoic acid, 2-bromo-3'-nitroacetophenone, 2-bromo-4'-nitroacetopheno- ne, 2-bromo-4'-methylacetophenone, 2-bromo-3'-methoxyacetophenone), Fluka (3-methylstyrene, 2-bromo-4'-methoxy-acetophenone, styrene oxide), and Merck (sodium borohydride) South-Africa. All other reagents used were of standard analytical grade.

Reactions were monitored and enantiomeric purities analysed by chiral gas chromatography (ThermoFinnigan® Focus GC equipped with a FID and a Thermo Electron AS 3000 Series auto sampler) on a fused silica cyclodextrin Chirasil-DEX-CB Varian® capillary column, utilising H_2 as carrier gas.

2.2 Methods

2.2.1 Synthesis of styrene oxide derivatives

All reactions were performed under atmospheric pressure using clean air-dried glassware. The commercially available reagents were used as received without further purification. The structure of the styrene oxides were numbered as shown in **Appendix 2**. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini-300 spectrometer in $CDCl_3$ at 75,462 and 300,075 MHz respectively. EI (electron impact) mass spectra were recorded on a VGA70-70E mass spectrometer fitted with an Ion Tech B11N saddle field gun and a VG11-250J data system and Infrared spectra as a neat film on KBr plates with a Nicolet 470 FT-IR spectrophotometer.

2.2.1.1 meta-Nitrostyrene oxide (m-NO_2)

A mixture of 25 mL methanol and 35 mL THF was cooled to + 4 °C in an ice bath, after which 2,50 g 2-bromo-3'-nitroacetophenone (97 %) was added. After all solids were dissolved, 500 mg of sodium borohydride fine granules was gradually added. The mixture was kept at +4 °C and continuously stirred on a magnetic stirrer until completion. Hereafter 35 mL of 2 N NaOH was gradually added. After further stirring on the ice bath, and subsequent completion of the reaction, the mixture was quenched with acetic acid (pH ≈ 6), and extracted twice with 150 mL ethyl acetate. The organic extract was washed with saturated sodium bicarbonate and water, and dried over sodium sulphate (Wierenga *et al.*, 1984:441). The product obtained after evaporation of the solvent was purified by means of packed column chromatography using a 1:1 mixture of petroleum ether/diethyl ether.

The synthesis delivered 1,19 g (70 % yield) of a light straw-colored viscose liquid of which the structure was confirmed to be *m*-NO_2 by NMR, MS and IR; $C_8H_7NO_3$; m/z (%; EI) (**Spectrum 1**):165 (49,6), 135 (80,7), 105 (80,7), 89 (100), 63 (87,9), 39 (74,2); δ_H (**Spectrum 2**, 300,075 MHz, $CDCl_3$): 2,76 (dd; 1H; J = 5,4; 2,5 Hz; H-2), 3,20 (dd; 1H; J = 5,4; 4,0 Hz; H-1a/H-1b), 3,96 (dd; 1H; J = 4,0; 2,5 Hz; H-1a/H-1b), 7,48 (m; 1H; H-5), 7,60 (m; 1H; H-4), 8,14 (m; 2H; H-6/ H-8); δ_C (**Spectrum 3**, 75,462 MHz, $CDCl_3$): 51,31 (C-2), 51,34 (C-1), 120,52 (C-8), 123,03 (C-6), 129,52 (C-5), 131,42 (C-4), 140,10 (C-3), 148,52 (C-7); ν_{max} (**Spectrum 4**, KBr, cm^{-1}); 808,82; 857,35; 1338,23; 1529,41; 2947,18; 3000,00; 3073,25.

2.2.1.2 para-Nitrostyrene oxide (p-NO_2)

The racemic epoxide was prepared in the same way as the *meta* derivative, except that 2-bromo-4'-nitroacetophenone (97 %) was used, and the product was purified through recrystallisation form methanol.

The synthesis delivered 1,23 g (72 % yield) of light yellow crystals confirmed to be *p*-NO_2 by NMR, MS and IR; $C_8H_7NO_3$; m/z (%; EI) (**Spectrum 5**): 165 (59,7), 148 (87,1), 118 (90,3), 89 (100,0), 63 (87,1), 30 (87,9); δ_H (**Spectrum 6**, 300,075 MHz, $CDCl_3$): 2,74 (dd; 1H; J = 5,52; 2,48 Hz; H-2), 3,20 (dd; 1H; J = 5,54; 4,11 Hz;H-1a/H-1b), 3,94 (dd; 1H; J = 5,54; 4,11 Hz; H-1a/H-1b), 7,42 (d; 2H; J = 8,52 Hz; H-4/H-8), 8,16 (d;2H; J = 8,79 Hz; H-5/H-7); δ_C (**Spectrum 7**, 75,462 MHz, $CDCl_3$): 147,70 (C-6), 5,37 (C-2), 51,55 (C-1), 123,72 (C-5/C-7), 126,17 (C-4/C-8), 145,22 (C-3); ν_{max} (**Spectrum 8**, KBr, cm^{-1}); 850,00; 1340,90; 1463,63; 1518,00; 1600,00.

2.2.1.3 meta-Methylstyrene oxide (m-Me)

For the synthesis of *m*-Me 60 mL dichloromethane (DCM) was cooled down to ± -2 °C in an ice bath containing $CaCl_2$. 3-Methylstyrene (5,55 mL) was added to the cooled DCM while being continuously stirred. To this mixture 9,85 g *m*-chloroperbenzoic acid (≤ 77 % mCPBA) was gradually added. The reaction mixture was stirred at 4 °C for another 72 hours until the reaction was complete. After completion of the oxidation reaction the formed *m*-chlorobenzoic acid and excess unreacted mCPBA was removed by repeated extraction with a 10 % (w/v) NaOH solution, and the organic phase washed with water until free from alkali and then dried over anhydrous Na_2SO_4. The product obtained after evaporation of the solvent was purified by means of packed column chromatography using a 1:1 mixture of petroleum ether/diethyl ether.

The synthesis delivered 2,80 g (47 % yield) of a viscose light straw-colored liquid of which the structure was confirmed to be *m*-Me by NMR, MS and IR; $C_9H_{10}O$; m/z (%; EI) (**Spectrum 9**): 134 (48,4), 119 (100), 118 (99,2), 105 (91,1), 91 (92,7), 77 (80,0); δ_H (**Spectrum 10**, 300,075 MHz, $CDCl_3$): 2,86 (s; 3H; H-9), 2,80 (dd; 1H; J = 5,54; 2,56 Hz; H-2), 3,12 (dd; 1H; J = 5,54; 4,09 Hz; H-1a/H-1b), 3,84 (dd; 1H; J = 4,01; 2,56 Hz; H-1a/H-1b), 7,08 (m; 3H; H-4/H-6/H-8), 7,25 (m; 1H; H-5); δ_C (**Spectrum 11**, 75,462 MHz, $CDCl_3$): 21,29 (C-9), 51,01 (C-1), 52,32 (C-2), 122,64 (C-4), 125,99 (C-8), 128,36 (C-5), 128,90 (C-6), 137,509 (C-3), 138,17 (C-7); ν_{max} (**Spectrum 12**, KBr, cm^{-1}); 750,00; 792,00; 1280,00; 1727,50; 2865,00; 2925,00; 3030,00.

2.2.1.4 para-Methylstyrene oxide (p-Me)

The racemic epoxide was prepared in the same way as the *m*-NO_2 derivative, except that only 1 g 2-bromo-4'-methylacetophenone (97 %) was used as starting material. All other reactant amounts were adjusted according to the initial acetophenone weight added. No further method of purification (crystallization or chromatography) was needed. The synthesis was repeated to produce a higher quantity of the epoxide.

The synthesis rendered 0,52 g (81 % yield) of a dark bright yellow liquid of which the structure was confirmed to be *p*-Me by NMR, MS and IR. $C_9H_{10}O$; m/z (%; EI) (**Spectrum 13**): 134

(42,7), 119 (87,9), 105 (100,0), 103 (57,3), 78 (37,5), 28(75); δ_H (**Spectrum 14**, 300,075 MHz, $CDCl_3$): 2,34 (s; 3H; H-9), 2,77 (dd; 1H; J = 5,48; 2,61 Hz; H-2), 3,09 (dd, 1H; J = 4,08; 5,49 Hz; H-1a/H-1b), 3,82 (dd; 1H; J = 4,05; 2,57 Hz; H-1a/H-1b), 3,14 (m, 4H, H-4/H-5/H-7/H-8); δ_C (**Spectrum 15**, 75,462 MHz, $CDCl_3$): 21,42 (C-9), 51,02 (C-1), 52,32 (C-2), 125,47 (C-4/C-8), 129,17 (C-5/C-7), 134,53 (C-3), 137,96 (C-6) ; ν_{max} (**Spectrum 16**, KBr, cm^{-1}); 823,53; 969,12; 1463,24; 1617,65; 2867,65; 2926,47; 3014,71.

2.2.1.5 *meta-Methoxystyrene oxide (m-MO)*

The racemic epoxide was again prepared in the same way as the *m*-NO_2 derivative, except that only 1 g 2-bromo-3'-methoxyacetophenone (97 %) was used as starting materail. All other reagent amounts were adjusted according to the initial acetophenone weight added. Again, no further method of purification was needed. The synthesis was repeated to produce a higher quantity of the epoxide.

The synthesis rendered 0,58 g (89 % yield) of a bright amber liquid of which the structure was confirmed to be *m*-MO by NMR, MS and IR. $C_9H_{10}O_2$; m/z (%; EI) (**Spectrum 17**): 150 (74,2), 121 (62,1), 119 (40,3), 91 (65,3), 77 (34,7), 28 (100,0); δ_H (**Spectrum 18**, 300,075 MHz, $CDCl_3$): 2,76 (dd; 1H; J = 5,59; 2,57 Hz; H-2), 3,12 (dd; 1H; J = 5,54; 4,03 Hz; H-1a/H-1b), 3,76 (s; 3H; H-9), 3,85 (dd; 1H; J = 4,10; 2,60 Hz; H-1a/H-1b), 6,55 (m; 3H; H-4/H-6/H-8) 7,24 (m, 1H; H-5); δ_C (**Spectrum 19**, 75,462 MHz, $CDCl_3$): 51,03 (C-1), 52,24 (H-2), 55,18 (C-9), 110,53 (C-8), 113,89 (C-6), 117,92 (C-4), 129,51 (C-5), 139,31 (C-3), 159,92 (C-7); ν_{max} (**Spectrum 20**, KBr, cm^{-1}); 772,72; 840,90; 1045,45; 1242,42; 1500,00; 1606,06; 2962,12; 300,00; 3045,45.

2.2.1.6 *para-Methoxystyrene oxide (p-MO)*

The racemic epoxide was again prepared in the same way as the *m*-NO_2 derivative, except that only 2 g 2-bromo-4'-methoxyacetophenone (97 %) was used as starting material. All other reagent amounts were adjusted according to the initial acetophenone weight added. Due to the instability of the compound, breakdown occurred when attempting to further purify through column chromatography - both silica gel and active neutral aluminum oxide were tested as stationary phases. Thus, no further method of purification was used.

The synthesis rendered 1,28 g (97 % yield) of a bright straw coloured liquid confirmed by NMR, MS and IR to be *p*-MO. $C_9H_{10}O_2$; m/z (%; EI) (**Spectrum 21**):150 (85,9), 134 (86,7), 121(100,0), 91 (79,8), 71 (86,7), 77 (77,4); δ_H (**Spectrum 22**, 300,075 MHz, $CDCl_3$): 2,75 (dd; 1H; J = 5,39; 2,62 Hz; H-2); 3,07 (dd; 1H; J = 5,37; 4,04 Hz; H-1a/H-1b), 3,78 (s; 3H; H-9), 3,92 (dd; 1H; J = 7,51; 5,31 Hz; H-1a/H-1b), 6,82 (d; 2H; J = 8,79; H-5/H-7), 7,15 (d; 2H; J = 8,62 Hz; C-4/C-8); δ_C (**Spectrum 23**, 75,462 MHz, $CDCl_3$): 50,75 (C-1), 52,05 (C-2), 55,12 (C-9), 113,87

(C-5/C-7), 126,71 (C-4/C-8), 129,35 (C-3), 159,58 (C-6); ν_{max} (**Spectrum 24**, KBr, cm^{-1}); 835,91; 1028,57; 1250,00; 1514,28; 1621,42; 2914,28; 2964,28.

2.2.2 Cultivation and preparation of yeast cells

The yeast *Rhodotorula glutinis* was grown at 27 °C in a light protected Labcon® rotary platform shaking incubator (148 rpm). All media solutions were prepared using single distilled water and autoclaved for 20 minutes at 121 °C. The growth media were left at room temperature for 24 hours to cool down before inoculation. YM medium pre-inoculum cultures (0,5 % yeast extract, 2,0 % malt extract, 0,5 % peptone, w/v) supplemented with 1,5 % (w/v) glucose was grown for 24 hours in 250 mL shake flasks after which 5 mL of the 50 mL pre-inoculum was sterilely transferred to 1 L shake flasks containing 200 mL YM growth media (supplemented with 2,0 % (w/v) glucose).

The flask culture was left to grow to a maximum for 108 hours after which cells were harvested by centrifugation (3500×*g*, 5 minutes) and washed with phosphate buffer (KH_2PO_4/K_2HPO_4, 50 mM, pH 7,3). The yeast cells were resuspended (25 %, w/v) in phosphate buffer containing 10 % (v/v) glycerol (pH 7,3), frozen and stored below −18 °C in 50 mL centrifuge tubes until hydrolytic reactions were conducted within three weeks after harvest.

2.2.3 Kinetic resolution

The hydrolytic kinetic resolution of the synthesized styrene oxide derivatives were followed over time until >98 % *e.e.* was reached, or until a decrease in the % *e.e* was observed (*p*-NO_2) for the epoxide substrates. Stock solutions (1 M) in DMSO were prepared of all SO derivatives.

The frozen cell suspensions (of the previously grown and harvested *R. glutinis*) were thawed, centrifuged for 5 minutes at 3500 g and resuspended (25 % w/v) in a 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7,3) containing 5 % (w/v) hydroxypropyl-β-cyclodextrin (HPB). 500 µL of the cell suspension was aliquoted into 1,5 mL micro-centrifuge tubes and the epoxide (10 µL of the 1 M stock) added to a final concentration of 20 mM. The reaction mixtures were incubated at 15 °C with continuous shaking (190 rpm). Reactions were terminated at certain time intervals by submerging the centrifuge tubes in liquid nitrogen. The residual epoxide was extracted through the addition of 270 µL ethyl acetate to the frozen sample. The mixture was vortexed until completely defrosted, and subsequently vortexed for another 3 minutes to ensure complete extraction of the epoxides, after which it was centrifuged for 3 minutes at 10 000 rpm. The ethyl acetate was extracted and dried over anhydrous Na_2SO_4 before analysis and quantification of the residual epoxide on chiral GC.

2.2.4 Chemical hydrolysis of *p*-NO_2

For the chemical hydrolysis of *p*-NO_2, *p*-NO_2 stock solution (10 µL) was dissolved in 500 µL phosphate buffer solution (pH 7,3) containing 5 % (m/v) HPB without cells. The reaction mixtures were incubated at 15 °C with continuous shaking (190 rpm). Reactions were terminated at different time intervals as was described for the kinetic resolution. Extraction and preparation of the sample for analysis of the residual substrate was conducted as described for the kinetic resolution.

2.2.5 Semi-preparative chiral hydrolytic kinetic resolutions

Semi-preparative kinetic resolution was conducted on three of the synthesized substrates (*m*-NO_2, *m*-Me and *m*-MO). Previously harvested frozen cell suspensions were thawed and centrifuged for 5 minutes at 3500 g. Cell pellets were resuspended (25 % w/v) in 50mM KH_2PO_4/K_2HPO_4 buffer (pH 7,3) containing 5 % (w/v) HPB. A total of 90 mL cell suspension was aliquoted into 200 mL closed glass reaction vessels. The reaction vessels with cell suspension were incubated at 15 °C in a water bath for 5 minutes to ensure that the suspension was at the reaction temperature. The pure SO derivatives were added to the cell suspensions to a total of 20 mM. The reaction mixtures were incubated at 15 °C with continuous shaking, and monitored to ensure that the time to reach a % *e.e.* of >98 % was the same as was found for the previously conducted small volume kinetic resolutions. The reaction was terminated through the addition of 90 mL ethyl acetate when the 2nd enantiomer peak could not be observed through chiral GC analysis.

The styrene oxide derivatives were extracted from the reaction mixtures through washing the cell/reaction volume twice with ethyl acetate. The organic phase was washed numerous times with $H_2O_{(d)}$ to remove any cell debris. For final removal of cell debris and water from the organic phase, the organic phase was centrifuged at 3500 g for 1 minute and the top organic phase extracted. The organic phase was dried over anhydrous Na_2SO_4. The products obtained after evaporation of the solvent was purified using packed column chromatography. DCM was used as mobile phase and silica as stationary phase for all substrates.

2.2.6 Analysis

All chiral GC analysis, except for SO, were conducted isothermally. Chiral GC analysis of the isolated substrates (**Appendix 1**) were carried out as follows: *m*-NO_2, 105 °C, tR (1) 86,80 min and tR (2) 91,18 min, *p*-NO_2, 130 °C, tR (R) 27,24 min and tR (S) 29,43 min, *m*-Me, 90 °C, tR (1) 16,58 min and tR (2) 17,72 min, *p*-Me, 115 °C, tR (1) 5,06 min and tR (2) 5,26 min, *m*-MO, 82 °C, tR (1) 73,35 min and tR (2) 77,00 min, and p-MO at 120 °C; tR 10,06 min. For all these compounds the inlet and detector temperatures were respectively set at a 170 °C and 190 °C,

except for *p*-Me where the inlet temperature was set at 120 °C. For all these analysis the split flow was set at 50 mL.min^{-1}, with a split ratio of 33. The analysis of SO was done non-isothermally with a initial temperature of 130 °C which was kept for 0,50 min, after which the temperature decreased at a rate of 10 °C.min^{-1} up to 110 °C which was followed by further isothermal conditions at 110 °C with tR (R) 5,26 min and tR (S) 5,65 min. The inlet and detector temperatures were again 170 °C and 190 °C respectively, with a split flow of 50 mL.min^{-1} and a split ratio of 33. DMF and DMSO were used as internal standards.

2.2.7 VCD analysis

2.2.7.1 Experimental

IR and VCD spectra were measured on a modified Chiral*IR* VCD spectrometer (BioTools, Wacaunda, IL) with 4 cm^{-1} resolution. The instrument was optimised at 1400 cm^{-1} for a 3 to 9 mg sample in 100 µL $CDCl_3$ solvent. A 100 µm pathlength cell with BaF_2 windows was used, while the sample collection time varied between 6 and 9 hours.

2.2.7.2 Calculations

Optimised geometries, vibrational frequencies, and IR and VCD intensities were calculated for the (*S*)-enantiomer of each compound at the DFT level (B3LYP functional/6-31G(d) basis set) with Gaussian 03 (Gaussian, Inc., Wallingford, CT). Calculated frequencies were scaled by 0,97 and the calculated intensities converted to Lorentzian bands with 6 cm^{-1} half-width for comparison to the experimental spectra.

3 Results and Discussion

3.1 Hydrolytic kinetic resolution

The EH mediated biocatalytic resolution of a number of styrene oxide derivatives was examined using resting cells of the yeast *R. glutinis* (UOFS Y-0563) for possible preparative separation of these enantiomers in order to determine the absolute configuration of the residual enantiomers. With the GC columns to our disposal, the diol products of the SO derivatives could not be analysed, only the decrease in the epoxide enantiomer concentrations for SO and the SO derivatives were tracked and are presented in **Figure 4-2**. We have presented the data in such a way as to clearly show for which compounds the absolute configuration of the residual epoxide (thus the selectivity) has been determined and published to date for *R. glutinis*.

With the available GC columns, the chiral analysis of *p*-MO unfortunately not be conducted, and thus the decrease in total epoxide concentration is given (**Figure 4-2 *p*-MO**). Initially, it seemed as if the enzymatic hydrolyses of this substrate took place at an exceptionally fast rate. To confirm if the total decrease in the epoxide could be contributed to the enzymatic mediated

hydrolysis, the spontaneous chemical hydrolysis of this substrate was followed under the same conditions without any biocatalyst.

It is clear that more than half of the reduction of *p*-MO observed during enzymatic hydrolysis can be contributed to spontaneous chemical breakdown (hydrolysis) of this substrate. Due to the extensive contribution of the achiral chemical hydrolysis, the reaction would have to be conducted at a much lower temperature in order to reduce the chemical, non-selective breakdown (hydrolysis) of this substrate (*p*-MO).

These experiments were conducted to determine the optimum reaction time for the hydrolytic kinetic resolution of these substrates, under these conditions, thus to produce the pure residual enantiomer (unreacted epoxide) in the highest possible yield and with the highest possible enantioselectivity. Therefore, there were not corrected for chemical hydrolysis for the other substrates.

The enantioselectivity of *R. glutinis* EH towards both SO and *p*-NO_2, thus the absolute configuration of the unreacted SO and *p*-NO_2 enantiomers for this reaction has previously been assigned (Yeates *et al.*, 2003:679; Yeates, 2001:68). *R. glutinis* preferentially, but not selectively, hydrolyses (R)-SO leading to the formation of the (R)-1-phenyl-1,2-ethane diol in excess, thus rendering the unreacted (S)-(-)-epoxide. For *p*-NO_2 it was also found that the (R)-epoxide is preferentially hydrolysed at low temperatures. The same preferential selectivity for *p*-NO_2, thus also rendering the (S)-(+)-epoxide, was previously observed with the EH of various strains of *Aspergillus niger*, but reached a much higher % *e.e.* of between 97 and 100 (Jin *et al.*, 2004:410; Nellaiah *et al.*, 1996:76) with a maximum yield of 42 %.

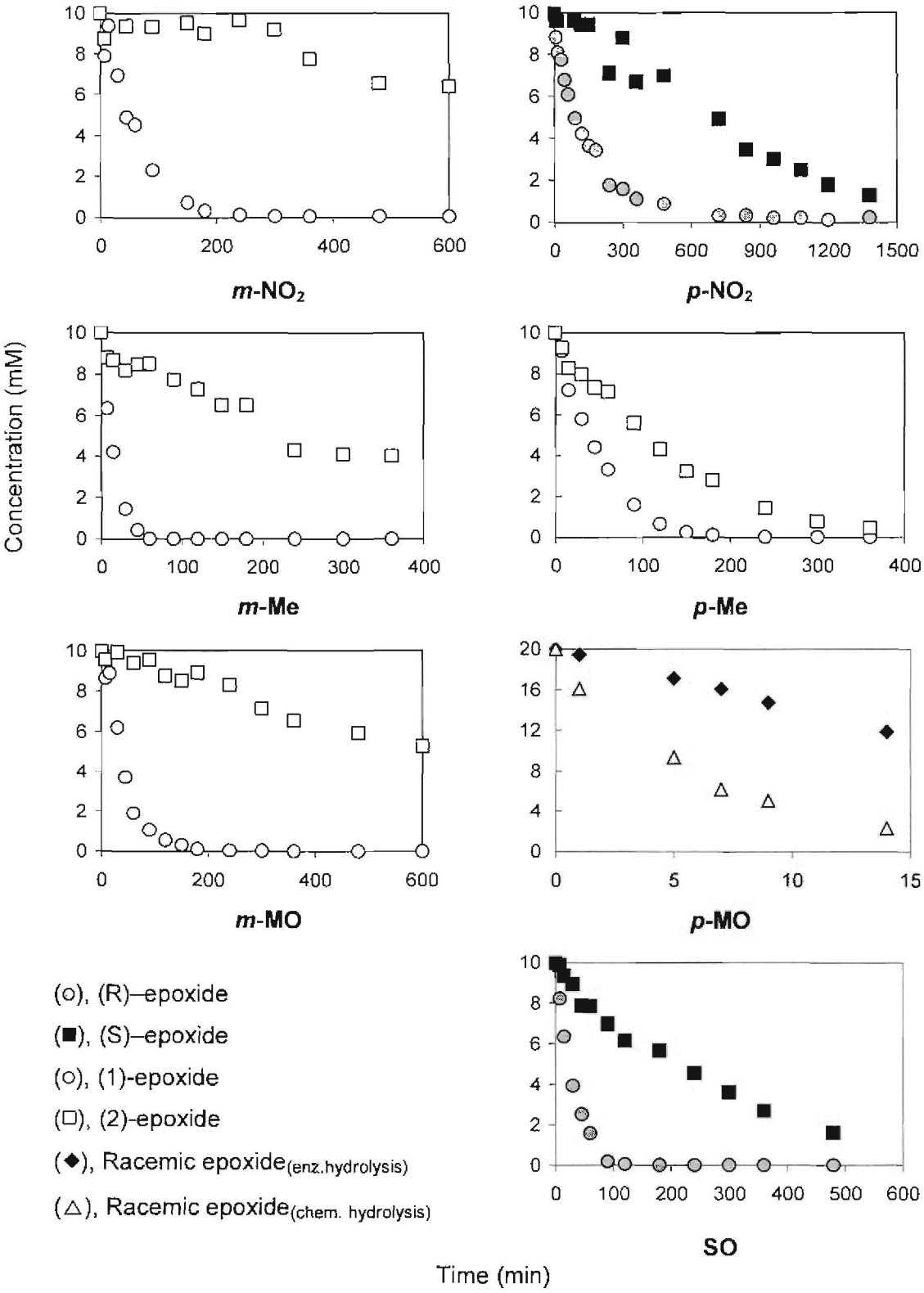


Figure 4-2 Enantioselective hydrolysis of SO and SO derivatives over time.

Yeates *et al.* (2003:679) also reported the kinetic resolution of *m*-NO_2 by *R. glutinis* EH, but the absolute configuration of the residual epoxide was not resolved. The only other example for the

kinetic resolution of *m*-NO_2, according to our knowledge, was again reported by Jin *et al.* (2004:410) with a % *e.e.* of 55 by *Aspergillus niger*. Although the absolute configuration of the residual epoxide was noted as (S)-(+)-*m*-NO_2, the method for the determination of the absolute configuration was not reported.

A very fast reaction rate with extremely high selectivity is observed for *m*-Me that reached a % *e.e.* of >98 within 60 minutes, compared to a % e.e. of >98 for *p*-Me, only after 240 minutes. It was previously shown that *Aspergillus niger* is also selective towards the (R)-epoxides during the kinetic resolution of both *m*-Me and *p*-Me, and thus rendering the (S)-epoxides in excess. % *E.e.* and yields of 50 and 19 % and >95 and 21 % was respectively reported for *m*-Me and *p*-Me. In their study the absolute configuration was assigned from the elution order from chiral GC, with respect to standards of enantiopure (R)-styrene oxide (Grogan *et al.*, 1997:255). This method of assignment of the absolute configuration of enantiomers is however risky, and has to be used with discretion since a switch in the elution order of a homologous series of compounds is frequently observed (Schurig, 2001:287).

Moussou *et al.* (1998b:3537) reported the kinetic resolution of *p*-Me by *Syncephalastrum racemosum*. The absolute configuration of the residual enantiopure epoxide was determined through chemical correlation after conducting a two-step chemical synthesis of the pure enantiomers. *Syncephalastrum racemosum* selectively hydrolysed the the (R)-epoxide, and thus renedered the residual (S)-(-)-epoxide.

The kinetic resolution of *m*-MO rendered 41,5 % epoxide with a % *e.e.* of 98 %. According to our knowledge, *m*-MO has not previously been used as a substrate for biocatalytic kinetic resolution by EHs.

In **Table 4-1** a summary of the data in **Figure 4-2** is presented. It can be seen that the EH from *R. glutinis* hydrolysed all SO derivatives. a % *E.e.* of more that 98 was reached for all substrates within 5 hours, except for *p*-NO_2 that reached a maximum of % *e.e.* of 88 after a reaction time of 12 hours.

Table 4-1 Hydrolysis of various epoxides by *R. glutinis*.

	e.e. (%)	Yield (%)	Reaction time (min)	Residual epoxide configurateion
m-NO_2	>98	45,9	300	
p- NO_2	88	24,6	720*	(+)(S)
m-Me	>98	42,5	60	
p-Me	>98	7,2	240	
m-MO	98	41,5	240	
SO	>98	28,4	180	(-)(S)

* Time where % *e.e.* was highest or above 98 %.

The highest selectivity was exhibited towards *m*-Me and the lowest towards *p*-Me. The EH enzyme exhibited higher selectivity for both *m*-Me and *m*-NO_2 epoxides in comparison to their *para* substituted counterparts.

From this data, it can be seen that the time taken for these substrates to reach the highest possible % *e.e.* or % *e.e.* above 98 is in the order of *m*-Me > SO > *p*-Me ≈ *m*-MO > *m*-NO_2 > *p*-NO_2.

High yields (>42 %) and % *e.e.* (≥98) are observed for all *meta* substituted derivative with much lower yields observed for the *para* substituted and unsubstituted styrene oxides. The effect of substitution on the reaction rate and enantioselectivity will be discussed in more detail in **Chapter 5**.

The semi-preparative production of the residual epoxide of *p*-Me was not attempted due to the extremely low theoretical yield (7,2 %) of pure enantiomer that was attained at an enantiomeric excess of 98 %. Thus, the absolute configuration of the residual *p*-Me could not be determined through VCD analysis.

3.2 Vibrational Circular Dichroism analysis

A large number of papers as well as review articles have been published in recent years demonstrating the reliability of VCD as a method for the determination of the absolute configuration of chiral molecules (Solladié-Cavallo *et al.*, 2001:2606; Monde *et al.*, 2005:5208; Freedman *et al.*, 2003:746; Nafie, 1995:83). VCD is the difference in the IR absorbance, A, of a specific molecule for left versus right circulatory polarized radiation during a vibrational transition and can be quantified as $\Delta A = A_{left} - A_{right}$. All molecules have a distinctive IR absorption

pattern, but only chiral molecules exhibit VCD absorption patterns over the IR spectrum (Nafie, 2004:1).

