BIOCATALYTIC RESOLUTION OF EPOXIDES

EPOXIDE HYDROLASES AS CHIRAL CATALYSTS FOR THE SYNTHESIS OF ENANTIOMERICALLY PURE EPOXIDES AND *VIC* DIOLS FROM α-OLEFINS

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by

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Promoter: Prof. M.S. Smit
Co-Promoter: Prof. D. Litthauer

For my parents, Carel and Maggie du Plessis

Their love gave me wings

Contents

PREFACE

This research was conducted under the auspices of the Sasol Center for Biotechnology in an ongoing effort to synthesize value-added products from bulk substrates produced by the petrochemical industry. These bulk substrates comprise aromatic and aliphatic hydrocarbons and α-olefins. Selective functionalization of the alkyl component of aromatic compounds such as toluene, and of aliphatic hydrocarbons, can only be achieved by means of oxygenase catalyzed introduction of oxygen. Once this is achieved, a whole array of products can be synthesized by chemical or biocatalytic means. Many oxygenases are however integral, membrane bound multi-enzyme complexes. Oxygenases require co-factors, as well as prosthetic groups. The use of whole cells or co-factor recycling is thus indicated for biotransformation processes. Furthermore, the more soluble alcohol product is toxic to the cells, so that only low concentrations of product is accumulated in the medium due to product inhibition and downstream enzymes involved in the metabolic pathway. The use of biphasic systems employing organic solvents led to some biotechnological improvement, but large-scale laboratory or industrial production is still difficult to achieve. A practical breakthrough in the biotransformation of these substrates is actively explored because of the difficulty of achieving selective oxygen incorporation by traditional organic synthesis.

Oxygen incorporation into α-olefins, however, is easily achieved by direct chemical epoxidation with 3-chloroperbenzoic acid (*m*-CPBA) or hydrogen peroxide (H_2O_2). Racemic epoxides obtained by epoxidation of olefins are relatively inexpensive substrates. A simple and efficient process for the resolution of these racemic epoxides into enantiopure epoxides would greatly increase their value. The recent surge of chiral technology in the pharmaceutical and agrochemical industries, and the many applications of enantiopure epoxides as intermediates in the synthesis of these compounds as single enantiomers, indicated that the exploration of a biocatalytic approach for the resolution of racemic epoxides, would be relevant.

MOTIVATION

Synthesis of chiral pharmaceuticals and agrochemicals in enantiopure form has distinct advantages

The synthesis of chiral pharmaceuticals in an enantiopure form had become increasingly important in the last few years. Today, an estimated 80% of all drugs in development are single enantiomers of chiral drugs. The rationale for this is that the two isomers can have very different pharmacological effects. Due to the inherent chirality of biological systems, the interaction of isomers of the same drug with the chiral receptors and proteins, differ in their pharmacokinetic and/or pharmacodinamic nature. Synthesis of only the biologically active enantiomer, usually increases the efficacy and allows lower and more accurate dosage, as well as removing the potentially harmful side effects. While regulatory authorities do not enforce the registration of single isomers of chiral drugs, the FDA regards each isomer as a different molecule. Therefore, toxicology on essentially three products - both isomers as well as the racemate - is required. It is thus cheaper in terms of registration to develop enantiomerically pure drugs.

This same trend is now found in the synthesis of agrochemicals. As a result of the need to produce more efficient and environmentally friendly agrochemicals, more specific and thus more complex (chiral) molecules are being developed in enantiomerically pure forms. An estimated 25% of all commercial agrochemicals contain a chiral centre, but only 10% of these are available in optically active form. It is interesting that in the area of agrochemicals, where cost considerations are much greater, molecular biology and biocatalytic approaches are considered to drive manufacturing costs down to levels below that of the pharmaceutical sector. (European Chemical News, 9-15 June, 1997: 14 – 36)

Epoxides and diols are key chiral intermediates in the production of enantiopure pharmaceuticals and agrochemicals

Epoxides, due to their high reactivity with a large number of reagents, and *vic* diols, employed as their corresponding cyclic sulfates or sulfites as reactive intermediates, are versatile chiral synthons in the synthesis of many bioactive compounds. Extensive research efforts have thus been directed towards the chemical synthesis

of optically active epoxides and *vic* diols. Various biocatalytic methods for the production of chiral epoxides have been evaluated as *via*ble alternatives to these chemical catalysts. Patents held for the biocatalytic production of optically active epoxides are based on mono-oxygenases, toluene dioxygenases, chloroperoxidases, lipases and epoxide hydrolases. All the chincona alkaloid ligands developed by Sharpless for the asymmetric dihydroxylation of olefins are sold under license from ChiRex. ChiRex also licensed all three industrial enantioselective chemical processes based on salen complexes that were developed by Jacobsen: asymmetric epoxidation, asymmetric opening of meso-epoxides with trimethylsilyl azide, and the most recent hydrolytic kinetic resolution of terminal epoxides.

Kinetic resolution allows access to both enantiomerically pure epoxides, diols, amino-alcohols and azido-alcohols

Kinetic resolution of racemic epoxides by epoxide hydrolases has recently emerged as a very attractive strategy for the synthesis of enantiopure epoxides. The inherent disadvantage of kinetic resolution that only a 50% theoretical yield can be obtained, is offset by the fact that the diol product is also a valuable chiral synthon, and that the enantiomerically pure diol and epoxide can be readily separated and chemically interconverted without racemization. Access to both enantiomers of the epoxide as well as access to both enantiomers of the diol is thus achieved. The use of non-natural nucleophiles to open the epoxide ring, giving access to chiral amino- and azido-alcohols, further enhances the synthetic potential of this approach.

Epoxide hydrolases are useful catalysts for organic synthesis

Microbial epoxide hydrolases are ubiquitous in nature, and can easily be produced in large quantities. They are stable, constitutive, monomeric and co-factor independent enzymes, and no noticeable loss of activity is observed when they are stored either as whole cells or enzyme extracts. Furthermore, they can act in the presence of organic solvents, allowing the use of insoluble substrates.

Terminal epoxides are inexpensive and readily available substrates

Terminal epoxides are arguably the most important subclass of epoxide building blocks in organic synthesis. The absence of other functional groups avoids competing side-reactions, allowing the use of whole cells or crude enzyme extracts.

They are available very inexpensively as racemic mixtures. Terminal aliphatic epoxides are derived from α-olefins, which are bulk substrates produced by the petrochemical industry. Epoxidation of α-olefins is readily achieved using *m*-CPBA. A practical method for epoxidation of terminal olefins with 30% H_2O_2 under halide-free conditions without organic solvents, had recently been developed. Kinetic resolution employing lipases require a bifunctional molecule with both an epoxide and an ester moiety. Other indirect biocatalytic approaches use halohydrins derived from ketones, which generates a vast amount of salt waste.

Yeasts are excellent bioctalysts and potential sources of epoxide hydrolases

The biotechnological applications of *Saccharomyces* are well known. Non-conventional yeasts are attracting increasing attention in basic research and biotechnological applications. Due to their exceptional metabolic pathways, they have been used in various biotechnological processes for producing foods or food additives, drugs and a variety of biochemicals (Wolf, 1996; Sudbery, 1994). Epoxide hydrolases are ubiquitous in nature, and had been found in mammalian cells, plants, insects, bacteria and filamentous fungi. Extensive research had been conducted on mammalian epoxide hydrolases. Recently, organic chemists realized the potential of microbial epoxide hydrolases for the production of enantiopure epoxides and focused their attention on bacteria and fungi as potential sources of these enzymes. Yet, the epoxide hydrolase of only one yeast, *Rhodotorula glutinis*, had been investigated (Weijers, 1997).

Chapter 1

Chemical and biological catalysts for the production of enantiopure epoxides and *vic* diols from α-olefins

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Chemical Reviews (Submitted)

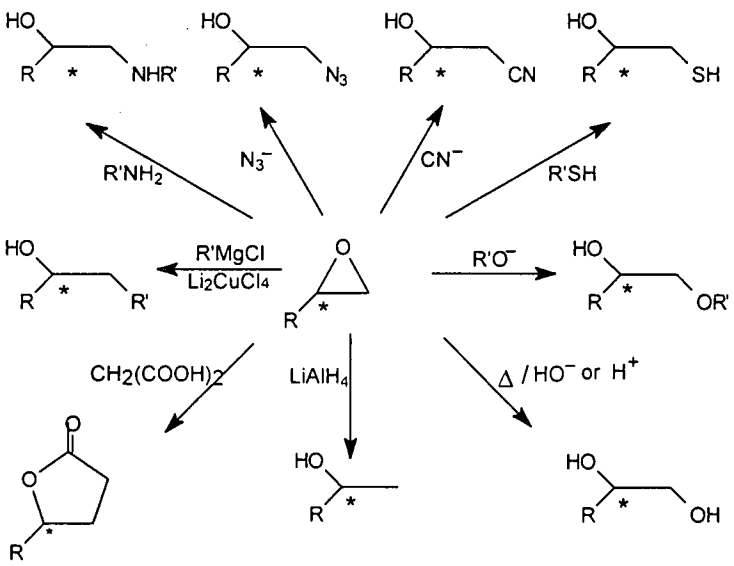
CONTENTS

Chemical and biological catalysts for the production of enantiopure epoxides and *vic* diols from α-olefins

1. EPOXIDES AND *VIC* DIOLS: REACTIVE INTERMEDIATES AND BIOACTIVE COMPOUNDS

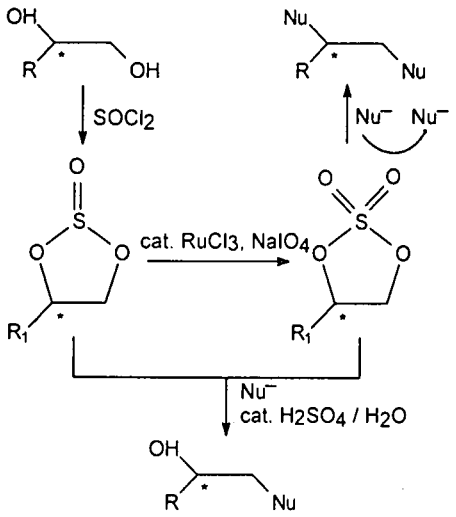
1.1 Epoxides and *vic* diols as reactive intermediates in organic synthesis

Epoxides are highly reactive electrophiles because of the strain inherent in the three-membered ring and the electronegativity of the oxygen. Epoxides react readily with various O-, N-, S-, and C-nucleophiles, acids, bases, reducing and oxidizing agents, allowing access to bifunctional molecules (Leak *et al.*, 1992) (Scheme1).



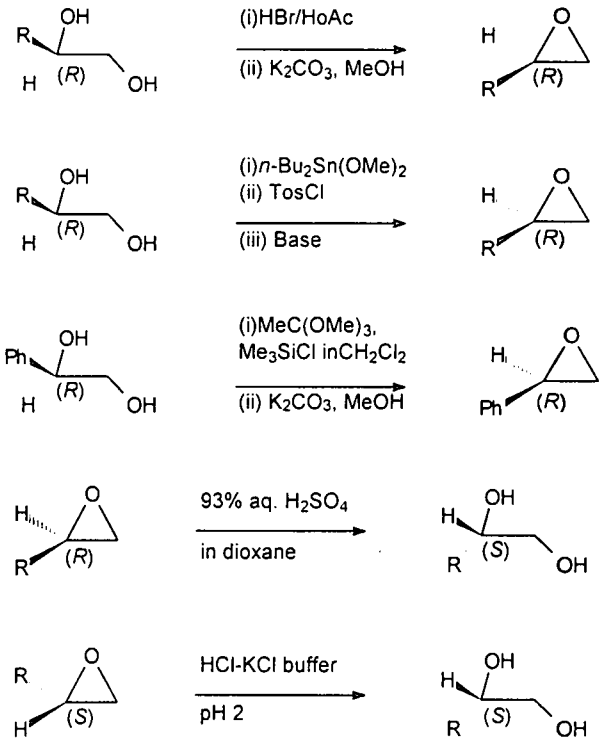
Scheme 1. Reactions of epoxides with nucleophiles, acids, bases, reducing and oxidizing agents. (Adapted from Leak *et al.*, 1992)

Vic diols, employed as their highly reactive cyclic sulfites and sulfates, act like epoxide-like synthons (Lohray, 1992) with a broad range of nucleophiles. The possibility of double nucleophilic displacement reactions (Lohray *et al.*, 1989) with amidines and azide, allow access to dihydroimidazole derivatives, aziridines, diamines and diazides (Scheme 2).



Scheme 2. Cyclic sulfites and sulfates of diols as synthetic equivalents of epoxides. (Adapted from Kolb *et al.*, 1994)

Various methods exist for the stereospecific conversion of enantiomerically pure *vic* diols to epoxides (Kolb *et al.*, 1994) and stereospecific opening of the oxirane ring with inversion (Orru *et al.*, 1998a) or retention (Zhang *et al.*, 1991) of configuration (Scheme 3).



Scheme 3. Stereospecific interconversion of epoxides and diols

1.2 Epoxides and *vic* diols as bioactive compounds

Due to their reactivity, enantiopure epoxides and *vic* diols not only serve as versatile chiral building blocks in the synthesis of enantiopure bioactive compounds, but also have numerous important biological activities. Since enantiopure epoxides and diols can be stereospecifically interconverted, they can be regarded as synthetic equivalents. The synthetic applications of 1,2-diols and epoxides were summarized in an excellent review by Kolb *et al* (1994). Some examples of bioactive compounds derived from optically active epoxide and 1,2-diol intermediates are listed in Table 1, and bioactive epoxides and diols are listed in Table 2.

Table1. Bioactive compounds accessible *via* optically active epoxides and 1,2-diols.

(a) β-adrenergic agonists and antagonists

β-blocker	Synthesis	References
(*S*)- Propranolol	Sharpless AD	(Wang *et al.*, 1993)
	Lipases	(Kamal *et al.*, 1992)
(*S*)- Atenolol	Monooxygenases	(Johnstone *et al.*, 1987)
(*S*)- Metoprolol	Monooxygenases	(Johnstone *et al.*, 1987)
(*R*)- Nifénalol	Epoxide hydrolase	(Pedragosa-Moreau, 1997)
(*S*)- Timolol	Lipases	(Kloosterman *et al.*, 1988)
(*S*)-Penbutolol	Lipases	(Kloosterman *et al.*, 1988)
(*S*)- Levobunolol	Lipases	(Kloosterman *et al.*, 1988)
Foradil® ((R,R)-formoterol): bronchodilator	Asymmetric catalytic borane reductions of epoxide	(Hett *et al.*, 1997)

(b) Insect pheromones

Pheromone	Synthesis	References
(2*S*,5*S*)-*trans*-2-methyl-5-hexanolide: carpenter bee	Addition of organocopper reagents to cyclic sulfites	(Kang *et al.*, 1992)
(-)-frontalin	Sharpless AE	(Hosokawa *et al.*, 1985)
(Pine beetle)	Lipases	(Chen and Fang, 1997;
	Lipases	Ferraboschi *et al.*, 1993)
(2*R*,5*R*)-Pityol (elm bark beetle)	Lipases	(Mischitz *et al.*, 1994)
(2*R*,5*S*)-pityol (spruce bark beetle)	Epoxide hydrolase	(Archelas and Furstoss, 1992)
(6*R*)-Bower's compound (insect JH analog)	Epoxide hydrolases	(Archelas *et al.*, 1993)
Western corn rootworm pheromone	Sharpless AD	(Sinha *et al.*, 1993)
Endo-brevicomin (Bark beetles)	Lipases	(Kim *et al.*, 1995)
(-)-exo-isobrevicomin (mountain pine beetles)	Sharpless AD	(Taniguchi *et al.*, 1998)
(+)-disparlure (gypsy moth)	Epoxide hydrolase	(Otto and van der Willigin, 1988)
4-dodecanolide (rove beetle)	Chiral pool	(Chattopadhyay *et al.*, 1990)
6-alkyl-α,β-unsaturated δ-	Lipases	(Haase and Schneider,

lactones		1993)
Stegobiol and stegobinone (Drugstore beetle)	C_2 symmetrical diols	(Kolb *et al.*, 1994)

(c) Antibiotics

Antibiotic	Synthesis	References
(-)-*cis*-Fosfomycin	Microbial oxidation	(Itoh *et al.*, 1995)
Chloramphenicol	Sharpless AE	(Rao *et al.*, 1992)
Antibiotic (-)-A 26771B	Sharpless AD	(Sinha *et al.*, 1993)
(+)Aspicillin	Sharpless AD	(Sinha and Keinan, 1994)
β-lactams	Sharpless AD	(Kim and Sharpless, 1990)
L-Rhodinose antibiotics: streptoglydin, vineomycin B_2, landomycin A	Tandem Sharpless AD and lipases KR	(Sobti and Sulikowski, 1995)
Azole antifungals	Sharpless AD	(Kolb *et al.*, 1994)
Antifungal Sch 42427/SM 9164	Chiral epoxide converted to α-hydroxy aryl ketones	(Gala and Dibenedetto, 1994)
(+)-Mibemycin β_3 (antiparasitic macrocyclic lactone spiroacetal)	Chiral pool, aldol reaction	(Street *et al.*, 1985)
(-)-Patchoulenone (anti-malaria, anti-fungal)	Toluene dioxygenase	(Banwell and Mcleod, 1998)
Alternaric acid	Ruthenium-catalyzed addition of terminal alkenes with alkynes	(Trost *et al.*, 1998)
Mycalamides	Sharpless AD	(Hoffmann and Schlapbach, 1993)
Cryptophycins	Isolated from Nostoc	(Golakoti *et al.*, 1995)
α-Bisabolol	Epoxide hydrolases	(Chen *et al.*, 1993)
Antiviral (*R*)-tritylglycidol-derivatives	Lipases	(Kim and Choi, 1992)

(d) Anti-tumor pharmaceuticals

Drug	Synthesis	References
(2*R*,3*S*)- Taxol and Taxotère (Paclitaxel)	Sharpless AE of *trans*-cinnamyl alcohol	(Bonini and Righi, 1994)
	Jacobsen AE	(Deng and Jacobsen, 1992)
	Sharpless AD	(Song *et al.*, 1998)
	Lipases	(Mori *et al.*, 1991)

	Lipases	(Kierkels and Peeters, 1995)
	Lipases	(Gou *et al.*, 1993)
Camptothecin and analogs	Sharpless AD	(Fang *et al.*, 1994)
	Amano PS-30 lipase	(Henegar *et al.*, 1997)
Cryptophycins	Isolated from Nostoc	(Golakoti *et al.*, 1995)
	Chiral pool, PTSA	(Rej *et al.*, 1996)
	Pd-cat ring opening of E-alkenyloxyranes	(Furuyama and Shimitzu, 1998)
Bromoxone	Lipases	(Johnson and Miller, 1995)
Pancratistatin	Toluene dioxygenases	(Hudlicky *et al.*, 1996b)
Castanospermine	Sharpless AD	(Kim *et al.*, 1993)
(2*R*,2'*S*)-1O-(2'-hydroxy-hexadecyl)glycerol	Chiral epoxyalkene	(Baskaran *et al.*, 1996)
Cyclophellitol	Toluene dioxygenases	(Hudlicky and Thorpe, 1996a)
Triptolide	Diterpenoid with a triepoxide structure, from herbs	(Yang *et al.*, 1998)

(d) HIV-1 Protease inhibitors

Drug	Synthesis	References
Crixivan® (Indinivar)	Various HIV-1 protease inhibitors have at least one side chain derived from enantiopure epoxides	(Senanayake *et al.*, 1996)
Norvir (ritonavir)		(Askin *et al.*, 1994)
Inverase (saquinavir)		(Ng *et al.*, 1995)
VX-478		(Maligres *et al.*, 1996)

(e) Ca^{2+}- and K_{ATP} -channel agonists and antagonists

Agonist/antagonist	Synthesis	References
(2*R*,3*S*)- Diltiazem	Jacobsen AE	(Jacobsen *et al.*, 1994)
Ca^{2+}-blocker;		(Mori *et al.*, 1991)
Antihypertension		(Kierkels and Peeters, 1995)
		(Gou *et al.*, 1993)
Cromakalin	Jacobsen AE	(Lee *et al.*, 1991)
K_{ATP}-channel opener	Lipases	(Patel *et al.*, 1995)
BMS-180448	Lipases	(Patel *et al.*, 1995)
K_{ATP}-channel opener	Ring-opening of epoxide with cyanoguanidine dianions	(Chen *et al.*, 1998)

(f) Natural products and drugs of fatty acid metabolism

Compound	Synthesis	References
Arachidonic acid metabolites		
Leukotriene A_4:	Jacobsen AE	(Chang *et al.*, 1993)
Leukotriene B_4	From (*R*)-glycidol	(Avignon-Tropis *et al.*, 1991)
Leukotrien antagonist SKF-104353	Sharpless AD	(Kolb *et al.*, 1994)
Fatty acid metabolism	Sharpless AD	(Kolb *et al.*, 1993)
(*R*)- carnitine (Vit. B_T)	From epichlorohydrin	(Kabat *et al.*, 1997)
L-carnitine	Lipases	(Kloosterman *et al.*, 1988)
Vit D_3 metabolite (treatment of osteoporosis)	Lipases	(Chen and Fang, 1997)
Antidiabetic and antiobesity CL-316,243	From 2-amino-1-phenylglycidic ethanol	(Furukawa *et al.*, 1998)
Anorressants		
(*R*)-2-benzylmorpholine	Baker's yeast, epoxide	(D'Arrigo *et al.*, 1998)
chiral amino-alcohols	From 1,2-diols	(Chang and Sharpless, 1996)
(*R*)-mevalonic acid	Chloroperoxidase	(Lakner and Hager, 1996)
Biosynthetic precursor for isoprenoids, vitamins and sterols; regulation of HMG-CoA reductase	Epoxide hydrolase	(Orru *et al.*, 1998b)

(g) Miscellaneous bioactive compounds derived from epoxides and 1,2-diols

Compound	Synthesis	References
(*R*)-reticuline: Morphine precursor	Jacobsen AE	(Hirsenkorn, 1990)
	Sharpless AD	(Kolb *et al.*, 1993)
GABOB: Antiepileptic, hypotensive	Sharpless AD	(Kolb *et al.*, 1993)
Prostaglandins	Jacobsen AE	(Leighton and Jacobsen, 1996)
	Monooxygenases	(Takahashi *et al.*, 1989)
	Lipases	(Chen and Fang, 1997)
Platelet aggregation factor: Phospholipid mediater of platelet aggregation, inflammation and anaphylaxix	Lipases	(Kloosterman *et al.*, 1988)
N-methyl-D-aspartic acid	(2R)-glycidyl triflate	(Sagnard *et al.*, 1995)

receptor agonist: plant growth hormones		
Conduritols	Dioxygenase	(Yan *et al.*, 1997)
	Sharpless AD	(Takano *et al.*, 1994)
Pyrrolidine alkaloids	Sharpless AD	(Takahata *et al.*, 1992)
Nucleoside analogs: anticancer and antiviral	Reaction with epoxide	(Yang *et al.*, 1998)
Histrionicotoxin: blocker of neuromuscular nicotinic channels	Sharpless AD	(Devaux *et al.*, 1998)
Immunosuppressant FK 506	Epoxide intermediate	(Ireland *et al.*, 1990)
Phyllodulcin: New class of low calorie sweeteners, antimicrobial activity	Sharpless AD	(Ramacciotti *et al.*, 1996)

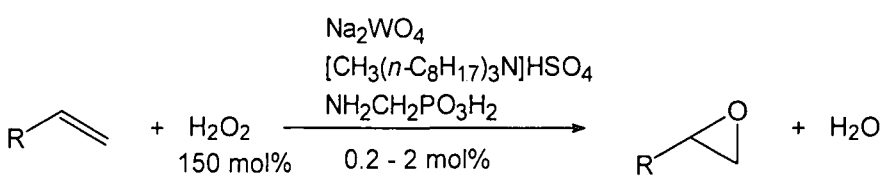
Table 2. Bioactive epoxides and 1,2-diols

Biological function	Compound	Reference
Expectorant	(*S*)- Guaifenesin	(Wang *et al.*, 1993)
Muscle relaxant	(*S*)- Mephenesin	(Wang *et al.*, 1993)
Antifungal	(*S*)- Chlorphenesin	(Wang *et al.*, 1993)
D_1 dopamine receptor agonist	(*R*)-(+)- SKF-38393	Sigma catalogue
Steroidal phytohormones	22*R*,23*R* brassinosteroids	(Lichtblau *et al.*, 1999)
Insect hormones	Insect juvenile hormones	(Mori, 1994)
	Insect moulting hormone	
	(7*R*,8*S*)-(+)-disparlure	
	C16- juvenile hormone	
	Epofenonane: insect growth regulator	Fluka catalogue
Treatment of osteoporosis	(24*R*)-24,25-dihydroxyvitamin D_3 (Cholecalciferol)	(Kolb *et al.*, 1994)
Fungal metabolite; Cytotoxic, antitumor	Diacetoxyscirpenol (Anguidin)	Fluka catalogue
Antifungal and anti-protozoa	HC-toxin	(Ahn and Walton, 1998)
Hypoglycemic, inhibitor of fatty acid oxidation	Methyl-(2*R*)-tetradecylglycidate	(Jiménez *et al.*, 1997)
Defense against rice-blast disease	C_{18} polyoxygenated fatty acids	(Honda and Mizutani, 1999)

Other applications of epoxides and 1,2-diols include their use as chiral auxiliaries for other asymmetric transformations, phase transfer catalysts, radiolabelling of steroids and sugars and ferro-electric liquid crystals. The applications of cyclic sulfates and sulfites in research and industry had been documented by Lohray (Lohray, 1992) .