The application of VCD to the assignment of absolute configuration as well as the identification of the dominant solution conformations entails the comparison of observed spectra with calculated spectra for a specific configuration and conformation of the molecule or a suitable fragment of that molecule (Freedman *et al.*, 2003:746). VCD and IR spectra of one enantiomer are measured, and the *ab initio* calculations of the VCD and IR spectra of a randomly chosen enantiomer are carried out. The IR and VCD spectra of the enantiomer with known configuration (calculated) are compared to the measured IR and VCD spectra. If the calculated VCD bands have the same sign as the experimental VCD spectral bands, then the absolute configuration of the measured sample is the same as the absolute configuration of the molecule configuration chosen for the calculations. Thus, when the spectral bands on the measured and calculated spectra are opposite, the chosen absolute configuration of the compounds for the calculations is opposite of the measured sample configuration (Solladié-Cavallo *et al.*, 2001:2606).

The absolute configuration of the residual epoxide enantiomers of *m*-NO_2, *m*-Me and *m*-MO was thus determined by comparing the measured VCD spectra of the samples with that of the *ab initio* quantum calculation of the enantiomers. In all three cases the (S)-enantiomer was chosen for the calculations of the IR and VCD spectra.

3.2.1 *m*-Nitrostyrene oxide

Two samples (9,1 and 3,2 mg *m*-NO_2 in 100 µL $CDCL_3$) were prepared for the experimental VCD and IR spectra analysis at a resolution of 4 cm^{-1} (**Figure 4-3**). The high IR intensity of the nitro group stretches introduced large VCD noise for the more concentrated sample, so a more diluted sample was also analysed.

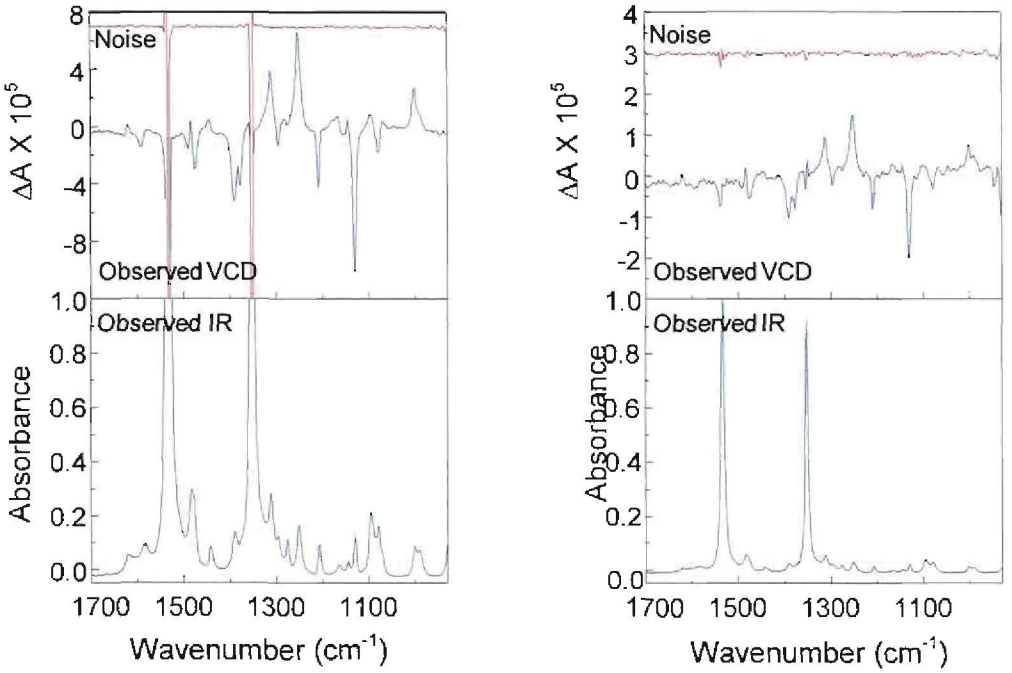
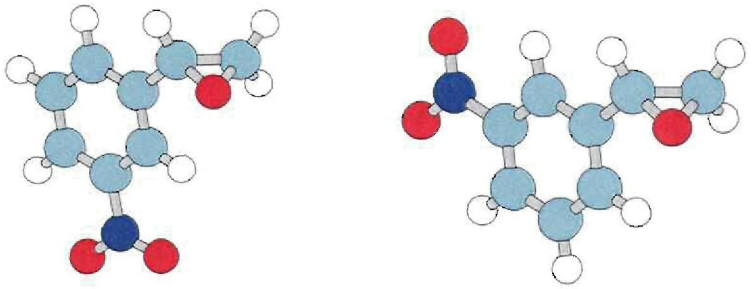


Figure 4-3 Experimental IR (lower frames) and VCD (upper frames) spectra for 9,1 mg/100 µL CDCl3 (left) and 3,2 mg/100 µL $CDCl_3$ (right) samples of *m*-NO_2.

Two conformers of the *m*-NO_2 molecule in the (S)-configuration were calculated with Gaussian 03 at the DFT level (B3LYP functional/6-31 G(d) basis set). These conformations differed with a 180° orientation of the substituted phenyl ring and presented an energy difference of 0,31 kcal.mol^{-1}. The optimised calculated geometries, relative energies and Boltzmann populations are portrayed in **Figure 4-4**.



(*S*)-*m*-NO_2 Conf. a
+0,31 kcal.mol^{-1}; 37 %

(*S*)-*m*- NO_2 Conf. b
0,0 kcal.mol^{-1}; 63 %

Figure 4-4 Calculated optimised geometries, relative energies and Boltzmann populations for conformers of (S)-*m*-NO_2.

VCD an IR spectra were also calculated at the DFT level (B3LYP functional/6-31 G(d) basis set). The observed experimental spectra for the concentrated sample in **Figure 4-3** are compared to the sum of the calculated spectra of conformers a and b weighted by their Boltzmann populations ([a]*0,37 + [b]*0,63) (**Figure 4-5**).

The comparison between the calculated Boltzmann-population-weighted composite IR and VCD spectra for the (S)-enantiomer and the experimental measurement for the more concentrated sample establishes the absolute configuration of the experimental sample as (S)-*m*- NO_2. Comparison with the experimental spectra for the diluted sample indicates weak negative VCD for the higher frequency NO_2 stretch in both the experimental and calculated spectra.

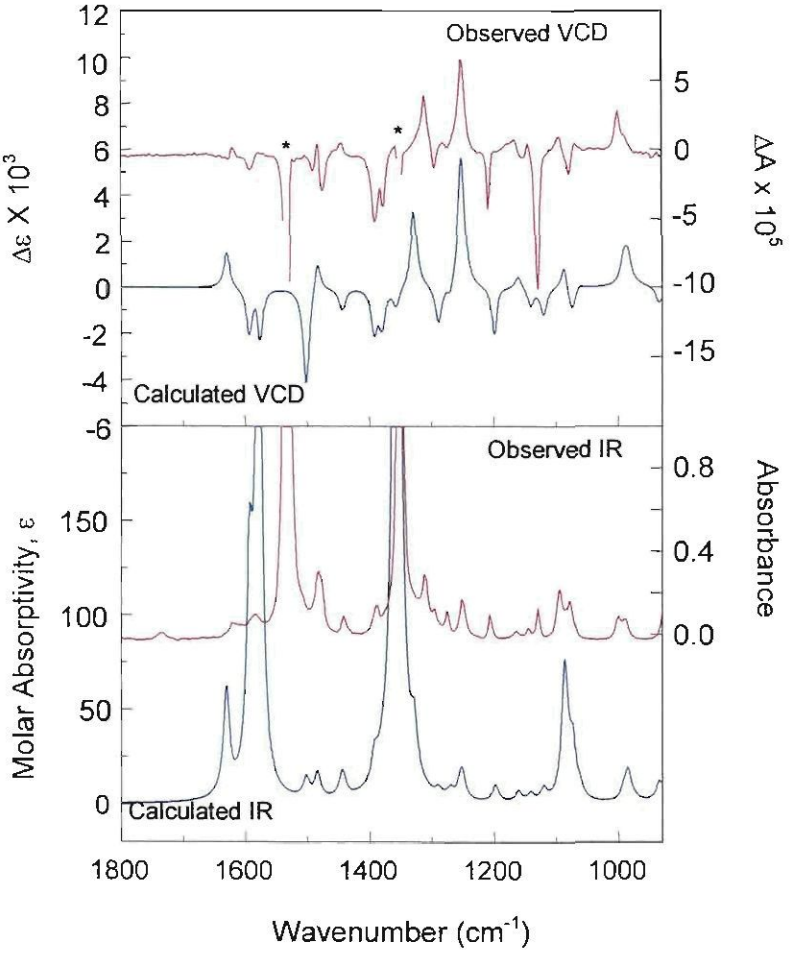


Figure 4-5 Calculated (left axis) and observed (right axis) IR (lower frame) and VCD (upper frame) spectra for (*S*)-*m*- NO_2.

3.2.2 *m*-Methylstyrene oxide

VCD and IR spectra were experimentally measured from a 6,0 mg enantiomer sample dissolved in 100,0 µL $CDCL_3$ at a resolution of 4 cm^{-1} (**Figure 4-6**). The observed VCD noise was minimal and is indicated in the upper frame.

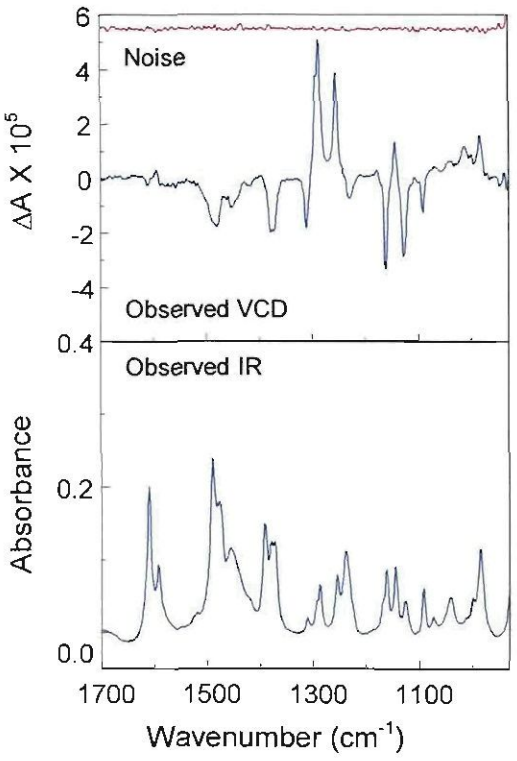
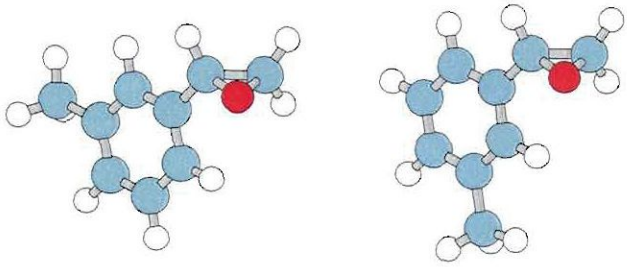


Figure 4-6 Experimental IR (lower frame) and VCD (upper frame) spectra for *m*-Me.

Two conformers of the *m*-Me molecule in the (S)-configuration as well as the IR and VCD spectra were calculated as described for *m*-NO_2. These conformations also differed with a 180° orientation of the substituted phenyl ring and presented an energy difference of only 0,06 kcal/mol. The optimised calculated geometries, relative energies and Boltzmann populations are portrayed in **Figure 4-7**.



(*S*)-*m*-Me Conf. a
+0,06 kcal.mol^{-1}; 48 %

(*S*)-*m*-Me Conf. b
0 kcal.mol^{-1}; 52 %

Figure 4-7 Calculated optimised geometries, relative energies and Boltzmann populations for (*S*)-*m*-Me.

The observed experimental spectra in **Figure 4-6** was compared to the sum of the calculated spectra of conformers a and b weighted by their Boltzmann populations ([a]*0,48 + [b]*0,52) (**Figure 4-8**).

Comparing the calculated IR and VCD spectra for the (*S*)-enantiomer and the experimental spectra measurement, the signs of the bands between the experimental and calculated VCD spectra was in agreement, establishing the absolute configuration of the experimental sample to be (*S*)-*m*-Me.

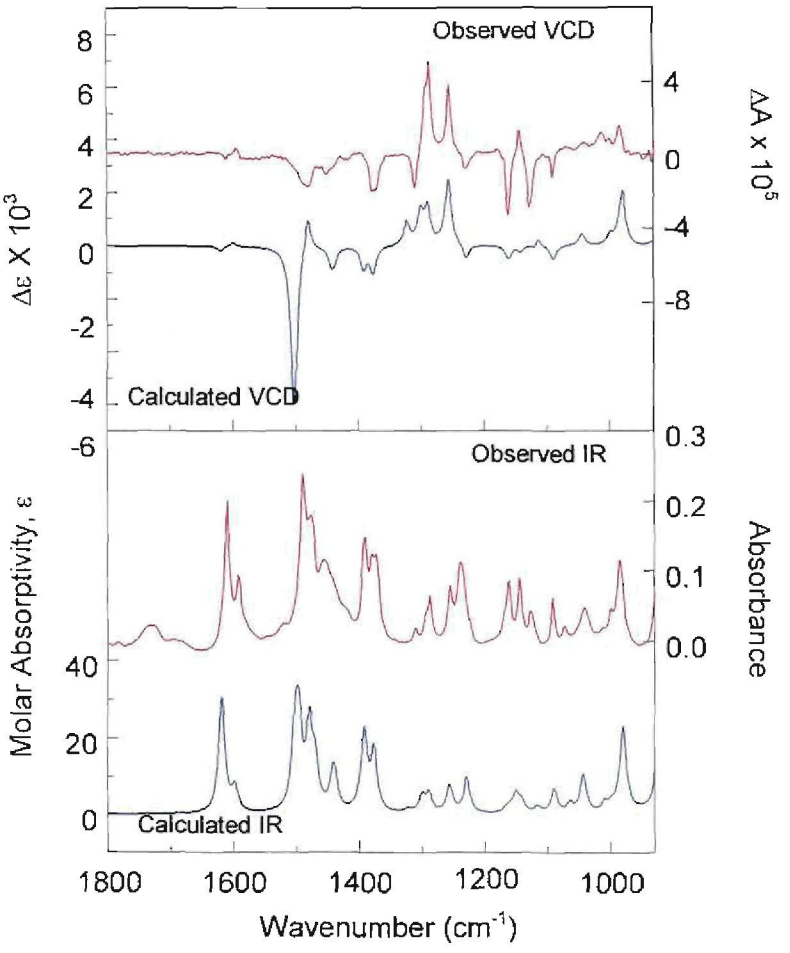


Figure 4-8 Calculated (left axis, 48 % conformer a + 52 % conformer) and observed (right axis) IR (lower frame) and VCD (upper frame) spectra for (*S*)-*m*- Me.

3.2.3 *m*-Methoxystyrene oxide

VCD and IR spectra for *m*-MO were also experimentally measured from a 6,0 mg enantiomer sample dissolved in 100,0 µL $CDCL_3$ at a resolution of 4 cm^{-1} (**Figure 4-9**). VCD noise trace was minimal and is indicated in the upper frame.

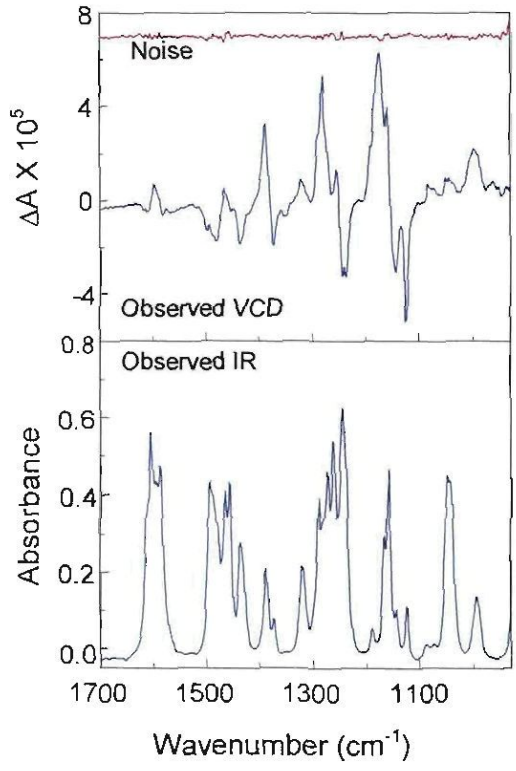


Figure 4-9 Experimental IR (lower frame) and VCD (upper frame) spectra for *m*-MO.

Two conformers of the *m*-MO molecule in the (S)-configuration as well as the IR and VCD spectra were calculated as described for *m*-NO_2. These conformations did not only differ by a 180° orientation of the substituted phenyl ring as was found with the different conformers with *m*-NO_2 and *m*-Me, but also with a 180° orientation of the methoxy group. The optimised calculated geometries, relative energies and Boltzmann populations are given in **Figure 4-10**.

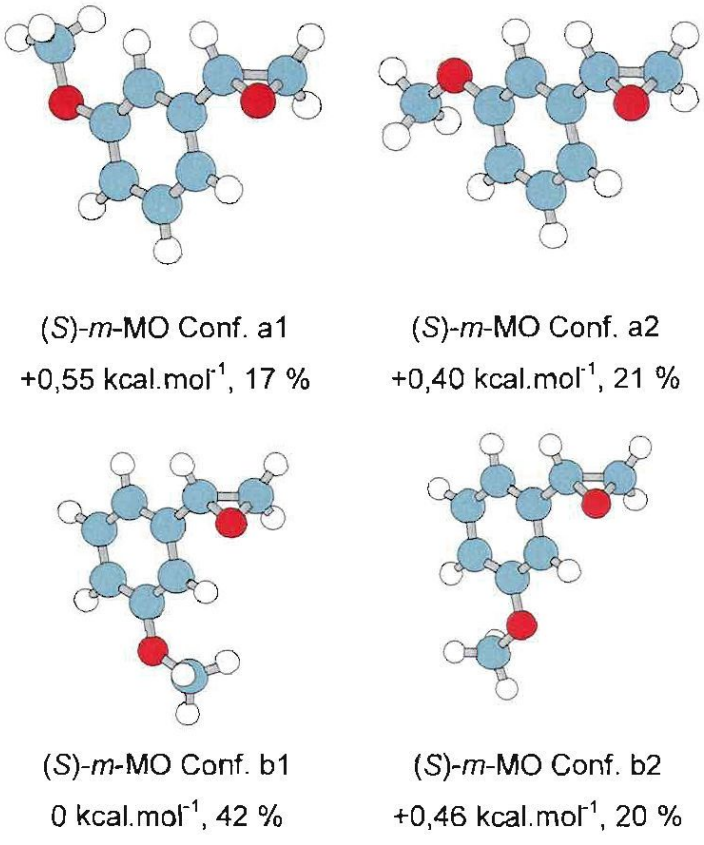


Figure 4-10 Calculated optimised geometries, relative energies and Boltzmann populations for (*S*)-*m*-MO.

The observed experimental spectra in **Figure 4-9** are compared to the sum of the calculated spectra of conformers a (a1 and a2) and b (b1 and b2) weighted by their Boltzmann populations ([a1]*0,17 + [a2]*0,21 +[b1]*0,42 +b2*0,2) (**Figure 4-11**).

Comparing the calculated IR and VCD spectra for the (S)-enantiomer and the experimental spectra measurement, the signs of the bands between the experimental and calculated VCD spectra was in agreement, establishing the absolute configuration of the experimental sample to be (S)-m-MO.

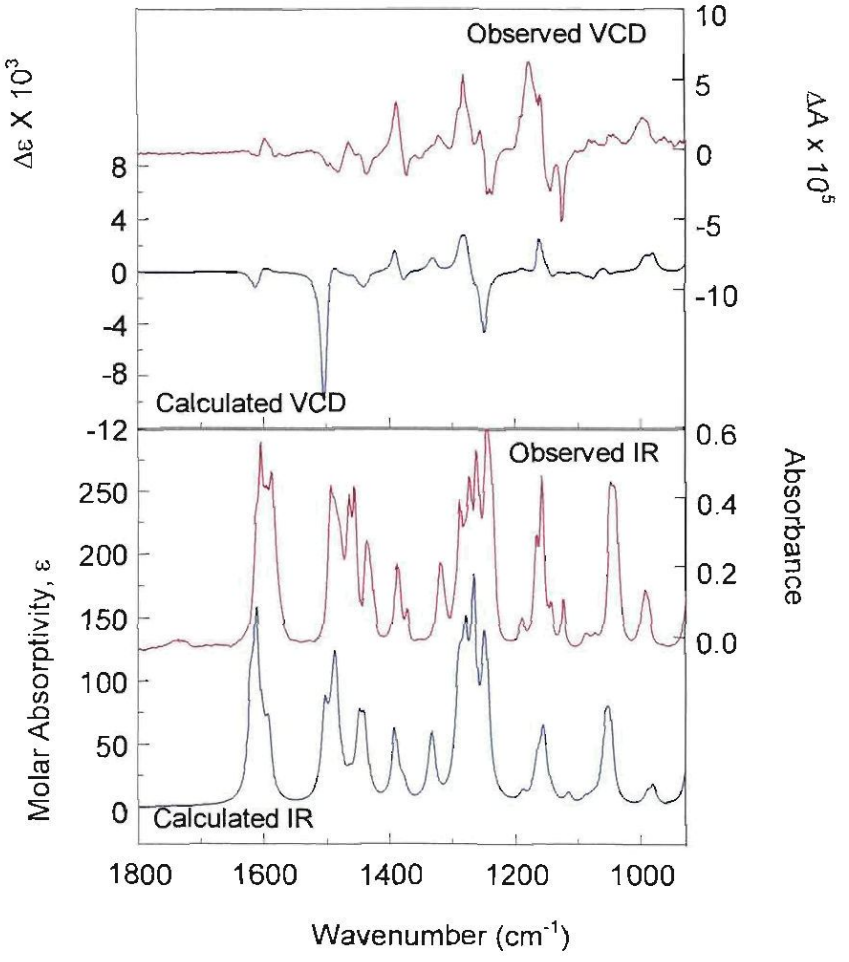


Figure 4-11 Calculated (left axis) and observed (right axis) IR (lower frame) and VCD (upper frame) spectra for (*S*)-*m*- MO.

For all three residual epoxides, the absolute configuration of (S) was assigned. Thus, *R. glutinis* EH preferentially hydrolyses the (R)-epoxide in all cases.

From these results and the previously determined absolute configurations already mentioned, the selectivity of *R. glutinis* EH towards SO and SO substituted derivatives at 15 °C has been proven to be towards the (R)-epoxide, thus rendering the unreacted residual (S)-epoxides. Although the absolute configuration of p-Me has not been determined, it is expected that the resultant epoxides would also be of the (S)-configuration due to the so far proven selectivity of *R. glutinis* towards these substrates as well as the GC elution order of the two enantiomers.

4 Conclusion

In conclusion, it was demonstrated that *R. glutinis* epoxide hydrolases exhibits high activity towards *meta* and *para* substituted methyl and methoxy substituted styrene oxide derivatives with a decreasing activity in the order of *m*-Me > SO > *p*-Me ≈ *m*-MO > *m*-NO_2 > *p*- NO_2. The highest activity and selectivity was exhibited towards *m*-methylstyrene oxide that reached a % *e.e.* ≥98 within a reaction time of 60 minutes. The enantiopure *meta* substituted styrene oxide derivatives were also successfully produced in high yields (≥ 41 % theoretical yield). *R. glutinis* epoxide hydrolases exhibited lower activity as well as selectivity towards the *para* substituted styrene oxide derivatives, with yields as low as 7,2 % for *p*-Me at a % *e.e.* of 98.

The absolute configuration of the pure *meta* substituted SO derivative enantiomers were also successfully determined as (S) by comparing theoretical calculated and observed VCD spectra. Thus, from these results it was shown that the EH from *R. glutinis* preferentially hydrolyses the (R)-enantiomer of these substituted styrene oxide derivatives (m-NO_2, m-Me and m-MO) as was also previously observed for SO and *p*-NO_2 (Yeates *et al.*, 2003:679; Yeates, 2001:68). From the VCD analysis the lowest energy solution state conformations of the (S) *meta*-substituted SO derivatives was also determined.

As far as is known, this is the first time that the absolute configuration of these compounds has been resolved by VCD analysis.

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Chapter 5

Solubility and modeling of the chemical and enzymatic hydrolysis of substituted styrene oxides

Abstract

The effect of hydroxypropyl-β-cyclodextrin (HPB) on the solubility of a range of *meta* and *para* substituted styrene oxides and the effect of HPB on *R. glutinis* epoxide hydrolase (EH) enzymatic activity on a reference compound was investigated. Furthermore, we attempt to correlate both the chemical and *R. glutinis* EH mediated enzymatic hydrolysis of styrene oxide derivatives to the electronic properties of their substituents and the spatial arrangement of the substrates in relation to the EH catalytic triad. Linear increase in the solubility and epoxide hdrolase enzyme activity was observed for *p*-nitrostyrene oxide, and a solubility increase of 2,9 to 6,3 times with a 5 % HPB additive concentration for the range of substrates. The chemical and enzymatic reaction rate of the microsomal EH of *Rhodotorula glutinis* exhibited a linear relationship to the Mulliken charge distribution over the protonated C7 (most substituted) and neutral/non-protonated C8 (least substitutes) epoxide carbons respectively. Modeling and docking of the EH binding site of *Aspergillus niger* visibly demonstrated a closer and more preferential fit of the (R)-epoxides which is the faster reacting epoxide for both *A.niger* and *R.glutinis*.

Keywords: epoxide hydrolases, *R. glutinis*, Mulliken, electronic properties and catalytic triad

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1 Introduction

Substantial interest exists in the development of methods for the synthesis of enantiopure epoxides due to their capability to easily undergo stereospecific ring opening (Besse & Veschambre, 1994:8885). Biocatalytic epoxide ring opening is either accomplished by direct attack of the nucleophile on the epoxide ring or via a two-step mechanism involving the formation and hydrolysis of a covalent intermediate (Lacourciere & Armstrong, 1993:10467) in which there is a covalent link between the enzyme and the substrate, also known as covalent catalysis (De Vries & Janssen, 2003:414). Epoxide hydrolase (EH) enzymes exhibit the capability to enantioselectively catalyse the hydrolysis of epoxides to the corresponding diols and thus, kinetically resolving the epoxide enantiomers (Arand *et al.*, 2003:2583).

Generally, the activity of an enzyme towards a specific substrate or substrate range is measured by screening. This is where a range of enzymes and mutants are tested either against a specific substrate or range of substrates (Genzel *et al.*, 2002:219, Yeates *et al.*, 2003:677). By completely understanding the reaction mechanism, thus all factors affecting both the enantioselectivity and reaction rate of hydrolyses by EHs, the possibility might exist to predict and possibly control these enzymes` choice of substrates and enantioselectivity. This might bring about benefits such as rendering time consuming practices such as full screenings unnecessary.

Previously, the reaction rate and mechanism of a number of bacterial and fungal EH mediated reactions have been correlated with the electronic properties of the substituent groups with varying levels of success (Spelberg *et al.*, 2002:980; Moussou *et al.*, 1998:3532). In the present study we optimised the HPB additive concentration for this range of substrates through the use of a model substrate. Furthermore we investigated the effect of substrate substitution on the enzyme activity and attempted to correlate the enzymatic reaction rate as well as the enantioselectivities displayed for this range of nitro, methyl and methoxy substituted styrene oxides to both the electronic properties of the substituent groups and spatial arrangement of the substrate in the enzyme active site.

2 Experimental

2.1 General

All reagents and the yeast strain of *Rhodotorula glutinis* (UOFS Y-0563) were purchased from the same suppliers as presented in Chapter 3 and 4.

2.2 Methods

2.2.1 Synthesis of styrene oxide derivatives

All styrene oxide (SO) derivatives were synthesised as described in Chapter 4.

2.2.2 Solubility of styrene oxide and derivatives in HPB

An excess amount of epoxide was added to 1 mL of 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7,3) containing a range of % hydroxypropyl-β-cyclodextrin (HPB) concentrations (% w/v). The mixtures were continuously shaken at 15 °C for 24 hours after which it was centrifuged for 5 minutes at 10 000 rpm in order to attain a clear supernatant. The supernatant was slowly removed with a Hamilton® syringe and extracted with ethyl acetate (2:1 (v/v) ethyl acetate: supernatant). The ethyl acetate was dried over anhydrous Na_2SO_4 before analysis and quantification of the epoxide concentration as described in Chapter 4. All solubility experiments were repeated in triplicate with an average deviation of between 0,6 and 3,5 %.

2.2.3 Chemical hydrolysis of styrene oxide and derivatives

Specific amounts of SO and SO derivatives were aliquoted into 1,5 mL micro-centrifuge tubes to a total concentration of 40 mM in 500 µL water (pH 3,6, HCl). The reaction mixtures were incubated at 15 °C while continuously shaken at 190 rpm for 20 minutes. At certain time intervals the chemical hydrolyses were stopped through neutralisation by the addition of 1 µL 2 M KOH and 100 µL saturated NaCl solution. The residual epoxide was immediately extracted through the addition of 290 µL ethyl acetate. The mixtures were vortexed for 1 minute, centrifuged for another 1 minute at 10 000 rpm and the ethyl acetate layer immediately extracted. Before analysis the ethyl acetate was dried over anhydrous Na_2SO_4.

2.2.4 Cultivation and preparation of yeast cells

The yeast *R. glutinis* was grown at 27 °C in a light protected Labcon® rotary platform shaking incubator. All media solutions were prepared, and the organism grown, harvested and stored as described in Chapter 4.

2.2.5 Kinetic resolution

The *R. glutinis* EH mediated hydrolytic kinetic resolution of the synthesised styrene oxide derivatives was followed for 35 minutes in order to determine the reaction rate. Stock solutions (1 M) were prepared of all substrates in DMSO.

The frozen cell suspensions were thawed, centrifuged for 5 minutes at 3500 g and resuspended (25 % w/v) in a 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7,3) containing 5 % (w/v) hydroxypropyl-β-cyclodextrin (HPB). 500 uL of the cell suspensions were aliquoted into 1,5 mL micro-centrifuge

tubes and the epoxide (10 µL of the 1 M stock) added to a final concentration of 20 mM. The reaction mixtures were incubated at 15 °C with continuous shaking at 190 rpm. Reactions were terminated at certain time intervals by submerging the centrifuge tubes in liquid nitrogen. The residual epoxide was extracted through the addition of 270 µL ethyl acetate to the frozen sample. The frozen reaction mixture and added ethyl acetate were vortexed until completely defrosted. Subsequently, this mixture was vortexed for another 3 minutes, after which it was centrifuged for 3 minutes at 10 000 rpm. The ethyl acetate was extracted and dried over anhydrous Na_2SO_4 before analysis and quantification of the residual epoxide on chiral GC. These experiments were respectively conducted in duplicate for the effect of HPB on p-NO_2 and in triplicate for the correlation of the complete series of SO derivatives with the substituent electronic properties. Average deviations of under 5 % were obtained for the duplicate analysis and average deviations of between 2,2 and 19,6 % were observed for the triplicate values. Even though a deviation of 19,6 % is extremely high (observed for *p*-Me), it did not affect the overall trend of the EH activity towards these substrates.

2.2.6 Analysis

Chiral GC analysis of the isolated products was conducted as was described in Chapter 4.

2.2.7 *Ab-initio* calculations

Mulliken charges, of both the protonated (acid) as well as the neutral (base or neutral catalysis) SO and the substituted SO derivatives, were calculated on DFT (B3LYP, 6-31G*) and on a semi-empirical (AM1) level using Spartan.

2.2.8 Conformer docking

Possible conformers for both the (R)- and (S)-enantiomers of the range of epoxides were generated using Catalyst by MSI. The EH binding site of *Aspergillus niger*, consisting of the catalytic triad, was located, and conformer docking carried out with MSI Cerius II.

3 Results and discussion

3.1 Effect of HPB on solubility and enzyme activity

According to the FDA, cyclodextrins are generally recognized as being safe additives in pharmaceutical formulations, and have been approved as a food additive in Japan (Loftsson & Duchêne, 2007:9). This non-toxicity makes their advantage as "co-solvent" or rather solubility increasing additive quite apparent in comparison to other possibly toxic organic solvents such as TMF, DMSO, DMF and other longer chain alcohols (Leščič et al., 2001:551; Lotter *et al.*, 2004:1194) for use in the production of pharmaceutical compounds where low solubility of the starting materials or intermediate products might lead to difficulties in the synthetic process.

During a previous study conducted in our laboratories, hydroxypropyl-β-cyclodextrin (HPB) was found to be a highly useful tool to increase the solubility of poor water soluble organic substrates (Yeates *et al.*, 2007:234). Although it was reported that the addition of equal amounts of HPB in comparison to DMSO lead to a higher decrease in activity, a much lower percentage HPB was needed to achieve equal solubility. Thus, the amount of HPB needed to increase the solubility of a substrate up to a specific point had a lower negative effect on enzyme activity in comparison to the effect of other organic solvents to reach the same solubility.

In the range of styrene oxide and synthesised styrene oxide derivatives (*meta*-nitrostyrene oxide (*m*-NO_2), *para*-nitrostyrene oxide (*p*-NO_2), *meta*-methylstyrene oxide (*m*-Me), *para*-methylstyrene oxide (*p*-Me) and *meta*-methoxystyrene oxide (*m*-MO), *p*-NO_2 was chosen as model substrate in order to get reliable results on the effect of HPB on the solubility of the epoxides and activity of *R. glutinis* EH. *p*-NO_2 exhibits the lowest water solubility in the range of styrene oxides tested, and exhibits the highest resistance towards chemical hydrolysis.

An increase in the HPB concentration (% w/v) lead to a substantial increase in both the solubility as well as enzyme activity (**Figure 5-1**). A non-linear fit (second order polynomal), accurately describes the solubility of *p*-NO_2 with an increasing HPB concentration. This increase in solubility could also be described as linear (Yeates *et al.*, 2007:230), however, the increase in solubility starts to flatten out with an increasing HPB concentration. A more than double increase in the solubility of *p*-NO_2 is observed with the addition of only 1,0 % HPB, and an increase from 0,38 $g.L^{-1}$ (0 % HPB) to 6,59 $g.L^{-1}$ is demonstrated with the addition of 20 % HPB in comparison to the solubility observed in the phosphate buffer without any solubility increasing additives. An increase in the reaction rate, (decrease in epoxide concentration ($mM.min^{-1}$) calculated over 35 minutes), is also observed. Initially (0-5 % HPB), this increase in solubility is linear, but levels off between a solubility of 2,4 $g.L^{-1}$ (14,4 mM) and 3,9 $g.L^{-1}$ (23,8 mM). Since reactions were done with 20 mM (3,3 $g.L^{-1}$) *p*-NO_2, this leveling of is probably due to the saturation concentration of *p*-NO_2 increasing above the amount of *p*-NO_2 in solution and that an increase in the solubility of the substrate should not cause a further increase in the substrate concentration available for hydrolysis. This is again confirmed by the results attained by Yeates *et al.*, (2007:231) where an increase in the HPB concentration, and thus solubility, above the substrate concentration, lead to a decrease in the reaction rate.

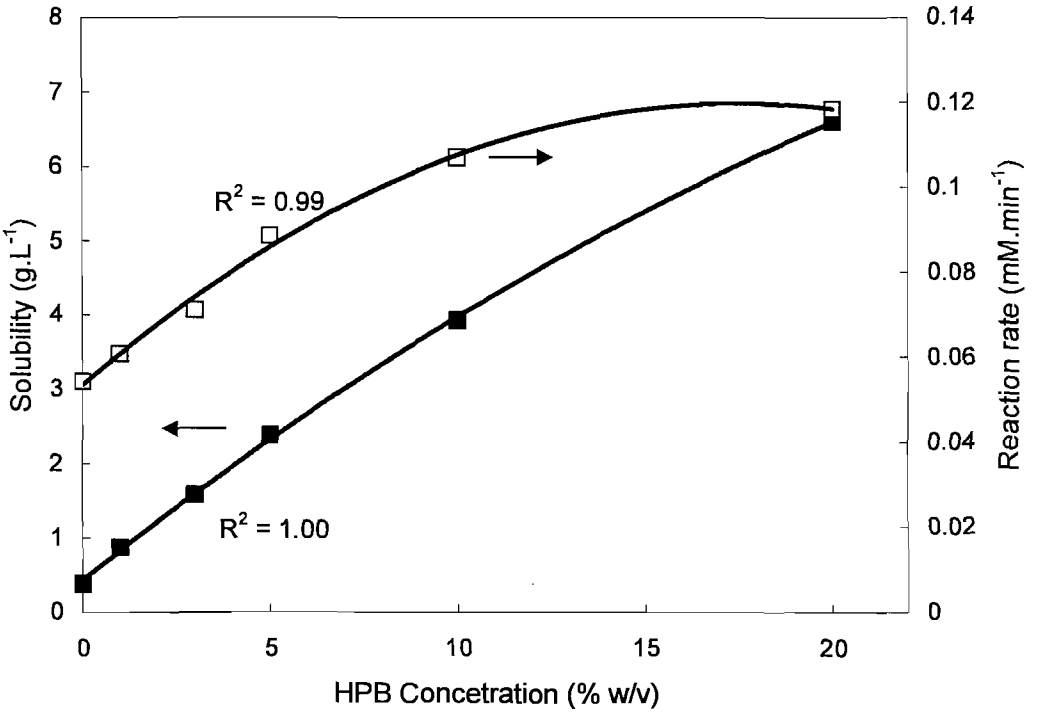


Figure 5-1: The effect of HPB on *p*-NO_2 solubility (■) and initial enzymatic reaction rate (□).

The biggest disadvantage of HPB is the cost in comparison to other more widely used organic solvents. Therefore, a compromise needs to be made when choosing the "optimum" additive concentration. Even though a definite increase in reaction rate can still be observed up to 10 % HPB concentration, a 5 % HPB additive concentration was chosen for all subsequent experiments due to the leveling of the reaction rate observed with higher HPB concentrations.

The effect of the addition of 5 % HPB (w/v) to the buffer solution on the solubility of all the substrates is illustrated in **Figure 5-2**. A substantial increase in solubility is observed in all cases, with the highest increase observed for *p*-NO_2 (which has the lowest solubility in comparison to the other compounds without any additives) and the lowest increase observed for SO, which exhibits the highest initial solubility. This increase in solubility of the substrates in a buffer solution in comparison to the buffer/HPB solution was between 2, 9 and 6,3 times. This clearly demonstrates the significant effect that HPB has on the solubility of low soluble organic compounds. All subsequent experiments were conducted with a 5 % HPB additive concentration.

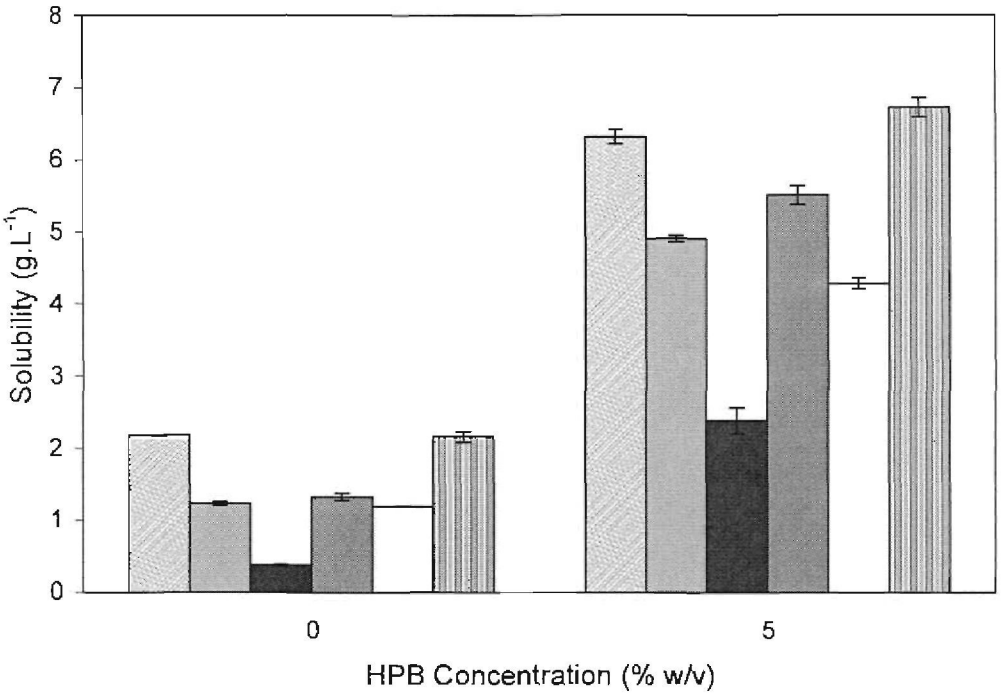


Figure 5-2 Solubility of SO and derivatives in the presence of 0 and 5 % HPB (▨, SO; ▩, *m*-NO_2; ■, *p*-NO_2; ▩, *m*-Me; □, *p*-Me; ▥, *m*-MO).

3.2 Effect of styrene oxide substitution on the hydrolytic reaction rate

3.2.1 The Hammett constant and epoxide hydrolysis

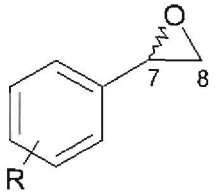
Hammett postulated that for benzene derivatives, the effect that a substituent in the *meta* or *para* position of the benzene ring has upon the rate, or equilibrium, of a reaction in which the reacting group is in a side chain attached to the ring, may be represented by a simple formula which is applicable within a reasonable accuracy for a diverse range of chemical reactions (Hammett, 1937:96).

This effect of the substituent at a *meta* or *para* location on a benzene ring can be quantified in terms of the substituent constant (σ) (**Table 5-1**). The Hammett substituent constant, determined by the difference between the logarithm of the ionization constant of a substituted benzoic acid and the logarithm of the ionization constant of benzoic acid, gives a numerical value to the inductive and resonance effects of a substituent group (Hammett, 1937:96, 103; Hansch *et al.*, 1991:166), and is thus a quantitative representation of the electron donating or electron withdrawing properties of a substituent group attached to a benzene ring.

Table 5-1 Electronic substituent effects on an aromatic system.

Substituent	σ	Inductive effect	Mesomeric effect
H	0		
m-NO_2	0,71	-I	
p-NO_2	0,78	-I	-M
m-Me	-0,07	+I	
p-Me	-0,17	+I	+M
m-MO	0,12	-I	

One of the reactions for which Hammetts` postulate holds true is chemical hydrolysis. The rate of hydrolyses is controlled by the electronic properties of the molecule. If the inductive as well as resonance effect, thus the effect of a substituent group on the partitioning of electrons over the rest of the molecule, e.g. styrene oxide (**Figure 5-3**), is considered, it is expected that the electron withdrawing effect of the substituent should be in the order of *p*-NO_2 > *m*-NO_2 > *m*-MO > SO > *m*-Me > *p*-Me, and that the Hammett constant, σ, should have a linear logarithmic relationship to the equilibrium or the rate constant for this reaction.



1. R = H
2. R = *meta*-NO_2
3. R = *para*-NO_2
4. R = *meta*-CH_3
5. R = *para*-CH_3
6. R = *meta*-OCH_3

Figure 5-3 SO and synthesised SO derivatives.

When the logarithmic values of the first order rate constant, k (min^{-1}) of the acid induced chemical hydrolysis of SO and its derivatives are plotted against σ (**Figure 5-4**), a definite negative linear relationship is observed. This relationship reflects the sensitivity of the reaction to the electronic effects of the substituent group (Chiu *et al.*, 2005: 286), which clearly indicates that the acid induced hydrolysis of these styrene oxide derivatives are favored by an increase in the electron donating properties of the substituent groups.

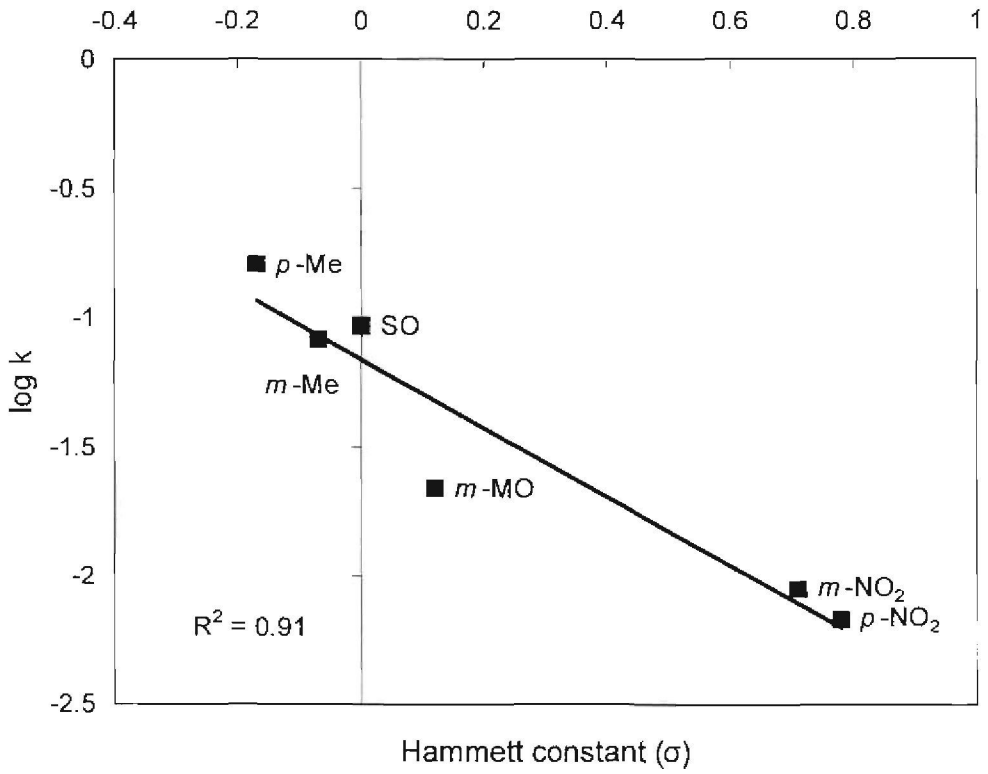


Figure 5-4 Hammett plot of the logarithmic chemical rate constant (k; min^{-1}) versus the substituent constant (σ) for the acid mediated hydrolysis of SO and substituted SO derivatives.

If the electron withdrawing or donating effect of the substituent on the aromatic ring affects the rate limiting step of enzymatic hydrolysis, a clear correlation should also be observed in a Hammett plot where the enzymatic reaction rate is plotted against the Hammett constant (De Vries & Janssen, 2003:415). In **Figure 5-5** the logarithmic value of the enzymatic hydrolysis reaction rate ($mM.min^{-1}$) from SO and the different derivatives are plotted against the Hammett constant (σ). Even though the linear correlation between the logarithmic value of the enzymatic reaction rate and σ is low, an increase in the reaction rate seems to correlate with an increase in the electron donating properties of the substituent group.

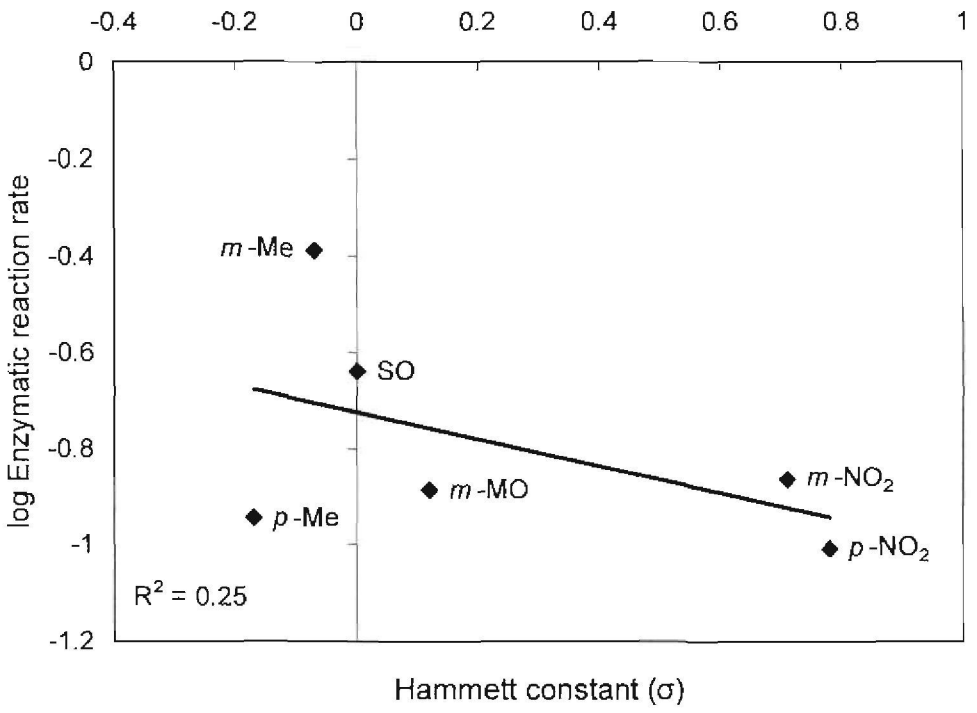


Figure 5-5 Hammett plot of logarithmic enzymatic reaction rate ($mM.min^{-1}$) versus σ for the EH mediated hydrolysis of SO and substituted SO derivatives.

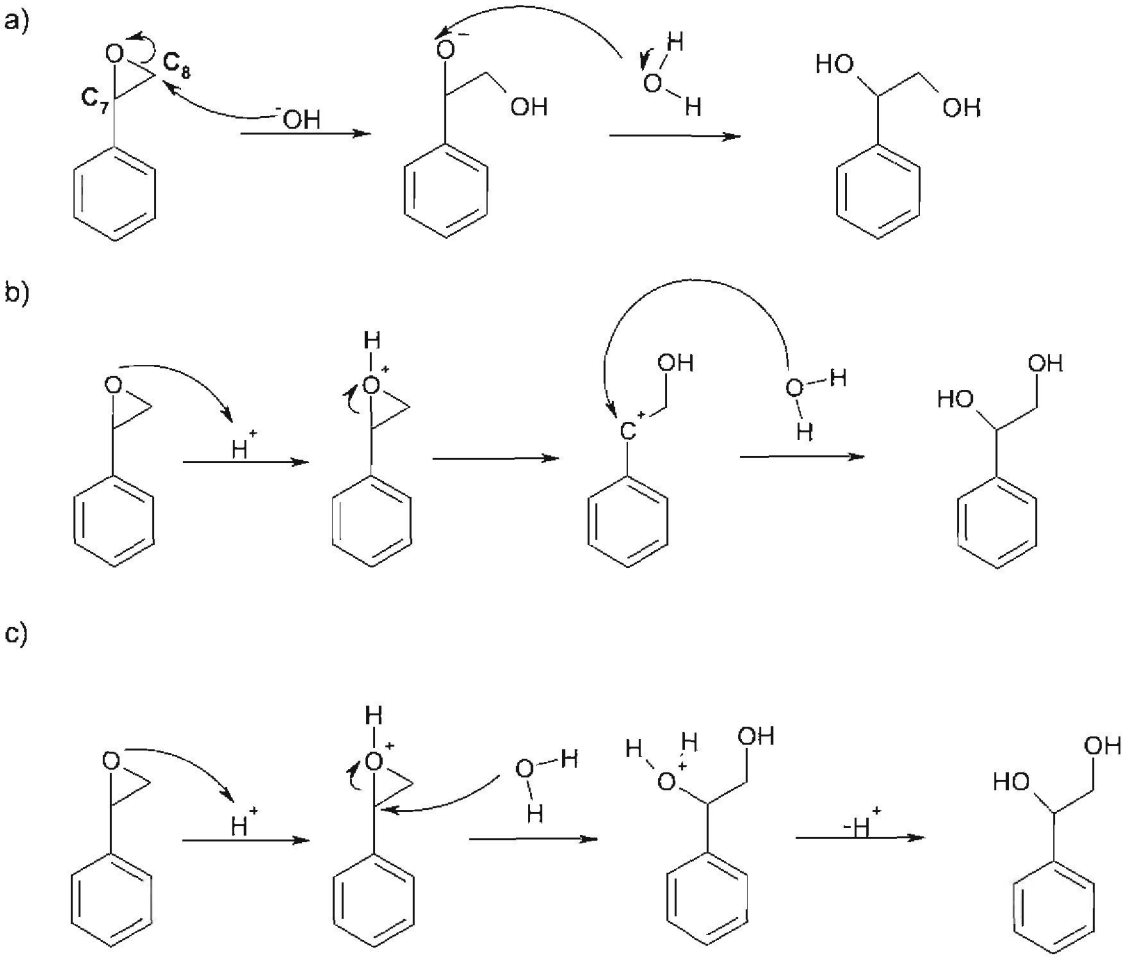
The low linear correlation could have been caused by any number of factors. It was previously observed that the Hammett correlation of two enantiomers is not identical, and seeing that a racemate was used for this experiment it might have contributed to the lack in correlation (Spelberg *et al.*, 2002:982). Another possible contributing factor is that the enzymatic reaction rate is the specific reaction rate observed with the addition of a constant initial substrate concentration, and not the maximum reaction rate (V_{max}) at the optimum individual substrate concentration for every compound as was done in the study by Moussou *et al.* (1998:3535). A number of studies also contributed a low linear correlation between the Hammet constant and the enzymatic reaction rate to the possibility that the initial nucleophilic attack on one of the epoxide carbons might not be the rate limiting step (Morisseau & Hammock, 2005:315, Orru & Faber, 1999:16).

3.2.2 Mechanism of epoxide hydrolysis.

3.2.2.1 Chemical hydrolyses

In principal, the chemical hydrolysis of SO and substituted SO's under basic and neutral conditions follow a different mechanism in comparison to hydrolysis under acidic conditions. According to March (1992:369) there is one possible mechanism for the basic induced or neutral spontaneous hydrolysis of epoxides (SN2) (**Scheme 5-1a**), but, in an acidic environment, the protonated epoxide can undergo either a SN1 (**Scheme 5-1b**) or SN2

(**Scheme 5-1c**) mechanistic hydrolysis. The ring opening during acid catalysis prior to the attack of the water molecule (S_N1) or during the attack of the water molecule (S_N2) occurs at the cyclic carbon which is most suitable to accommodate the positive charge, and this results in the intermediate with the lowest energy being formed (Hanzlik & Westkaemper, 1980:2465). Thus, the reaction is characterized by nucleophilic attack by the solvent (water) at the most positively charged oxirane carbon.



Scheme 5-1 Mechanistic representation of the a) base or neutral (SN1) and acid induced b) SN1 or c) SN2 chemical hydrolysis of styrene oxides.

Therefore, it can be said that during chemical hydrolysis the nucleophilic attack will preferentially take place on the C8 (least substituted) epoxide carbon under basic or neutral conditions, but on the C7 (most substituted) epoxide carbon under acidic conditions.

3.2.2.2 Enzymatic hydrolyses

As previously mentioned, all enzymes in the α/β-hydrolase family have a nucleophile-His-acid catalytic triad. According to Holmquist (2000:212) the reaction mechanism follows two catalytic

steps which involve the formation of a covalent intermediate. Styrene oxide type oxiranes posses a benzylic carbon atom which facilitates the formation of a carbo-cation which is stabilised by the neighboring aromatic moiety. As a consequence, attack at this position is electronically facilitated (**Figure 5-6**), although sterically hindered, and mixed regiochemical pathways (i.e. attack at both oxirane carbon atoms) are particularly easy within this group.

Figure 5-6 Effect of substitution on nucleophilic attack on SO type epoxides.

The implication of attack on both epoxide carbon atoms is that the absolute configuration may be retained or inverted, depending on the substituent arrangement on the attacked carbon atom.

3.2.3 Ab-initio correlation of hydrolytic reaction rates

In an attempt to correlate the enzymatic reaction rate with the electronic properties of SO and its derivatives the Mulliken charges of both the protonated (acid) as well as the neutral (base or neutral catalysis) SO and the substituted SO`s was calculated on DFT level (B3LYP, 6-31G*) as well as on a semi-empirical level (AM1) with Spartan.

When observing the LUMO (lowest unoccupied molecular orbital) for *p*-NO_2 calculated on the AM1 semi-empirical level (**Figure 5-7**) the effect of protonation of the epoxide in an acid environment (**Figure 5-7a**) and the shift of the LUMO in a neutral or basic (**Figure 5-7b**) environment can be observed. Under acidic conditions the epoxide–ring oxygen is protonated, which causes the LUMO to be situated around the most substituted epoxide carbon (C7), where in a neutral or basic environment no oxygen protonation occurs which leads to the location of the LUMO in the area of the least substituted carbon (C8). The greater the atom contribution to the LUMO, the greater the electrophilicity for that specific atom which results in a higher probability of nucleophilic attack on that specific atom (Ward, 1996:2837). The localisation of the LUMO in both the protonated and neutral epoxides clearly support the mechanisms (**Scheme 5-1**) and site of nucleophilic attack for both the acid (on C7) and base or neutral (on C8) induced epoxide hydrolysis.

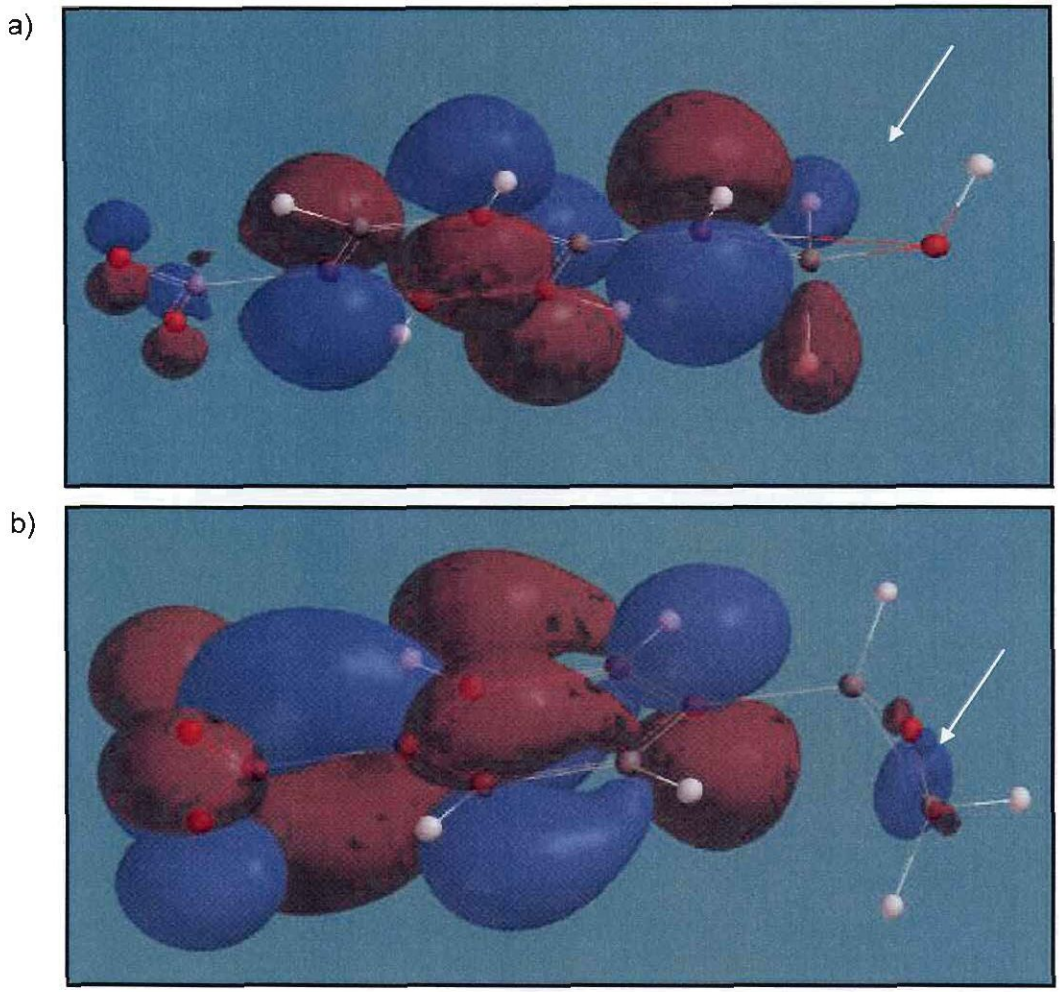


Figure 5-7 LUMO for both (a) protonated and (b) neutral *p*-NO_2.