1.3 Olefins and rac epoxides are inexpensive substrates for the production of enantiopure epoxides and *vic* diols

Racemic epoxides are relatively inexpensive compounds, prepared from the corresponding alkenes. α-Olefins are produced as bulk chemicals by the petrochemical industry, and as such represent the most abundant of the six possible configurations of olefins. Introduction of terminal functionalities to this class of olefins, such as epoxide moieties and 1,2-diols, also provide some of the most versatile chiral building blocks. Yet, this proved to be the most elusive class of olefins for asymmetric functionalization by chemical catalysts. Since *rac* terminal epoxides can be conveniently prepared from α-olefins, kinetic resolution became an attractive alternative for the synthesis of enantiopure epoxides, and major advances had been made since 1995 toward general methods for their synthesis by both bio- and chemical catalysts. Procedures for the epoxidation of terminal olefins with 30% hydrogen peroxide under halide-free conditions (Sato *et al.*, 1996) with < 0.5 mol% catalyst (Scheme 4) and without organic solvents, had recently been developed. High yields (>94%) and short reaction times are obtained. These new methods offer *via*ble alternatives to other methods, such as epoxidation with *m*--CPBA in dry CH_2Cl_2.



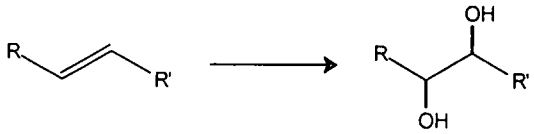
Scheme 4. Epoxidation of terminal olefins with 30% H_2O_2. (Taken from Sato *et al.*, 1996)

2. SYNTHETIC AND BIOCATALYTIC ROUTES TO OPTICALLY ACTIVE EPOXIDES AND *VIC* DIOLS FROM OLEFINS

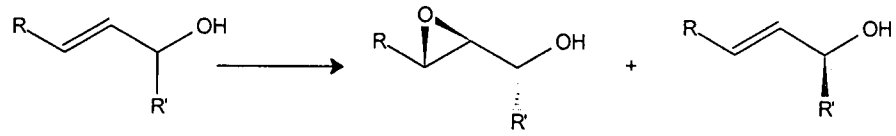
The search for generally useful catalysts for the asymmetric synthesis of epoxides and *vic* diols had been an important focus of research of both synthetic organic chemists and biochemists for the past two decades and many routes had been explored. Asymmetric epoxidation (AE) and asymmetric dihydroxylation (AD) of prochiral olefins, and kinetic resolution (KR) of olefins and racemic epoxides, offer direct routes for the synthesis of enantiopure epoxides and *vic* diols. Asymmetric reduction (AR) of prochiral α- or β-haloketones to halohydrins offers an indirect route (Scheme 5), and had been reviewed elsewhere (Singh, 1991; Deloux and Srebnik, 1993). Only those strategies that utilise olefins as starting materials and afford high enantiomeric purities (>90%) are discussed here. Chemical methods employing stoichiometric metal or metalloid based reagents are not included; only metal-mediated synthesis employing catalytic (<10mol%) amounts of ligands are considered. An overview of the most effective chemical and enzymatic catalysts is presented, and the advantages and disadvantages of the various methods are discussed.

Asymmetric epoxidation

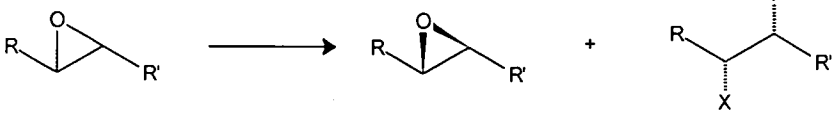
Asymmetric dihydroxylation



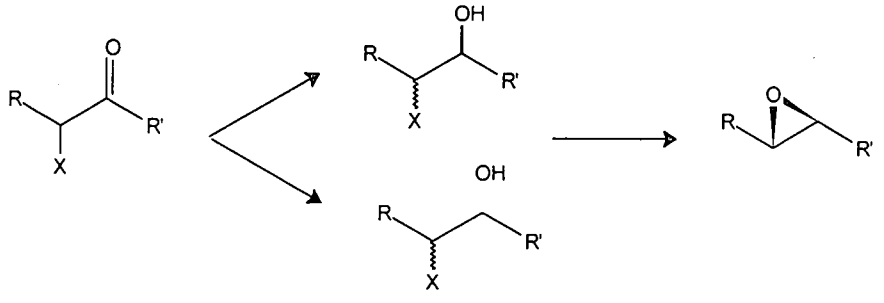
Kinetic resolution of olefins



Kinetic resolution of *rac* epoxides



Reduction of haloketones to halohydrins



Scheme 5. Routes to enantiopure epoxides

2.1 CHEMICAL CATALYSTS THAT GIVE DIRECT ACCESS TO ENANTIOPURE EPOXIDES AND VIC DIOLS VIA OLEFINS

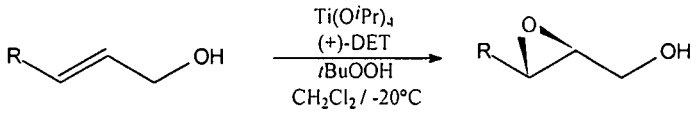
The development of a highly selective synthetic Ti based catalyst with broad specificity for AE of primary alylic alcohols and KR of secondary allylic alcohols in 1980 by Proff. K. Barry Sharpless and Tsutoma Hatsuki had a dramatic impact on synthetic chemistry. The goal of finding catalysts with enantiofacial selectivity with no requirement for auxiliary functionality to act as a tether, was first realized in 1987, when Sharpless developed Os based chiral catalysts for AD of olefins. The scope of AD was subsequently broadened through ligand variation. The utility of this method was further enhanced by the development of simple procedures for the stereospecific conversion of 1,2-diols to epoxides. The usefulness of the Sharpless *bis*-cinchona AD ligands was recently extended to asymmetric aminohydroxylation (AA) of olefins. A practical direct method for AE of unfunctionalized olefins by chiral (salen)Mn complexes was developed by Prof. Eric N. Jacobsen in 1991. Other researchers explored the potential of chiral Fe and Mn porphyrin complexes (Schurig and Betschinger, 1992) modeled on the iron porphyrin active site of cytochrome P-450, but their scope was limited, since they were either unstable under the oxidative conditions used, or did not accept electron deficient olefins as substrates. In 1995, Jacobsen used chiral (salen)Cr and Co complexes for the enantioselective ring opening of epoxides with various non-H_2O nucleophiles. He subsequently used the same type of (salen)Co catalyst, activated *in situ* by acetic acid in air to form the reactive species (salen)Co(III)OAc, for the hydrolytic kinetic resolution (HKR) of racemic epoxides in the absence of solvent.

2.1.1 Directed asymmetric epoxidation of functionalized olefins: Sharpless AE and KR

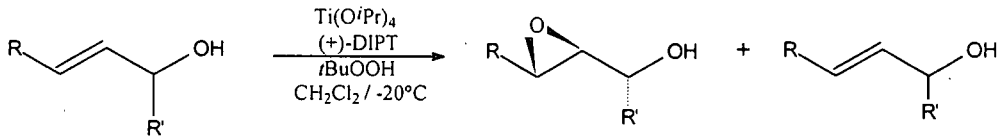
The Sharpless procedure (Katsuki and Sharpless, 1980) for the epoxidation of alyllic and homoallylic alcohols with titanium tetraisopropoxide/diethyl tartrate as Lewis acid catalyst and *tert*-butyl hydroperoxide (*t*-BHP) as oxidant (Scheme 6a) was the first practical method to obtain epoxides in high yields (>70%) and excellent enantiomeric purities (ee >90%). The resultant glycidols are derivatized to produce both (*R*)- and (*S*)-glycidyltosylates, glycidyl 3-nitrobenzenesulfonates, glycidyl 4-nitrobenzoates, 2-methylglycidyl 4-nitrobenzoates and 3,3-dimethylglycidyl 4-nitrobenzoates, as well as

(2*S*,3*S*)-2-methyl-3-phenylglycidol and (2*S*,3*S*)- and (2*R*,3*R*)-3-phenylglycidol. These optically active epoxides are licensed under US Patent 4,471,130 (Katsuki and Sharpless, 1984). The (*S*)-glycidyltosylates are employed in the synthesis of (*S*)-β-blockers and ferro-electric liquid crystals, while the (*R*)-enantiomers are employed in the synthesis of L-carnitine, phospholipids, platelet aggregation factor, anti-viral S-HMPA and ferro-electric liquid crystals. The stereochemical outcome of the epoxidation is controlled by the diethyl tartrate used. The addition of activated molecular sieves reduced the amount of reagent used to catalytic (5-14 mol%) quantities, by protecting the catalysts from inactivation by water. The reagents are relatively inexpensive and the catalyst is easily prepared. Kinetic resolution of various secondary alcohols (Scheme 6b) was achieved with the use of diisopropyl tartrate and dicyclododecyl tartrate.

(a) Asymmetric epoxidation of allylic alcohols



(b) Kinetic resolution of secondary allylic alcohols



Scheme 6. Asymmetric epoxidation and kinetic resolution of functionalized olefins

Since the epoxidation is independent of the substitution pattern of the allylic alcohol, this reaction has been successfully applied to the synthesis of a variety of optically active compounds such as vinyl carbinols (Martin *et al.*, 1992) and Taxol's C-13 side chain (Bonini and Righi, 1994). However, this method is applicable only if the olefin bears a directing functional group in the allylic position to achieve the precoordination required for high enantioselectivity. The reaction is also sensitive to preexisting chirality and steric hindrance in the case of secondary alcohols. The reaction is by no means universally

applicable (Gao *et al.*, 1987), and some structural classes of allylic alcohols (*cis*-disubstituted) are epoxidized very slowly (2 days) and with low enantioselectivity (<86%), while others (low molecular weight) are highly reactive, but unstable under the reaction conditions (anhydrous citric acid to remove excess titanium) used. Acid-catalysed ring opening of sensitive epoxides results in inactivation of the chiral complex by the formed diol, and low product concentrations (<0.2M) must be used. The titanium-tartrate complex is not stable, especially in the presence of molecular sieves, and must be freshly prepared at -20°C. Stringent control of the reaction temperatures (-10°C to -40°C) is required, and must be adjusted for different substrates. Product contamination by the hydroperoxide complicates the work-up procedure. The ratio of isooctane (solvent for oxidant) to dichlormethane (reaction solvent) may affect the polarity of the solvent, and result in slower reaction rates and/or decrease in ee. Furthermore, the use of toxic and carcinogenic chlorinated hydrocarbon solvents should be discouraged. Recent advances are aimed at the synthesis of heterogeneous ligands for the complexation of $Ti(O^iPr)_4$ and the recycling of the insoluble polymer catalysts by filtration (Karjalainen *et al.*, 1998).

2.1.2 Asymmetric dihydroxylation: Sharpless AD

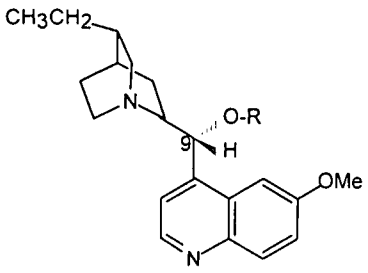
The osmium-catalyzed asymmetric dihydroxylation developed by Sharpless is characterized by the requirement of ligand variation for the dihydroxylation of the 6 different structural classes of olefins to obtain high optical purity. Ligands derived from the chiral cinchona alkaloid diastereomers dihydroquinidine (DHQD) or dihydroquinine (DHQ) (Scheme 7a) lead to diols of opposite configuration, but the ee's are usually not identical (Kolb *et al.*, 1994). More than 350 cinchona-based ligands have been tested for the AD reaction. The first generation ligands had single cinchona alkaloid units (Sharpless *et al.*, 1991), with the various 9-*O*-substituents (MEQ, CLB, PHN and IND) depicted in Scheme 7b and c. They were superceded by the second generation ligands (Sheme 7c) which have two independent cinchona alkaloid units attached *via* the 9-*O* to a heterocyclic (PHAL and PYR) (Crispino *et al.*, 1993) spacer. They are supplemented by the recent addition of the heterocyclic DPP and DP-PHAL (Becker *et al.*, 1995) or anthraquinone (AQN) spacers (Scheme 7d) to the arsenal of ligands. Other ligand variations include replacement of the ethyl group at C-3 of $[DHQ(D)]_2$ with alkoxy substituents to improve enantioslectivity for aliphatic terminal olefins (Arrington *et al.*, 1993). In the case of the dimeric ligands, only one alkaloid moiety is directly involved in

the reaction of OsO_4 with the alkene, while the other, in combination with the spacer, provide a chiral binding pocket for the olefin. The recommended use of the most versatile ligands for each olefin class is presented in Table 3 while the specific applications of the other ligands are given in Table 4. The commercially available ligands are sold under license from ChiRex. The monomeric ligands DHQ(D)MEQ, DHQ(D)CLB, DHQ(D)PHN are commercially available, since they still serve specific olefin dihydroxylations best. The DHQ(D)IND ligand, for dihydroxylation of *cis*-olefins, are not commercially available, probably because *cis*-olefins are poor substrates for Sharpless AD and ee's in excess of 80% are not achieved. The dimeric ligands $[DHQ(D)]_2PHAL$, $[DHQ(D)]_2PYR$ and the more expensive $[DHQ(D)]_2AQN$ are commercially available, while the $[DHQ(D)]_2DPP$ and $[DHQ(D)]_2DP$-PHAL are not (yet) available.

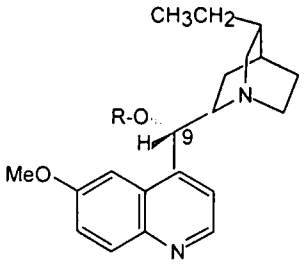
Sharpless AD has been applied to the synthesis of numerous enantiopure intermediates and bioactive compounds (Kolb *et al.*, 1994). The development of efficient methods to convert enantiopure *vic* diols to enantiopure epoxides, cyclic sulfates and sulfites, broadened the scope of AD reactions further. However, no efficient ligands exist for dihydroxylation of *cis*-olefins in the enantiomeric purity required for single enantiomer pharmaceuticals. This is compensated for by the recently discovered osmium catalyzed AA with $[DHQ(D)]_2$-PHAL, using chloramine-T, which acts as reoxidant and reservoir of TsN, as the key stoichiometric reagent. This reaction allows the addition of two different heteroatoms over the double bond of symmetric *cis*-disubstituted olefins, as well as *trans*-disubstituted olefins. Although the ee's obtained are low, the hydroxysulfonamide products are usually crystalline, and pure enantiomers are obtained by recrystallization from MeOH. AA of methyl cinnamate was applied to produce a Taxol side chain precursor.

Terminal aliphatic epoxides cannot be obtained in ee's >90%, (ee for C-10 is 90% and ee decreases sharply with a decrease in chain length) despite numerous efforts to find suitable ligands (Becker *et al.*, 1995; Kolb *et al.*, 1994). Tetrasubstituted olefins react very slowly, even in the presence of 3 equivalents $MeSO_2NH_2$, and the catalytic turnover of this class of olefins are rare. Electron-deficient olefins, such as α,β-unsaturated carbonyl compounds react very slowly, and require higher concentrations of ligand and catalyst.

(a) Cinchona alkaloid ligands for AD

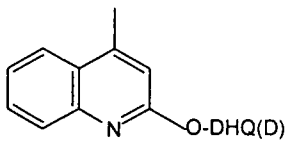


DHQ

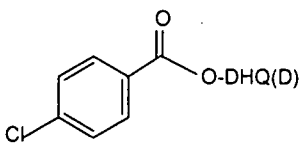


DHQD

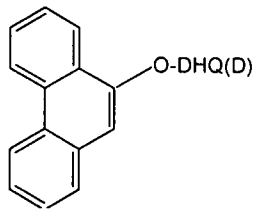
(b) First generation ligands with single chincona alkaloid units



[DHQ(D)]MEQ

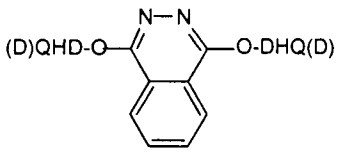


[DHQ(D)]CLB

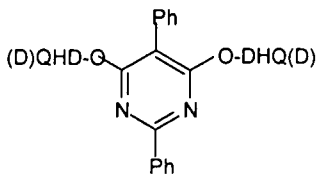


[DHQ(D)]PHN

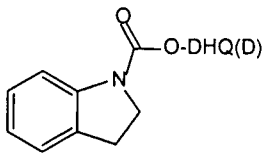
(c) Second generation ligands with dimeric chincona alkaloid units and heterocyclic spacers, and IND ligand for *cis*-olefins



[DHQ(D)]$_2$PHAL

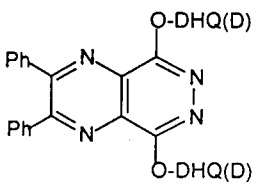


[DHQ(D)]$_2$PYR

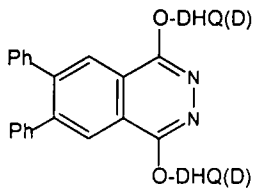


[DHQ(D)]IND

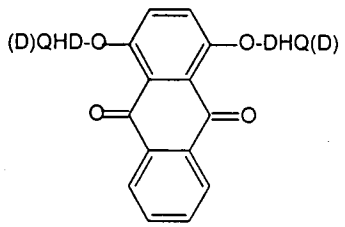
(d) New ligands with dimeric chincona alkaloid units with special applications



[DHQ(D)]$_2$DPP



[DHQ(D)]$_2$DP-PHAL



[DHQ(D)]$_2$AQN

Scheme 7. Sharpless asymmetric dihydroxylation ligands

Table 3. Recommended use of the most versatile AD ligands for each olefin class

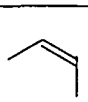
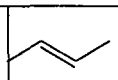
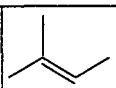
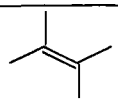
Olefin substitution pattern						
	Mono-	*Gem*-di-	*Cis*-di-	*Trans*-di-	Tri-	Tetra-
Preferred ligand	PYR PHAL AQN DPP	PHAL DP-PHAL	IND	PHAL DP-PHAL	PHAL	PYR PHAL
Ee range	30-97 %	70-97 %	20-80 %	90-99 %	90-99%	20-97%

Table 4. Choice of ligands for special classes of olefins

Ligand	Application
$[DHQ(D)]_2PHAL$	Gem di-substituted, *trans*-disubstituted trisubstituted and tetrasubstituted olefins, especially with aromatic substituents, aryl allyl ethers
$[DHQ(D)]_2PYR$	Monosubstituted terminal aliphatic olefins with α-branching or cyclic substituents, tetrasubstituted olefins
$[DHQ(D)]_2DPP$	Monosubsituted terminal aliphatic olefins, especially those with aromatic substituents, e.g. styrene
$[DHQ(D)]_2DP$-PHAL	Gem-disubstituted aliphatic olefins and trisubstituted olefins
$[DHQ(D)]_2AQN$	Monosubstituted terminal olefins, especially with allylic heteroatoms, but not for olefins with aromatic groups
DHQ(D)IND	*Cis*-olefins
DHQ(D)MEQ	Terminal and *trans*-disubstituted olefins with aromatic groups
DHQ(D)CLB	Allyl silanes, *trans*-disubstituted olefins
DHQ(D)PHN	Acrolein acetals, terminal aliphatic and *trans*-disubstituted olefins

The main drawbacks however, are the low concentration of olefin (100 mM) in the H_2O/*t*-BuOH (1:1) reaction medium, and the amount of waste generated by the AD reaction. Despite the catalytic efficiency of the reaction (0.2 - 1 mol% of the non-volatile $K_2OsO_2(OH)_4$, and 1 – 5 mol% ligand), several additives must be added to the reaction

mixture. $K_3Fe(CN)_6$ (3 equivalents) must be added as reoxidant, K_2CO_3 (3 equivalents), pH 12.2, to establish heterogeneous conditions to suppress the secondary catalytic cycle, $NaHCO_3$ (3 equivalents), pH 10.3 to buffer the reaction medium in the case of base-sensitive epoxides, and $MeSO_2NH_2$ (1 equivalent) to enhance the rate of catalytic turnover. $MeSO_2NH_2$ is added in all cases, except with terminal olefins, to reduce reaction times to 6-24 hours. Thus, 1.75 kg salt waste is generated for every mol alkene that is dihydroxylated. For an average alkene such as octene (M_r 112 g) this means that 15.6 gram of waste is generated for every gram of alkene (Table 5). For large-scale operations, this may have serious implications.

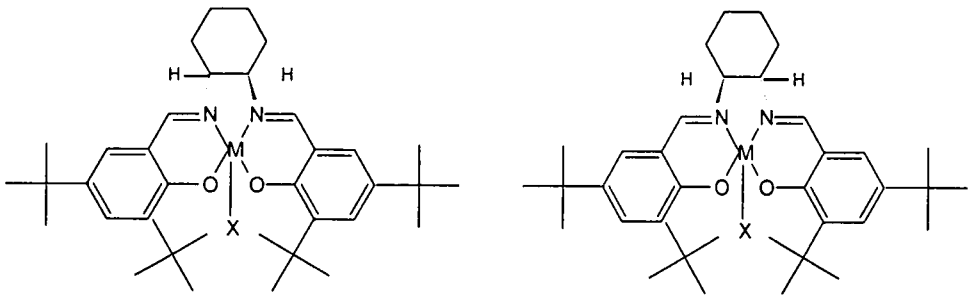
Table 5. Waste generated by additives for Sharpless AD

Additive	M_r	Eq. added	Waste/mol alkene (g)
$K_3Fe(CN)_6$	329.26	3	987.78
K_2CO_3	138.21	3	414.63
$NaHCO_3$	84.01	3	252.03
$MeSO_2NH_2$	95.12	1	95.12
Total			1749.56

2.1.3 Undirected asymmetric epoxidation: Jacobsen's AE with chiral (salen)Mn(III) complexes

Jacobsen's catalyst for AE of unfunctionalized olefins (Larrow and Jacobsen, 1994) is a Mn complex (Scheme 8a) of the chiral Schiff base of either (*R*,*R*)-or (*S*,*S*)-*trans*-1,2-diaminocyclohexane with 3,5-di-*tert*-butylsalicylaldehyde. The O-atom sources employed are oxidants such as NaOCl, *n*-Bu_4IO_4, iodobenzene and more recently dimethyldioxirane (Adam *et al.*, 1998). A donor ligand such as 4-phenylpyridine *N*-oxide (4-PPNO) or 4-(3-phenylpropyl)pyridine N-oxide (P_3NO) in a concentration of 20 mol% is required to stabilize and activate the catalyst. The amount of catalyst is dependent on the reactivity of the olefin, and ranges from 1.5 mol% to 16 mol%. The reactions can be performed at 0°C - 4°C in chlorobenzene or dichloromethane. Although stereoselectivity relies on nonbonded interactions and thus no formal functional group is required, both steric and electronic effects govern the enantio- and diastereoselectivity of epoxidation

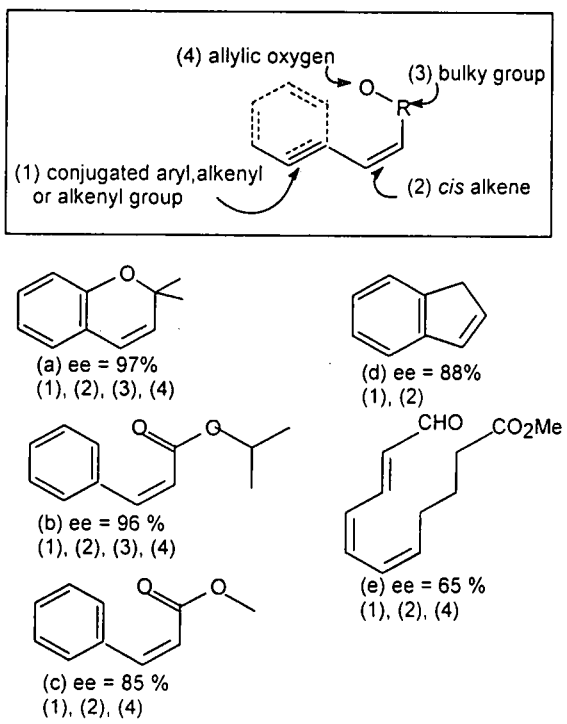
by (salen)Mn complexes (Jacobsen *et al.*, 1994). High enantioselectivity is obtained only if the olefins have the following structural properties (in order of importance): (1) aryl, alkenyl or alkynyl conjugated to the alkene, (2) a *cis* double bond linkage, (3) bulky allylic groups and (4) an allylic oxygen substituent. This methodology was successfully employed in the synthesis of Diltiazem (98% ee) from *cis*- isopropyl 4-methoxycinnamate (Scheme 9b), which satisfies all the above structural requirements. The ee obtained in the epoxidation of a key intermediate in the synthesis of Leukotriene A_4 (83% ee) methyl ester (Chang *et al.*, 1993) from a protected alcohol of the conjugated *cis*-disubstitiuted polyene (Scheme 9e), which do not have all the structural features required for high enantiofacial selectivity, was less satisfactory. Cyclic 1,3-dienes, which were predicted to be excellent candidates for Jacobsen's AE, were epoxidized with only moderate enantioselectivity. The enantioselectivities were slightly improved by introducing sterically hindered and electron donating $OSi(iPr)_3$ substituents in the place of the *tert* butyl groups in the 5,5' positions of the ligand (Chang *et al.*, 1994). Despite efforts to optimize reaction conditions (2M NaOCl containing 0.3M NaOH as O atom source and the expensive 4-(3-phenylpropyl)pyridine N-oxide (P_3NO) as donor ligand), indene oxide could be obtained in only 88% ee from indene (Scheme 9c) at Merck Research Laboratories (Senanayake *et al.*, 1996) with Jacobsen's AE catalyst. The strict structural requirements for high ee thus severely limit the scope of Jacobsen's AE. However, Katsuki synthesized several (salen)Mn(III) complexes with chiral salicylaldehyde and chiral ethylenediamine moieties (Katsuki, 1995), and obtained indene oxide in 98% ee using the derivatized catalyst in Scheme 10. This suggests that, like with Sharpless AD, ligand variation is required to broaden the scope of Jacobsen's AE. *Trans*-olefins are epoxidized with only moderate enantioselectivity, and terminal epoxides were not included in the published results, presumably because they were poor substrates.