The semi-empirical as well as DFT level Mulliken charges on C7 of the protonated epoxide were plotted against the logarithmic reaction constant (k) for the acid induced chemical hydrolysis of these substrates (**Figure 5-8**). In both instances a clear linear correlation is observed between the Mulliken charge on C7 and the chemical hydrolysis reaction constant. The best correlation (R^2 = 0,91) was found on a semi-empirical level. The modeled values confirm the theoretically expected increase in negativity of the charge value on C7 with an increase in the electron donating properties of the substituent groups and an increase in the chemical hydrolytic reaction rate.

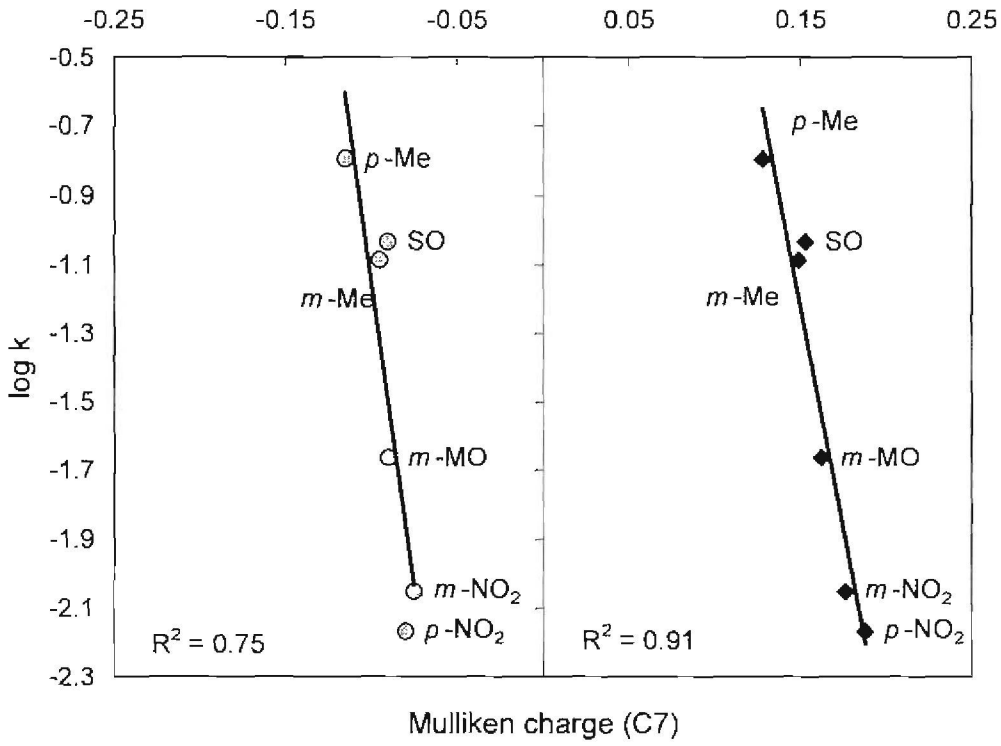


Figure 5-8 Mulliken charges calculated on DFT (o) and semi-empirical (◆) level for the protonated epoxide C7 atom versus logarithmic acid induced hydrolysis rate constant (min^{-1}) for SO and SO derivatives.

Thus, it can be seen that both the Hammett constant (Hammett, 1937:103) as well as Mulliken charges on the reacting carbon can be utilised to represent the effect of a substituent in the *meta* or *para* position of the benzene ring upon the chemical hydrolytic reaction rate in which the reacting group is in a side chain attached to the ring.

Even though nucleophilic attack can take place on either epoxide carbon atoms during the enzymatically catalysed reaction, this attack preferentially occurs on the least substituted epoxide carbon atom through a general base catalysed mechanism (Smit & Labuschagne, 2006:1151). Thus, the enzymatic reaction rate should show a higher correlation with the Mulliken charge on the neutral C8 epoxide atom in comparison to the neutral C7 atom or the protonated C7 atom.

As suspected, no real correlation can be observed between the Mulliken charge distribution on the most substituted carbon atom (C7) and the logarithmic value of the enzymatic reaction rate (**Figure 5-9**) on either the semi-empirical level or the DFT level. Again it can be observed that the distribution of the Mulliken charges is different for the two mathematical levels of calculation.

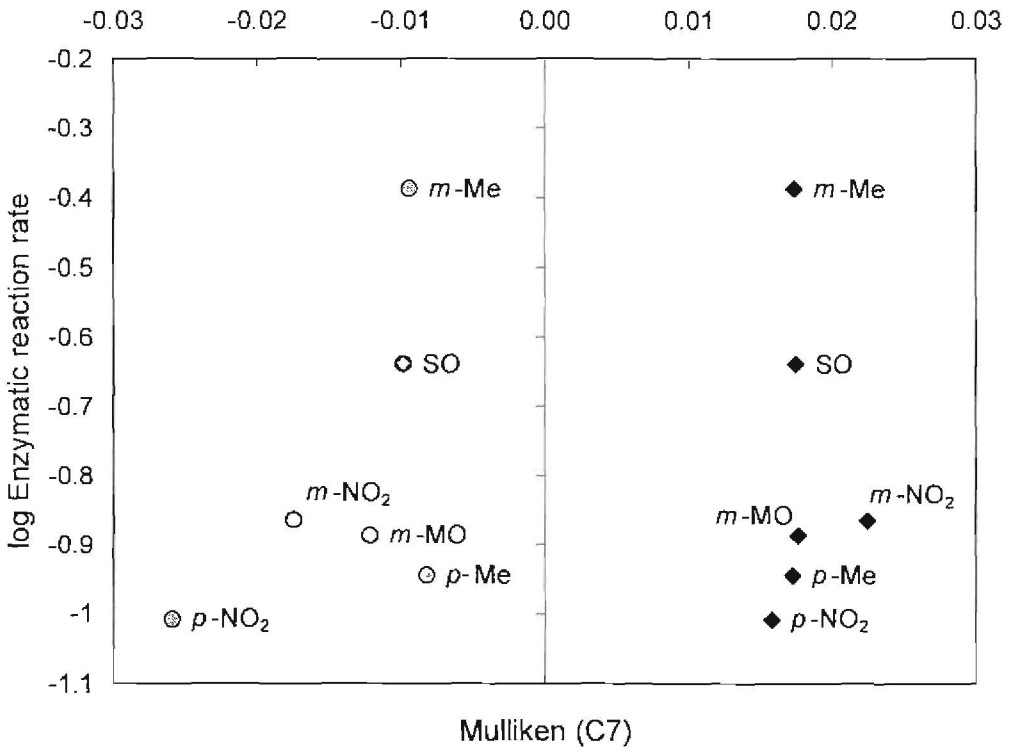


Figure 5-9 Mulliken charges calculated on DFT (○) and semi-empirical (◆) level for the neutral epoxide C7 atom versus the logarithmic enzymatic reaction rate (mM.min $^{-1}$) for SO and SO derivatives.

Although rare, there have been cases of microbial EH enzymes acting on a general acid catalysed mechanism (Moussou et al., 1998:3536) and not the general base catalysed mechanism. If this was the case for *Rhodotorula glutinis* EHs, one would expect to find a correlation between the Mulliken distributions over the C7 highest substituted epoxide carbon atom when the epoxide ring is protonated. As can be seen from **Figure 5-10**, no linear relationship can be observed between the C7 protonated epoxide Mulliken charge calculated on DFT or on semi-empirical level.

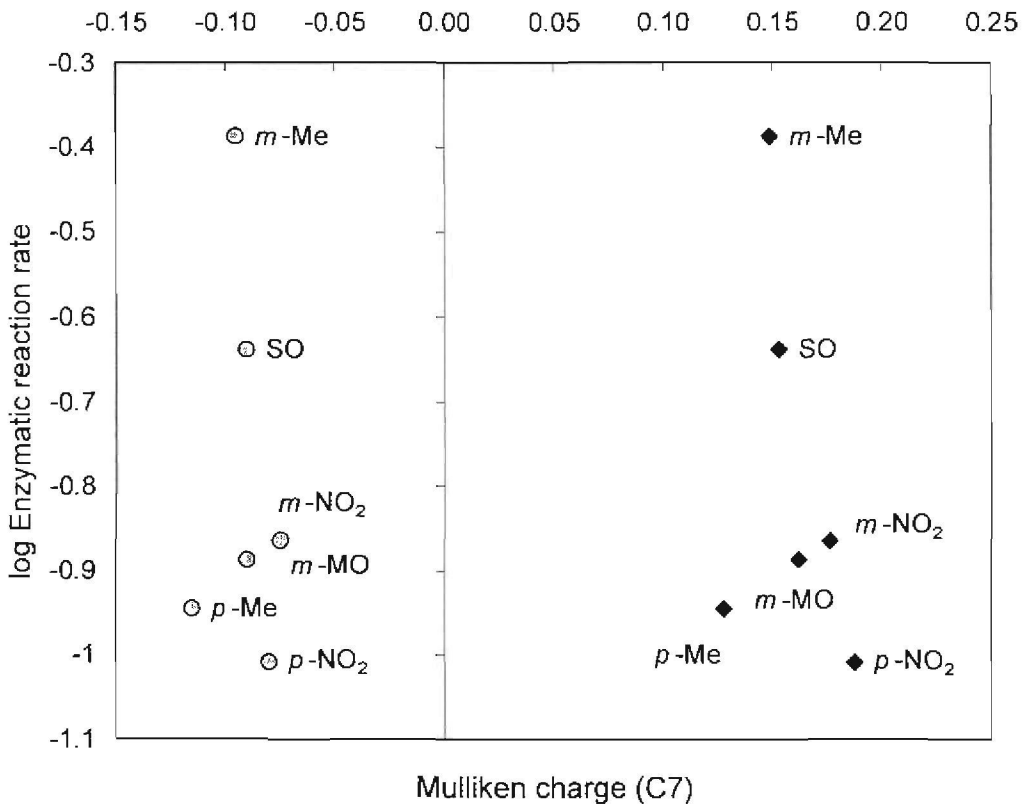
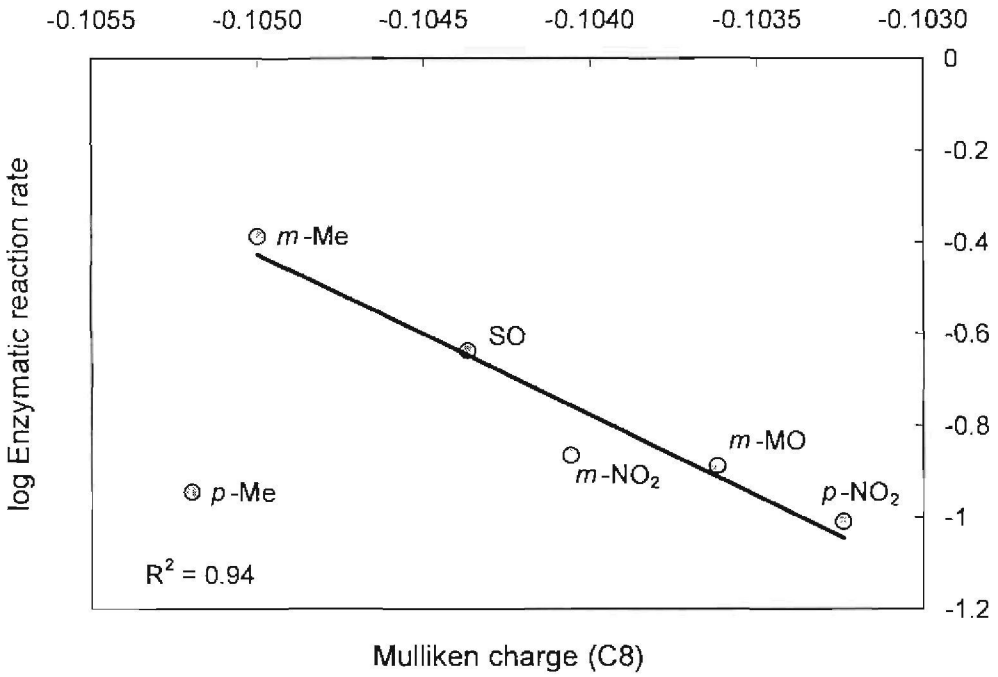


Figure 5-10 Mulliken charges calculated on DFT (⊙) and semi-empirical (◆) level for the protonated epoxide C7 atom versus the logarithmic enzymatic reaction rate (mM.min $^{-1}$) for SO and SO derivatives.

The DFT-level Mulliken charges on C8 of the neutral epoxide (**Figure 5-11a**), however, did give a linear correlation of R^2 = 0,94 for all substrates for the EH mediated enzymatic reaction rate, when excluding the *para*-methyl substituted SO. This linear correlation would be expected if nucleophilic attack preferentially takes place on the least substituted epoxide carbon (C8). It is unknown why the *p*-Me substituted epoxide does not comply with this correlation and it might be due to steric interference in the EH reaction site, or the possibility that enzymatic nucleophilic attack shifted to take place preferentially on C7 instead of C8 seeing this nucleophilic attack is extensively influenced by electronic effects.

(a)



(b)

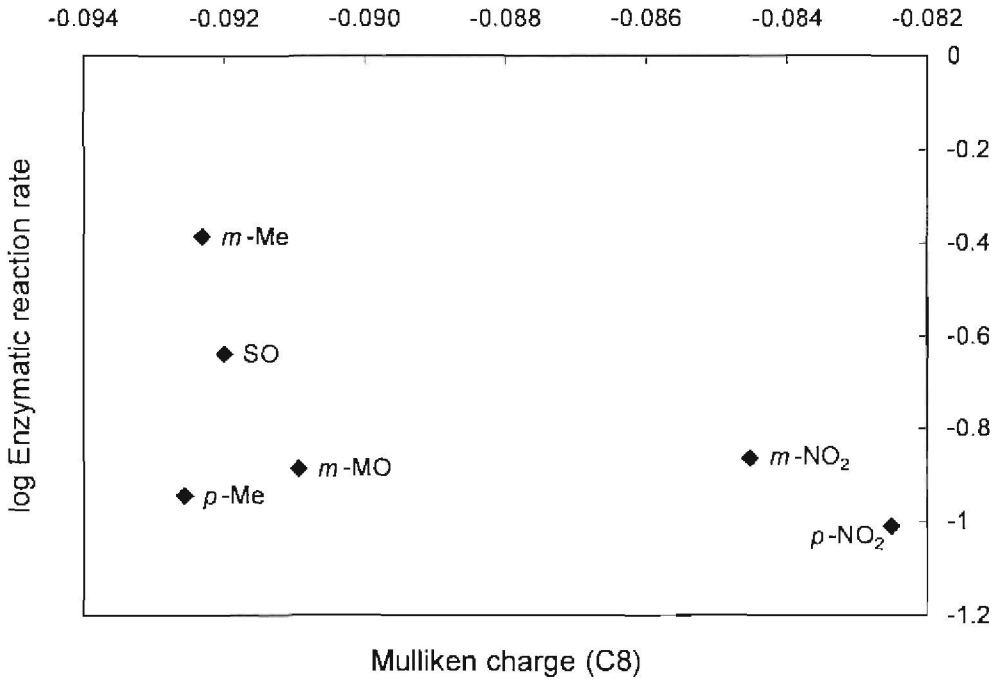


Figure 5-11 Mulliken charges calculated on (a) DFT and (b) semi-empirical level for the neutral epoxide C8 atom versus the logarithmic enzymatic reaction rate (mM.min $^{-1}$) for SO and SO derivatives.

Interestingly enough, C8 neutral epoxide Mulliken charges calculated on a semi-empirical level (**Figure 5-11b**) did not give such a good correlation with the enzymatic reaction rate, contrary to the chemical hydrolysis where Mulliken charge distribution calculated on a semi-empirical level gave the better correlation.

When comparing the enzymatic hydrolytic reaction rate for these substrates with the correlating chemical reaction or chemical parameters, one has to realise that the enzymatic reaction takes place in a chiral environment, which implies that both enantiomers aren't hydrolysed at equal rates and are competing for interaction with the enzymatic reaction site, and thus that the sum of two competitive reactions (reaction rate for the (R)-enantiomer and for the (S)-enantiomer) are used to calculate the reaction rate for the compound racemate, where during chemical hydrolysis both enantiomers react at the same rate.

The correlation observed between the enzymatic reaction rate and the DFT-level Mulliken charges on C8 of the non-protonated epoxide molecules confirm that *R. glutinis* EH follows a basic mechanism of hydrolysis of the epoxide ring as previously reported (Pedragosa-Moreau *et al.*, 1996:4599), and thus that nucleophilic attack preferentially takes place on the least substituted epoxide carbon (C8).

3.3 Binding site docking

Based on the amino acid sequence homology between the EH from *A. niger* and *Rhodotorula glutinis* (Kronenburg & De Bont, 2001:215) as well as the fact that both presented the same enantioselectivity towards the same range of substrates (Jin *et al.*, 2004:41; Yeates, 2003:678) it is fair to assume that these two EH enzymes are closely related and demonstrate the same mechanism of action. There is, however, one major difference, which is that the acid residue in the catalytic triad of *A. niger* (**Figure 5-12**) is an aspartame residue, but a glutamate in *R. glutinis* (Smit, 2004:127; Visser, 2000:418).

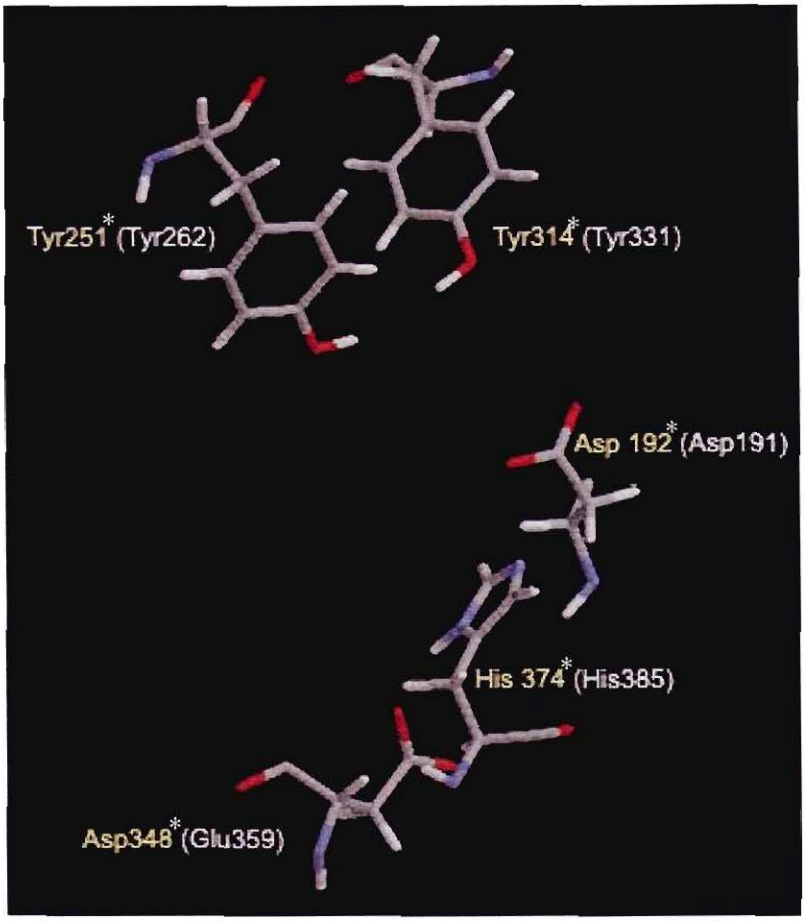


Figure 5-12 Catalytic triad of *A. niger** (yellow) and *R. glutinis* (white) EH.

Due to the difficulties experienced with crystallising membrane associated proteins such as the EH from *R. glutinis* (Kronenburg *et al.*, 1999:521), the structure of the bonding site is not available. The EH from *A. niger which* also belongs to the same family of microsomal EHs, could however be crystallized and thus the X-ray structure determined due to its soluble nature (Zou *et al.*, 2000:112). In view of the close proximity, the three-dimensional structure of *A. niger* EH was utilised instead for the simulated docking of the styrene oxide type epoxides.

Possible conformers for both the (R)- and (S)-enantiomers of the range of epoxides were generated using Catalyst by MSI. The EH binding site of *A. niger*, consisting of the catalytic triad, was identified by performing a possible site search using MSI Cerius II and then locating the three amino acids Asp192, His374 and Asp348 (Zou et al., 2000:116) present in the catalytic triad.

By docking both enantiomers of the range of epoxide substrates into the active site (**Appendix 3**) it can be observed that in all cases the (R)-enantiomer lies in a more optimal position relative to the aspartame residue of the catalytic triad in comparison to the (S)-epoxide. In all instances

the (R)-enantiomer epoxide oxygen (Tyr314-O and Tyr251-O) was also in a closer proximity to the tyrosine residues (involved in stabilisation of the epoxide) in comparison to the (S)-enantiomer epoxide oxygen (**Table 5-2**).

The same can be observed for the locality of the aspartame residue relative to C8. For all epoxides C8 in the (R)-enantiomer was in a closer proximity to the aspartame residue than it was for the (S)-enantiomer.

When looking at the proximity of C7 and C8 to the aspartame residue, C8, which is preferentially attacked, was in a closer vicinity to the aspartame residue than C7 in all instances except for (R)- and (S)-*p*-Me and (S)-*m*-NO_2. For both (R)- and (S)-*p*-Me C7 lies closer to the aspartame residue than C8 . Even though nucleophilic attack is electronically controlled, steric effects and spatial arrangement plays a major role in EH activity (Pedragosa-Moreau *et al.*, 1996:4599). This might explain why *p*-Me did not fit the linear relationship between the Mulliken charges on C8 and the enzymatic reaction rate (**Figure 5-11a**), and that nucleophilic attack is favored on C7 instead of C8 due to the spatial arrangement. Even though we can not prove this conclusion at this stage, a switch in regioselectivity has previously been observed for a range of styrene oxide type substrates with the EH of the fungus *Syncephalastrum racemosum* (Moussou *et al.*, 1998:3533).

As was expected, no relationship could be observed between the difference in interaction distances and the specific enantioselectivity over the whole range of substrates, but unfortunately no correlation could be found between the enantiomeric excess of some substrates of *A. niger* either. The difference between the EH activity and degree of selectivity of *A. niger* and *R. glutinis* was previously attributed to the difference in the active site residues (Zou *et al.*, 2000:111).

Table 5-2 Distance (Armstrong) between the catalytic triad amino acids of *A. niger* and the interacting epoxide atoms, correlated to the % *e.e.* and EH activity of *R. glutinis*.

	Tyr314 - O	Tyr251 - O	Asp192 - C8	Asp192 - C7	*R. glutinis*	*A. niger**	
					%ee	Time (min)	%ee
(S)-SO	4,18	4,95	3,47	4,45	66	215	75
(R)-SO	1,6	1,96	2,83	3,2			
(S)-*m*- NO_2	3,37	3,33	5,81	5,15	43	104	55
(R)-*m*- NO_2	1,58	1,99	3,01	3,13			
(S)-*p*- NO_2	2,34	3,11	2,84	3,05	26	109	94
(R)-*p*-NO_2	1,83	1,73	2,77	3,21			
(S)-*m*-Me	4,33	5,04	3,81	4,75	>98		
(R)-*m*-Me	1,45	2,18	2,75	3,13			
(S)-*p*-Me	3,82	4,73	3,02	4,01	48	43	70
(R)-*p*-Me	1,78	1,6	3,26	3,25			
(S)-*m*-MO	2,63	3,14	4,79	3,98	37		
(R)-*m*-MO	1,24	2,1	3,18	3,32			

Notes: (O) - epoxide oxygen

%ee for *R. glutinis* determined after a 60 minute reaction with a 20 mM initial epoxide concentration.

*: Jin *et al.*, 2004:410

The best enantioselectivity was observed for *R. glutinis* with *m*-Me. From substrate docking it can clearly be seen that the spatial arrangement is optimal for (R)-*m*-Me (**Figure 5-13**). The epoxide oxygen lies both in close proximity and almost in the center of the two tyrosine residues. According to the modeling results, the distance between the epoxide C8 and the aspartame group is also the smallest of all the substrates tested.

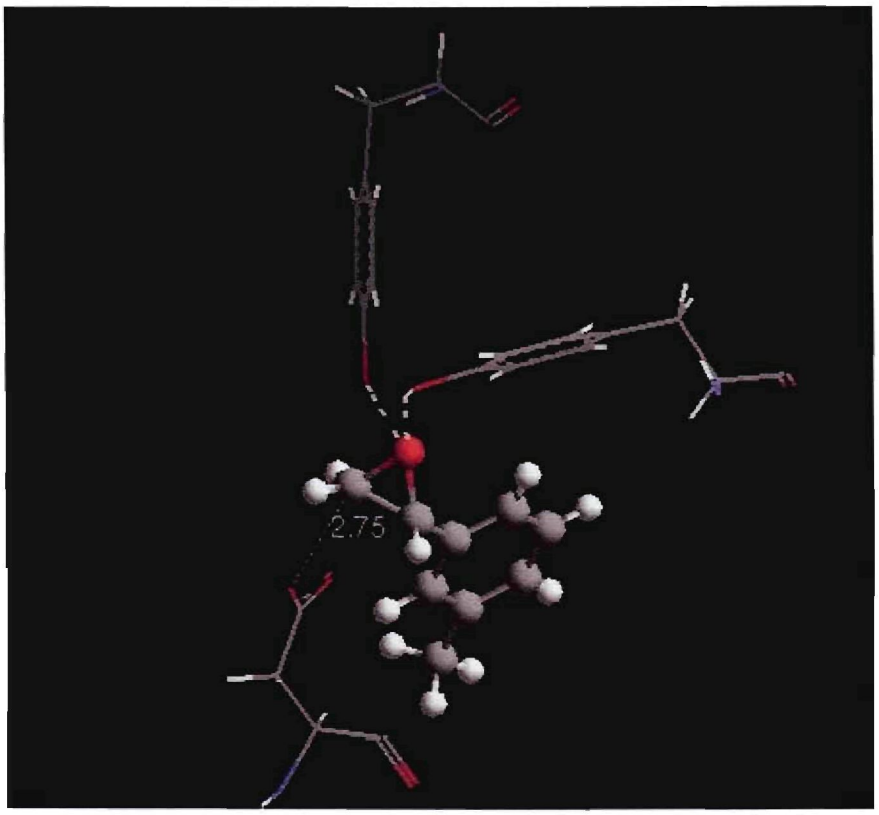


Figure 5-13 (R)-*m*-Me docked in the *A. niger* EH active site.

The (S)-*m*-Me epoxide oxygen on the other hand lies much further from the stabilising tyrosine groups (**Figure 5-14**) than the (R)-*m*-Me epoxide oxygen. The same can be observed for the distance between C8 and the aspartame residue. The distance between the stabilising tyrosine groups and the epoxide oxygen is the furthest for all the substrates tested. The difference observed between the proximity of the reacting epoxide moiety and the active site residues give an indication why the (R)-enantiomer is preferentially hydrolysed.