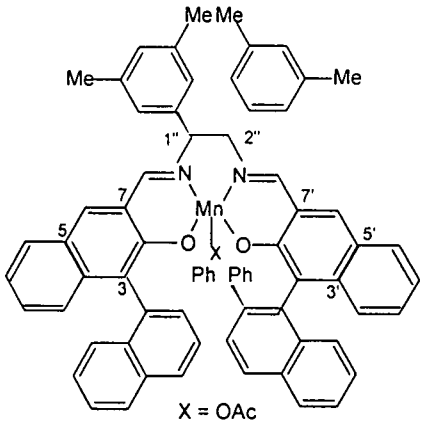
(*R*,*R*) -1 (*S*,*S*) -1

(a) M = Mn, X = Cl
(b) M = Cr, X = N_3
(c) M = Co, X = $(PhCO_2^-)_2$
(d) M = Co, X = $(OAc)(H_2O)$

Scheme 8. Jacobsen's chiral salen complexes



Sceme 9. Structural requirements of substrates for enantiofacial selectivity with Jacobsen's chiral (salen)Mn(III) catalyst for asymmetric epoxidation of unfunctionalized olefins



Scheme 10. Modified (salen)Mn(III) catalyst synthesized by Katsuki

2.1.4 Kinetic resolution of terminal epoxides and asymmetric ring-opening of meso-epoxides with $TMSN_3$: Jacobsen's chiral (salen)Cr(III) complex

Despite the usefulness of the Sharpless and Jacobsen catalysts for AE of several structural classes of olefins, enantiopure terminal epoxides were not accessible *via* these catalysts. The availability of a wide range of *rac* terminal epoxides at low cost and the importance of enantiopure terminal epoxides as intermediates, made kinetic resolution a *via*ble alternative strategy. Jacobsen obtained terminal alkyl-substituted epoxides in high ee with the (salen)Cr(III)N_3 complex (Hansen *et al.*, 1996) in Scheme 8b, *via* enantioselective ring opening with Me_3SiN_3.

The resultant ring-opening products (azido silyl ether) give access to enantiopure 1-amino-2-alkanols, which is not available from the chiral pool (reduction of α-amino acids). This process was applied to the synthesis of 2-(*S*)-propranalol and (*R*)-9-[2-(phosphonomethoxy)propyl]adenine (PMPA) (Larrow *et al.*, 1996). The same system also afforded asymmetric ring opening of *meso*-epoxides of five-membered rings (Martínez *et al.*, 1995) with excellent ee. Other applications include the synthesis of an *O*-protected (*R*)-4-hydroxy-2-cyclopentenone (Leighton and Jacobsen, 1996), an important prostaglandin building block.

This process constituted several important advances in synthetic catalysts for the synthesis of enantiopure epoxides. Direct access to terminal epoxides, which is probably the most versatile of epoxide building blocks, was achieved. Furthermore, ring opening of *meso*-epoxides was achieved under solvent–free conditions, which allowed maximum volumetric productivity, and with catalyst recycling. The reactivity was however low, and long reaction times (18-50 hr) and low temperatures (0-2°C) are required. It is also interesting to note that resolution of subterminal epoxides proceed with lower enantioselectivity.

2.1.5 Hydrolytic kinetic resolution of terminal epoxides and ring opening of meso-epoxides with carboxylic acids: Jacobsen's chiral (salen)Co(III) complex

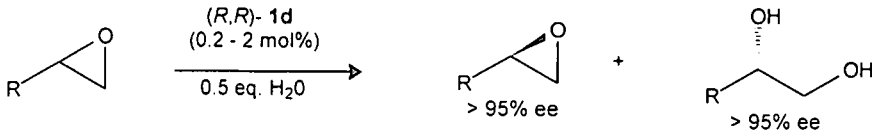
Ring-opening of *meso*-epoxides with benzoic acid catalyzed by the (salen)Co(III)$(PhCO_2^-)_2$ complex (Scheme 8c), generated *in situ* by aging of the Co(II) species with benzoic acid in air, afforded only moderate enantioselectivities, which could be improved by recrystallization of the resultant benzoates (Jacobsen *et al.*, 1997). Reactivity was low (40 – 144 hr), and the amine base *i*-Pr_2NEt had to be added in stoichiometric quantities to confer high solubility to benzoic acid in *tert*-butyl methyl ether (TBME), the non-polar reaction solvent. However, this catalyst led to the development of a highly efficient process for the hydrolytic kinetic resolution (HKR) of terminal epoxides (Scheme 11a) (Tokunaga *et al.*, 1997).

The (salen)Co(III)(OAc) catalyst (Scheme 8d), generated *in situ* by aging of the Cr(II) species with acetic acid in air, afforded C-3, C-6 and C-8 1,2-epoxides and 1,2-diols with excellent enantioselectivity (E>200) and reactivity (reaction time 6 – 12 hr). Useful resolutions of styrene oxide, epichlorohydrin and 1,2-epoxybutene (E >20) were obtained, although reactivities were low for styrene oxide (reaction time 44 hr) and 1,2-epoxybutene (reaction time 68 hr). Recycling of the catalyst, a solvent-free system and catalytic efficiency (0.2-1 mol% catalyst) made the development of this process a major advance in synthetic catalysts for the production of enantiopure terminal epoxides and 1,2-diols. This protocol was subsequently optimized to obtain C-3 building blocks (epihalohydrins and glycidol) in high enantiomeric purity (Furrow *et al.*, 1998). Epihalohydrins are invariably susceptible to racemization. Racemization of epichlorohydrin was very slow relative to the rate of hydrolysis under hydrolytic

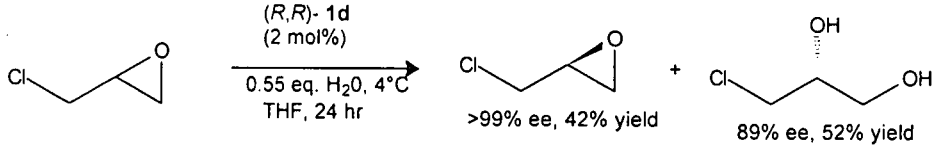
conditions with the Co catalyst, and could be suppressed by using THF as solvent (Scheme 11b). In contrast, racemization of epibromohydrin was rapid under the reaction conditions used, and dynamic kinetic resolution was effected using excess water (1.5 mol%) in THF as reaction solvent (Scheme 11c). Glycidol, while not a good substrate for HKR due to oligomerization, could be obtained in high enantiomeric purity from the optically active bromopropane diol obtained by the dynamic KR of epibromohydrin (Scheme 11d). Several glycidol derivatives were however excellent substrates for HKR, and benzylglycidyl ether, glycidyl butyrate as well as *tert*-butyldimethylsilyl glycidyl ether could be obtained in excellent enantiomeric excess (>99%) and yields (44 – 48%) (Scheme 11e).

An adapted procedure (1 mol% catalyst in Et_2O as solvent) allowed the HKR of long-chain (C-10 – C-20) alkyl epoxides, which cannot be conveniently prepared by other methods (Savle *et al.*, 1998). These long-chain alkyloxiranes are valuable chirons in the synthesis of enantiopure natural products, biomimetic molecules, chiral lipids and surfactants.

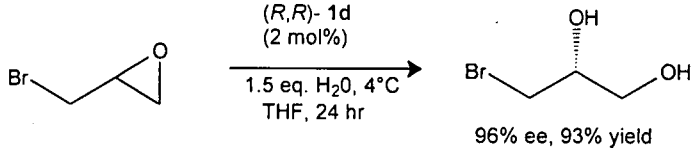
(a) Hydrolytic kinetic resolution



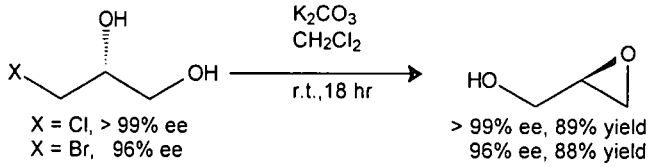
(b) THF suppresses racemization of epichlorohydrin



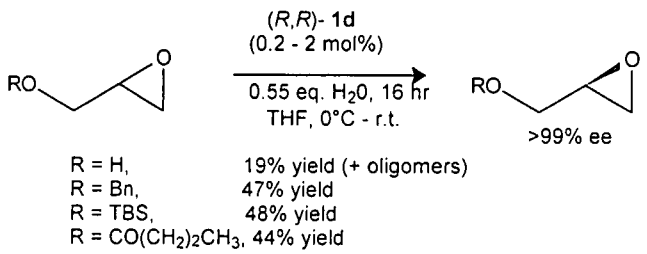
(c) Dynamic kinetic resolution of epibromohydrin



(d) Synthesis of glycidol from chloro- and bromopropanol



(e) Resolution of glycidol derivatives



Scheme 11. Synthesis of C-3 building blocks *via* Jacobsen's hydrolytic kinetic resolution

2.1.6 Asymmetric ring-opening of meso-epoxides with anilines

C_2 symmetric chiral ligands formed by derivatives of (*R*)-(+)- or (*S*)-(-)-1,1'-bi-2-naphtol had been used extensively in asymmetric synthesis. Applications included enantioselective reduction of unsymmetrical ketones (Singh, 1991; Deloux and Srebnik, 1993), asymmetric Diels-Alder reactions, asymmetric Claisen rearrangements (Maruoka *et al.*, 1995), asymmetric hydrocarboxylations (Alper and Hamel, 1990), dipolar cycloadditions (Pirrung and Zhang, 1992) and resolution of amines. Recently, derivatives of chiral lanthanide triflates formed from binaphtol had been used for the catalytic (10 mol% ligand) asymmetric ring opening of *meso*-epoxides with anilines (Hou *et al.*, 1998), allowing the direct synthesis of β-amino alcohols. The ligand was prepared from $Yb(OTf)_3$ and (*R*)-BINOL in the presence of a tertiary amine, and ring-opening of the epoxide with aniline was accomplished at -78°C. Useful enantioselectivities were obtained only with cyclohexene epoxide in the presence of the tertiary amine Ph_2NBn. The usefulness of this approach remains to be established.

2.1.7 Dimethyldioxirane oxidation of flavones and flavanols

The oxidation of enolate derivatives of flavone by dimethyldioxirane (DMD) to *trans*-3-hydroxyflavones as major diastereoisomer has been applied to the synthesis of oxygen-containing heterocycles from chalcone epoxides, such as *trans*- and *cis*-dihydroflavanols and *trans*-3-hydroxyflavones (Patonay *et al.*, 1996; Adam *et al.*, 1998)

2.1.8 Enzyme mimetics

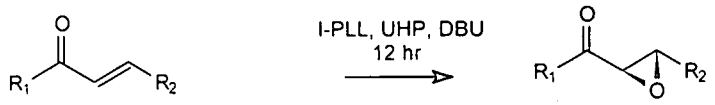
2.1.8.1 Catalytic antibodies

The employment of catalytic antibodies raised against haptens to promote selected transformations is still restricted to specialist laboratories (Roberts, 1998). Rate enhancement is generally low, in the order of 10^4, although rate enhancements of 10^6 had been achieved with aldolase antibody 38C2 (Fluka) (Roberts, 1999). Catalytic antibodies found for the enantioselective epoxidation of unfunctionalized olefins had a rate enhancement (k_{cat}/k_{uncat}) of only 40 to 50, which is too low to be of practical value (Koch *et al.*, 1994).

2.1.8.2 Polyleucine catalyzed epoxidation of α,β-unsaturated ketones

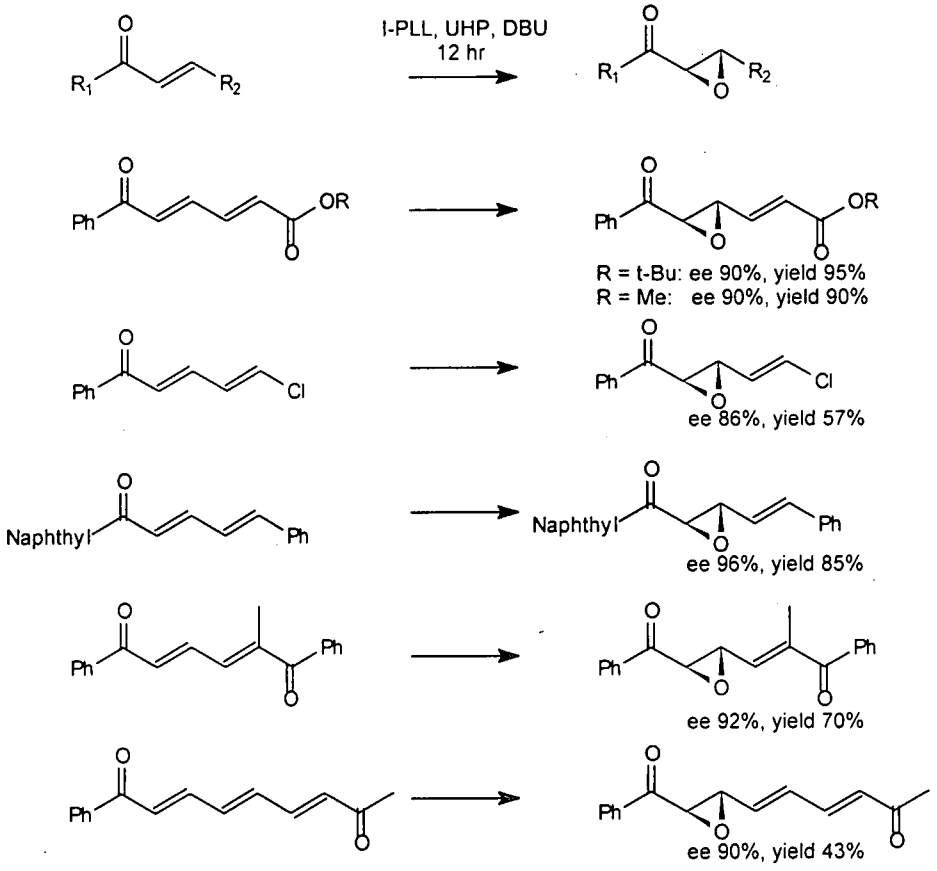
Poly-leucine catalyzed epoxidation of α,β-unsaturated ketones is effected in a two-phase system consisting of urea hydrogen peroxide (UHP) in THF or *tert*-butyl methyl ether (TBME) containing diazabicycloundecene (DBE) and immobilized poly-L-leucine (I-PLL) as insoluble catalyst. Either enantiomer of an epoxyketone can be accessed by using L- or D-leucine. Several enones are efficiently epoxidized with this catalytic system (Scheme 12a). Epoxidation of the enone (R_1 = *t*-Bu, R_2 = Ph) in Scheme 12a had been applied to the synthesis of diltiazem and the C-13 side chain of Taxol (Adger *et al.*, 1997). The marked difference in reactivity of different alkenes had been employed to distinguish between the conjugated double bonds of dienes (Scheme 12b) and was applied to the synthesis of γ-lactones.

(a) Enantioselective epoxidation of enones catalyzed by poly-L-leucine



R_1	R_2	Yield (%)	ee (%)	Time (h)
Ph	Ph	85	>95	0.3
Naphtyl	CH=CHPh	85	>98	2
Ph	cyclohexyl	91	89	3
Me	Ph	70	80	6
t-Bu	Ph	76	94	12
t-Bu	p-Me-Ph	90	96	28

(b) Relative differences in reactivity employed to discriminate between the different double bonds of dienes and trienes



Scheme 12. Polyleucine catalyzed epoxidation of α,β-unsaturated ketones

2.2 BIOCATALYSTS THAT GIVE DIRECT ACCESS TO ENANTIOPURE EPOXIDES AND VIC DIOLS VIA OLEFINS

The synthesis of enantiopure epoxides through various direct and indirect biocatalytic approaches had been reviewed extensively (Archelas and Furstoss, 1997; de Bont, 1993; Leak *et al.*, 1992; Besse and Veschambre, 1994). In analogy with the previous section, only those approaches that involve the direct stereospecific epoxidation of alkenes, and kinetic resolution strategies of *rac* epoxides and *vic* diols, will be considered here. Furthermore, only microbial enzymes, which can be prepared in large quantities, will be included. One exception is porcine pancreatic lipase, since it is commercially available at lower prices than most microbial lipases.

2.2.1 EPOXIDATION OF OLEFINS

2.2.1.1 Mono-oxygenases as enantioselective epoxidation catalysts

Various microbial mono-oxygenase (MO) systems had been studied, in particular with respect to their role in the biodegradation of xenobiotics such as polyaromatic hydrocarbons. Mono-oxygenases function mainly as hydroxylation enzymes, a reaction that is notoriously difficult to perform with traditional chemical methods. Terminal hydroxylation of *n*-alkanes by MO is widely used in the commercial production of C-13 to C-16 dioic acids for musk-like perfumes (Furuhashi, 1986). However, in the absence of steric constraints, epoxidation of terminal olefins may be kinetically more favourable than hydroxylation. This phenomenon has been exploited for the commercial production of various optically active epoxides from terminal olefins. All applications make use of whole cells, since MO's are complex multi-component systems that require co-factors (NADH) and O_2. Two-liquid phase fermentations are employed to reduce enzyme inhibition by the substrate and products, and to facilitate product recovery from the organic phase.

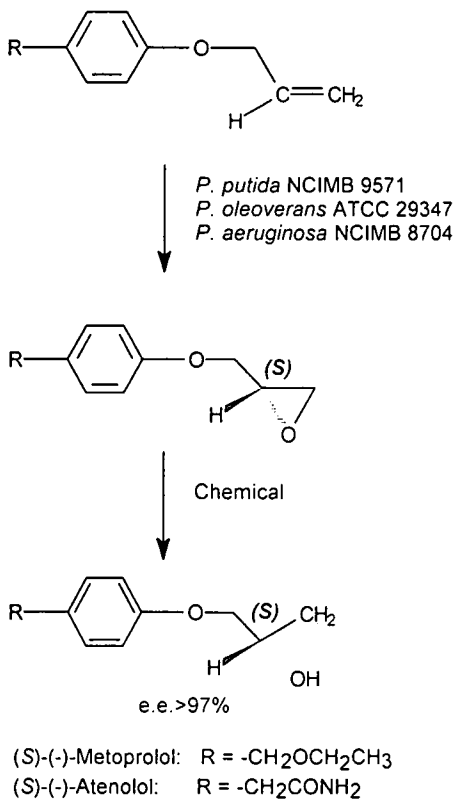
Bacterial mono-oxygenases

Heme-dependent cytochrome P-450 mono-oxygenases, although abundant, have found no synthetic applications. Similarly, the heme-independent methane mono-oxygenases

(MMO) of methylotrophic bacteria such as *Methylosinus trichosporium* produce epoxides of low enantiomeric purity. However, various other bacterial mono-oxygenases have been used for the synthesis of optically active epoxides on an industrial scale.

ω-hydroxylases

The ω-hydroxylase of *Pseudomonas oleoverans* and various other *Pseudomonas* spp. are capable of both epoxidation and hydroxylation of straight chain terminal olefins, resulting in a mixture of products. In the case of α,ω-dienes, stereoselective epoxidation at both terminals favor formation of the (*R*)-di-epoxide. ω-Hydroxylases also accept allylbenzenes and allylphenyl ethers as substrates, but do not catalyze epoxidation of cyclic substrates, subterminal olefins, styrene and allylic alcohols. This distinction was elegantly exploited for the industrial synthesis of the β-blockers Metoprolol® and Atenolol® (Johnstone *et al.*, 1987) (Scheme 13).



(*S*)-(-)-Metoprolol: R = $-CH_2OCH_2CH_3$
(*S*)-(-)-Atenolol: R = $-CH_2CONH_2$

Scheme 13. Synthesis of Metoprolol® and Atenolol® employing stereospecific epoxidation by *Pseudomanas* spp.

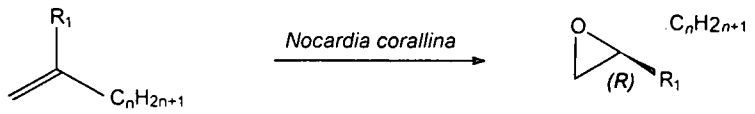
Xylene oxygenase (XO)

The XO system of *Pseudomonas putida* hydroxylates aromatic substrates, and does not usually epoxidize olefins. However, this enzyme catalyzes the stereospecific epoxidation of styrene to (*S*)-styrene oxide (93% ee). *Escherichia coli* recombinants expressing the XO genes from the TOL plasmid of *P. putida* mt-2 were used to produce (*S*)-enantiomers of styrene oxide (93% ee) and 3-chlorostyrene oxide (97% ee) in two-liquid phase fermentations, with *n*-octane as second phase (Wubbolts *et al.*, 1994). Methyl-substituted styrenes were preferentially hydroxylated at the methyl moiety, and 4-chlorostyrene oxide was obtained in low enantiomeric purity.

Alkene mono-oxygenases

These enzyme systems are usually induced by growth on alkenes, and do not display competing hydroxylation activity. Although excellent ee's are obtained, the substrate ranges are usually narrow and different organisms have to be employed as biocatalysts for the production of the various optically active epoxides. Various bacterial strains grown on specific alkenes can produce (*R*)-1,2-epoxypropane, (*R*)-1,2-epoxybutane, (*S*)-1-chloro-1,2-epoxypropene and (*R,R*)-trans-2,3-epoxybutane in excellent enantiomeric excess (99%) (Weijers *et al.*, 1988). *Corynebacterium equi* produces optically pure (*R*)-1,2-epoxyhexadecane (ee 100%) (Furuhashi, 1986).