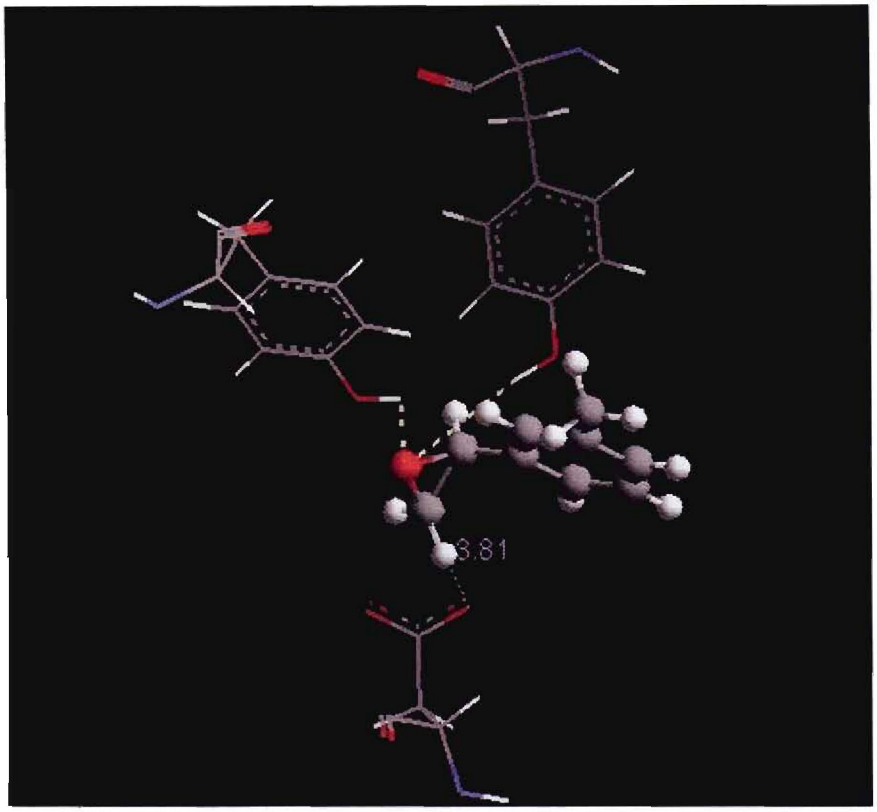


Figure 5-14 (S)-*m*-Me docked in the *A. niger* EH active site.

4 Conclusions

An increase in the HPB concentration (% w/v) lead to a substantial increase in both the solubility as well as epoxide hydrolase enzyme activity towards *para*-nitrostyrene oxide (p-NO_2) up to a critical HPB concentration. A significant increase of between 2,89 and 6,28 times was observed for the substrate range with the addition of 5 % HPB in comparison to a buffer solution which clearly displays the capacity of HPB to not only increase the solubility of organic compounds, but that an increase in the substrate solubility also has a positive effect on the substrate available for hydrolysis and thus increases the EH activity.

The chemical and enzymatic reaction rate of the microsomal EH of *R. glutinis* exhibited a negative linear correlation with the Hammett constant. Furthermore, was a linear relationship was observed between the Mulliken charge distribution over the most substituted epoxide carbon calculated on a semi-empirical level over the protonated epoxides (in an acid environment) and the chemical hydrolytic reaction rate for the series of substituted styrene oxides. This correlation corresponds with the mechanism of acid induced chemical hydrolysis, where the preferential site of nucleophilic attack is on the most substituted oxirane carbon atom (Hanzlik & Westkaemper, 1980:2465).

The same linear relationship was observed for the charge distribution over the neutral/non-protonated least substitutes epoxide carbon (excluding *p*-methylstyrene oxide). Even though nucleophilic attack can take place on either epoxide carbon atoms during the EH mediated enzymatically catalysed reaction, this linear relationship corresponds with the preferential nucleophilic attack of the least substituted epoxide carbon atom through a general base catalysed mechanism (Smit & Labuschagne, 2006:1151).

Docking of the possible conformers of the (R)- and (S)-enantiomers of these *meta* and *para* substituted styrene oxides into the EH binding site of the closely related *Aspergillus niger* displayed a closer and more preferential fit of the (R)-epoxides which is the faster reacting epoxide for both *A. niger* as well as *R. glutinis* EHs.

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Chapter 6

Conclusion

Abstract

The aim of this study was to increase the understanding of the factors that influence red yeast epoxide hydrolase activity. Different growth media had varying effects on both the biomass production as well as the expressed epoxide hydrolase activity of *R. glutinis* and *R. toruloides*. For a range of *meta*- and *para*-nitro, methyl and methoxy substituted styrene oxides epoxide hydrolase selectively hydrolysed the (R)-enantiomers with the highest activity displayed towards *meta*-methylstyrene oxide which was correlated to the electronic properties of the methyl substituent.

Keywords: *R. glutinis*, *R. toruloides*, epoxide hydrolase, activity, selectivity

Table of Contents

1 Introduction

In order to increase the understanding of the factors that influence biomass production, the activity and selectivity of the expressed epoxide hydrolase (EH) of the two closely related red yeasts *Rhodosporidium toruloides* and *Rhodotorula glutinis*, the effect of different growth media, the selectivity of *R. glutinis* towards a range of substituted styrene oxide derivates and the relationship between the electronic properties of the styrene oxide substituent groups and enzyme activity was investigated.

2 Activity-growth relationship of epoxide hydrolysing biocatalysts

The different growth media (YM, YMvit and malt) had an explicit effect on the growth time and biomass production of *R. glutinis* with only a slightly more active EH enzyme produced in the YMvit medium, where an activity of 0,26 $mM.min^{-1}$ and selectivity of % *e.e.* = 28 was observed. The highest amount of biomass was attained from the YM medium (64,9 ± 1,3 $mg.mL^{-1}$). No decreased enzyme activity was observed during the stationary phase in any of the growth media over the observed times. Even though a higher enzyme activity was observed for the YMvit grown biocatalyst, the 30 % higher biomass production attained from the YM medium rendered this the medium of choice.

For *R. toruloides*, the YM medium also produced the highest amount of biomass (130,8 ± 4,8 $mg.ml^{-1}$), but the three media produced biocatalysts with almost equal EH activity (average conversion 0,75 ± 0,01) and selectivity (average % *e.e*; 93,57 ± 1,53). The single malt medium again delivered the lowest amount of biomass (75,7 ± 1,6 $mg.mL^{-1}$). Both the YM and YMvit media reached the optimum activity after depletion of the glucose supplement, in comparison to the activity maximum being reached in stationary phase for the malt medium.

The malt grown catalysts did not only deliver the lowest biomass for both *R. glutinis* and *R. toruloides*, but also delivered an enzyme with decreased activity (0,17 $mM.min^{-1}$) in comparison to the YM and YMvit media for *R. glutinis*. This illustrates that either glucose or the other ingredients of the YM medium (peptone and yeast extract) is clearly needed to attain *R. glutinis* EH enzymes with high activity.

From these results it can also be deduced that the growth medium producing the highest biomass does not necessarily render enzymes with the highest activity and selectivity. Seeing that the enzyme activity varied substantially throughout the growth period for these two red yeasts, it is clear that all parameters (growth time and growth media) have to be individually

optimised for even closely related organisms, not only to deliver high quantities of biocatalyst, but also high quality (active and enantioselective) biocatalysts.

The results also demonstrate that the addition of expensive additives such as the vitamin solution (YMvit) might not have a big enough advantage to justify the cost associated with it as can clearly be observed for *R. toruloides*.

To determine the effect of glucose on the growth, activity and selectivity of these organisms, the glucose concentration in the YM media was varied between 0 and 2,0 % (w/v). The growth and EH activity of the two biocatalysts were again not equally affected by the available energy sources.

R. glutinis displayed no real increase in the time for the growth to reach stationary phase between the different glucose concentrations (72 to 76 hours for all glucose concentrations), while the 0,5 and 1,0 % (m/v) glucose concentrations delivered 48,6 ± 1,1 and 48,4 ± 0,3 $mg.mL^{-1}$ biomass respectively and the 1,5 % glucose additive YM medium produced a much higher biomass concentration of 60,0 ± 0,9 $mg.mL^{-1}$ wet biomass. Interestingly, the highest glucose concentration 2,0 % (m/v) yielded a slightly lower (56,0 ± 1,7$mg.mL^{-1}$) wet biomass. This drop in biomass production with the 2,0 % (w/v) glucose concentration was also previously observed by Cantarella *et al.* (2002:409) where biomass production decreased after a critical glucose concentration, which was ascribed to glucose mediated growth repression.

For all glucose concentrations, EH activity increased after the stationary phase was reached with no significant drop in the enzyme activity even at 144 hours. During the stationary phase the activity stabilised with no considerable difference between EH activity for the lower glucose concentrations (0,24 $mM.min^{-1}$ for 0,5 % and 0,23 $mM.min^{-1}$ for both 1,0 and 1,5 % (w/v) glucose concentrations), while the highest concentration (2,0 % (m/v)) rendered a biocatalyst with an almost 20 % higher activity (0,29 $mM.min^{-1}$) at 144 hours growth time.

Thus, for a significantly faster reaction with high selectivity, but compromising on yield, 2,0 % (w/v) glucose would be the additive concentration of choice, while 1,5 % (w/v) glucose additive would lead to a slower reaction rate, and a higher biomass yield. Irrespective of the added glucose concentration, an increase in growth time had a positive effect on both enzyme activity as well as selectivity for *R. glutinis* EHs.

Both the growth and EH activity of *R. toruloides* were more explicitly influenced by the increased initial glucose concentration. The time needed to attain the stationary phase increased

progressively (40 to 96 hours) with an increase in glucose with maximum wet biomass weights of 64,3 ± 2,9, 127,2 ± 0,4, 130,8 ± 4,8 and 150,5 ± 3,5 $mg.mL^{-1}$ respectively for 0,5, 1,0, 1,5 and 2,0 % (m/v) initial glucose concentration at the stationary phase.

An increase in EH activity and selectivity was observed for all initial glucose concentrations between 12 and 36 hours with the optimum EH activity being reached between 24 and 36 hours followed by a drop in the activity between 36 and 144 hour growth times. The highest activity was observed for 1,0 % (m/v) initial glucose concentration between 24 and 72 hours. The most pronounced decrease in EH activity was observed for the 2,0 % (w/v) glucose concentration with a drop in conversion of almost 62 % between 36 and 144 hours growth time which can be related to glucose mediated catabolic repression, where after a critical point the glucose represses EH activity. Thus, an increase in glucose concentration leads to a higher concentration biomass, but the production of a less effective enzyme.

Even though the overall highest activity was observed with a 1,0 % (w/v) glucose concentration at 36 hours, the 1,5 % (w/v) glucose concentration grown biocatalyst delivered a conversion of 0,73 even after 144 hours growth time from 0,82 at 36 hours, in comparison to a conversion of 0,59 for the 1,0 % additive concentration grown biocatalyst at 144 hours. Thus, to attain the biocatalyst with the highest EH activity, *R. toruloides* should be harvested at around 36 hours growth time, but at a high loss of possible biomass, and in that case a very low glucose concentration 1,0 % (w/v) can be added. If more biomass is required the ideal % glucose added will be 1,5 %, and harvested after stationary phase when the maximum biomass is reached.

This difference observed for *R. glutinis* and *R. toruloides* under the influence of different glucose concentrations clearly emphasise the importance of glucose to attain a high biomass production, but also demonstrates the possible devastating effect that glucose might have on EH activity as observed for *R. toruloides*.

From these experiments YM medium with a 2,0 % w/v glucose concentration , and YM medium with a 1,5 % w/v added glucose was chosen as the optimum media for *R. glutinis* and *R. toruloides* respectively.

3 Production and VCD analysis of styrene oxide derivatives

In our laboratory, *R. glutinis* EH was previously shown to exhibit selectivity towards nitro substituted styrene oxides (Yeates *et al.*, 2003:678) and the question was raised if the substitution of the nitro substituent on the phenyl ring with more electron donating groups like

methyl and methoxy would positively influence the enzymatic reaction rate. Grogan *et al.* (1997:255) studied the hydrolysis of a series of methyl and chloro substituted styrene oxides by *Beauveria densa* EH and observed that reaction rate was greatly affected by substituent position, decreasing in the order *para* > *meta* > *ortho*. In order to investigate the effect of the substitution pattern and substituent groups on *R. glutinis* EH activity, a range of *meta*- and *para*-substituted styrene oxides were firstly synthesised (nitro-, methyl- and methoxystyrene oxide). *R. glutinis* EH mediated kinetic resolution (biocatalysts grown in YM medium with 2,0 % glucose concentration) was used to produce an enantiopure epoxide, and the absolute configuration of the resultant epoxides determined by VCD of the substrates that reached the highest enantiopurity. The relationship between substitution pattern and EH activity will be discussed later.

The range of *meta* and *para* substituted styrene oxides were successfully synthesised and *R. glutinis* EH was found to enantioselectively hydrolyse all tested substrates, except for *p*-methoxystyrene oxide which could not be chirally analysed and was found to undergo spontaneous hydrolysis at a rate faster than enzymatic hydrolysis which makes it not a suitable substrate for EH mediated hydrolysis under these conditions.

A very fast reaction rate with extremely high selectivity was observed for *m*-methylstyrene oxide that reached a % *e.e.* of >98 within 60 minutes, with an exceptionally high yield of 42,5 %, compared to a % e.e. of >98 for *p*-methylstyrene oxide, after 240 minutes, and a low yield of 7,2 %. The kinetic resolution of *m*-methoxystyrene oxide yielded 41,5 % pure epoxide with a 98 % *e.e.* within a reaction time of 240 minutes.

The time taken for the EH mediated biocatalytic reaction to reach the maximum possible enantiomeric excess or a % e.e. of more than 98 for the different substrates was in the order of *m*-Me > SO > *p*-Me ≈ *m*-MO > *m*-NO_2 > *p*-NO_2. Overall higher activity was observed towards the *meta* substituted epoxides in comparison to the *para* substituted epoxides which is in accordance with the results of Grogan *et al.* (1997:255), and higher activity towards methyl substituted styrene oxides than the nitro substituted derivatives.

The absolute configuration of the residual epoxide enantiomers of *m*-NO_2, *m*-Me and *m*-MO was determined by comparing the measured VCD spectra of the samples with that of the *ab initio* quantum calculation of the randomly chosen (S)-enantiomers. For all three *meta* substituted styrene oxide derivatives the comparison between the calculated Boltzmann-population-weighted composite IR and VCD spectra for the (S)-enantiomer and the experimental

measurements for the samples establishes the absolute configuration of the experimental sample as (S).

From these results and the previously determined absolute configurations of the resultant epoxide of styrene oxide (Yeates *et al.*, 2003:678) and *p*-nitrostyrene oxide (Yeates 2001:65), the selectivity of *R. glutinis* EH towards SO and SO substituted derivatives at 15 °C has been proven to be towards the (R)-epoxide, thus rendering the unreacted residual (S)-epoxides. Although the absolute configuration of *p*-methylstyrene oxide has not been determined, it is expected that the resultant epoxides would also be of the (S)-configuration due to the so far proven selectivity of *R. glutinis* towards these substrates by Yeates and co-workers, and during this study, as well as the GC elution order of the two enantiomers for *p*-methylstyrene oxide where the first eluded enantiomer decreases more rapidly for all the substrates tested.

These results also display the advantage of VCD analysis for the determination of absolute configuration of small molecules in the solution state, where X-ray crystallography would not have been possible due to the absence of a crystal.

4 Solubility and modeling of chemical and enzymatic hydrolysis

In an attempt to further increase the EH activity of *R. glutinis* towards the range of substrates, the effect of an increasing hydroxypropyl-β-cyclodextrin (HPB) concentration on both substrate solubility and EH activity was investigated.

Furthermore, as a number of observations were previously made regarding the activity of substituted styrene oxides (*meta* > *para* and methyl > nitro), as well as the selectivity of the enzyme ((R) > (S)), it was attempted to correlate the enzymatic reaction rate as well as the enantioselectivities displayed for the range of substrates to both the electronic properties of the substituents and the spatial arrangement of the substrate in the enzyme active site.

An increase in the HPB concentration (% w/v) lead to a substantial increase in both the solubility as well as enzyme activity of *para*-nitrostyrene oxide (p-NO_2) up to a critical HPB concentration where the increase flattened out. It is believed that the levelling of the reaction rate is due to the saturation concentration of *p*-NO_2 increasing above the amount of *p*-NO_2 in solution at that point, and that an increase in the solubility of the substrate should not cause a further increase in the substrate concentration available for hydrolysis. A significant increase of between 2,89 and 6,28 times was observed for the substrate range with the addition of 5 % HPB in comparison to a buffer solution. This clearly displays the capacity of HPB to not only

increase the solubility of organic compounds, but that an increase in the substrate solubility also has a positive effect on the substrate available for hydrolysis and thus increases the EH activity. The chemical and enzymatic reaction rate of the microsomal EH of *R. glutinis* exhibited a negative linear correlation with the Hammett constant. This negative correlation is in accordance with an increased chemical reaction rate with an increase in the electron donating properties of the styrene oxide substituent groups (*p*-Me > *m*-Me > SO > *m*-MO > *m*-NO_2 > *p*-NO_2). The Mulliken charge distribution over the most substituted epoxide carbon calculated on a semi-empirical level over the protonated epoxides (in an acid environment) gave a linear correlation to the chemical hydrolytic reaction rate for the series of substituted styrene oxides. This correlation corresponds with the mechanism of acid induced chemical hydrolysis, where the preferential site of nucleophilic attack is on the most substituted oxirane carbon atom (Hanzlik & Westkaemper, 1980:2465).

The same linear relationship was observed between the charge distribution over the neutral/non-protonated least substitutes epoxide carbon (excluding *p*-methylstyrene oxide) and the enzymatic reaction rate. Even though epoxide hydrolase mediated nucleophilic attack can take place on either epoxide carbon atoms, this linear relationship corresponds with the preferential nucleophilic attack of the least substituted epoxide carbon atom through a general base catalysed mechanism (Smit & Labuschagne, 2006:1151).

Docking of the possible conformers of the (R)- and (S)-enantiomers of these *meta* and *para* substituted styrene oxides into the EH binding site of the closely related *Aspergillus niger* displayed a closer and more preferential fit of the (R)-epoxides which is the faster reacting epoxide for both *A. niger* as well as *R. glutinis* EHs.

This study demonstrated the relationship between the electronic properties of the substituent groups of styrene oxide derivatives and the enzymatic and chemical hydrolytic reaction rates. An increase in the electron-donating properties of the styrene oxide substituent groups were correlated with an increase in both the chemical as well as the *R. glutinis* EH mediated hydrolysis reaction rates of the styrene oxide derivatives.

This could open up the possibility to correctly predict both the enantioselectivity as well as the activity of *R. glutinis* EHs, and possibly other yeast EHs, towards more complex epoxide substrates without the need of time consuming screenings.

5 Conclusion

The aim of this study was to increase the understanding of the factors that influence red yeast EH activity. The first objective was to investigate and optimise the growth media for the production of both *R. glutinis* and *R. toruloides* and their epoxide hydrolysing enzymes. It was clearly demonstrated that the effect of growth conditions and additives on the EH enzyme activity and selectivity as well as the biomass production vary substantially for even closely related organisms and need to be individually optimised to obtain the highest amount of biomass that express an enzyme with high activity.

Secondly, *R. glutinis* EH was found to catalyse the kinetic resolution of the whole range of *meta* and *para* nitro-, methyl-, and methoxysubstituted styrene oxides tested, with highest selectivity towards the (R)-epoxides and activity towards the *meta* substituted derivatives in the order of methyl > methoxy > nitro.

To our knowledge this was the first time that *m*-methoxystyrene oxide was utilised as a substrate for biocatalytic EH mediated kinetic resolution and that the absolute configuration of these compounds were determined by VCD analysis.

Thirdly, we succeeded in correlating the chemical and enzymatic hydrolysis reaction rates, as well as enzymatic enantioselectivity of the *R. glutinis* EH respectively to the electronic properties of their substituents and the spatial arrangement of the substrates in relation to the EH catalytic triad.

6 Future work

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

- Since this study has focused on the epoxide production, the absolute configuration of the diol products of the *R. glutinis* EH mediated kinetic resolution of *meta*- and *para*-nitro-, methyl-, and methoxystyrene oxide will have to be determined in order to verify the potential of this enzyme to produce enantiopure diol products.

- The kinetic resolution of both pure enantiomers will have to be conducted individually and the diol product analysed to confirm the regeoselectivity of *R. glutinis* EH.

- To verify the attained relationship and effect of substrate substitution over a range of substrates, kinetic resolution of the individual enantiomers will have to be conducted in order to counteract the competitive binding of the different substrate enantiomers to the EH binding site.

- In order to substantiate the use of quantum mechanical calculations for the prediction and correlation of the enzymatic reaction rate of EH activity, the same range of experiments should be conducted on another EH enzyme with the same series of substrates.

7 References

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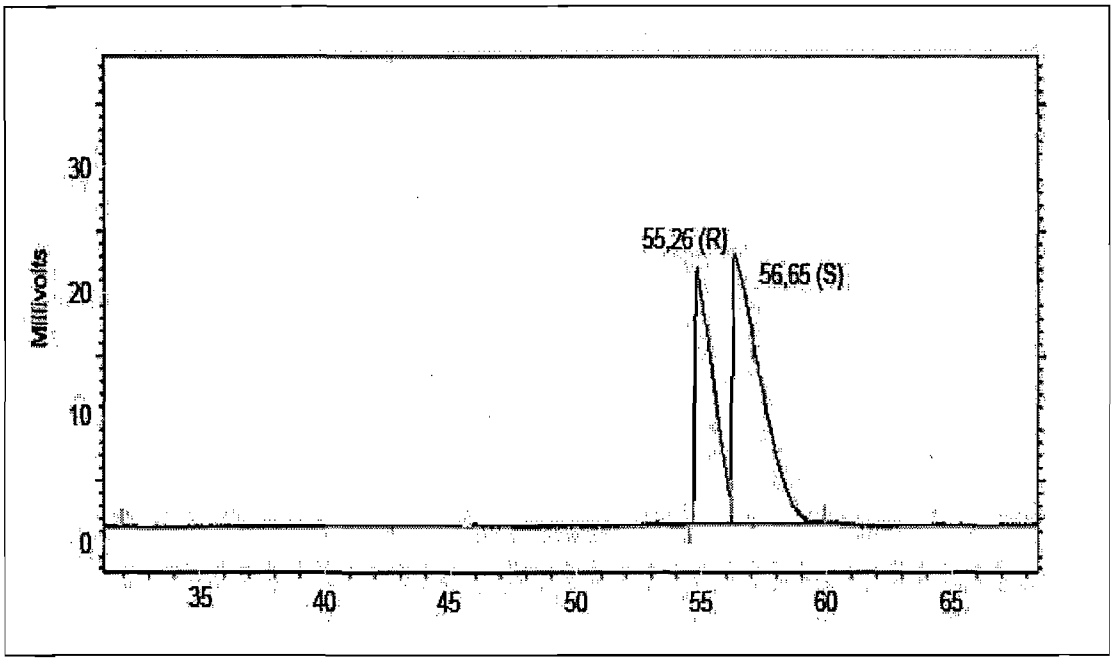
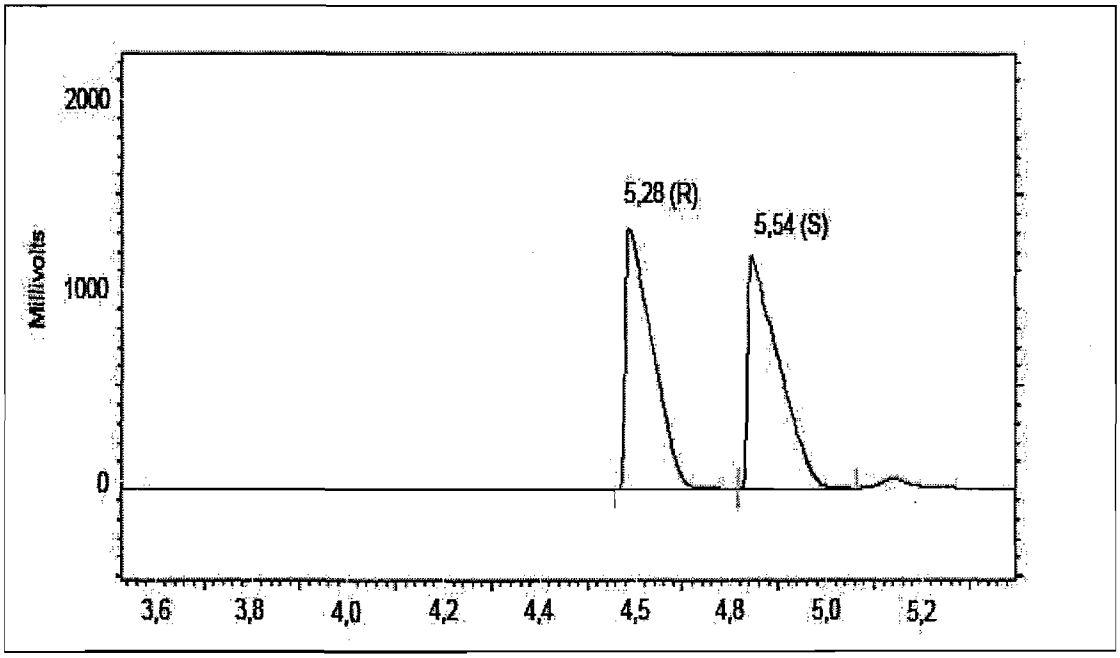
Appendix 1

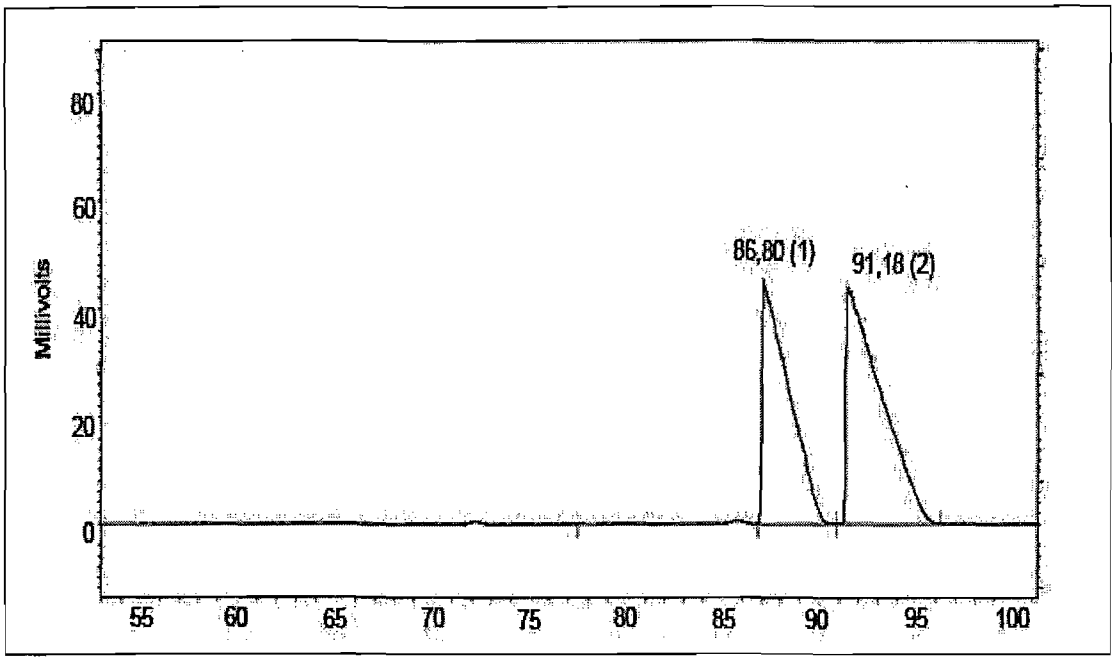
All the results shown were obtained using a ThermoFinnigan® Focus GC equipped with a FID and a Thermo Electron AS 3000 Series auto sampler on a fused silica cyclodextrin Chirasil-DEX-CB Varian® capillary column, utilising H_2 as carrier gas.



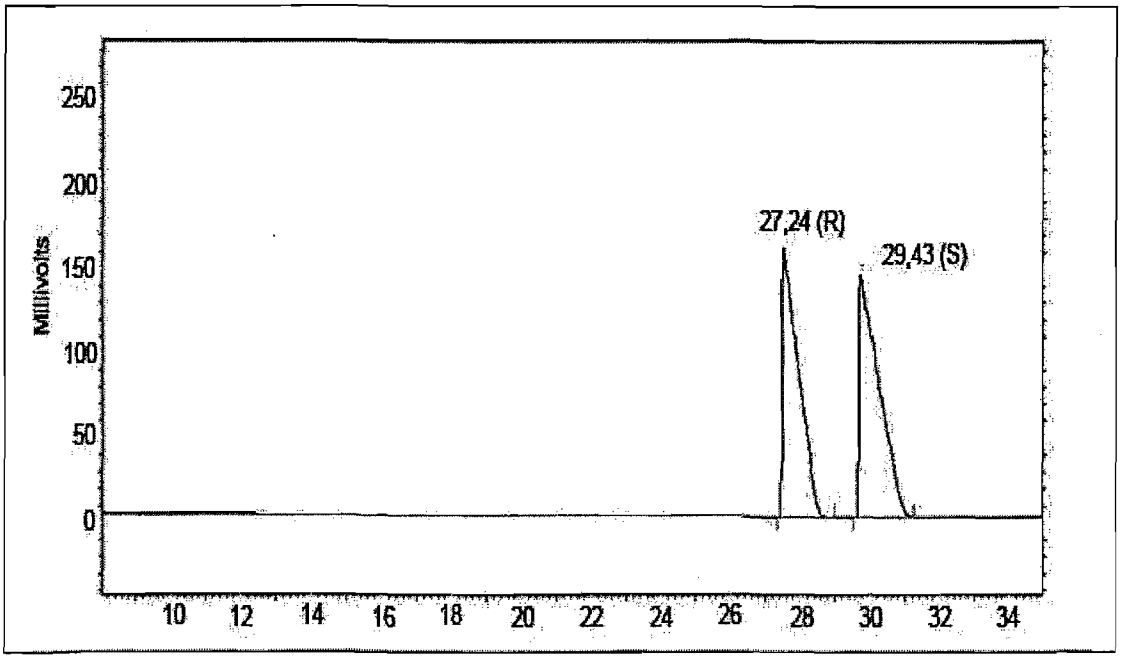
Chromatogram 1 Racemic mixture of 1,2-epoxyoctane.