One exception is *Nocardia corallina*, which constitutively expresses alkene MO with a remarkably broad substrate range (Scheme 14). This allows the organism to be grown on glucose, and to apply the same process for the production of a wide range of alkenes. Optically active (*R*)-1,2-epoxyalkanes produced by *Nocardia corallina* are sold by Nippon Mining Co. (Furuhashi, 1986). These epoxides are used as the chiral part of ferroelectric liquid crystals. *Nocardia corallina* also catalyze the stereospecific epoxidation of various 2,2-disubstituted terminal olefins to the corresponding (*R*)-epoxides, which were used as chiral building blocks to produce prostaglandin ω-chains (Takahashi *et al.*, 1989).

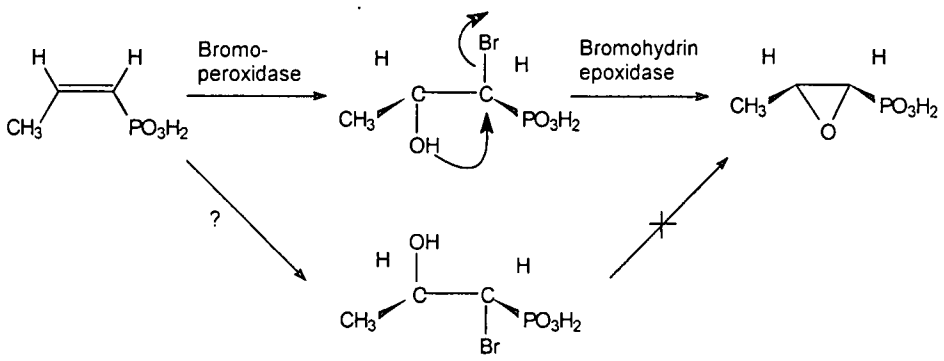


(a) R_1 = H ; n = {1...16} ee = 80 - 90 %
(b) R_1 = CH_3 ; n = 3,4,5 ee = 76 - 90 %

Scheme 14. Stereospecific epoxidation of a homologous range of mono- and *gem* disubstituted terminal olefins by *Nocardia corallina*

Miscellaneous microbial epoxidations

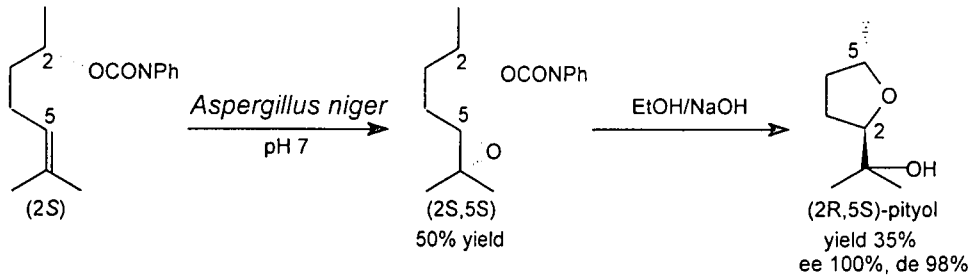
A number of *Penicillium* strains (White *et al.*, 1971), as well as the bacterium *Cellvibrio gilvus* (Uwajima *et al.*, 1989) were reported to produce the broad spectrum antibiotic fosfomycin [(-)-(1*R*,2*S*)-1,2-epoxypropylphosphonic acid) *via* mono-oxygenase catalysed epoxidation. However, *Flavobacterium esteroaromaticum* IFO 3751, and *Pseudomonas putida* IK-8, selected from fifteen strains capable of producing fosfomycin, were found to produce the antibiotic *via* a two enzyme system consisting of bromoperoxidase and bromohydrin epoxidase (Itoh *et al.*, 1995) (Scheme 15). One or both of the enzymes must be stereoselective to give the optically active epoxide formed *via* the bromohydrin intermediate.



Microorganism	Productivity (mg/ml)
Penicillium spinulosum	0.45
Cellvibrio gilvus	1.5
Flavobacterium esteroaromaticum	2.0

Scheme 15. Stereoselective microbial production of fosfomycin

In some rare cases, certain fungal strains accumulate epoxides without further degradation to *trans* diols. A (2*S*)-sulcatol derivative was stereospecifically epoxidized to the (2*S*,5*S*)-epoxy-ester (Archelas and Furstoss, 1992). The enantiomerically pure epoxy-ester was subsequently transformed (Scheme 16) to enantiopure (2*R*,5*S*)-pityol, the pheromone of the spruce bark beetle *Pityophthorus pityographus*. (See also the lipase-catalyzed formation of (2*R*,5*R*)-pityol (Mischitz *et al.*, 1994), the pheromone of the elm bark beetle *Pteleobus vittatatus*).



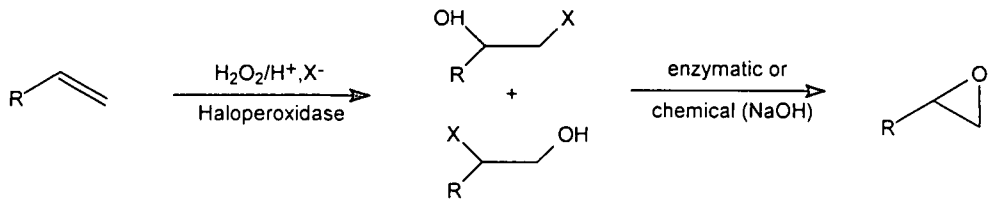
Scheme 16. Epoxidation of the phenylcarbamate of (*S*)-culcatol by the fungus *A. niger*. (2*R*,5*S*)-pityol was obtained by subsequent base-catalyzed rearrangement.

2.2.1.2 Haloperoxidases as enantioselective epoxidation catalysts

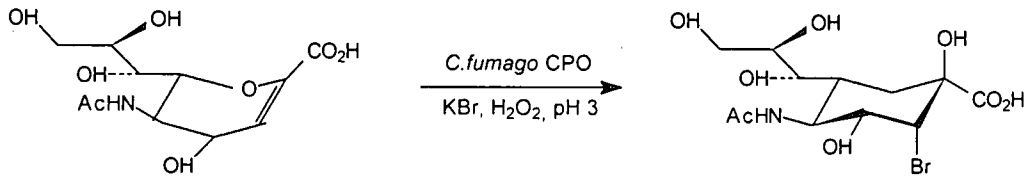
Indirect epoxidation *via* halohydrin intermediates

The acidic form of haloperoxidases catalyzes the formation of halohydrins in the presence of H_2O_2 and halide ions (Scheme 17a). This reaction usually produces racemic halohydrins, because hypohalous acid (XOH) is generated by the enzyme, which is added outside the active site to the olefinic substrate. The halohydrin can subsequently be transformed into epoxides by the action of various enzymes, such as halohydrin epoxidases and halohydrin hydrogen-halide lyases (Nakamura *et al.*, 1991), or chemically by base treatment. Unless the enzymes that catalyze the epoxidations are enantioselective, optically active epoxides can only be obtained by kinetic resolution of the racemic halohydrins *via* enantioselective degradation of one halohydrin enantiomer (Scheme 17c and 17d), followed by chemical ring closure.

(a) Halohydrin formation catalyzed by haloperoxidases in the presence of halide ions and conversion of halohydrin to epoxide



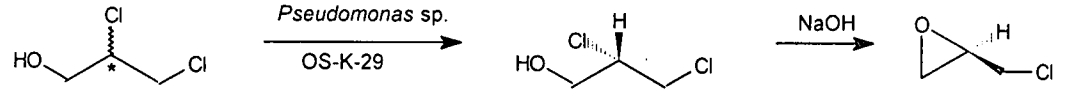
(b) Regio- and stereoselective bromohydration of 2,3-dehydrosialic acid



2,3-dihydrosialic acid

3-deoxy-3-bromosialic acid

(c) Enantioselective degradation of (*R*)-2,3-dichloropropanol employed in the synthesis of (*R*)-epichlorohydrin

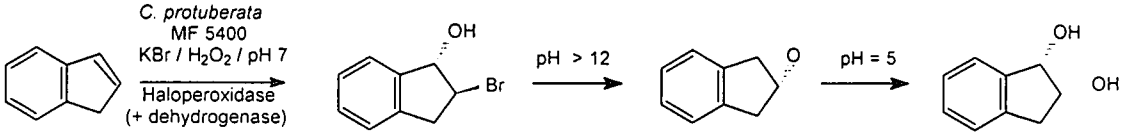


rac-2,3-dichloropropanol

(S)-2,3-dichloropropanol
ee 100%

(R)-epichlorohydrin
ee 99.5%

(d) Enantioselective bromohydration of indene by haloperoxidase and a stereoselective dehydrogenase in the synthesis of protease inhibitor intermediates



Scheme 17. Indirect epoxidation of olefins *via* halohydrin formation catalyzed by haloperoxidases

One exception to the formation of racemic halohydrins is the regio- and stereospecific bromohydration of the glycal 2,3-dehydroxysialic acid (Fang *et al.*, 1995) catalyzed by the chloroperoxidase (CPO) of the fungus *Caldariomyces fumago* (Scheme 17b). An example of an enantioselective halohydrin epoxidase is probably the formation of the optically active fosfomycin (Scheme 16), although the ee was not reported. Enantioselective degradation of only the (*R*)-enantiomer of 2,3-dichloro-1-propanol *via* assimilation by *Pseudomonas* sp. OS-K-29 (Kasai *et al.*, 1992) was applied to the synthesis of enantiopure (*R*)-epichlorohydrin (Scheme 17c). The fact that the halohydrin formation is generally not stereospecific, is exemplified by the patent (Chartrain *et al.*, 1997) registered for the quantitative conversion of indene to (1S,2R)-indene oxide and (1S,2R)-indanediol by combination of haloperoxidase and chemical steps (Scheme 17c). The acidic chloroperoxidase from *Calderiomyces fumago* catalyzed the direct epoxidation of indene, but formed racemic indene oxide. Under neutral conditions, indene was unreactive with several haloperoxidases, unless KBr was co-fed to the system. In the presence of KBr and H_2O_2 at neutral pH, *Curvularia protuberata* MF 5400 (ATCC 74332) haloperoxidase was thought to catalyze the formation of *trans*-(1*S*,2*S*)-bromoindanol, which could be converted to the desired (1*S*,2*R*)-indene oxide and (1*S*,2*R*)-indanediol. However, mechanistic studies later revealed that the stereoselectivity observed was probably due to a specific dehydrogenase activity present in this fungus (Zhang *et al.*, 1999).

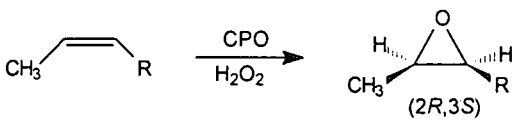
Direct enantioselective epoxidation catalyzed by CPO

The neutral form of haloperoxidases catalyzes the direct epoxidation of olefins in the absence of halide ions. The heme-containing chloroperoxidase (CPO) of the fungus *Caldariomyces fumago* is a versatile catalyst that diplays exquisite enantioslelectivity towards the epoxidation of a broad range of alkenes. Most heme-peroxidases have a histidine as 5th axial ligand of the iron (van Deurzen *et al.*, 1997). However, CPO of *Caldariomyces fumago* has a cysteine as 5th axial ligand, which strongly resembles the active site of the cytochrome P-450 family of enzymes. Indeed, this enzyme acts as a free cytochrome P-450 and catalyses the hydroxylation of many substrates (Zaks and Dodds, 1995). Enantioselective epoxidation of *cis*-2-alkenes (Scheme 18a) (Allain *et al.*, 1993; Zaks and Dodds, 1995) and *gem*-disubstitiuted alkenes (Scheme 18b) (Dexter *et*

al., 1995; Lakner and Hager, 1996; Lakner *et al.*, 1997; Lakner and Hager, 1997) proceeds smoothly without the formation of hydroxylated by-products. In the case of aliphatic substituents, the chain length is restricted to C-5 to C-8. Several classes of olefins are not suitable substrates. The epoxide formed during epoxidation of aliphatic terminal alkenes cause suicide deactivation through heme *N*-alkylation. *Trans*-disubstituted alkenes are unreactive, and epoxidation of 3-alkenes result in low yields or the formation of by-products. Styrenes only give moderate enantioselectivities. Synthetic applications include the synthesis of (*R*)-(-)-mevalanolactone (Scheme 18c) and a chiral α-methylamino synthon (Lakner and Hager, 1997).

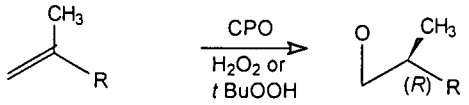
CPO of *Caldariomyces fumago* is a relatively stable, extracellular enzyme, which requires neither co-substrates nor co-factors, and is commercially available. CPO is quite sensitive to H_2O_2 and controlled addition of the oxidant is required. The catalase side reaction, which generates O_2 from H_2O_2, causes foaming and may reduce the yields of volatile products. The use of *t*-BuOOH as oxidant is thus more efficacious (Lakner *et al.*, 1997), but is not effective for the epoxidation of certain substrates (Allain *et al.*, 1993). The addition of co-solvents may also be beneficial.

(a) Epoxidation of *cis*-2 alkenes by CPO of *Caldariomyces fumago*



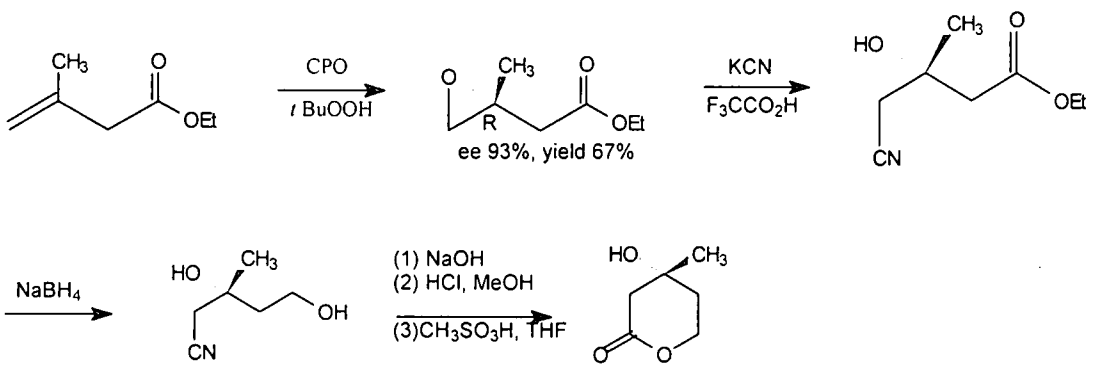
R	Ee (%)	Yield (%)
n-C_3H_7	97	12
n-C_4H_9	96	78
n-C_5H_{11}	92	82
i-C_4H_9	94	33
Ph	96	67

(b) Epoxidation of *gem*-disubstituted alkenes by CPO of *Caldariomyces fumago*



R	Ee (%)	Yield (%)
n-C_2H_4Br	88	93
n-C_3H_6Br	95	89
n-C_4H_8Br	87	33
n-C_5H_9	95	23
Ph	89	55
-CH_2OPh	89	22
CO_2Et	94	34
-CH_2CO_2Et	93	67
-CH_2NHCO_2Me	94	82

(c) Synthesis of (*R*)-(-)-mevalonolactone employing CPO of *Caldariomyces fumago*

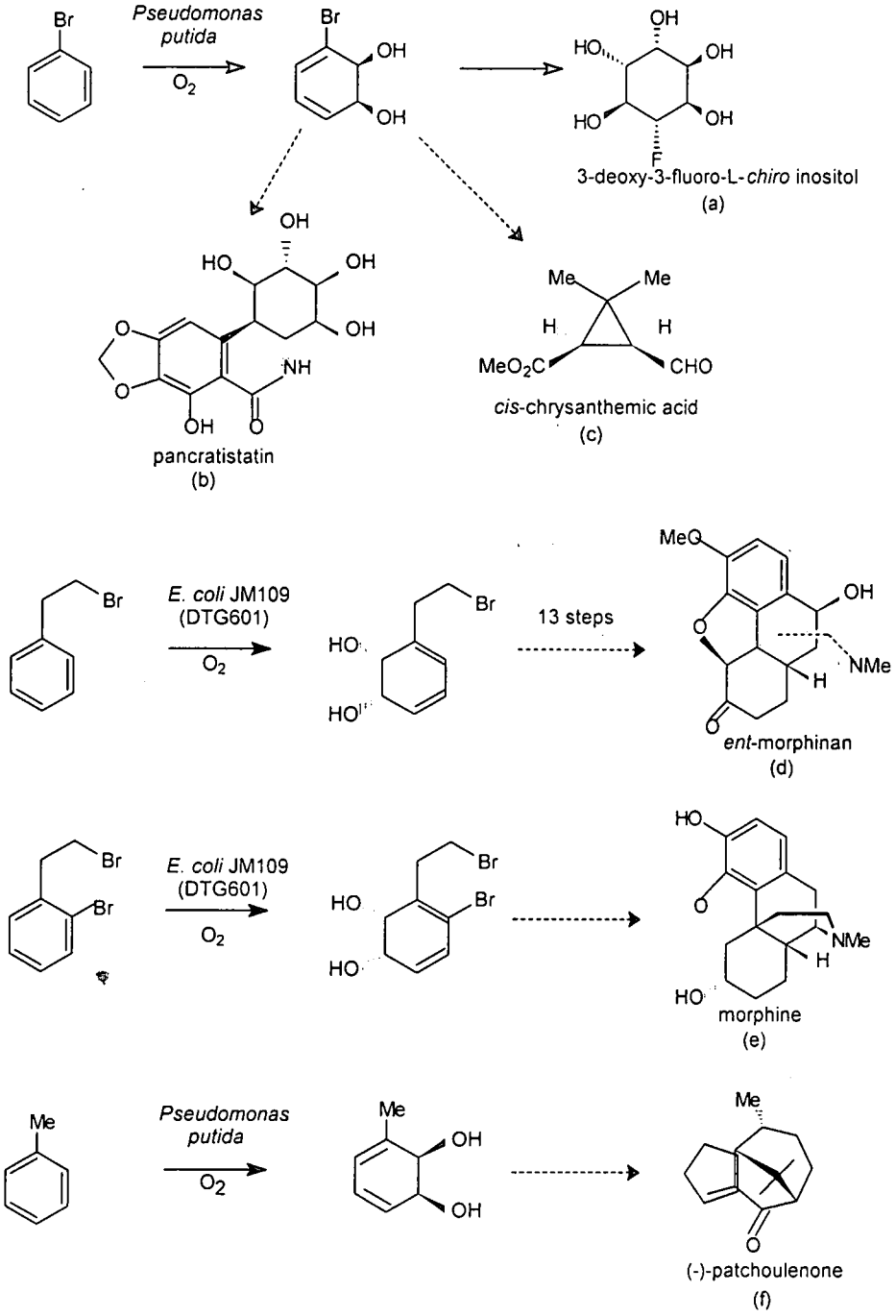


Scheme 18. CPO catalyzed enantioselective epoxidation of olefins

2.2.2 DIHYDROXYLATION OF OLEFINS

2.2.2.1 Dioxygenases as enantioselective arene dihydroxylation catalysts

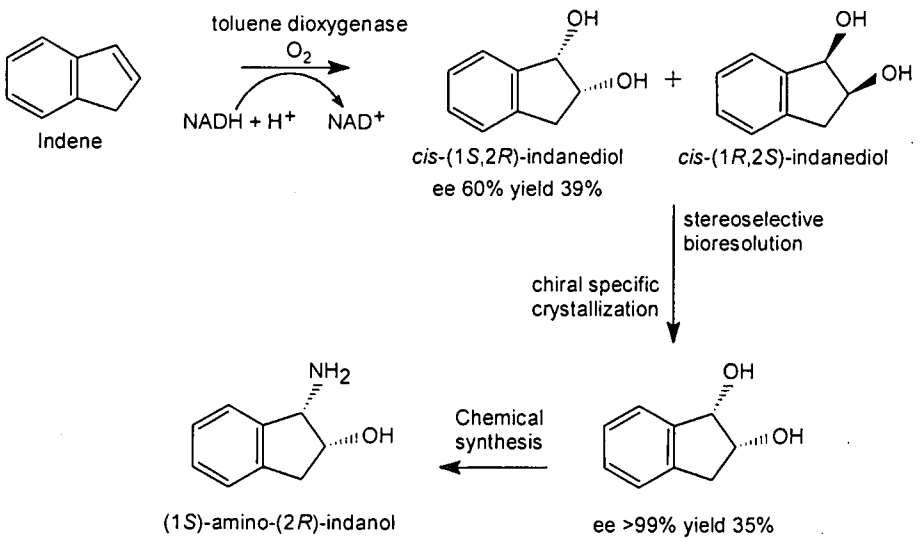
Dihydroxylations of arenes can only be effectively achieved by biological catalysts. The dihydroxylation of benzene, toluene, styrene and their 3-chloro or bromo-substituted derivatives by *Pseudomonas putida* give access to optically active (3-substituted) *cis*-cyclohexadiene-1,2-diols in high yields and with excellent ee values. These dienediols are extremely useful building blocks for natural products (Hudlicky and Thorpe, 1996). 3-Chloro and 3-bromo-cyclohexadiene *cis*-diols, produced by controlled microbial oxidation of the corresponding arene by *Pseudomonas putida* (Pp 39D) and *E. coli* JM109 (pDTG 601), had been used extensively in the synthesis of diverse natural products by the group of Thomas Hudlicky (Yan *et al.*, 1997). Notable work from this group includes the concise synthesis of cyclitols, aminocyclitols, aza-sugars, amino-sugars, several conduritols, and alkaloids. They also established a general methodology for the preparation of deoxymonosaccharides containing fluorine in any desired position, and applied this methodology to the synthesis of 5-deoxy-5-fluoro-lactol, an important intermediate in the synthesis of 3-deoxy-3-fluoro-L-*chiro*-inositol (Scheme 19a). Other work of this group include the synthesis of polydeuteriated mannose and pancratistatin (Scheme 19b), (Roberts, 1998), *ent*-morphinans (Scheme 19d) and morphine (Scheme 19e) (Endoma *et al.*, 1997). The group of Banwell employed the 3-bromo dienediol in the synthesis of *cis*-chrisanthemic acid (Scheme 19c), and the *cis*-1,2-dihydrocatechol derived from the oxidation of toluene in the synthesis of (-)-patchoulenone (Scheme 19f) (Banwell and Mcleod, 1998).



Scheme 19. Arene dihydroxylation catalyzed by dioxygenases in the synthesis of biologically active compounds

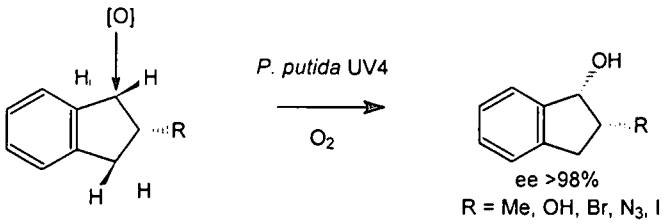
2.2.2.2 Dioxygenases as enantioselective dihydroxylation catalysts of indene

In their ongoing efforts to synthesize enantiopure building blocks for HIV protease inhibitors, Merck recently registered a patent for the synthesis of (1*S*)-amino-(2*R*)-indanol from indene (Buckland *et al.*, 1999). Three bacterial strains with enantioselective toluene dioxygenases were employed in the bioconversion of indene to give *cis*-(1*S*,2*R*)-indanediol in high enantiomeric excess. The ballast *cis*-(1R,2S)–indanediol could be fortuitously removed by the same organisms, that degraded the unwanted stereoisomer through enantioselective dihidrodiol dehydrogenase activity, thereby boosting the enantiomeric purity to >99.5% (Scheme 20). Alternatively, chiral specific crystallization was employed after the enantioselective dihydroxylation to give enantiopure *cis*-(1*S*,2*R*)-indanediol, which could be derivatized to enantiopure (1*S*)-amino-(2*R*)-indanol without tartaric acid resolution. The strains used were *Pseudomonas putida* F1 (ATCC 55688), which had a toluene dependent dioxygenase, and *Pseudomonas putida* 421-5 (ATCC 55687) and *Rhodococcus* sp. B264-1 (ATCC 55806), which constitutively expressed the dioxygenase.



Scheme 20. Enantioselective dihydroxylation of indene by toluene dioxygenase employed in the synthesis of building blocks for HIV protease inhibitors

Both (1*S*)-amino-(2*R*)-indanol and (1*S*,2*R*)-indanediol was also synthesized by the benzylic monohydroxylation of 2-substituted indan substrates, catalyzed by the toluene dioxygenase of *Pseudomonas putida* UV4 (Boyd *et al.*, 1996) (Scheme 21).