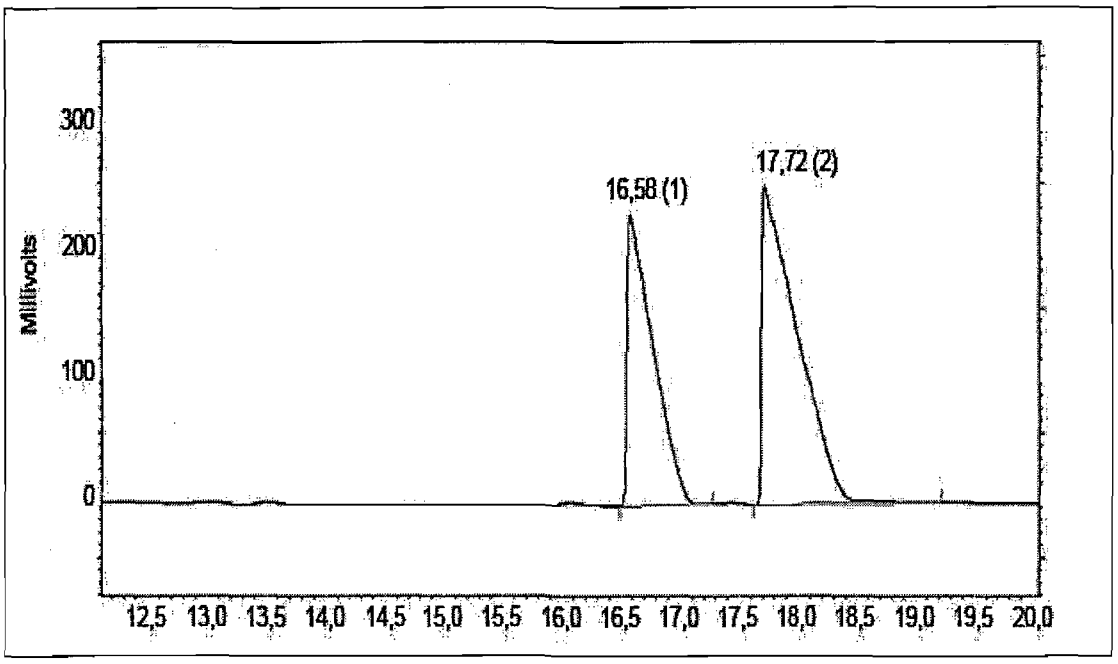
Chromatogram 2 Racemic mixture of styrene oxide.



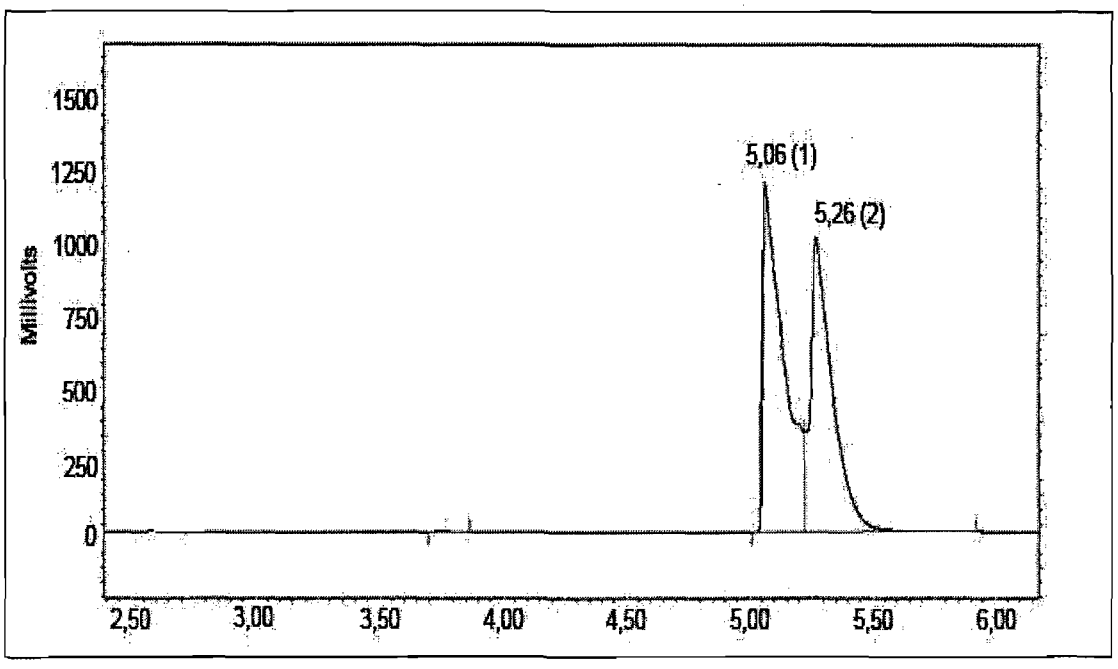
Chromatogram 3 Racemic mixture of *m*-nitrostyrene oxide.



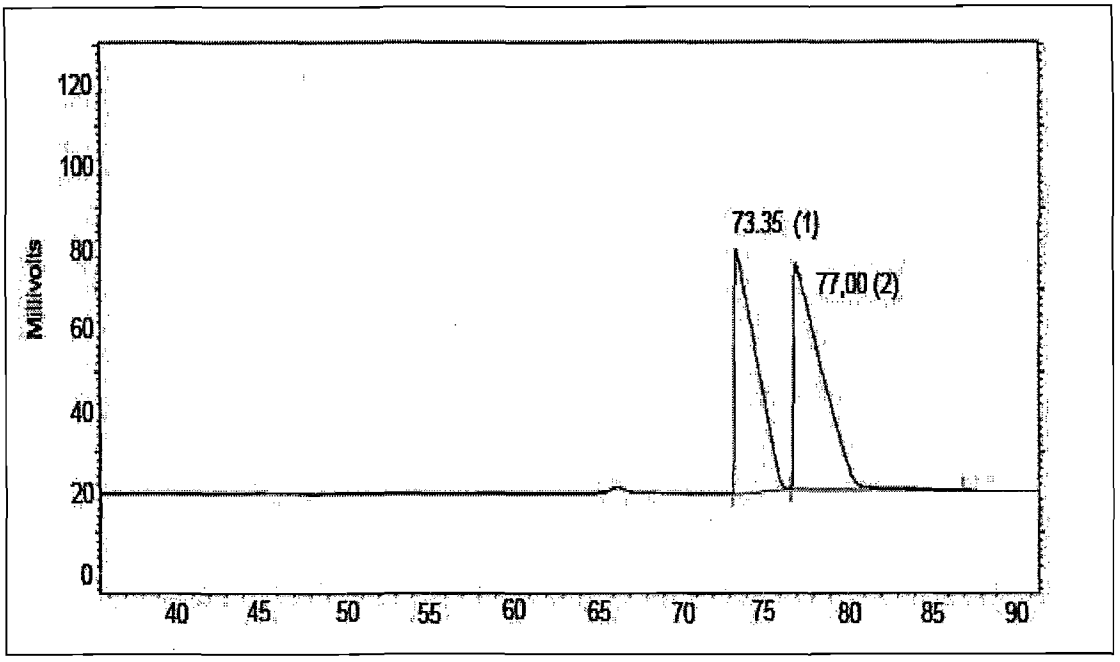
Chromatogram 4 Racemic mixture of *p*-nitrostyrene oxide.



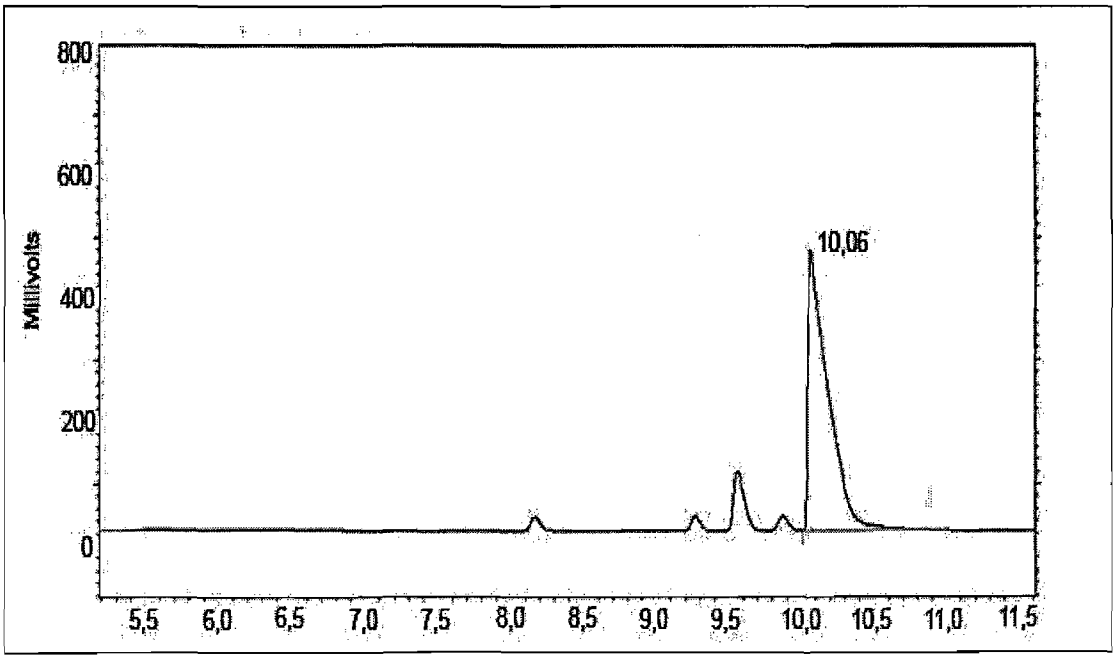
Chromatogram 5 Racemic mixture of *m*-methylstyrene oxide.



Chromatogram 6 Racemic mixture of *p*-methylstyrene oxide.



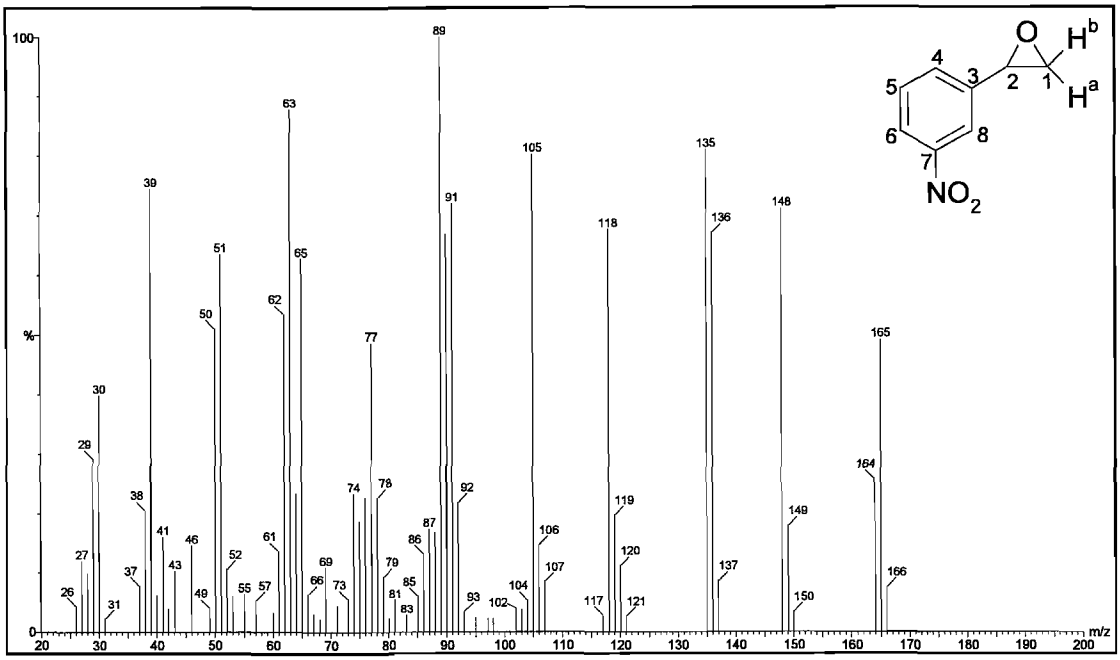
Chromatogram 7 Racemic mixture of *m*-methoxystyrene oxide.



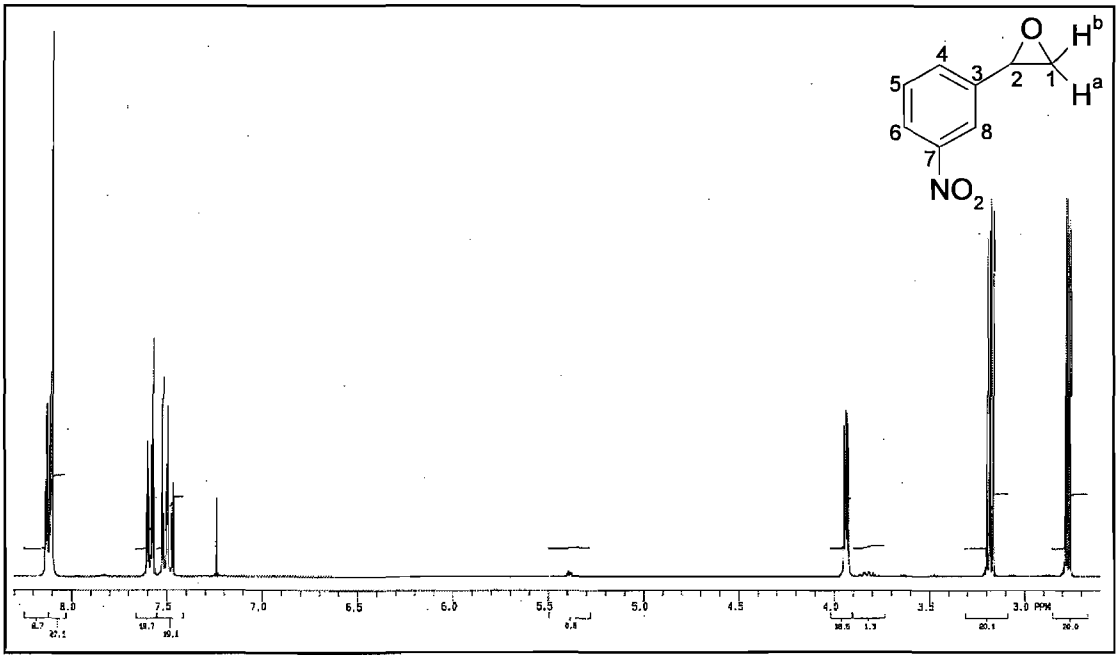
Chromatogram 8 Racemic mixture of *p*-methoxystyrene oxide.

Appendix 2

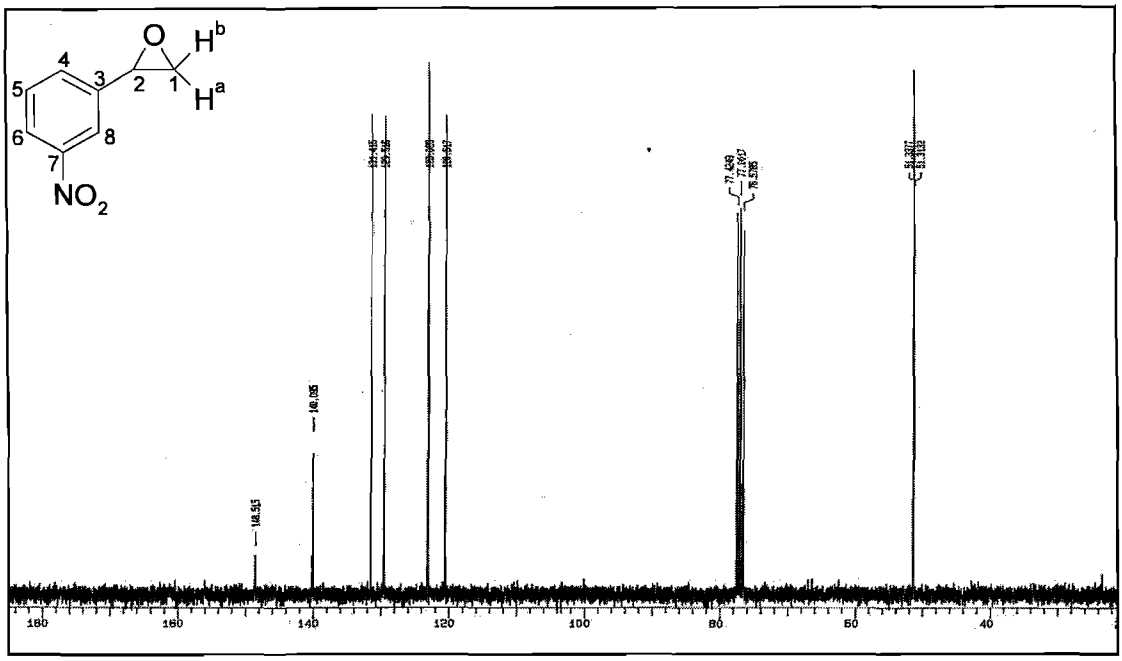
^{1}H and ^{13}C NMR spectra, MS and IR spectra of *meta*-nitrostyrene oxide (*m*-NO_2) oxide, *para*-nitrostyrene oxide (*p*-NO_2), *meta*-methylstyrene oxide (*m*-Me), *para*-methylstyrene oxide (*p*-Me), *meta*-methoxystyrene oxide (*m*-MO) and *para*-methoxystyrene oxide (*p*-MO).



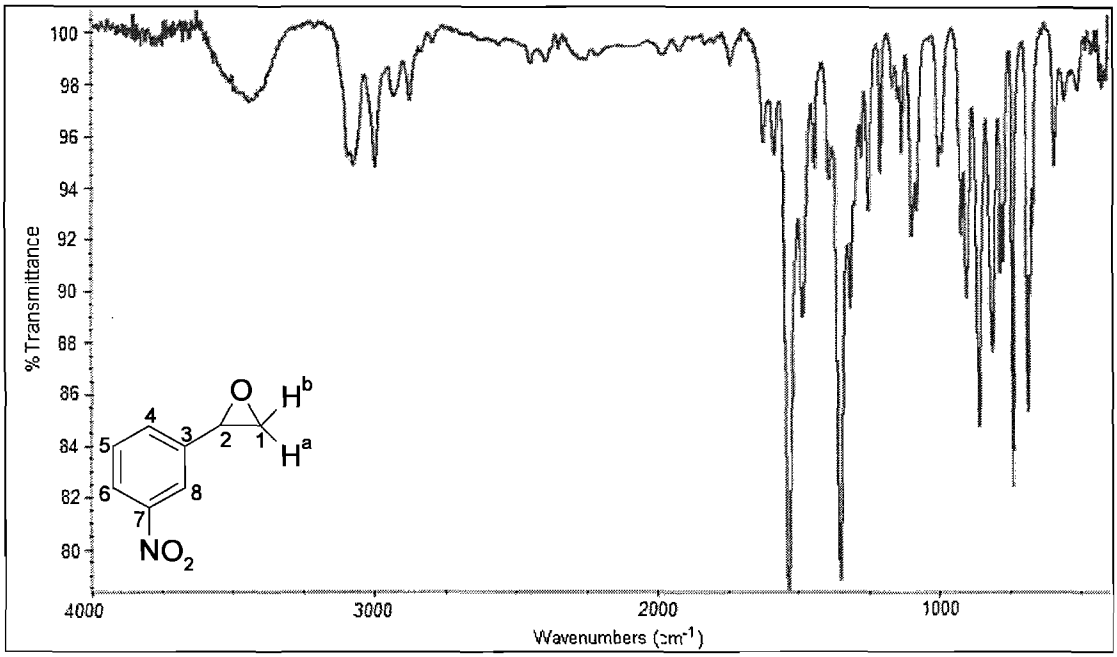
Spectrum 1 MS spectrum of *m*-NO_2.



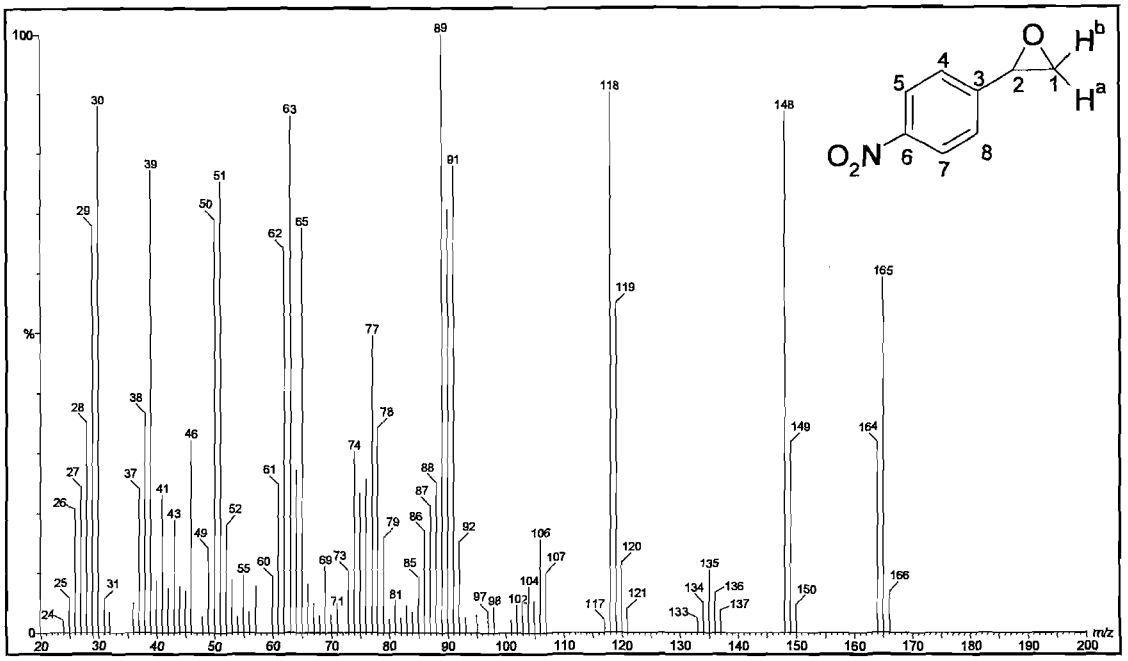
Spectrum 2 ^{1}H NMR spectrum of *m*-NO_2 ($CDCl_3$).



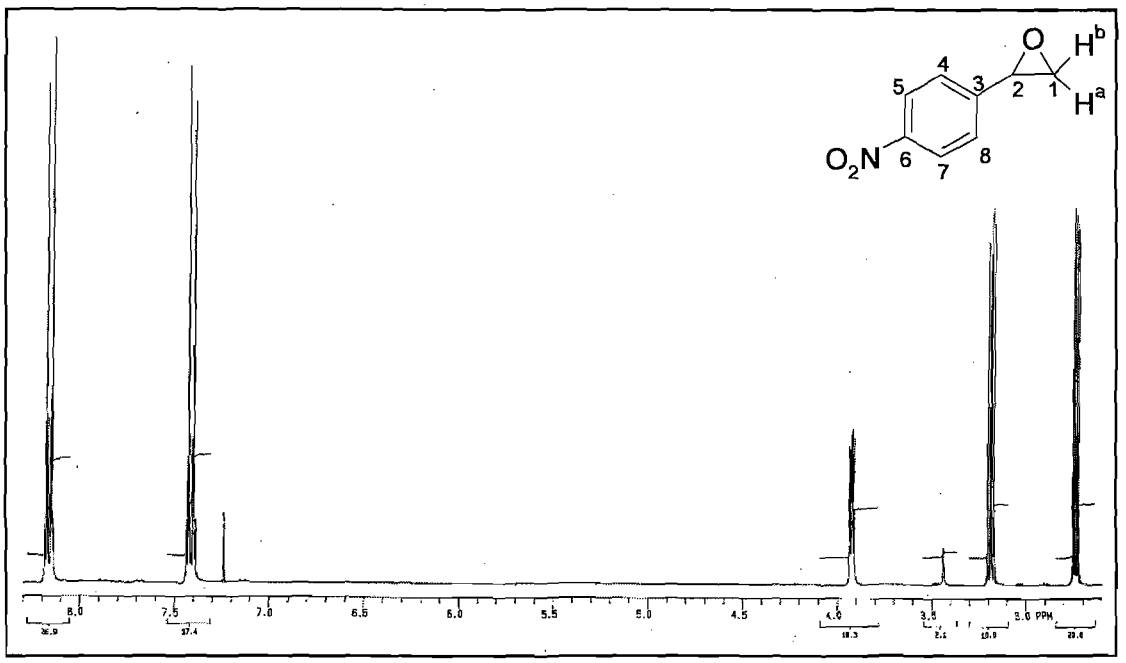
Spectrum 3 ^{13}C NMR spectrum of *m*-NO_2 ($CDCl_3$).



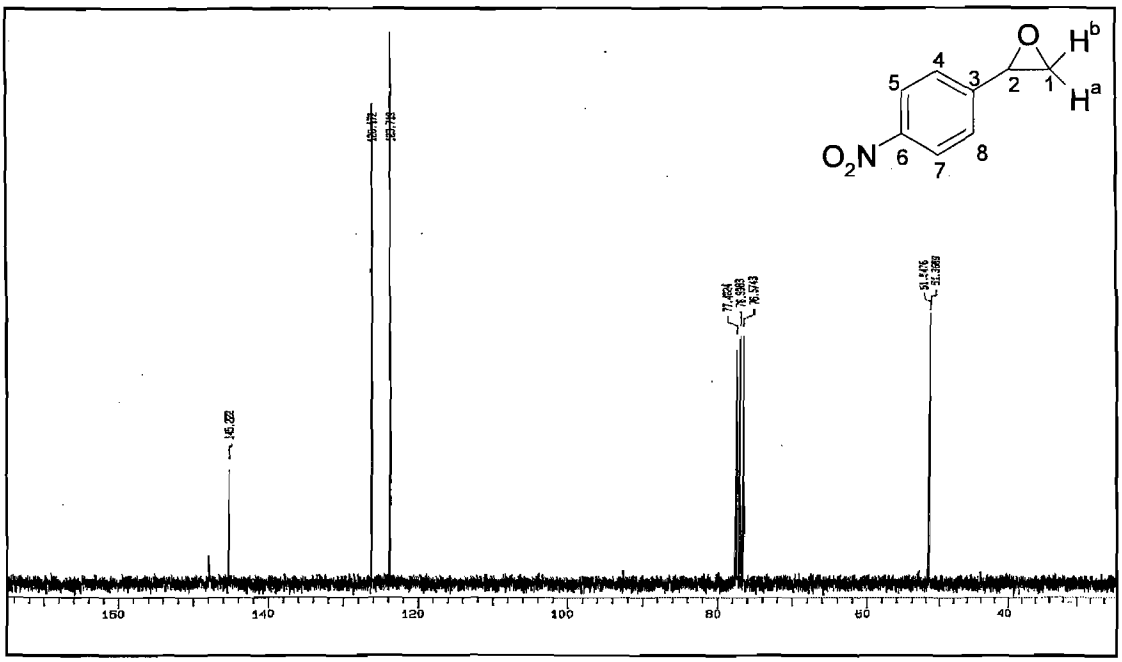
Spectrum 4 IR spectrum of *m*-NO_2.



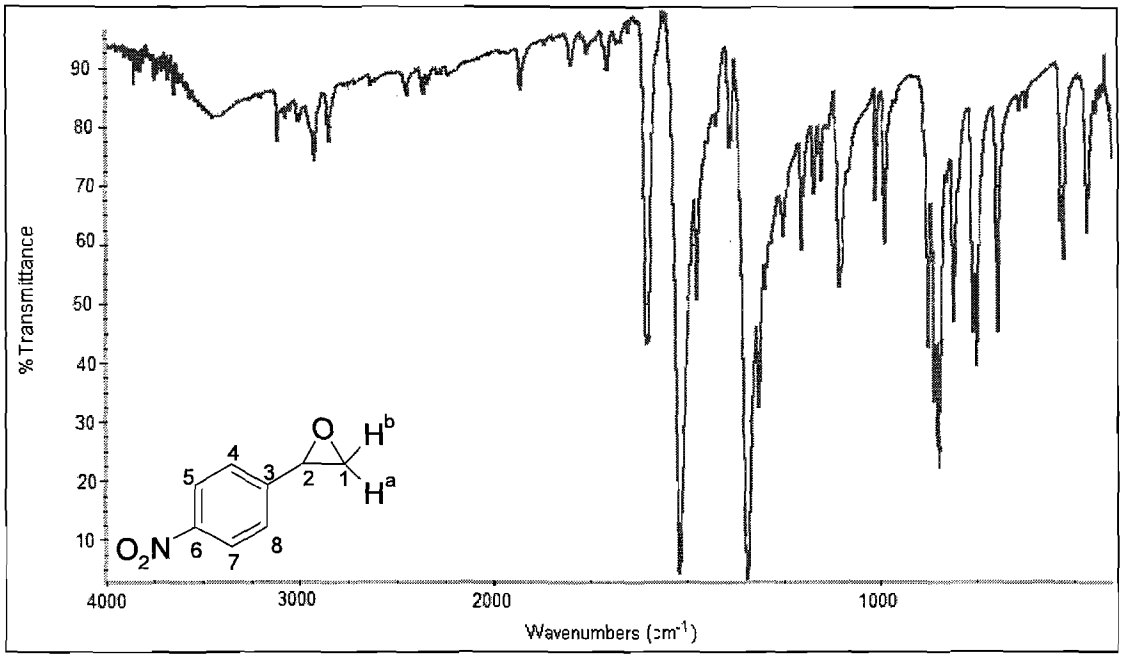
Spectrum 5 MS spectrum of *p*-NO_2.