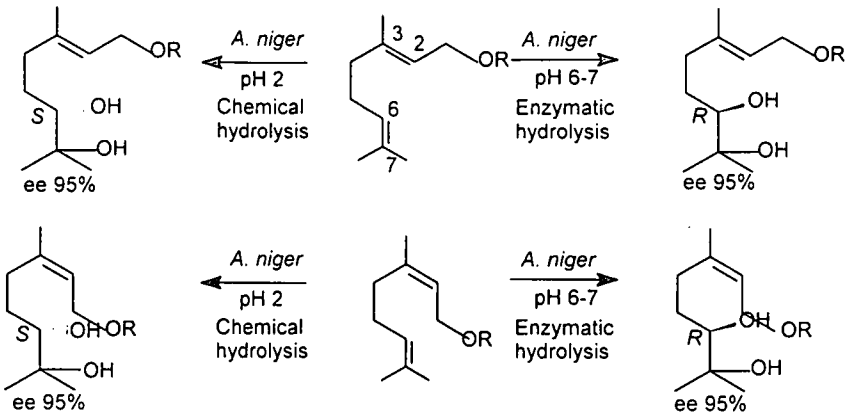


Scheme 21. Monohydroxylation of 2-substituted indane substrates by toluene dioxygenase

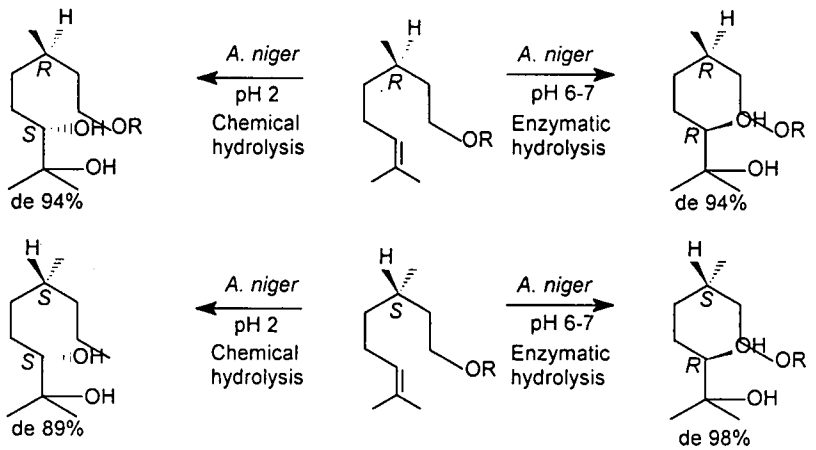
2.2.2.3 Dihydroxylations of monoterpenes catalyzed by fungi

Dihydroxylation of the *N*-phenylcarbamates of geraniol and nerol catalyzed by *Aspergillus niger* (Fourneron *et al.*, 1989), takes place exclusively at the remote C6=C7 double bond *via* a 6*S*-epoxide intermediate (Zhang *et al.*, 1991). This is in contrast with Sharpless AE of allylic alcohols, where epoxidation occurs exclusively at the C2=C3 double bond. Furthermore, the stereochemical outcome of the hydroxylation can be modulated by controlling the pH of the reaction medium (Scheme 22a). Similarly, stereoselective dihydroxylation of both the (*R*)- and (*S*)-citronellol enantiomers was achieved (Zhang *et al.*, 1992), giving access to all four diol isomers by controlling the pH of the medium (Scheme 22b).

(a) Dihydroxylation of the *N*-phenylcarbamates of geraniol and nerol



(b) Dihydroxylation of the *N*-phenylcarbamates of citronellol enantiomers



Scheme 22. Dihydroxylation of monoterpenes by *Aspergillus niger*. R = CONHPh

2.2.3 KINETIC RESOLUTION OF *RAC* EPOXIDES

2.2.3.1 Kinetic resolution of functionalized epoxides by lipases

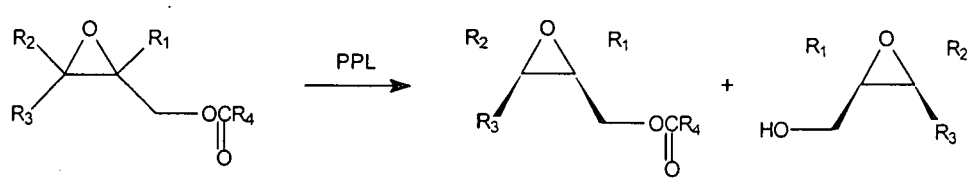
Hydrolases, and in particular lipases and esterases, have been the main contributors to the breakthrough of enzymatic methods in organic chemistry. These enzymes are remarkably stable under non-physiological conditions, have wide substrate spectra and are co-factor independent. At present, lipases and esterases constitute the largest group of synthetically useful enzymes. Lipases work well in organic apolar solvents, due to

their need for a lipophilic environment for catalytic activity. This allows suppression of the activity of the nucleophile H_2O, thus enabling esterification, transesterification and amide bond forming reactions. Lipases from numerous microbial as well as other sources such as hog pancreas, are commercially available as powders. The productivity (space, time, yield) of enzymatic processes are greatly improved by immobilization of the biocatalyst. Particularly useful is the incorporation of lipases in hydrophobic organic-inorganic hybrid materials (allyl silanes) with the sol-gel process. Sol-gel immobilization of lipases increase enzyme activity, improve enantioselectivity and enzyme stability, and allow convenient recovery by filtration or centrifugation (Fluka chiral nonracemic compounds catalogue, 1998/1999).

However, the use of lipases for the resolution of epoxides require a pre-existing functionality (hydroxyl or ester group) as the site of the reaction, while the epoxide moiety forms the basis for chiral recognition.

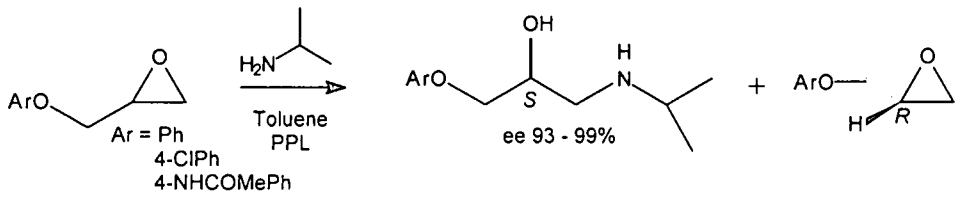
Resolution of racemic esters of epoxyalcohols

Porcine pancreatic lipase (PPL) resolves (substituted) glycidyl esters to give (*R*)-glycidyl esters and (*R*)-glycidols (Ladner and Whitesides, 1984) (Scheme 23). The PPL-catalyzed resolution of racemic (substituted) glycidyl esters is exploited on a commercial scale by Adeno-DSM (Kloosterman *et al.*, 1988) to produce (2*R*)-glycidylesters and (2*R*)-glycidols. The (*S*)-glycidols are employed in the synthesis of (*S*)-β-blockers and ferro-electric liquid crystals, while the (*R*)-enantiomers are employed in the synthesis of L-carnitine, phospholipids, platelet aggregation factor, anti-viral S-HMPA and ferro-electric liquid crystals.



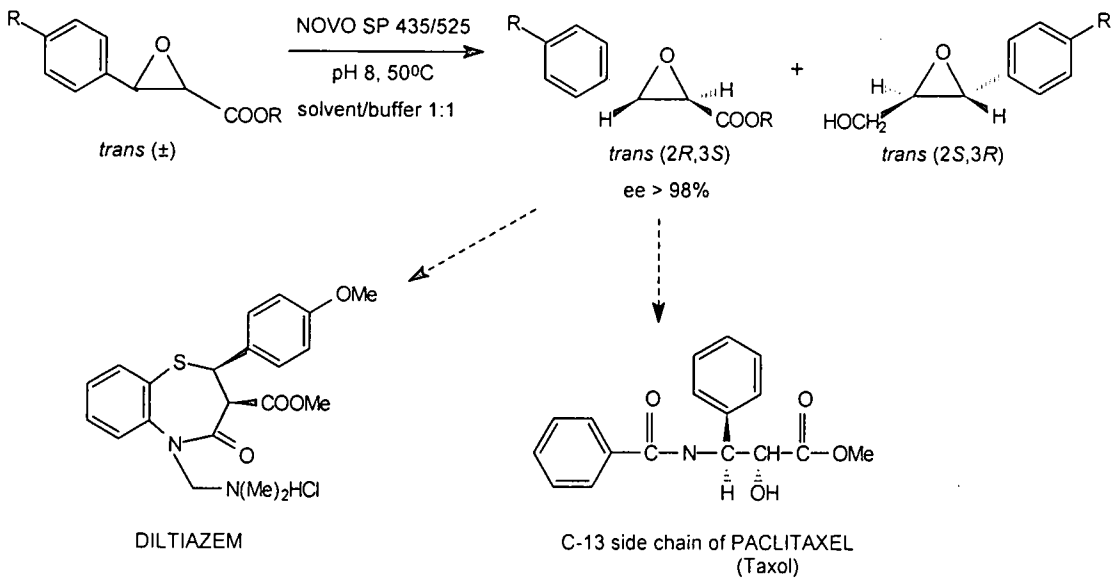
Scheme 23. Kinetic resolution of epoxides by enantioselective ester hydrolysis

PPL had also been employed to catalyze the stereoselective ring-opening of epoxides with 2-propylamine, which gave direct access to (*S*)-propanolamine β-blockers (Scheme 24).



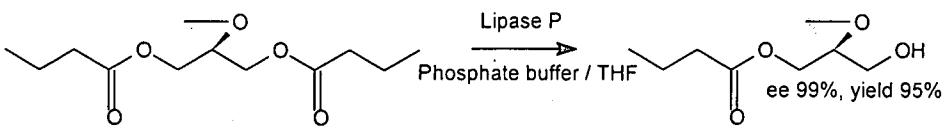
Scheme 24. Kinetic resolution of epoxides by enantioselective ring-opening with isopropylamine

Many patents for the production of optically active 3-phenylglycidic esters by stereoselective ester hydrolysis by microbial lipases had been registered. Optically active 3-phenylglycidic esters are important building blocks in the synthesis of benzothiazepines such as Diltiazem, and the side chain of benzazepines such as Taxol®. Stereoselective hydrolysis of the (2*R*,3*S*)-enantiomer by various bacteria, yeasts and fungi allow the recovery of (2*S*,3*R*)-3-phenylglycidic esters from the organic layer, while the alcohol remains in the aqueous layer (Mori *et al.*, 1991). *Micrococcus urea* 1AM 1010 produced the desired enantiomer in 3.4 mg/ml yield in 72 hours. Similarly, the (3*R*,3*S*)-3-phenylglycidic ester may be obtained by using enzymes from other microbial sources. Stereoselective hydrolysis using immobilized *Candida antarctica* lipases (NOVO SP 435 and 525), have distinct advantages (Kierkels and Peeters, 1995). Various solvents may be used, since the solvent do not influence the selectivity of the enzyme. The enzyme is stable at high temperatures, and the maximum operating temperature is determined by the stability of the substrate (80°C). Higher reaction temperatures (50°C) and pH (pH 8) increase the reaction rates 3-fold. The desired enantiomer is obtained in >98% ee at 55% conversion (Scheme 25).



Scheme 25. Enatioselective hydrolysis of *rac* 3-phenylglycidic esters by *Candida antarctica* lipases

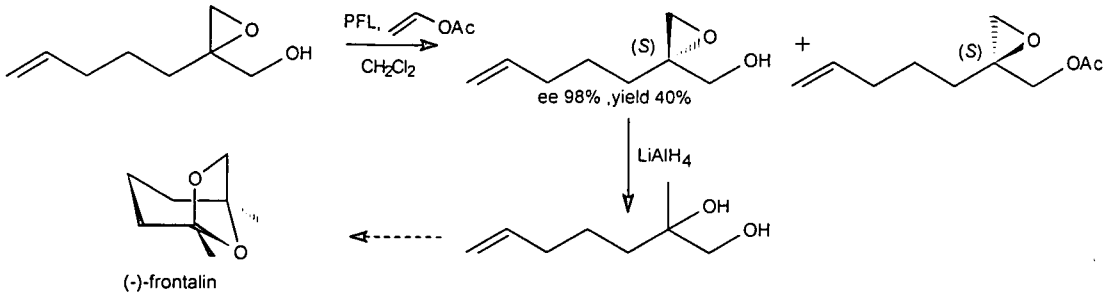
A precursor of (*R*)-2-butyryloxymethylglycidol was synthesized in high enantiomeric purity (ee 99%), by lipase-catalyzed asymmetric hydrolysis (Seu *et al.*, 1995) (Scheme 26). This compound is a versatile building block in the synthesis of enantiomerically pure natural products with a stereogenic quaternary carbon centre, such as α-tocopherol, bicyclomycin, sordidin and acacialactam.



Scheme 26. Synthesis of an enantiopure glycidol derivative *via* symmetry destruction of a prochiral substrate by lipase P

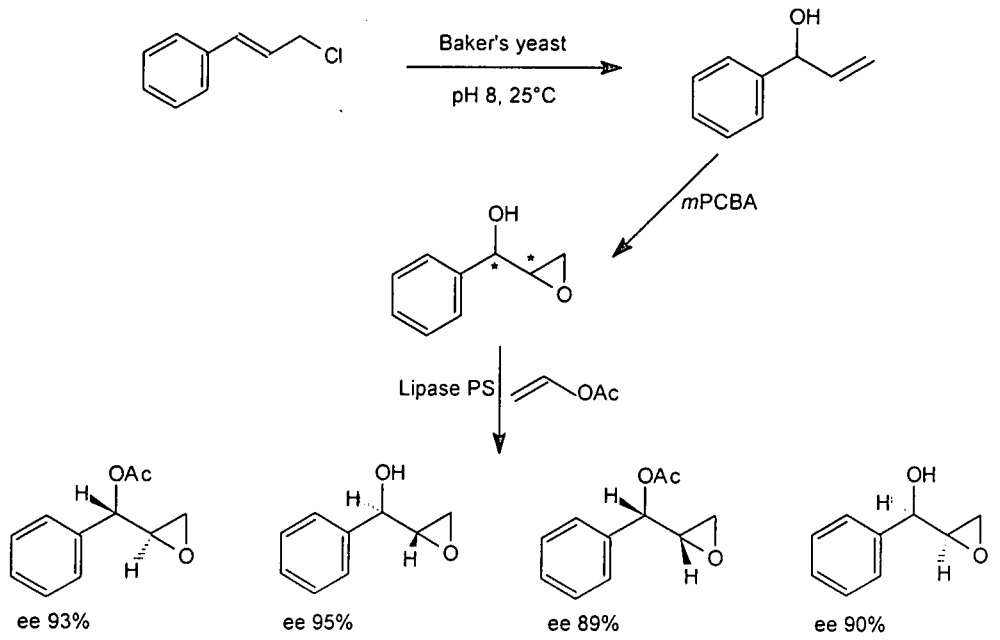
Enantioselective esterification of epoxyalcohols

Lipase-catalyzed transesterification was employed to resolve the enantiomers of 2,3-epoxy-2-(4-pentenyl)-propanol. The (*S*)-epoxyalcohol served as a building block for (1S,5R)-frontalin (Ferraboschi *et al.*, 1993) (Scheme 27).



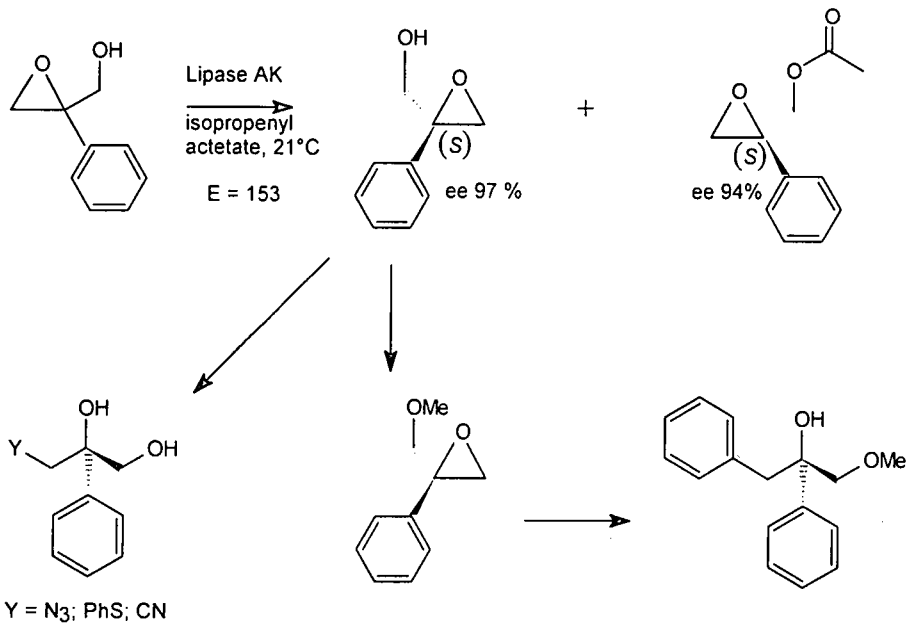
Scheme 27. Esterification of a primary alcohol by *Pseudomonas fluorescens* lipase employed in the synthesis of (-)-frontalin

Esterification of secondary epoxyalcohols was employed in the synthesis of optically active 1-phenylglycidols (Takeshita *et al.*, 1992). Baker's yeast was used to preprare 1-phenylallylalcohol, which was subsequently epoxidized with *m*-CPBA, and resolved in all four enantiomers using *Pseudomonas* lipase (Scheme 28).



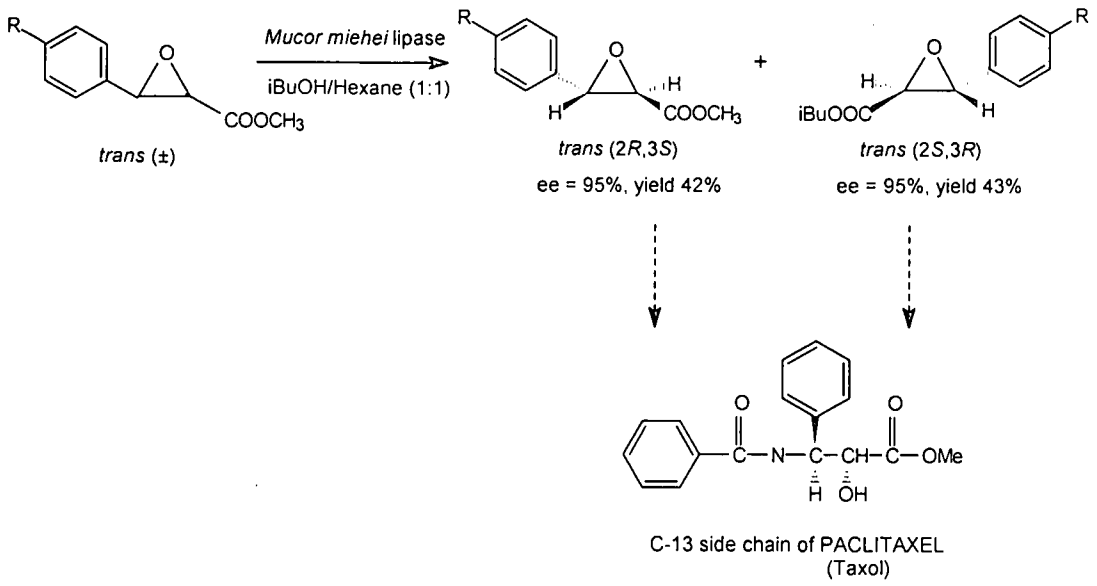
Scheme 28. Stereoselective esterification of secondary epoxyalcohols employed to give optically active 1-phenylglycidols

Enantiomerically pure tertiary alcohols and their derivatives are useful building blocks for many drugs and natural products, but their resolution by esterification is notoriously difficult to achieve, especially if they contain bulky groups. However, stereoselective esterification of 1,1-disubstituted 1,2-epoxides may be employed to obtain tertiary alcohols in excellent enantiomeric excess (Chen and Fang, 1997). Stereoselective ring opening with various nucleophiles gives highly functionalized tertiary alcohols (Scheme 29).



Scheme 29. Esterification of 1,1-disubstituted 1,2-epoxides with Lipase AK allows access to tertiary alcohols containing bulky substituents

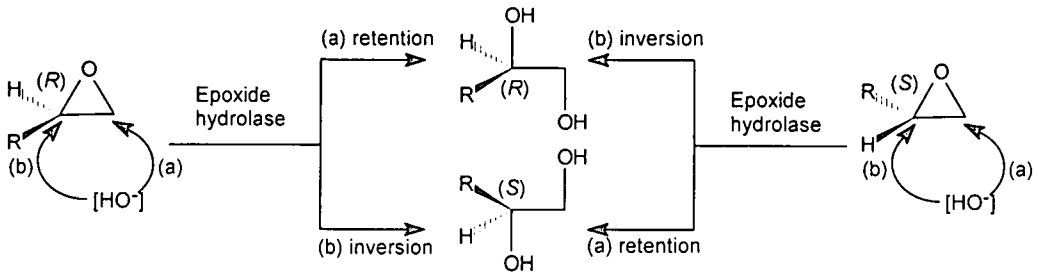
Transesterification of 3-phenylglycidic esters was also employed for the efficient synthesis of the C-13 side chain of Taxol. *Mucor miehei* lipase catalysed the stereospecific transesterification of the (2*S*,3*R*)-phenyl glycidic methylester to the corresponding isobutyl ester (Gou *et al.*, 1993). The esters of both the (2*S*,3*R*)- and (2*R*,3*S*)- enantiomers were thus obtained, and could be separated by fractional distillation. Recycling of the substrate allowed the recovery of both esters in high yield and enantiomeric excess (Scheme 30).



Scheme 30. Transesterification of phenylglycidic esters gives access to both enantiomers, which are employed in the synthesis of the C-13 side chain of Taxol®

2.2.3.2 Kinetic resolution of unfunctionalized epoxides by epoxide hydrolases

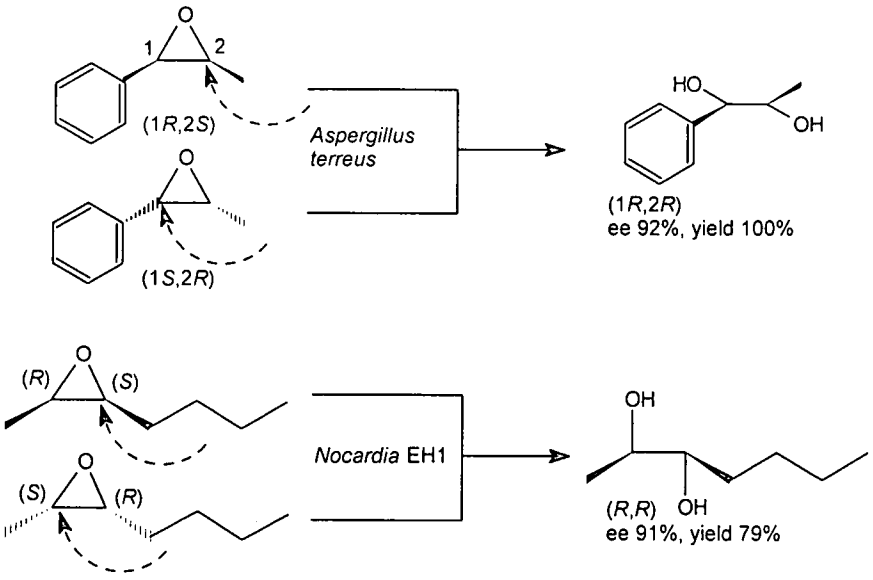
Epoxide hydrolases from microbial sources have recently emerged as promising enantioselective catalysts. They are, like lipases, esterases, haloperoxidases and dehalogenases, hydrolytic enzymes that belong to the α,β-hydrolase fold family of enzymes that catalyzes reactions *via* an acid-base-nucleophile catalytic triad (Misawa *et al.*, 1998; Rink *et al.*, 1997). Enantioselective hydrolysis results in an optically active (remaining) epoxide and the formed diol. While kinetic resolution of esters by lipases, esterases and proteases usually proceed with retention of the absolute configuration at the stereogenic centre, the enzymatic hydrolysis of epoxides may take place with either retention or inversion of configuration. Either carbon of the oxirane ring may be attacked during hydrolysis, depending on the regioselectivity of the enzyme involved and the substitution pattern of the epoxide (Scheme 31).



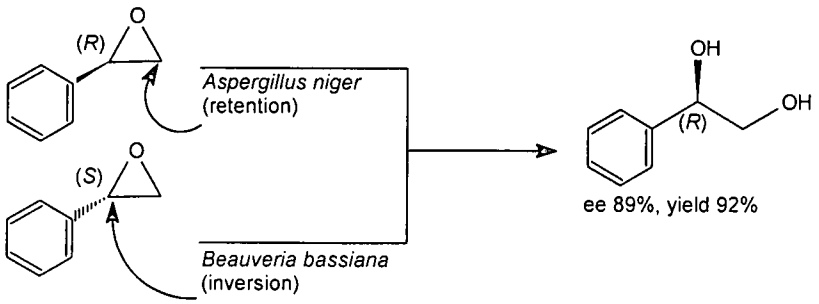
Scheme 31. Stereochemical pathways of epoxide hydrolases. Attack may occur at either of the carbons of the oxirane ring, depending on the enzyme involved and the substitution pattern of the epoxide

Besides classical kinetic resolution with which a maximum theoretical yield of 50% of enantiomerically pure epoxide and diol is obtained, epoxide hydrolases had also been employed to obtain enantiopure diols quantitively through enantioconvergent hydrolysis of the enantiomers of the racemic epoxide (Kroutil *et al.*, 1996a; Kroutil *et al.*, 1997; Archelas, 1998) (Scheme 32a). Combination of two enantiocomplementary fungal strains had also been employed to achieve deracemization of epoxides (Pedragosa-Moreau *et al.*, 1993; Pedragosa-Moreau *et al.*, 1996) (Scheme 32b). Enantioconvergent hydrolysis may also be achieved by combination of regioselective biohydrolysis and acid-catalyzed hydrolysis in a controlled resolution-inversion sequence (Archer *et al.*, 1996; Orru *et al.*, 1998a) (Scheme 32c).

(a) Enantioconvergent hydrolysis of epoxides

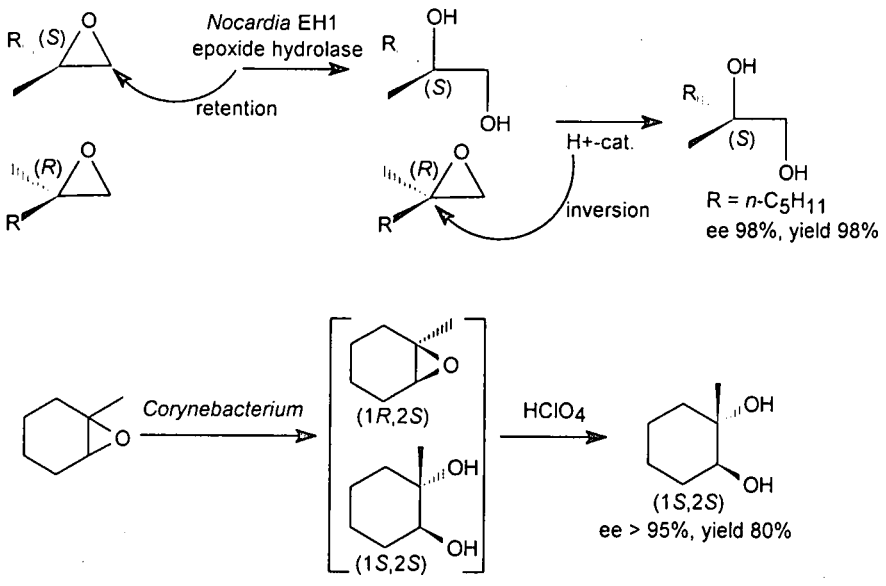


(b) Enantiocomplementary hydrolysis of styrene oxide by different fungal strains



1 151 551 83

(c) Chemo-enzymatic hydrolysis of epoxides

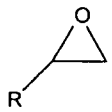
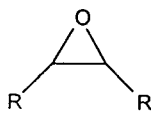
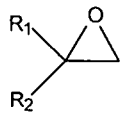
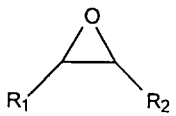
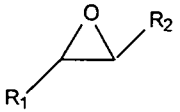
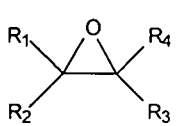
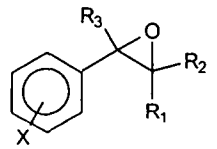


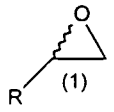
Scheme 32. Non-classic resolution strategies employing epoxide hydrolases

Stereoselectivities of microbial epoxide hydrolases

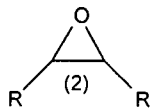
Enantioselectivities of epoxide hydrolases from yeasts, bacteria and fungi can be correlated to different structural classes of epoxides, with some overlapping for some epoxides (Table 6). Epoxides of the different structural classes which have been resolved by hydrolytic kinetic resolution, or from which optically active *vic* diols have been obtained by deracemization and asymmetrization, are given in Scheme 33.

Table 6. Stereoselective biocatalysts for different structural classes of epoxides

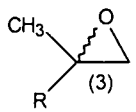
						
Mono-substituted	*Meso*-epoxides	2,2-disubstituted	*Cis*-2,3-disubstituted	*Trans*-2,3-disubstituted	Tri-substituted	Styrene oxides
Yeasts: *Rhodosporidium toruloides* *Rhodotorula glutinis* *Trichosporon mucoides* Fungi *Mortierella issabellina* *Diploida gossipina* Bacteria: *Chryseomonas luteala*	Yeasts: *Rhodotorula glutinis*	Bacteria: *Nocardia* EH1, TB1 and H8 *Rhodococcus ruber* *Mycobacterium paraffinicum* *Rhodococcus* NCIMB 11216	Bacteria: *Nocardia* EH1, TB1 and H8 *Rhodococcus ruber* *Pseudomonas* Yeasts: *Rhodotorula glutinis*	Bacteria: *Nocardia EH1* Yeasts: *Rhodotorula glutinis* Fungi: *Mortierella issabellina*	Fungi: *Aspergillus niger* Bacteria: *Corynebacterium Rhodococcus* NCIMB 11216 Yeasts: *Rhodotorula glutinis*	Yeasts: *Rhodotorula glutinis* Bacteria: *Agrobacterium radiobacter* Fungi: *Diploida gossipina* *Gilmaniella humicola* *Aspergillus terreus* *Beauveria bassiana,* *Beauveria densa* *Aspergillus niger*



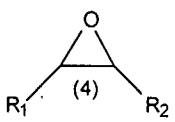
1a R = *n*-C_2H_5
1b R = *n*-C_3H_7
1c R = *n*-C_4H_9
1d R = *n*-C_5H_{11}
1e R = *n*-C_6H_{13}
1f R = $(CH_2)_2CH{=}CH_2$
1g R = $(CH_2)_4CH{=}CH_2$
1h R = *tert*-butyl
1i R = CH_2OCH_2Ph
1j R = cyclic acetal



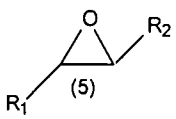
2a cyclopentene oxide
2b cyclohexene oxide
2c 2,3-epoxybutane



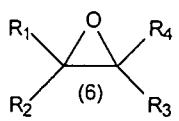
3a R = *n*-C_4H_9
3b R = *n*-C_5H_{11}
3c R = *n*-C_7H_{15}
3d R = *n*-C_9H_{19}
3e R = $(CH_2)_4Br$
3f R = $(CH_2)_3CH{=}CH_2$
3g R = CH_2Ph



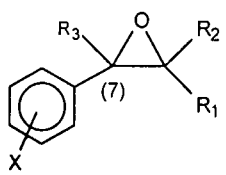
4a R_1 = *n*-C_4H_9, R_2 = CH_3
4b R_1 = C_2H_5, R_2 = CH_3
4b 9,10-epoxy-15-methyl-hexadecanoic acid



5a R_1 = *n*-C_4H_9, R_2 = CH_3
5b R_1 = C_2H_5, R_2 = CH_3



6a 4(*S*)-1,2-limonene oxide
6b 1-methyl-1,2-epoxycyclohexane
6c 4(*S*) and 4(*R*)-8,9-epoxylimonene
6d 6,7-epoxygeraniol derivatives
6e 2,3-epoxylinalyl acetate



7a Styrene oxide: R_1 = R_2 = R_3 = H
7b R_1 = R_2 = R_3 = H; X = 2-Cl or 3-Cl
7c R_1 = R_2 = R_3 = H ;
X = 4-Cl, 4-Fl, 4-Br, 4-NO_2, 4-CN, 4-Me
7d R_1 = Me, R_2 = R_3 = H
7e R_2 = Me, R_1 = R_3 = H
7f R_3 = Me, R_1 = R_2 = H
7g R_3 = Me, R_1 = R_2 = H; X = 4-Br
7h Indene oxide
7i Dihydronaphtalene oxide

Scheme 33. Epoxides of different structural classes which have been resolved by classic kinetic resolution, or from which optically active *vic* diols have been obtained in high enantiomeric excess by deracemization and asymmetrization

Monosubstituted epoxides

Monosubstituted epoxides represent highly flexible molecules that lack anchoring groups for chiral recognition by enzymes. As a consequence, no epoxide hydrolases from bacterial or fungal origin with useful enantioselectivities (E>30) for mono-substituted epoxides were found, despite extensive screening. Epoxide hydrolases from mammalian origin also did not display significant enantioselectivities for these elusive substrates (Bellucci *et al.*, 1989). Interestingly, this class of epoxides also posed the greatest challenge for synthetic chiral catalysts (Tokunaga *et al.*, 1997). It was only very recently that biocatalysts from yeast origin (Botes *et al.*, 1998b) were found for the efficient HKR of these epoxides. Highly enantioselective hydrolysis (E = 85 - 200) of mono-substituted straight chain aliphatic epoxides were restricted to yeasts from the genera *Rhodoturula* and *Rhodosporidium*. Moderate enantioslectivities (E>20) were displayed by the yeast *Trichosporon mucoides*, the bacterium *Chryseomonas luteola* (Botes *et al.*, 1998a) and the fungus *Mortierella isabellina* (Moussou *et al.*, 1998). The fungus *Diploida gossipina* (Mischitz *et al.*, 1995) required a more bulky *tert*-butyl substituent for chiral recognition. The (*R*)-epoxides were preferentially hydrolyzed by all the biocatalysts, except the glycidal acetal derivative, for which *Aspergillus niger* displayed enantiopreference for the (*S*)-enantiomer (Archelas, 1998). Although nucleophilic attack occurred predominantly at the least hindered carbon, resulting in retention of configuration of the formed diol, regioselectivity was not absolute. The enantiomeric purities of the formed diols were thus lower than that of the remaining epoxides at conversions approaching 50%, and E-values were calculated based on the ee's of the epoxides (Table 7).

The yeasts *Rhodosporidium toruloides* and *Rhodotorula glutinis* displayed exceptionally high reaction rates. The specific activities of the purified enzymes were 172 (Botes, 1999; Kronenburg *et al.*, 1999) and 67 μmol.min^{-1}mg protein^{-1} respectively, which translates to turnover frequencies (mol substrate processed per mol catalyst in one hour) of 566 000 and 181 000 (Botes, 1999). A turnover frequency of higher than 2000 is considered *via*ble for a commercial process (European Chemical News, May 19, 1997, p36). Furthermore, the catalytic activities of these enzymes are not influenced by a biphasic system, and enhanced enantioselectivity is obtained at high substrate concentrations.

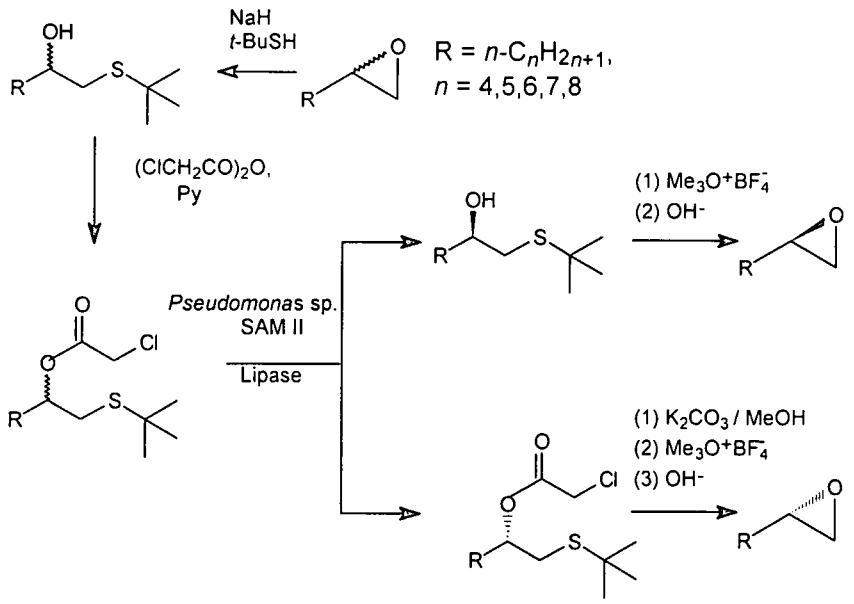
Table 7. Enantioselectivities of epoxide hydrolases for mono-substituted epoxides

Substrate	Biocatalyst	Selectivity (E)
1a	*Rhodotorula glutinis* CIMW 147	8
1b	*Rhodosporidium toruloides* UOFS Y-0471	23
1c	*Rhodotorula glutinis* UOFS Y-2042	103
1d	*Rhodosporidium toruloides* UOFS Y-0471	100
1e	*Rhodosporidium araucariae* UOFS-Y-0473	200
1f	*Rhodotorula glutinis* CIMW 147	23
1g	*Rhodosporidium toruloides* UOFS Y-0471	100
1h	*Diploida gossipina* ATCC 10936	18
1i	*Rhodotorula aurantiaca* UOFS 2049	9
1j	*Aspergillus niger*	ee 99%, yield 40%

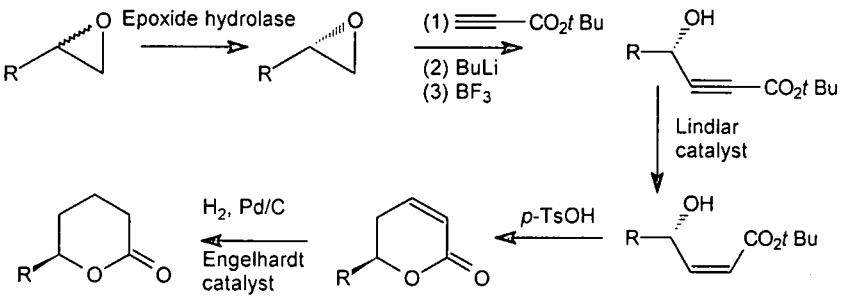
Synthetic applications of optically active mono-substituted epoxides

The production of optically active 1,2-epoxyalkanes for the chiral part of ferro-electric liquid crystals with yeast epoxide hydrolases certainly constitutes a viable alternative to the mono-oxygenase based process currently used. The mono-oxygenase process, using *Nocardia corallina* whole cells, is not trivial, and require high engineering skills, co-substrates and low substrate concentrations. In contrast with alkene mono-oxygenases, yeast epoxide hydrolases display exceptionally high catalytic activities, resting cells may be used, the enzymes are not inhibited by high substrate concentrations, and higher optical purities of the epoxides are obtained. Furthermore, enantiopure 1,2-epoxyalkanes produced by yeast epoxide hydrolases may be employed in the synthesis of enantiomerically pure δ-lactones (Scheme 34b). This would constitute a much more efficient synthesis than the route employed by Haase (Haase and Schneider, 1993) (Scheme 34a).

(a) Synthesis of optically pure 1,2-epoxyalkanes employing lipase



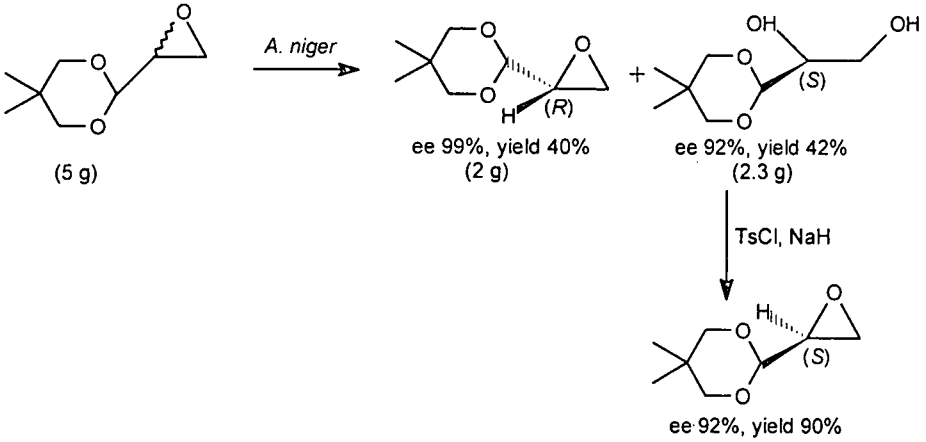
(b) Direct synthesis of optically pure 1,2-epoxyalkanes employed in the synthesis of δ-lactones



Scheme 34. A direct approach to the synthesis of enantiomerically pure δ-lactones from 1,2-epoxyalkanes employing yeast epoxide hydrolases rather than lipases eliminates several reaction steps

However, suitable catalysts for long-chain 1,2-epoxyalkanes had not been found, and the applicability is limited to C-5 to C-8 1,2-epoxyalkanes. Similarly, C-3 building blocks derived from glycidol or epihalohydrins are not yet accessible using epoxide hydrolases. However, glyceraldehyde synthons were recently prepared in excellent enantiomeric

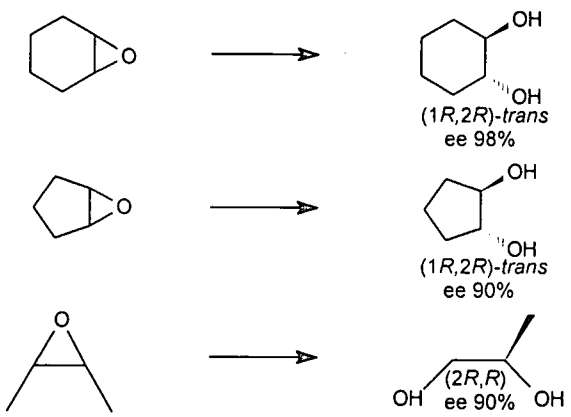
purity and yield (Archelas, 1998) (Scheme 35). The protected glyceraldehyde and glycidaldehyde serve as stable building C3 chirons, as well as building blocks for bioactive compounds such as azasugars and glycosidase inhibitors. An analogous synthesis of glyceraldehyde synthons was achieved with Sharpless AD of acrolein acetals (Oi and Sharpless, 1992).



Scheme 35. Synthesis of optically active glyceraldehyde synthons employing *Aspergillus niger* epoxide-hydrolase

Meso-epoxides

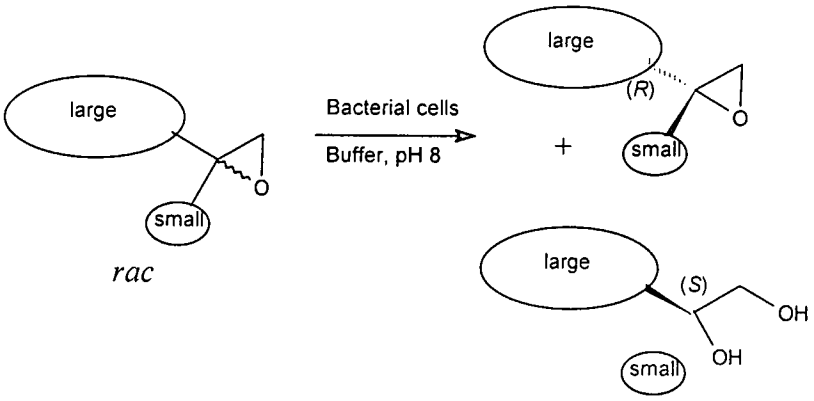
Asymmetrization of *meso*-epoxides could not be achieved with bacterial and fungal epoxide hydrolases, which require auxiliary anchoring groups for chiral recognition (Hechtberger *et al.*, 1993; Pedragosa-Moreau *et al.*, 1996). However, asymmetrization of *meso*-epoxides, which leads to (*R*,*R*)-*trans*-diols in almost quantitative yields, had been achieved with *Rhodotorula glutinis* (Weijers, 1997) (Scheme 36). This constitutes a *via*ble alternative to asymmetrization of *meso*-epoxides with Jacobsen's chiral (salen)Cr(III) complexes (Martínez *et al.*, 1995) for the synthesis of valuable C_2 symmetrical chiral diols. The only other report of the use of microbial enzymes to prepare optically active cyclohexanediols involves the double stereoinversion of (±)-*trans* and *cis* cyclohexane-1,2-diols to (S,S)-*trans* cyclohexanediol with whole cells of *Corynesporium casiicola* (Carnell *et al.*, 1994).



Scheme 36. Asymmetrization of *meso*-epoxides by *Rhodotorula glutinis*

2,2-Disubstituted epoxides

Epoxide hydrolases from several bacterial strains (Table 8) hydrolyze 2-methyl-2-(aryl)alkyloxiranes with virtually complete enantiopreference (E>200) for the (*S*)-epoxide and with absolute regioselectivity for the least hindered oxirane carbon atom. The enantioselectivities of all strains were dependent on the difference in relative size of the two substituents (Scheme 37). Substitution of the methyl group with an ethyl group resulted in a dramatic decrease in enantioselectivity (Orru *et al.*, 1998).



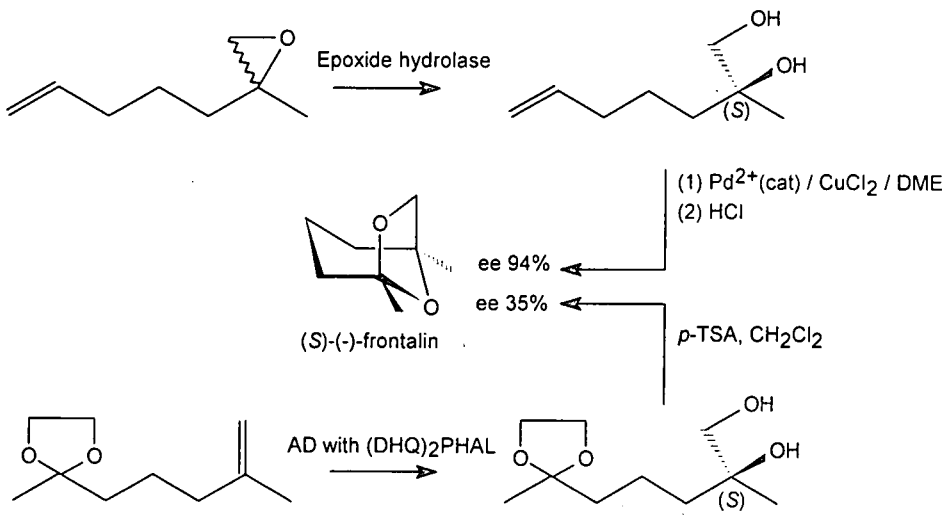
Scheme 37. Resolution of oxiranes by bacterial cells

Table 8. Enantioselectivities of bacterial epoxide hydrolases for 2,2-disubstituted epoxides

Substrate	Biocatalyst	Selectivity (E)
3a	*Nocardia* sp. TB1	>200
3b	*Nocardia* sp. EH1	>200
3c	*Rhodococcus equi* IFO 3730	>200
3d	*Rhodococcus* sp. NCIMB 11216	>200
3e	Nocardia sp. H8	>200
3f	Nocardia sp. EH1	>200
3g	*Nocardia* sp. EH1	123

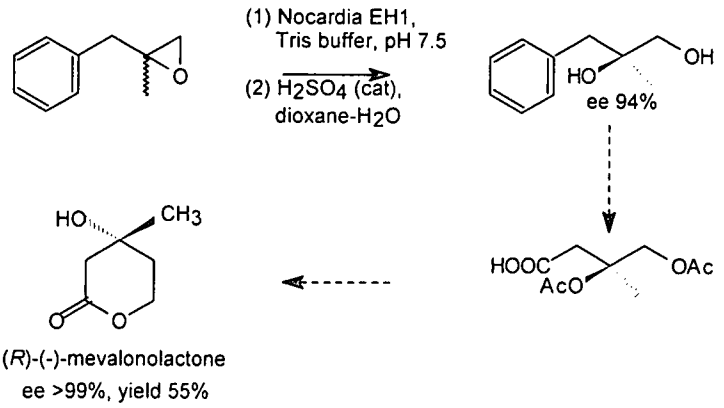
Synthetic applications of optically active 2,2-disubstituted epoxides

The pheromone (*S*)-(-)-frontalin was synthesized in 94% enantiomeric purity from **3f** employing lyophilized cells of *Rhodococcus equi* IFO 3730 (E=39) (Kroutil *et al.*, 1996b). A bacterial strain with much higher enantioselectivity (*Nocardia* sp. EHI, E = 123) was subsequently identified (Ospiran *et al.*, 1997). In an analogous Sharpless AD synthesis, (*S*)-(-)-frontalin was obtained in a much lower enantiomeric purity of 35% (Kolb *et al.*, 1994) (Scheme 38).