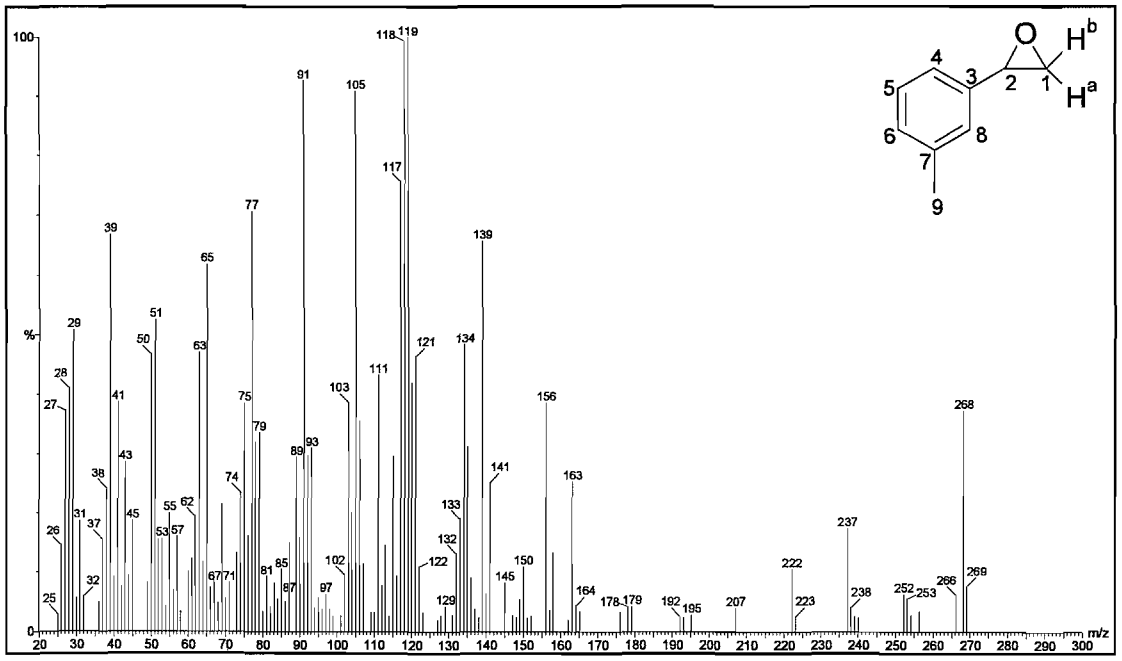
Spectrum 6 ^{1}H NMR spectrum of *p*-NO_2 ($CDCl_3$).



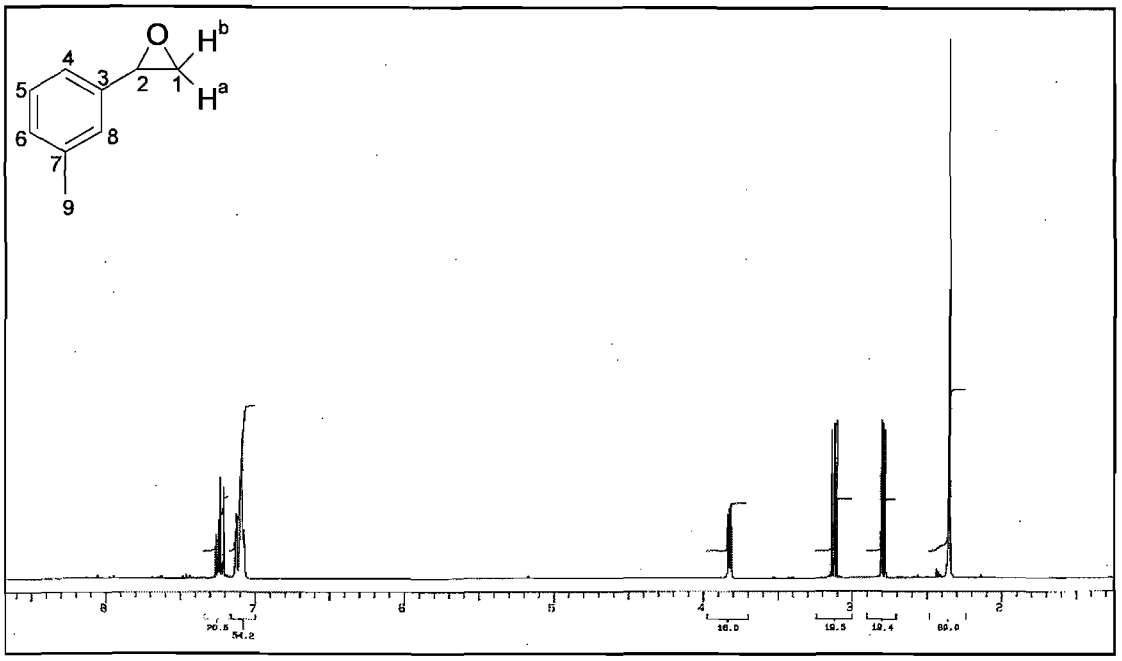
Spectrum 7 ^{13}C NMR spectrum of *p*-NO_2 ($CDCl_3$).



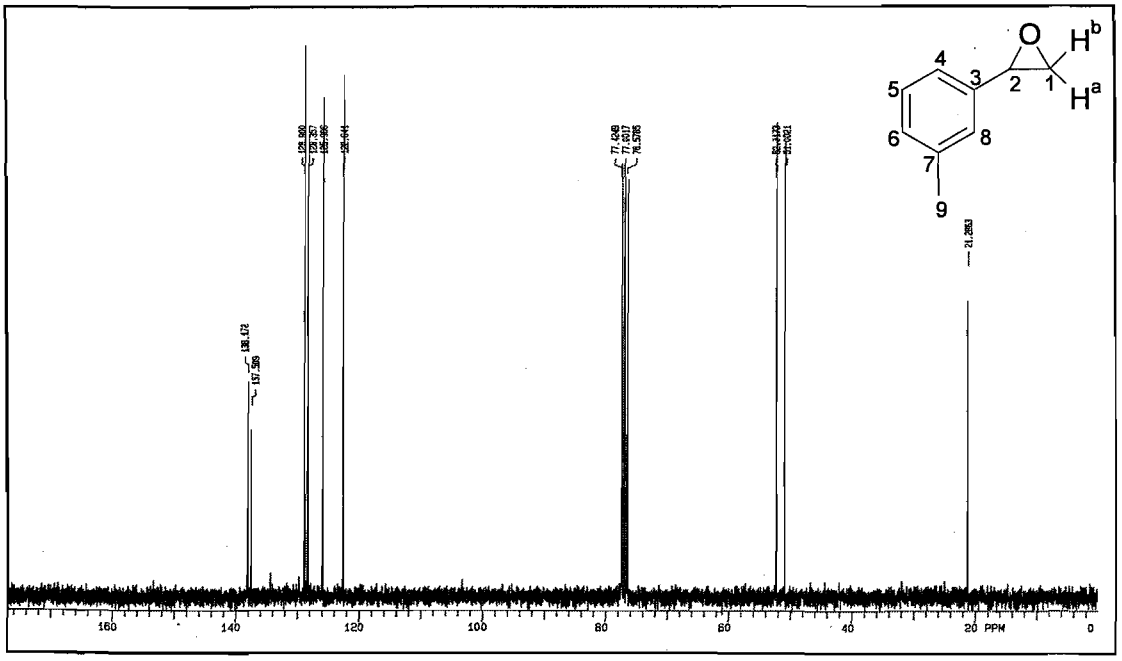
Spectrum 8 IR spectrum of *p*-NO_2



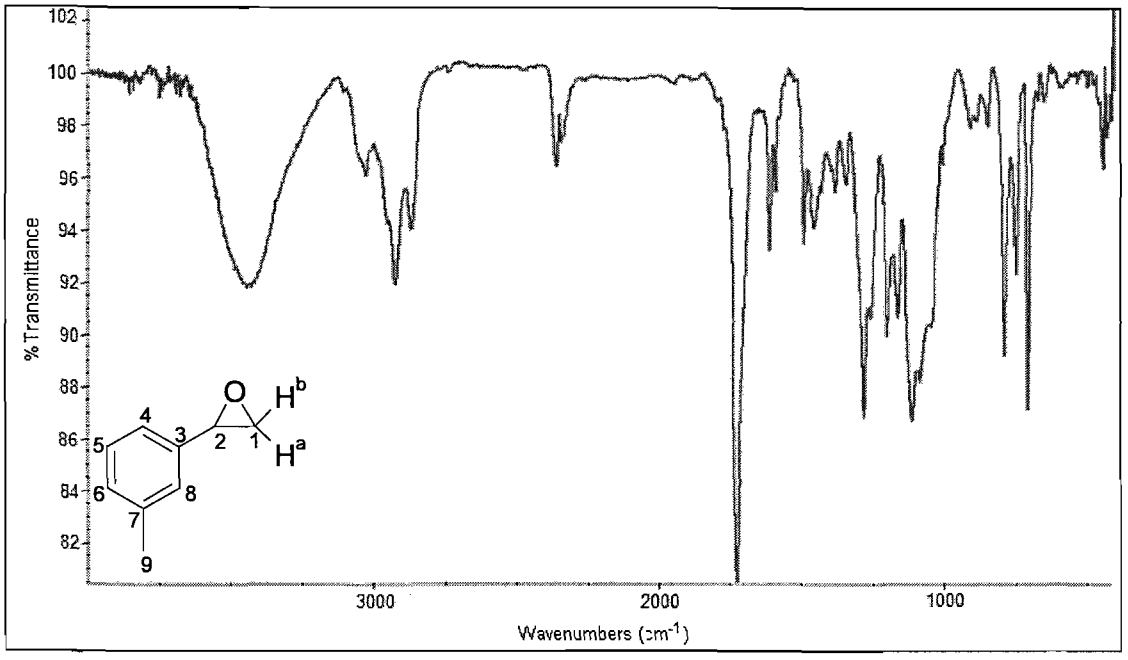
Spectrum 9 MS spectrum of *m*-Me.



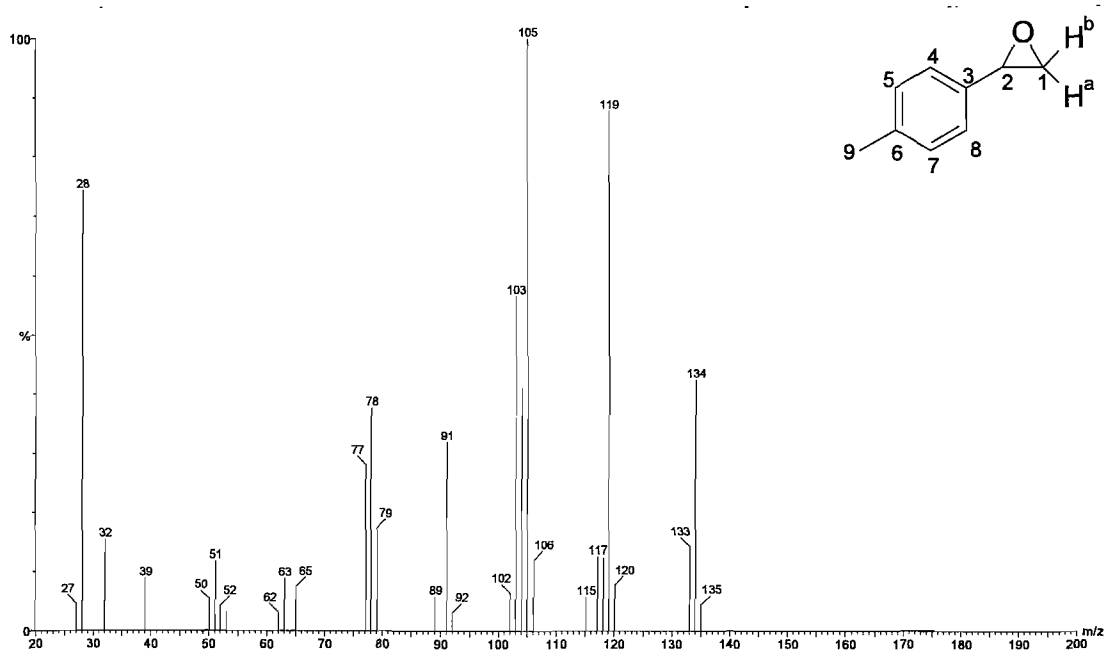
Spectrum 10 ^{1}H NMR spectrum of *m*-Me ($CDCl_3$).



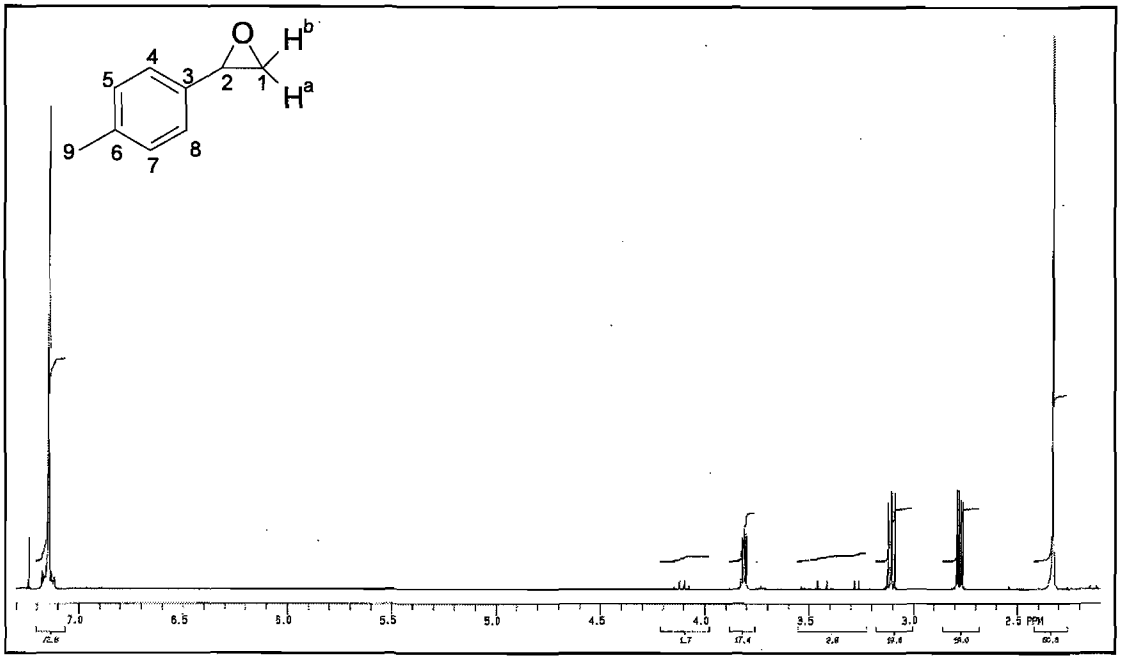
Spectrum 11 ^{13}C NMR spectrum of *m*-Me ($CDCl_3$).



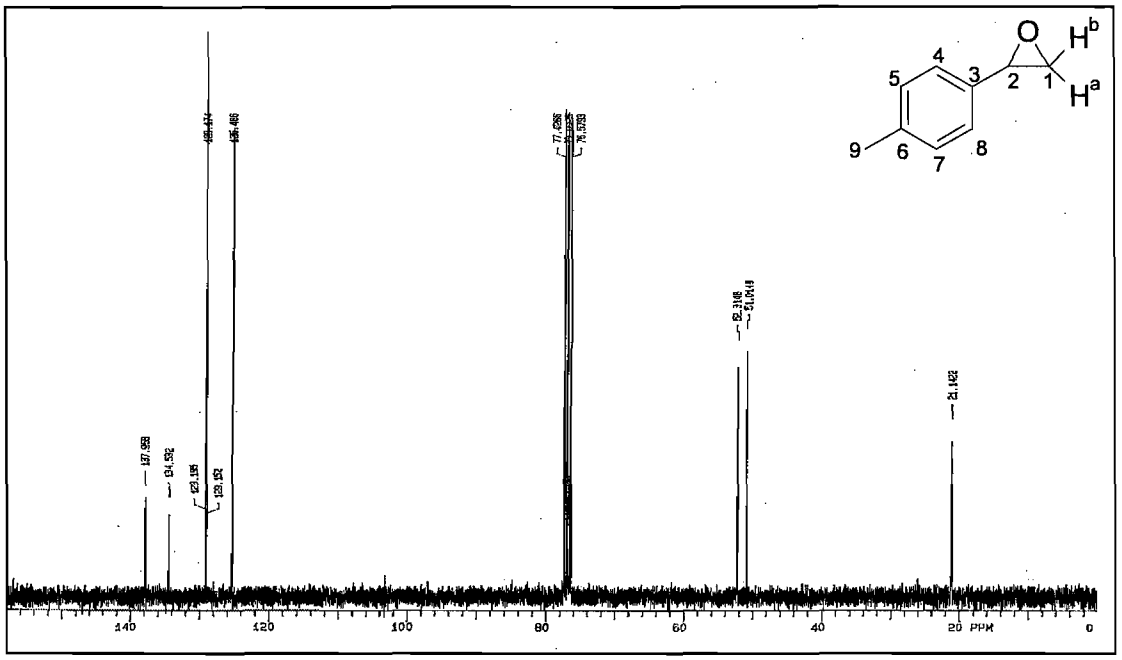
Spectrum 12 IR spectrum of *m*-Me.



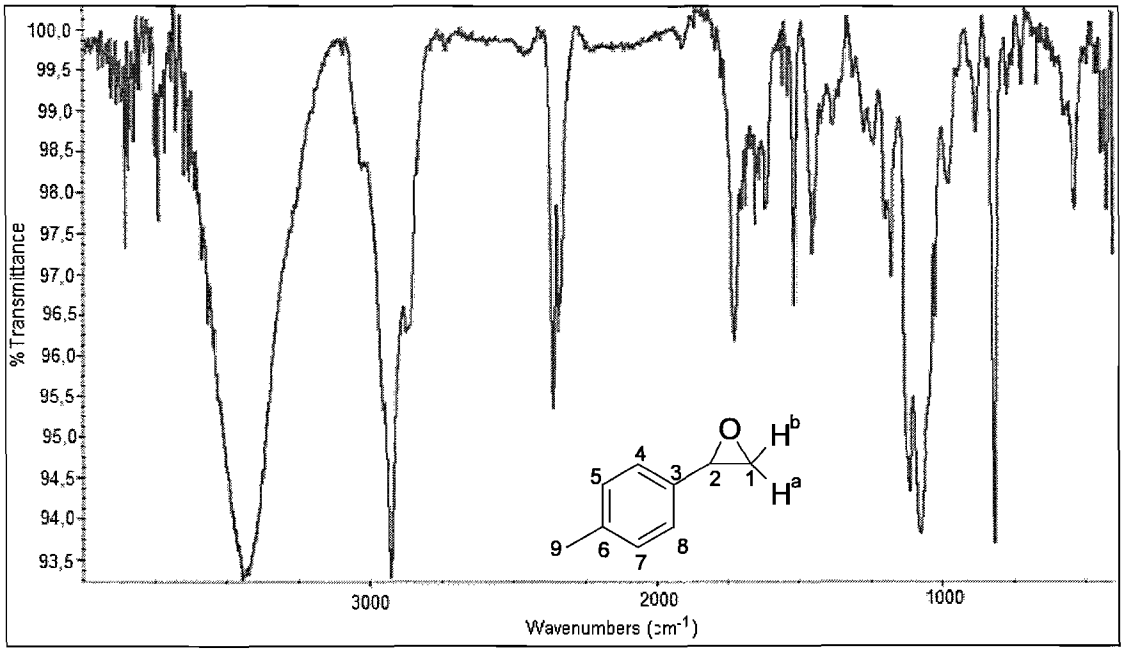
Spectrum 13 MS spectrum of *p*-Me .



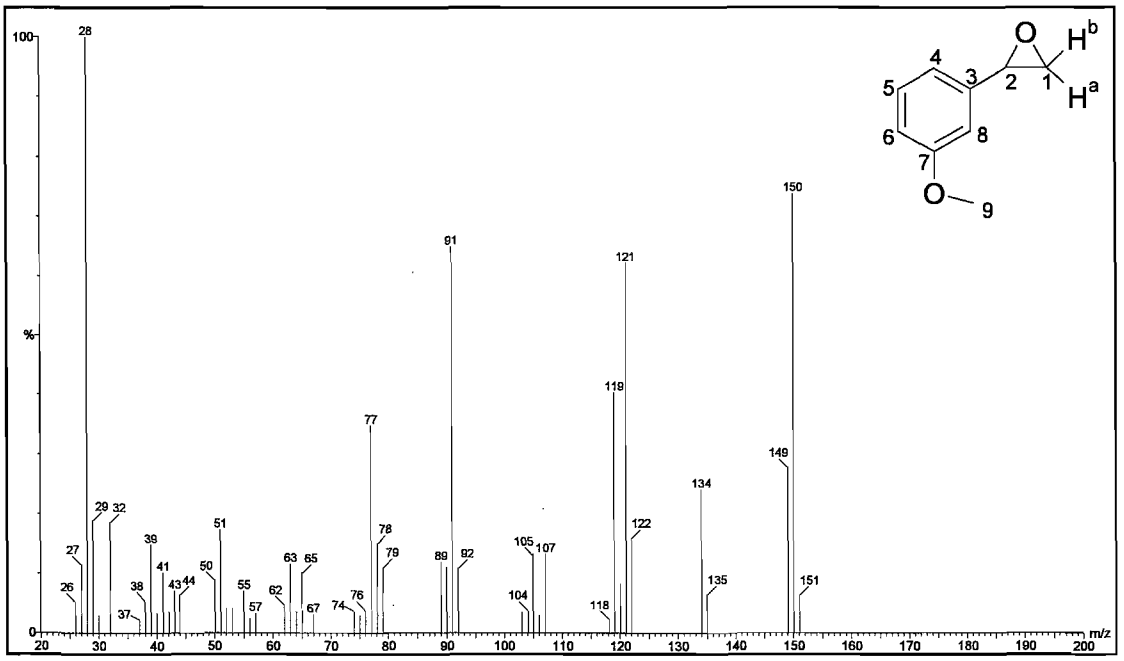
Spectrum 14 ^{1}H NMR spectrum of *p*-Me ($CDCl_3$).



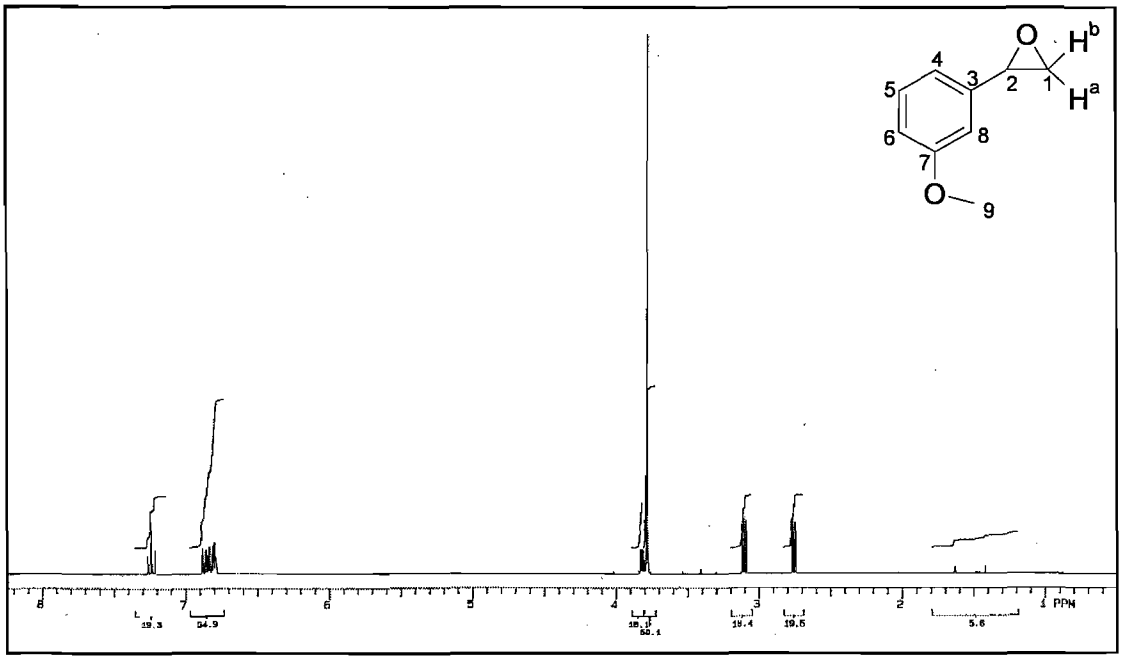
Spectrum 15 ^{13}C NMR spectrum of *p*-Me ($CDCl_3$).



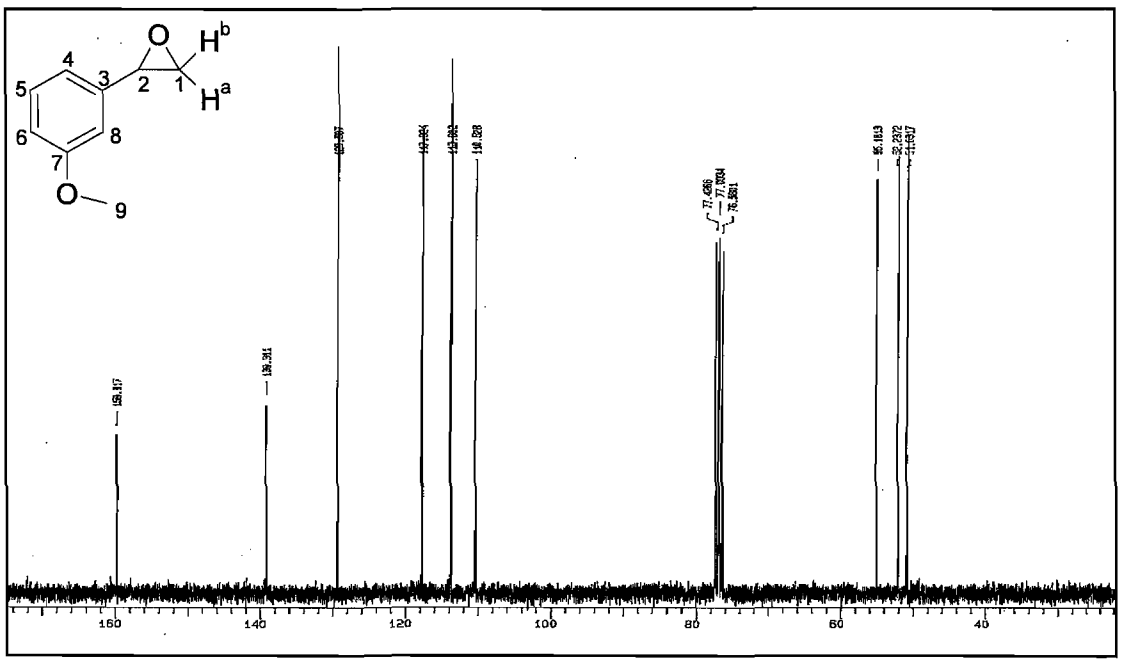
Spectrum 16 IR spectrum of *p*-Me.



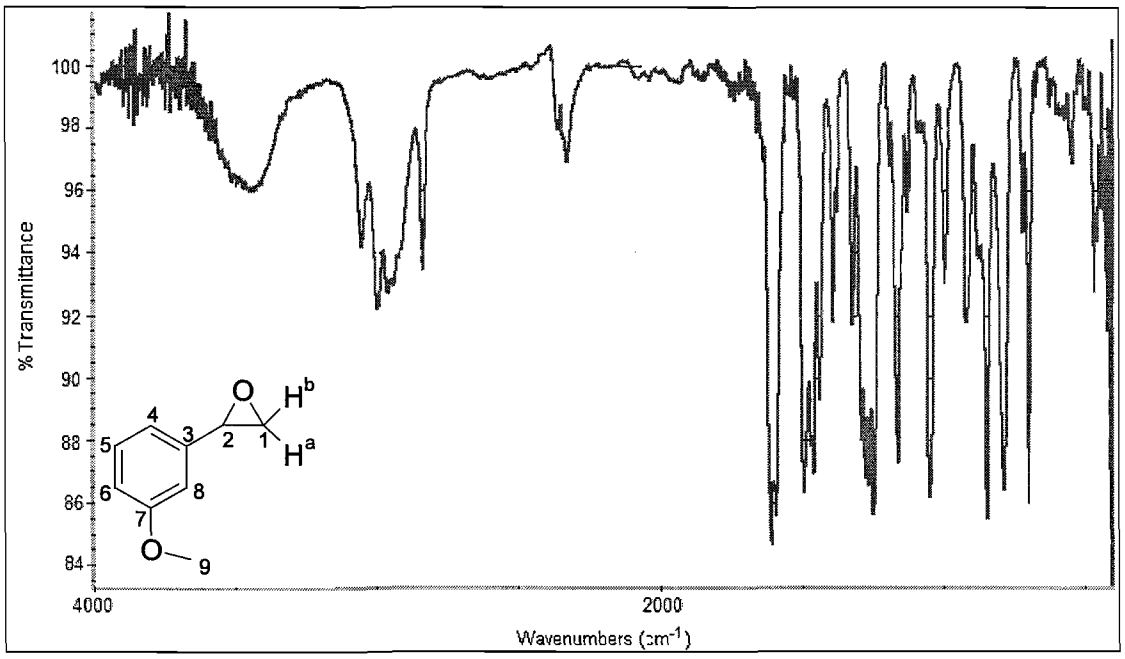
Spectrum 17 MS spectrum of *m*-MO.