Scheme 38. Synthesis of (*S*)-(-)-frontalin employing kinetic resolution by bacterial epoxide hydrolase and Sharpless AD

(*R*)-(-)-Mevalonolactone was synthesized from **3g** on a multigram scale (10 g) in excellent enantiomeric purity (ee>99%) and chemical yield (55%) (Orru *et al.*, 1998b) (Scheme 39). In an analogous synthesis using CPO of *Caldariomyces fumago*, an enantiomeric purity of 95%, and a chemical yield of 57% was obtained (see Scheme 18).

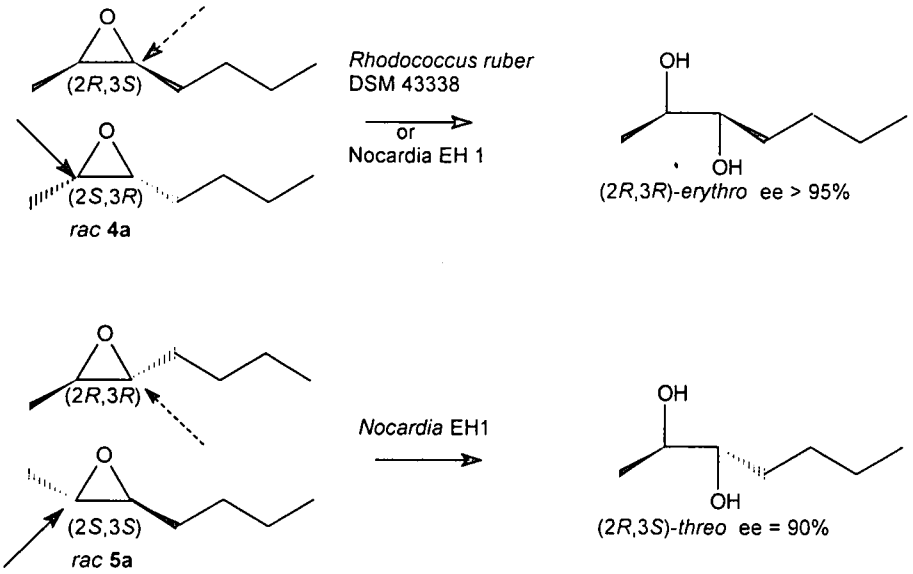


Scheme 39. Large scale synthesis of (*R*)-(-)-mevalonolactone employing kinetic resolution of 2,2-disubstituted oxiranes by bacterial epoxide hydrolase

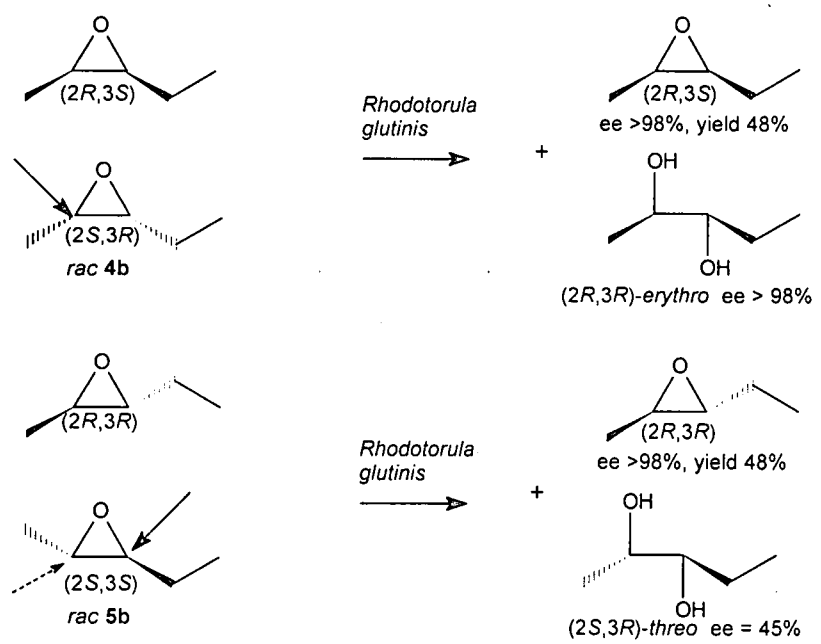
2,3-Disubstituted oxiranes

Most bacterial strains display non-classic resolution of *cis*- and *trans*-2,3-disubstituted oxiranes, and display a marked preference for attack on the (*S*)-configured carbon, regardless of the stereochemistry of the substrate. Thus, both enantiomers are hydrolysed by attack on the (*S*)-carbon, while the enantiomer with the least hindered (*S*)-carbon are hydrolyzed at a faster rate (Scheme 40a). The reaction proceed *via* a S_N-2-type hydrolytic ring opening of the substrate, leading to inversion of configuration at the carbon being attacked and enantioconvergent hydrolysis of the two enantiomers. Thus, the *threo*-diol (2*R*,3*S*) is obtained from the *trans*-epoxide **5a**, while the *erythro*-diol (2*R*,3*R*) is obtained from the *cis*-epoxide **4a** (Kroutil *et al.*, 1997).

(a) Enantioconvergent hydrolysis of 2,3-disubstituted epoxides by bacterial epoxide hydrolases



(b) Enantioselective kinetic resolution of 2,3-disubstituted epoxides by yeast epoxide hydrolases

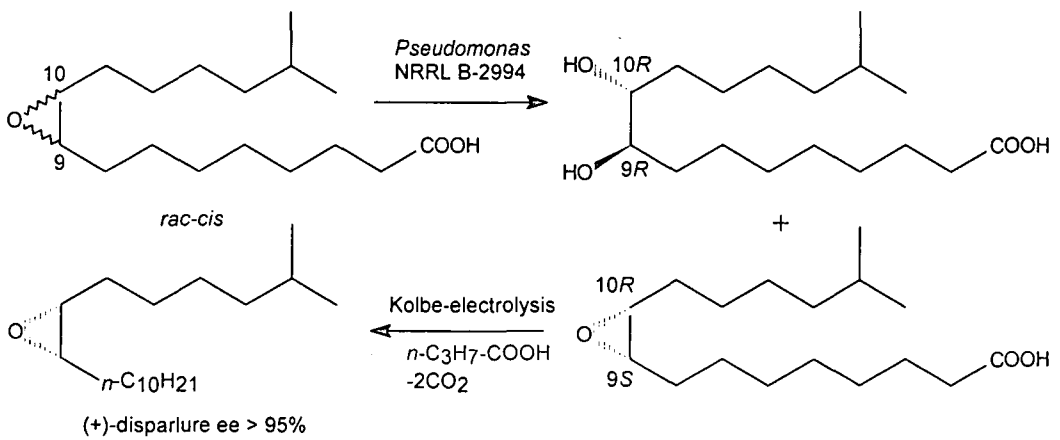


Scheme 40. Deracemization of 2,3-disubstituted oxiranes by bacterial and yeast epoxide hydrolases

In contrast to the bacterial epoxide hydrolases that hydrolyze both enantiomers of 2,3-disubstituted epoxides in an enantioconvergent process, the yeast *Rhodotorula glutinis* displays highly enantioselective classical kinetic resolution of 2,3-disubstituted epoxides (Weijers, 1997). The *cis*-2,3-epoxide **4b** is hydrolysed with absolute enantioselectivity for the (2*S*,3*R*)-enantiomer, and attack occurs exclusively at the (2*S*) oxirane carbon, yielding both the residual epoxide and formed diol in excellent enantiomeric excess. In the case of the *trans*-epoxide **5b**, the enzyme is highly enantioselective for the (2*S*,3*S*)-enantiomer, but regioselectivity is not absolute, resulting in enantiopure residual epoxide, but the formed diol is obtained in lower enantiomeric purity (Scheme 40b).

Synthetic applications of optically active 2,3-disubstituted epoxides

An epoxide hydrolase preparation of *Pseudomonas* sp. NRRL B-2994 showed absolute enantiopreference for the (9*R*,10*S*)-enantiomer of (±)-9,10-epoxy-15-methyl-hexadecanoic acid, and the reaction stopped at 50% conversion (Otto and van der Willigin, 1988). Attack on the 10(*S*) carbon of the oxirane ring with inversion of configuration yielded the (9*R*,10*R*)-*threo*-diol. The remaining (9*S*,10*R*)-epoxide was converted into (+)-disparlure, the sex pheromone of the gypsy moth, in a single step and > 95%ee (Scheme 41). An analogous synthesis with Sharpless AD involved a complex multistep procedure from a cyclic sulfate, and the resultant pheromone had an enantiomeric excess of 90% (Kolb *et al.*, 1994).



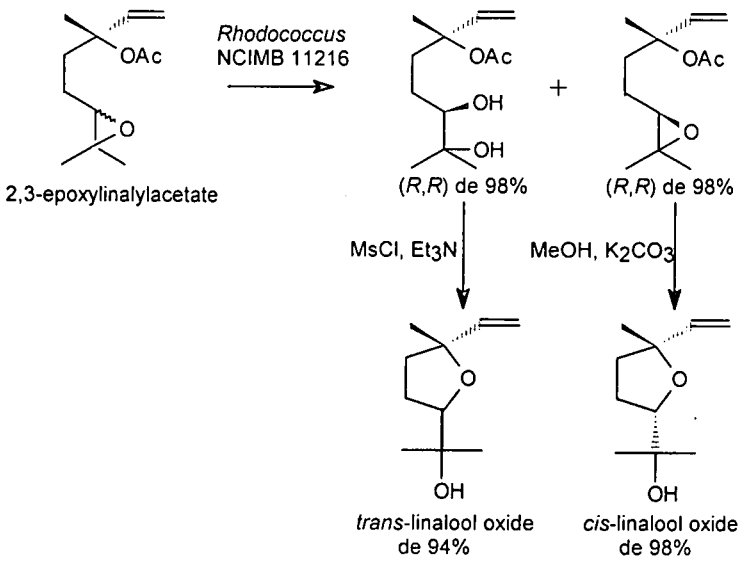
Scheme 41. Resolution of a *cis*-2,3-disubstituted epoxide employed in the synthesis of (+)-disparlure

Trisubstituted oxiranes

Fungal, bacterial as well as yeast epoxide hydrolases have been employed in the resolution of various trisubstituted epoxides. *Rhodotorula glutinis* hydrolyzes (4*S*)-(-)-1,2-limonene oxide (Scheme 33, **6a**) with excellent enantioselectivity, allowing access to the remaining (1*S*,2*R*,4*S*)-epoxide and the formed (1*R*,2*R*,4*S*)-diol in >98% ee at 48% conversion. Lower enantioselctivity was observed for (4*R*)-(+)-limonene (Weijers, 1997). *Corynebacterium* sp. C12 epoxide hydrolase preferentially hydrolyses the (1*R*,2*S*)-enantiomer of (±)-1-methyl-1,2-epoxycyclohexane (Scheme 33, **6b**) (Archer *et al.*, 1996).

Synthetic applications of trisubstituted epoxides

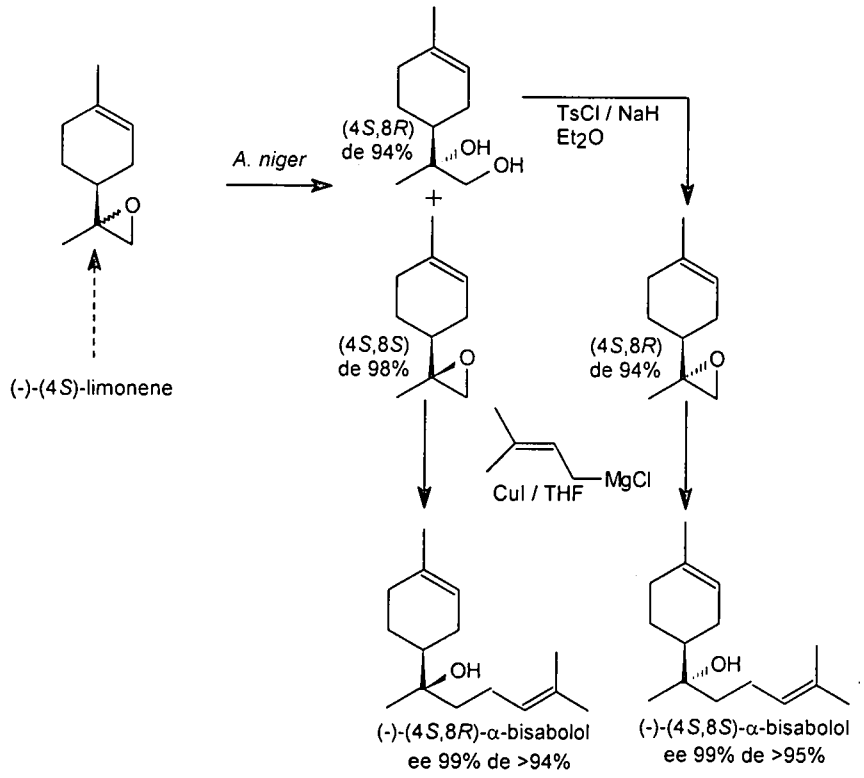
Optically pure *trans*-and *cis*-linalool oxides are aroma components of several plants. Both diastereomers were prepared from 2,3-epoxylinalyl acetate (Scheme 33, **6e**), employing an epoxide hydrolase preparation from *Rhodococcus* sp. NCIMB 11216 for the resolution of the diastereomers (Scheme 42) (Mischitz and Faber, 1996).



Scheme 42. Synthesis of *cis*- and *trans*-linalool oxide employing bacterial epoxide hydrolase

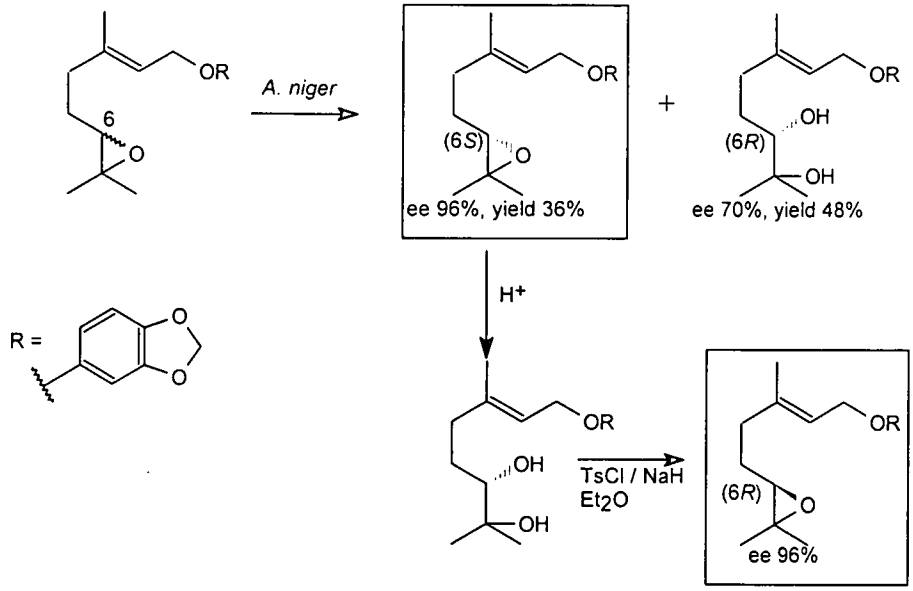
Fungal epoxide hydrolases have been employed in the synthesis of all four naturally occurring stereoisomers of bisabolol from (-)-(4*S*)- and (+)-(4*R*)-limonene-8,9-epoxide

(Scheme 33, **6c**) (Chen *et al.*, 1993). The (-)-(4*S*,8*S*)-α-bisabolol enantiomer (Scheme 43) is used on an industrial scale for the preparations of skin-care products, because of its antiinflammatory and bactericidal properties.



Scheme 43. Chemoenzymatic synthesis of α-bisabolol from (-)-(4*S*)-limonene. The other two enantiomers were synthesized from (+)-(4*R*)-limonene

In yet another application of fungal epoxide hydrolases, the enantiomers of the benzodioxole derivative of 6,7-epoxygeraniol (Scheme 33, **6d**) was resolved, allowing access to both enantiomers of Bower's compound. Bower's compound is a potent analogue of an insect juvenile hormone (Scheme 44) (Archelas *et al.*, 1993).



Scheme 44. Synthesis of (*R*)- and (*S*)-Bower's compound employing fungal epoxide hydrolase

Styrene oxide-type epoxides

The benzylic carbon of styrene oxide-type substrates facilitates the formation of a carbo-cation, which is stabilized by the adjacent aromatic moiety. Attack at the benzylic carbon is thus electronically facilitated, despite steric impediment. Regioselectivity may thus be strongly influenced by electronic factors. Since optically active styrene oxide and its derivatives are extremely useful intermediates in the synthesis of bioactive compounds, a lot of attention had been focused on the kinetic resolution of this type of epoxides employing microbial epoxide hydrolases. The most successful kinetic resolutions and enantioconvergent hydrolysis achieved are given in Table 9.

Table 9. Enantioselectivities of various biocatalysts for styrene oxide-type epoxides

Substrate (Scheme 33)	Biocatalyst	Ee / yield epoxide (%)	Ee / yield Diol (%)	Config. Epoxide	Config. Diol
7a	*Aspergillus niger*[1]	98, 28	51, 54	*S*	*R*
	Beauveria bassiana	98, 34	83, 47	*R*	*R*
	Agrobacterium radiobacter[2]	99, 33	NA	*S*	NA
7b	*Agrobacterium radiobacter*	99, 27 - 35	NA	*S*	NA
7c, 4-Me	*Agrobacterium radiobacter*	99, 36	NA	*S*	NA
	Aspergillus niger	95, 34	NA	*S*	NA
	Beauveria bassiana	98, 30	76, -	*R*	*R*
7c, 4-Cl	*Agrobacterium radiobacter*	99, 34	NA	*S*	NA
7c, 4-	*Aspergillus niger*	98, 35	81, -	*S*	*R*
Fl,Br	*Beauveria bassiana*	96, 25	78, -	*R*	*R*
7c, 4-NO_2	*Aspergillus niger*	98, 37	70, -	*S*	*R*
	Beauveria bassiana	20, 50	49, -	*S*	*R*
7c, 4-CN	*Aspergillus niger*	98, 38	76, -	*S*	*R*
	Beauveria bassiana	15, 59	50, -	*R*	*R*
7d	*Aspergillus terreus*[a,3]		92, 100		(*1R,2R*)
7e	*Rhodotorula glutinis*[4]	98, 45	98, -	(*1R,2R*)	(*1R,2S*)
	Beauveria bassiana	98, 30	90, 38	(*1R,2R*)	(*1R,2S*)
7f	*Agrobacterium radiobacter*	99, 27	NA	*S*	NA
	Beauveria bassiana[a]	20, 42	99, 42	(*1R,2S*)	(*1R,2R*)
7g	*Aspergillus niger*	100,39	96, 49	*S*	*R*
7h	*Diploida gossipina*[5]	100, -	NA	(*1S,2R*)	NA
	Gilmanniella humicola[5]	100, -	NA	(*1R,2S*)	NA
7i	*Beauveria bassiana*	98, 38	77, 49	(*1R,2S*)	(*1R,2R*)

a) Enantioconvergent hydrolysis

1) References for *Aspergillus niger* and *Beauveria bassiana*: (Pedragosa-Moreau *et al.*, 1996; Archelas, 1998; Cleij *et al.*, 1998)

2) Reference for *Agrobacterium radiobacter*: (Spelberg *et al.*, 1998)

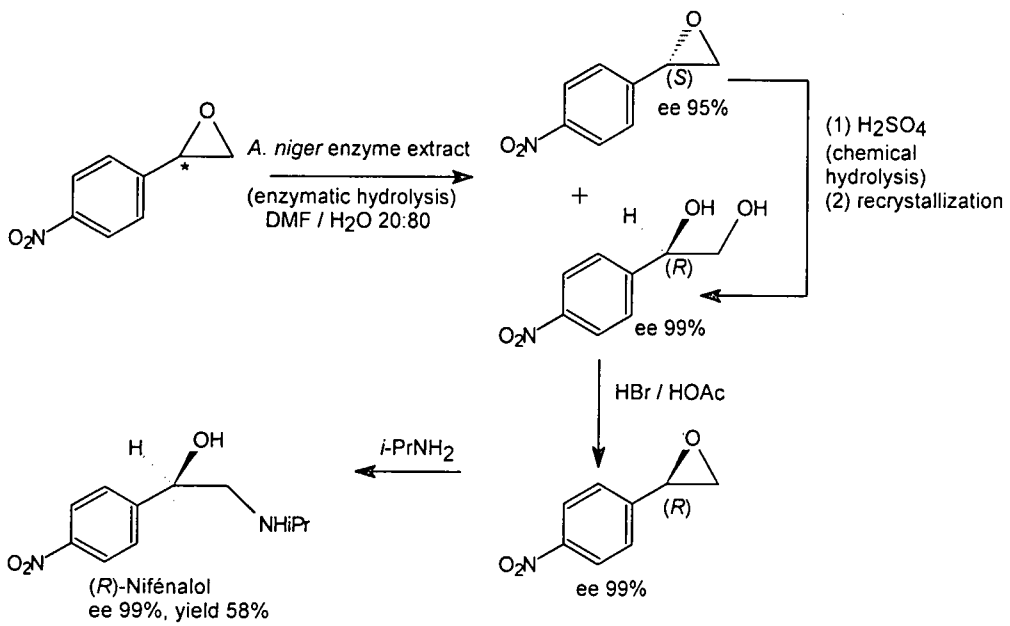
3) Reference for *Aspergillus terreus*: (Archelas, 1998)

4) Reference for *Rhodotorula glutinis*: (Weijers, 1997)

5) Reference for *Diploida gossipina* and *Gilmanniella humicola*: (Zhang *et al.*, 1995)

Synthetic applications of styrene oxide-type epoxides

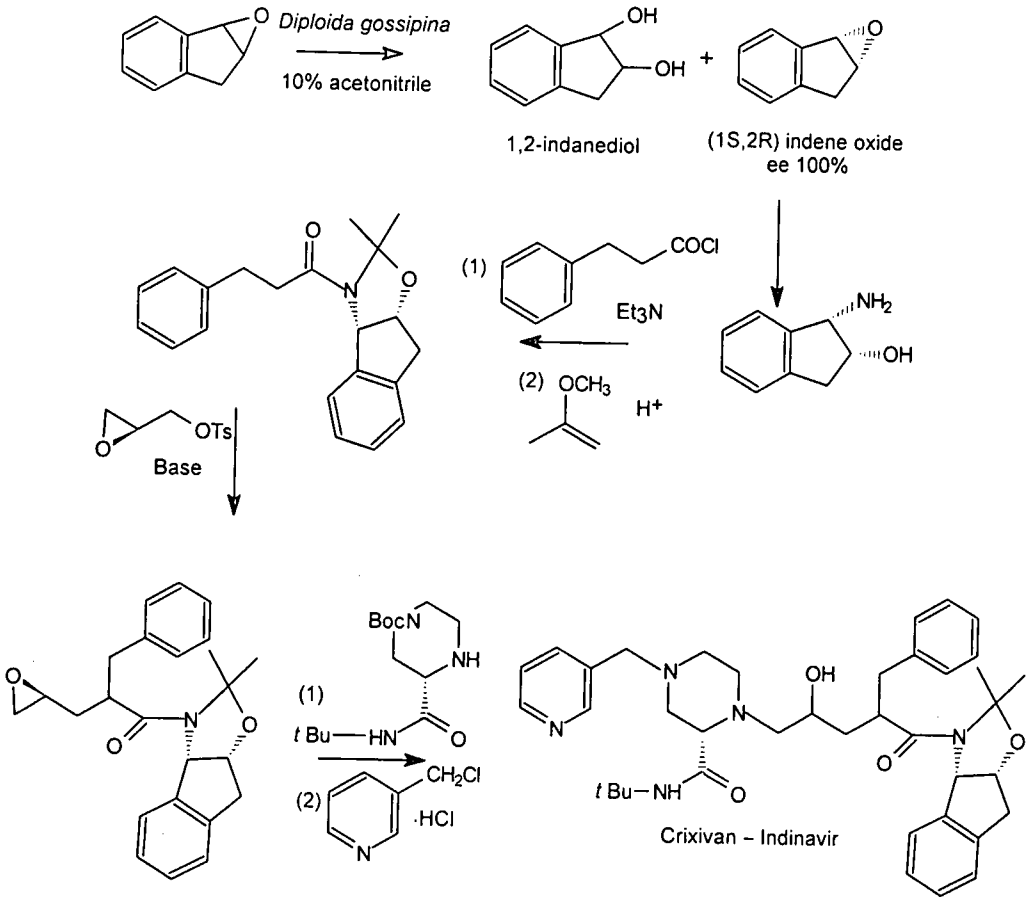
Synthesis of the biologically active enantiomer of the β-blocker (*R*)-Nifénalol® was synthesized in an enantioconvergent chemoenzymatic process in excellent enantiomeric excess and yield (Pedragosa-Moreau, 1997) (Scheme 45).