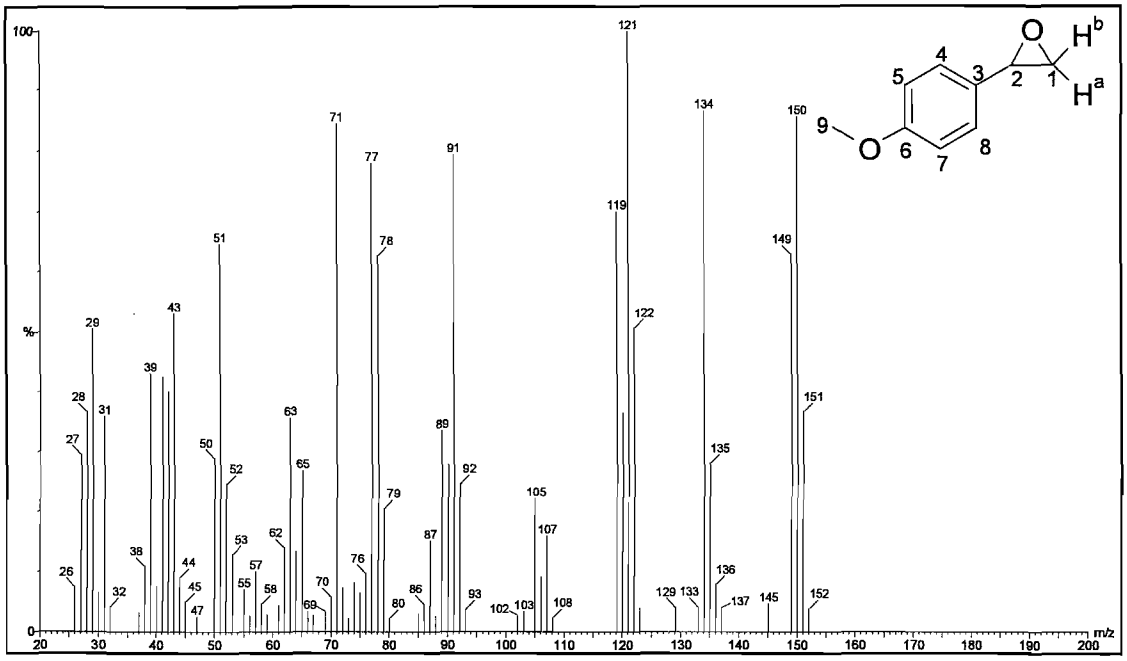
Spectrum 18 ^{1}H NMR spectrum of *m*-MO ($CDCl_3$).



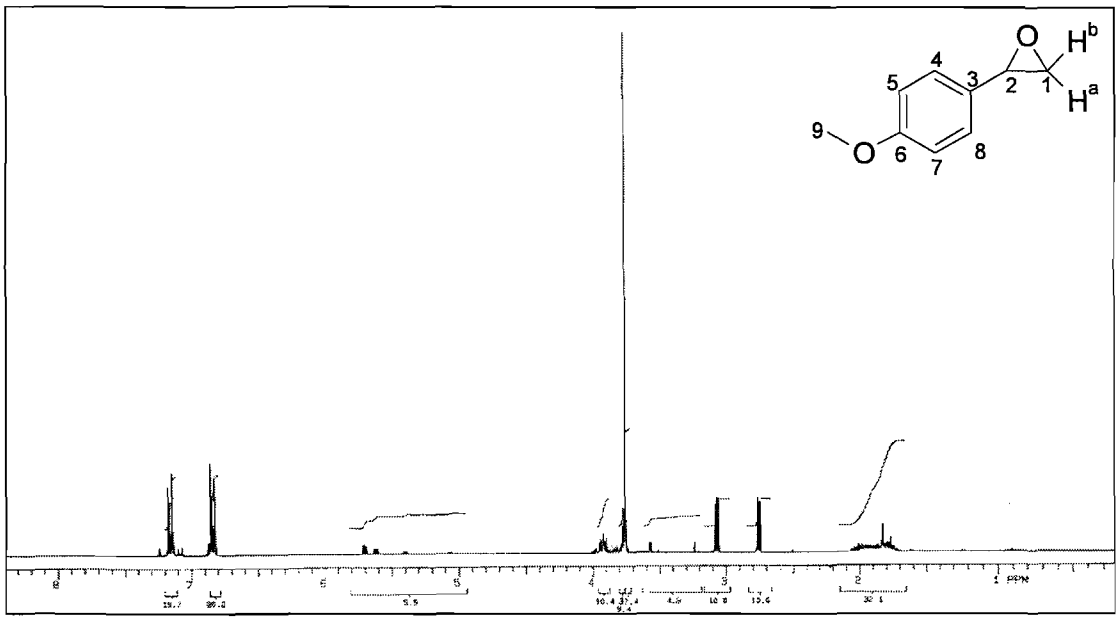
Spectrum 19 ^{13}C NMR spectrum of *m*-MO ($CDCl_3$).



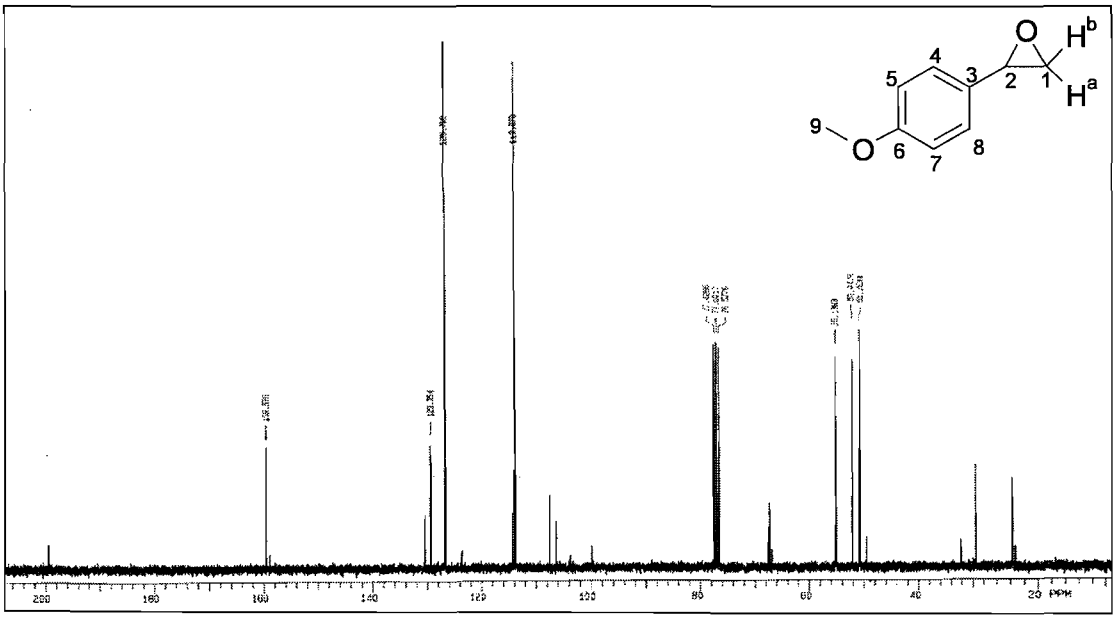
Spectrum 20 IR spectrum of *m*-MO.



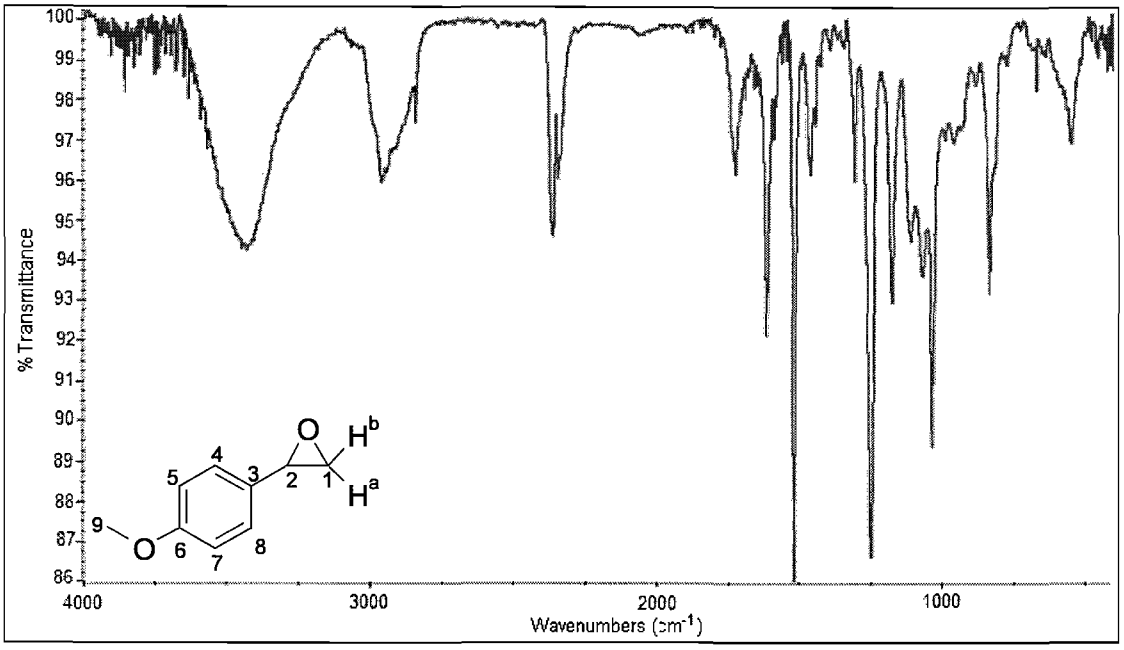
Spectrum 21 MS spectrum of *p*-MO.



Spectrum 22 ^{1}H NMR spectrum of *p*-MO ($CDCl_3$).



Spectrum 23 ^{13}C NMR spectrum of *p*-MO ($CDCl_3$).



Spectrum 24 IR spectrum of *p*-MOSO.

Appendix 3

Substrates docked into the active site of the enzyme of *Aspergillus niger* shown with the distances between the hydroxyl group of the Tyrosine residues, as well as the distance between C8 and the Aspartame residue.

Figure 1 (R)-SO docked in the *A. niger* EH active site.

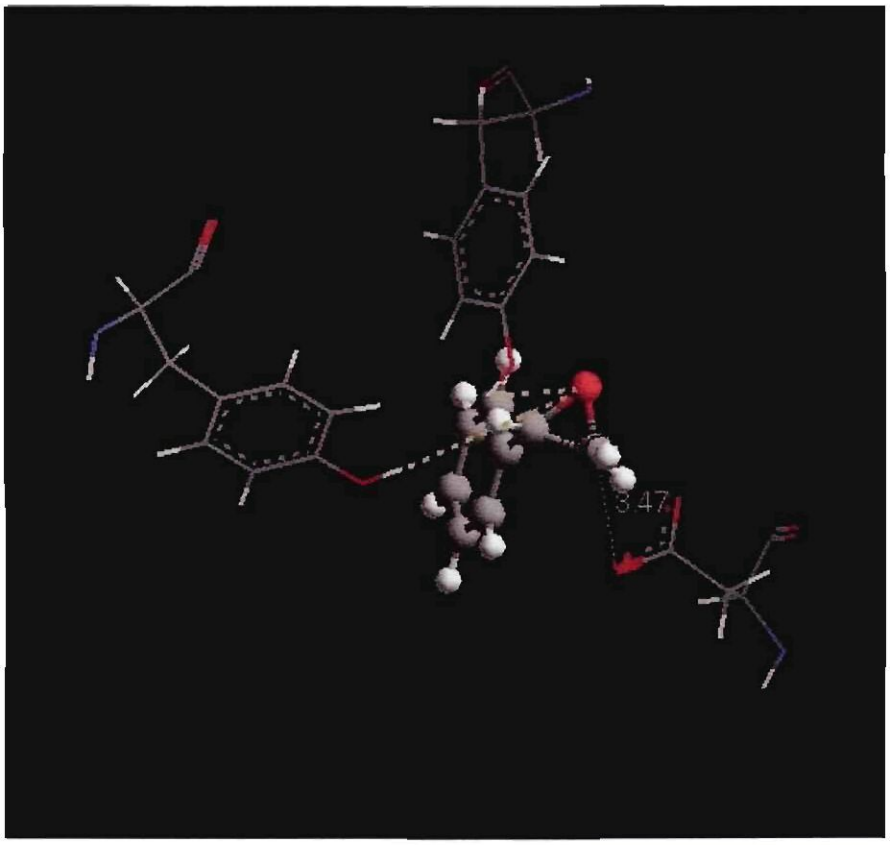


Figure 2 (S)-SO docked in the *A. niger* EH active site.

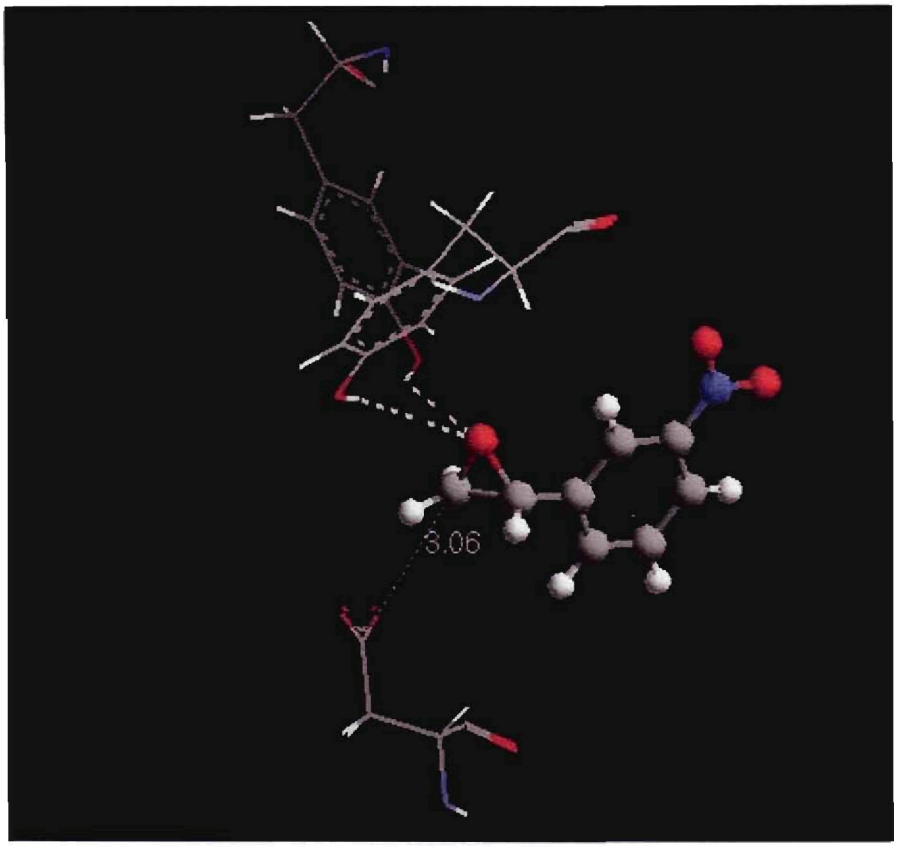


Figure 3 (R)-*m*-NO_2 docked in the *A. niger* EH active site.

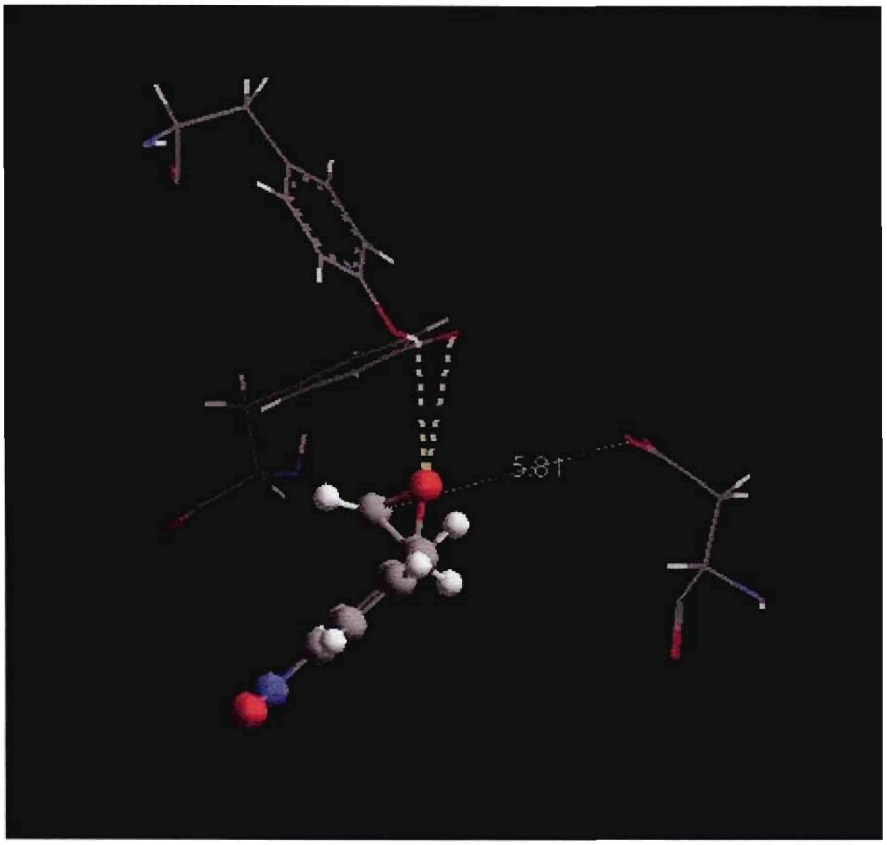


Figure 4 (S)-*m*-NO_2 docked in the *A. niger* EH active site.

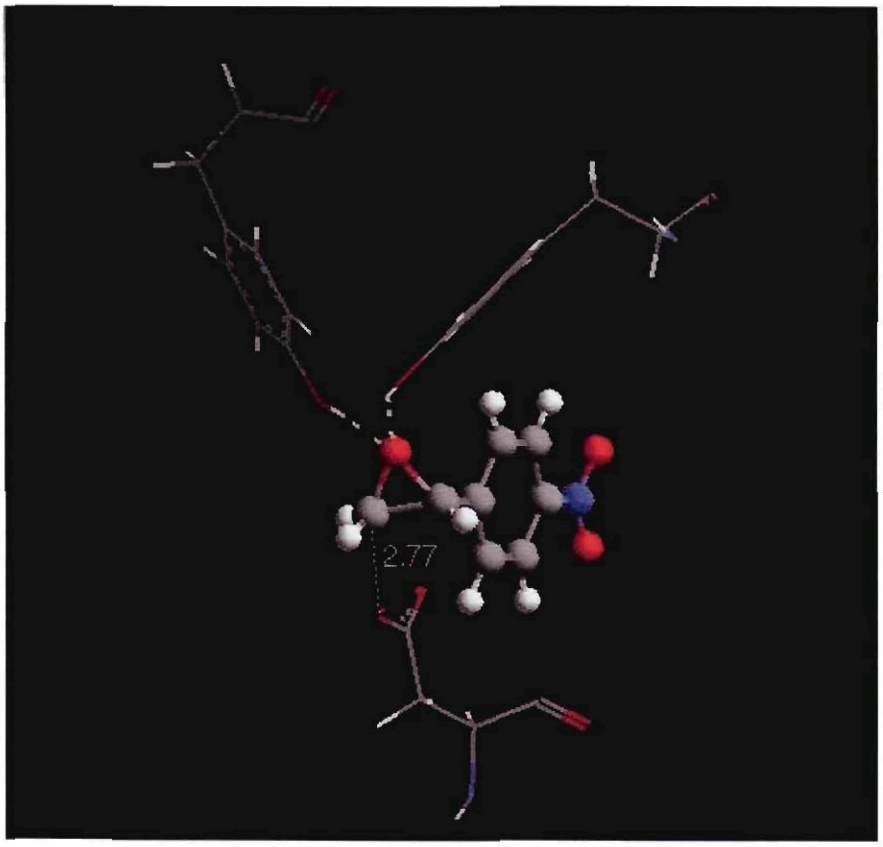


Figure 5 (R)-*p*-NO_2 docked in the *A. niger* EH active site.

Figure 6 (S)-*p*-NO_2 docked in the *A. niger* EH active site.

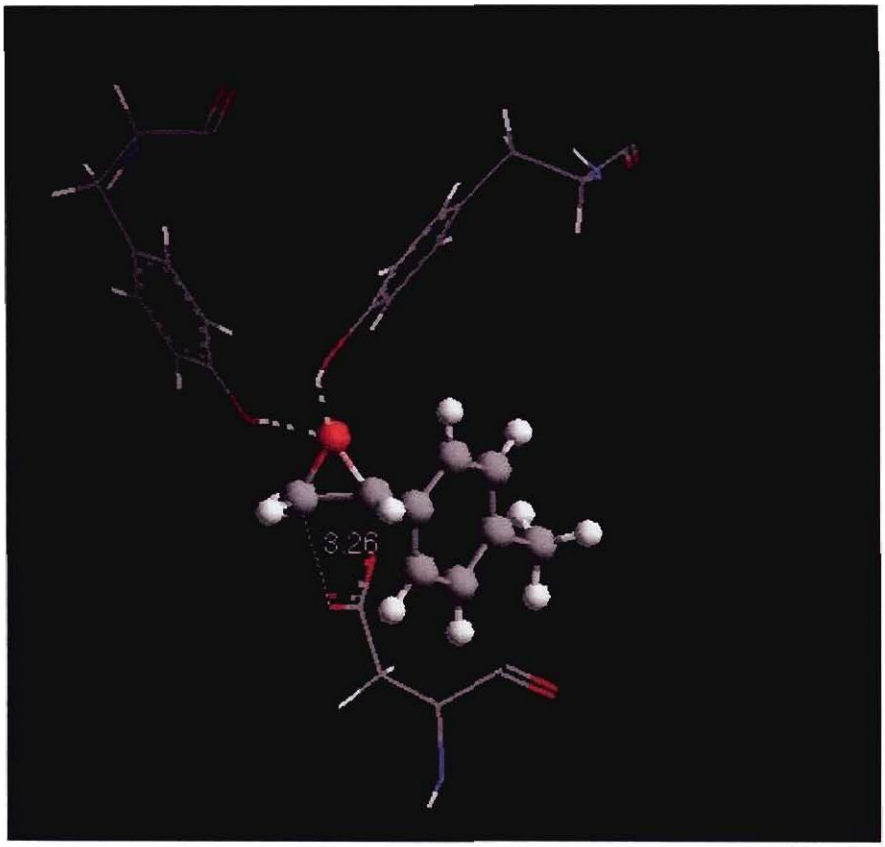


Figure 7 (R)-*p*-Me docked in the *A. niger* EH active site.

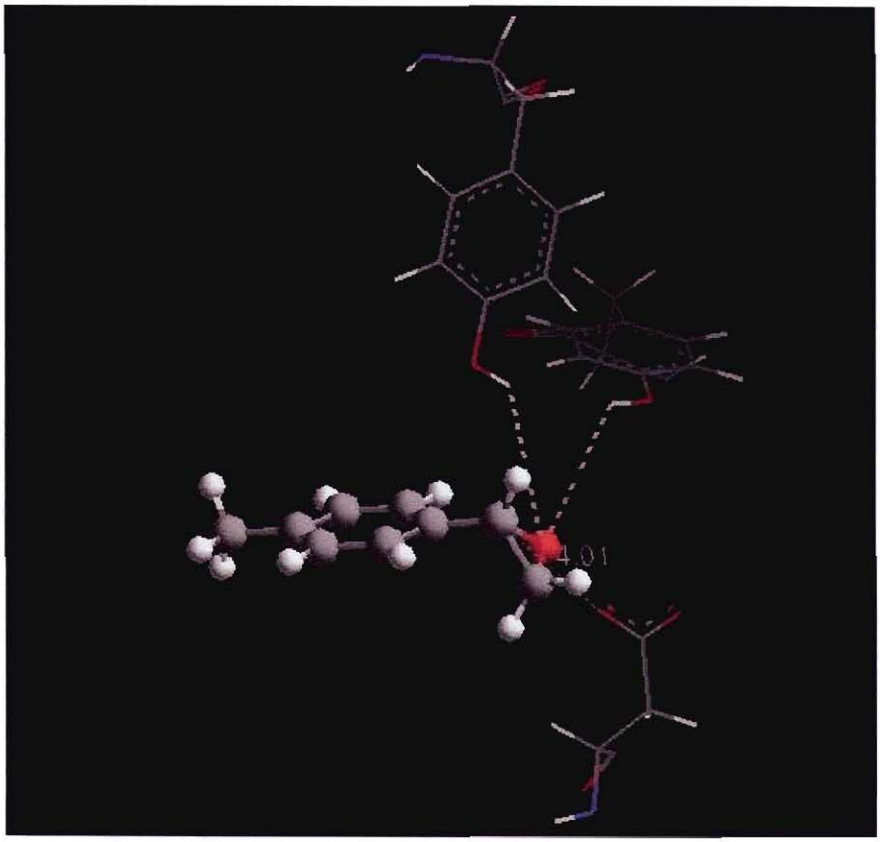


Figure 8 (S)-*p*-Me docked in the *A. niger* EH active site.

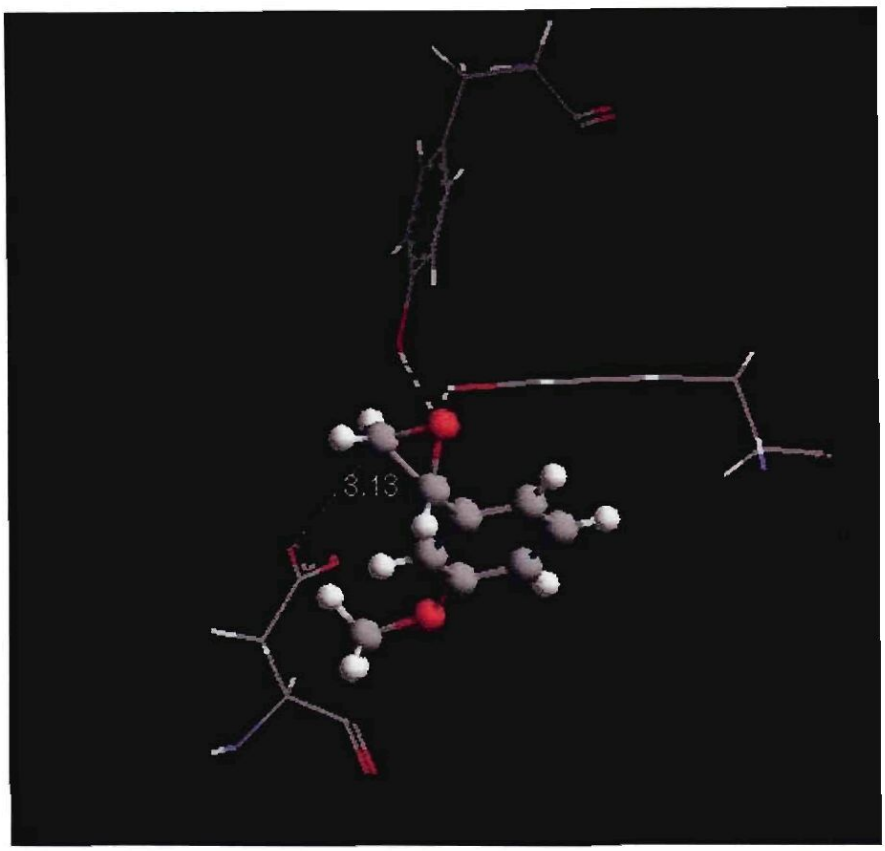


Figure 9 (R)-*m*-MO docked in the *A. niger* EH active site.

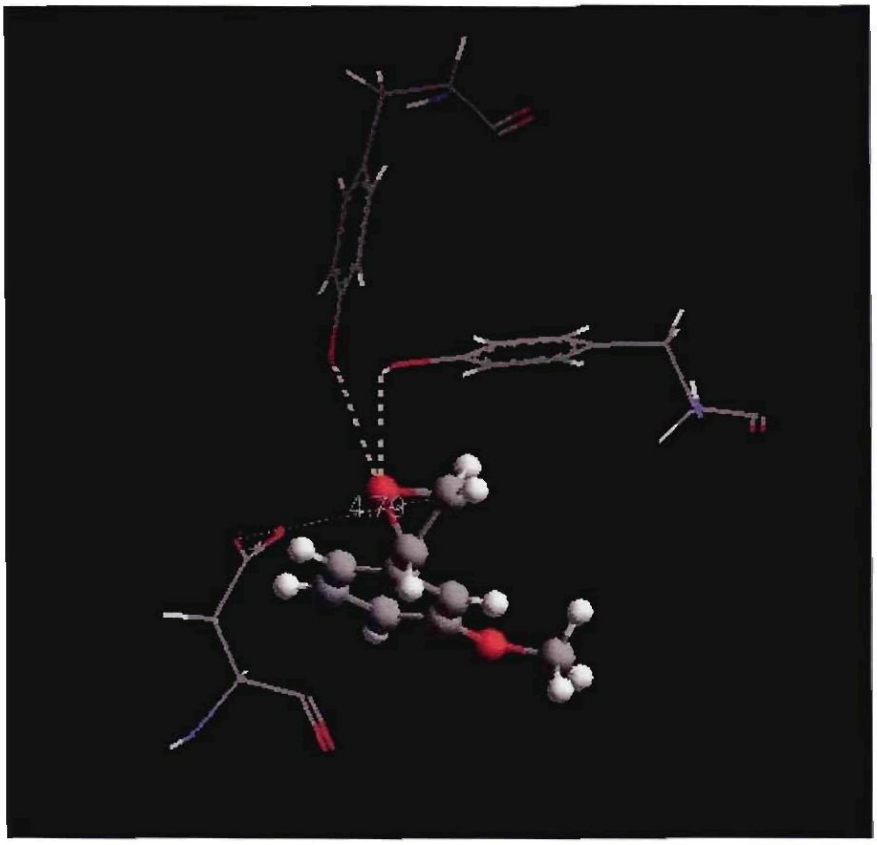


Figure 10 (R)-*m*-MO docked in the *A. niger* EH active site.