Scheme 45. Chemoenzymatic synthesis of (*R*)-Nifénalol® from *p*-nitro styrene oxide

In a recent study, enantioselective hydrolysis of *p*- bromo-α-methyl styrene oxide was achieved using a soluble enzyme extract of *Aspergillus niger* LCP 521 (Cleij *et al.*, 1998). This compound was identified as a possible precurser of α-aryl propionic acid based drugs such as ibuprofen (α-methyl-4-[isobutyl]phenylacetic acid).

Merck & Co registered a patent based on one of the first applications of epoxide hydrolases for the synthesis of pharmaceuticals (Chartrain *et al.*, 1997). Enatiopure (1*S*,2*R*)-indene oxide, a precursor to the side chain of the HIV protease inhibitor Crixivan® was obtained from *rac*-indene oxide using *Diploida gossipina* ATCC 16391 (Scheme 46).



Scheme 46. Synthesis of Crixivan® from (1*S*,2*R*)-indene oxide employing fungal epoxide hydrolase

2.2.3.3 Kinetic resolution *via* enantioselective epoxide degradation

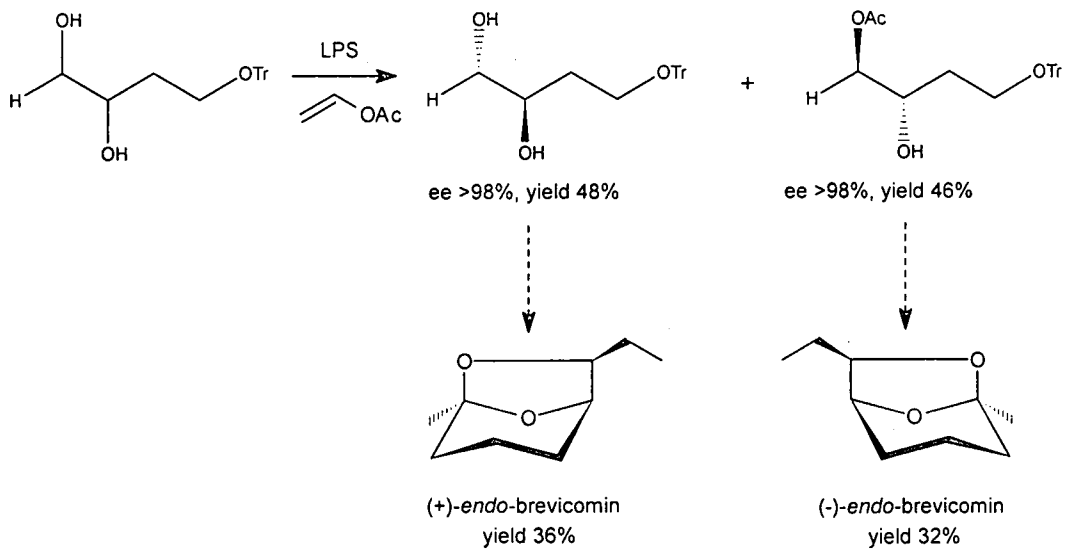
In all degradation routes involving an epoxide as a reaction intermediate, the epoxides to be degraded are formed by the action of monooxygenases. Several such degradation pathways had been reviewed recently (Swaving and De Bont, 1998). An oxide lyase specific only for α-pinene oxide had been identified in *Nocardia* and *Pseudomonas* strains. Styrene oxide isomerases had been identified in various microorganisms, and convert styrene oxide to phenylacetic acid (Nöthe and Hartmans, 1994). However, styrene oxide isomerases are not enantioselective.

Glutathione transferases depend on glutathione as co-factor. The epoxyalkane-degrading enzyme as characterized in *Xanthobacter* Py2 is able to degrade certain (2*S*)-2,3-epoxyalkanes with absolute enantioselectivity However, the enzyme system is very complex, and needs dithiol and NAD as co-factors. Consequently, it cannot be applied outside a metabolically active cell. Furthermore, the range of epoxide substrates accepted by these enzymes is very restricted. The ubiquotous, stable, co-factor independent epoxide hydrolases are thus more suitable asymmetric catalysts for organic synthesis.

2.2.4 KINETIC RESOLUTION OF *RAC VIC* DIOLS

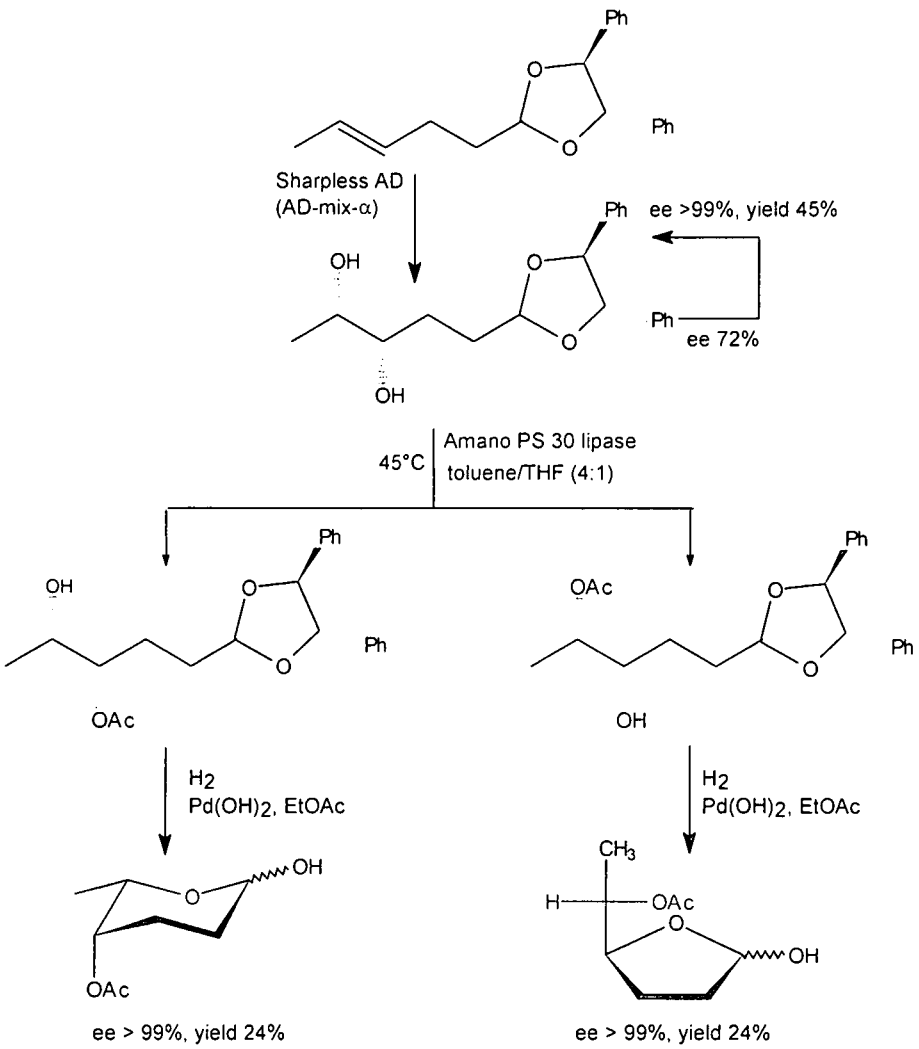
2.2.4.1 Kinetic resolution of *vic* diols by lipases

Discrimination between primary and secondary hydroxyl groups by lipase PS (LPS) from *Pseudomonas cepacia* in the presence of a bulky substituent, allowed the synthesis of both enantiomers of *endo*-brevicomin in excellent (ee > 98%) enantomeric purity (Kim *et al.*, 1995). The *anti*-1,2-diol and monoacetylated product were obtained as sole products (Scheme 47).



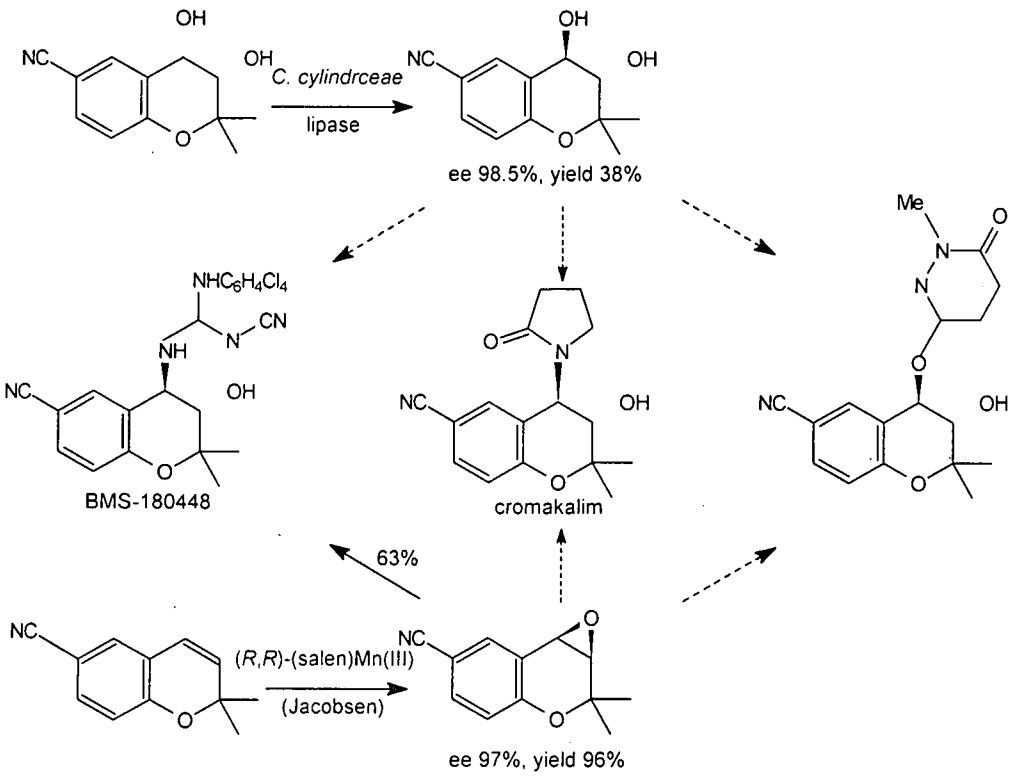
Scheme 47. LPS-catalyzed monoacetylation employed in the synthesis of both enantiomers of *endo*-brevicomin

Tandem Sharpless AD and lipase-catalyzed transesterification was used for the synthesis of the pyranose tautomer of L-rhodinose, which is featured in many naturally occuring antibiotics. Sharpless AD alone showed low enantioselectivity in the dihydroxylation step, and the distribution of the pyranose and furanose forms in the final product could not be controlled. However, transesterification of the diol obtained by AD with Amano PS-30 lipase, provided a separable mixture of the formed monoacetates, and allowed the synthesis of both tautomers in excellent enantiomeric excess (Sobti and Sulikowski, 1995). Substrate recycling augmented the yields of the monoacetates (Scheme 48).



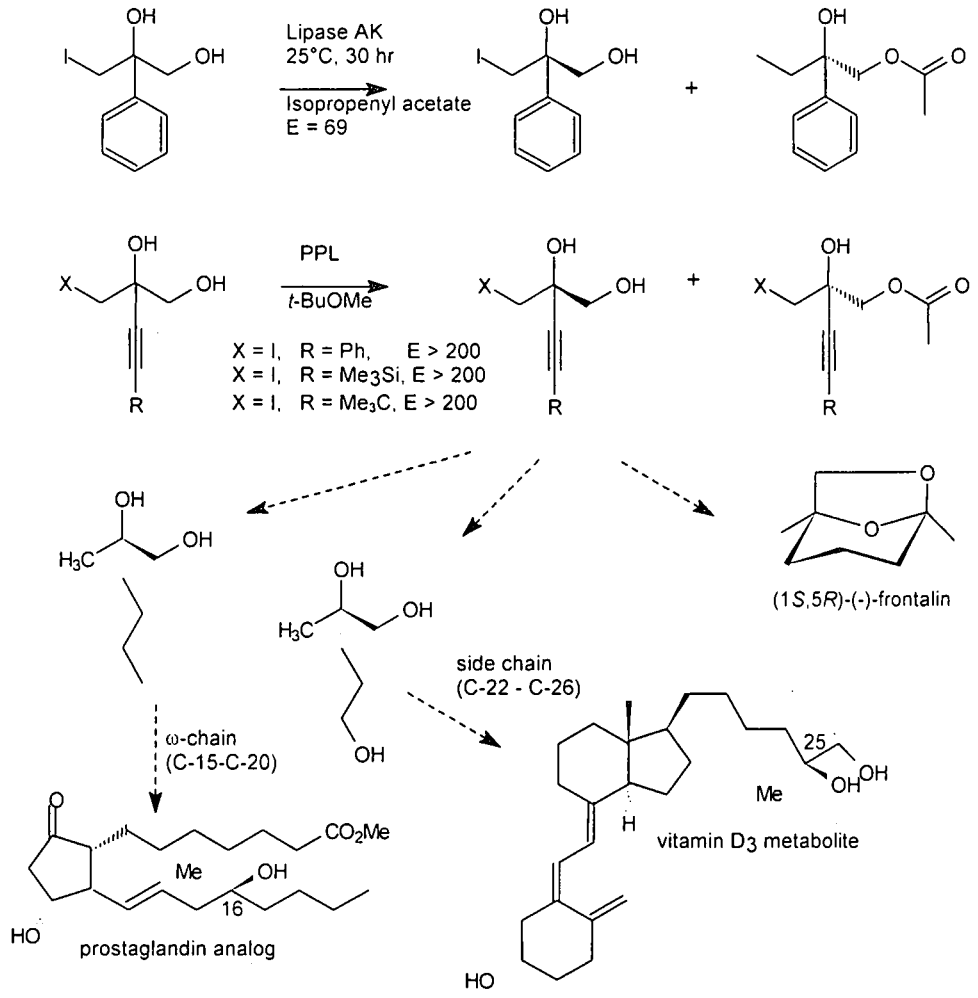
Scheme 48. Tandem Sharpless AD and lipase-catalyzed transesterification employed in the synthesis of L-rhodinose tautomers

Lipases from *Pseudomonas cepacia* (LPS-30) and *Candida cylindraceae* was employed in the synthesis of intermediates for potassium channel opener drugs (Patel *et al.*, 1995). Jacobsen AE with chiral (salen)Mn(III) catalysts provides an alternative route for the synthesis of these K_{ATP} openers (Lee *et al.*, 1991; Chen *et al.*, 1998) (Scheme 49).



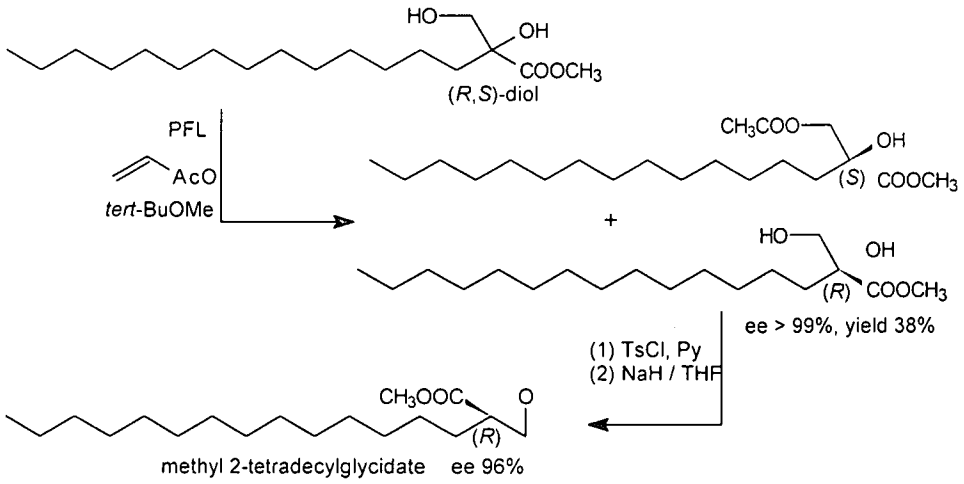
Scheme 49. Approaches to the synthesis of K_{ATP} openers

Lipase-catalyzed reactions of 1,1-disubstituted 1,2-diols were employed to obtain optically active tertiary alcohols as intermediates for the synthesis of drugs and natural products (Chen and Fang, 1997). The reactions occurred efficiently at the primary hydroxyl groups, while the enantioselectivity was controlled by the tertiary carbinyl centers (Scheme 50).



Scheme 50. Lipase-catalyzed resolution of 1,1-disubstituted 1,2-diols employed in the synthesis of natural products and drugs containing tertiary hydroxyl groups

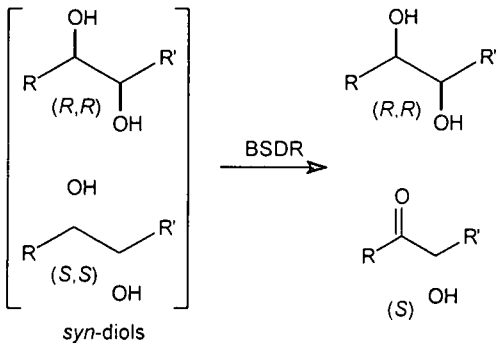
The (*R*)-enantiomer of methyl 2-tetradecylglycidate is a potent oral hypoglycemic and antiketogenic agent. This potent specific inhibitor of long-chain fatty acid oxidation was resolved through enantioselective acylation. Sequential kinetic resolution using *Pseudomonas fluorescens* lipase, afforded the biologically active (*R*)-enantiomer in excellent enantiomeric purity (ee >99%) (Scheme 51). In an analogous synthesis, an enantiomeric purity of 95% was achieved using Sharpless AD with $(DHQD)_2PHAL$ as asymmetric catalyst (Jiménez *et al.*, 1997).



Scheme 51 . Sequential kinetic resolution using PFL employed in the synthesis of optically active methyl 2-tetradecylglycidate

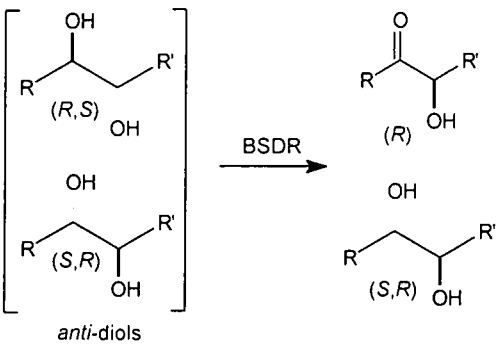
2.2.4.2 Kinetic resolution of *vic* diols by diacetyl reductase

Kinetic resolution of *syn*- and *anti*-1,2-diols was achieved by enzymatic oxidation with *Bacillus stearothermophilus* diacetyl reductase (BSDR). Enantiomerically pure (*R*,*R*)-*vic*-diols were obtained from racemic *syn*-diols bearing aliphatic, aromatic and cyclic substitents. Enantiomerically pure (*R*,*S*)-*vic*-diols, which are difficult to obtain in high ee by Sharpless AD of *cis*-olefins, were obtained from racemic *anti*-diols with aliphatic substituents (Scheme 52). Recovery of the (*S*)- and (*R*)-α-hydroxyketones was low, except for *meso*-1,2-cyclohexanediol, for which desymmetrization to the homochiral product was achieved. The NADH produced by the enantioselective oxidation is recycled in the presence of pyruvate and lactate dehydrogenase (Bortolini *et al.*, 1998).



syn-diols

R = CH_3, R' = *n*-C_3H_7; *n*-C_5H_{11}; Ph
R = R' = Et
R,R' = -$(CH_2)_3$- and -$(CH_2)_4$-



anti-diols

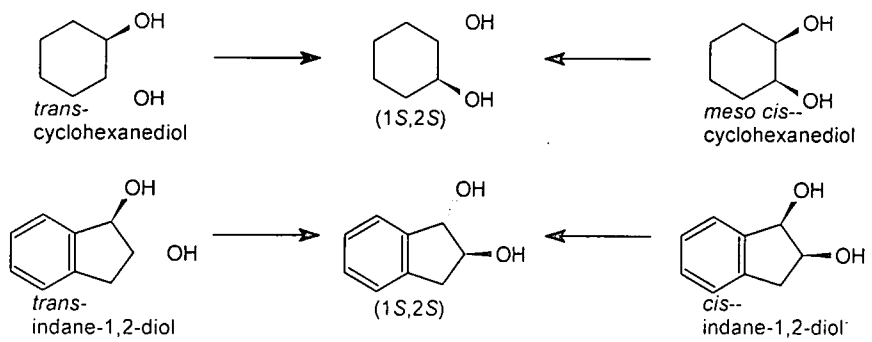
R = CH_3, R' = *n*-C_3H_7; *n*-C_5H_{11}
R,R' = -$(CH_2)_4$-

Scheme 52. Kinetic resolution of *syn*- and *anti*-1,2-diols by BSDR

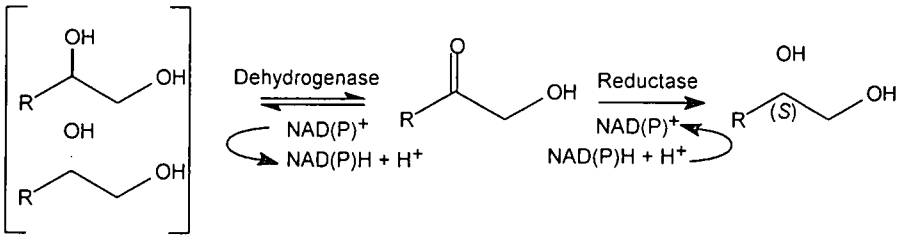
2.2.5 STEREOINVERSION OF *VIC* DIOLS

Biocatalytic stereoinversion of 1,2-diols holds great synthetic potential, but has not yet been widely explored. Stereoinversion of aliphatic 1,2-diols by the yeast *Candida parapsilosis* (Hasegawa *et al.*, 1990) had been reported (Table 10). Enantiomerically pure (ee>99%) (+)-(1*S*,2*S*)-cyclohexanediol was obtained from both (±)- *trans*- and *meso cis*-cyclohexane 1,2-diol by the fungus *Corynesporium casiicola* (Carnell *et al.*, 1994) (Scheme 53a). Complete stereoinversion of *trans*-indane-1,2-diol and 1-hydroxy-2-bromo-indane to (1*S*,2*S*)-indane-1,2-diol was subsequently achieved with the same fungus (Scheme 53a), while *cis*-indanediol was converted at a slower rate (Table 11) (Page *et al.*, 1998). Stereoinversion takes place *via* two consecutive oxidation-reduction reactions, and no co-factor recycling is thus necessary. The high enantiomeric purities (>97%) obtained in these stereoinversions, implies that one (or both) of the redox-reactions must be irreversible (Scheme 53b). In the case of diols containing two stereogenic centres, double-stereoinversion of both centres proceeds through four individual steps *via* the corresponding *oxo*-alcohols, and the *meso*-form, respectively (Stecher and Faber, 1997).

(a) Stereoinversion of 1,2-diols by *Corynesporium casiicola* DSM 62475



(b) Stereoinversion of 1,2-diols proceed with internal co-factor recycling. At least one of the redox reactions is irreversible



Scheme 53. Stereoinversion of 1,2-diols

Table 10. Stereoinversion of 1,2-diols by *Candida parapsilosis*

R (Scheme)	Config.	Yield (%)	Ee (%)
n-C_3H_7	S	93	100
n-C_4H_9	S	95	100
n-C_5H_{11}	S	97	98
n-C_6H_{13}	S	95	97
$(CH_3)_2CHCH_2$	S	96	97
Ph	S	100	100
$PhCH_2$	S	100	100
CH_3SCH_2	*R*[a]	65	98

[a] Switch in sequence rule

Table 11. Stereoinversion of 1,2-diols by *Corynesporium casiicola*

Substrate	Config.	Yield (%)	Ee (%)
1,2-cyclohexanediol	1*S*,2*S*	83	>99
Trans-indane-1,2-diol	1*S*,2*S*	82	>99
cis-indane-1,2-diol	1*S*,2*S*	26	>99

SUMMARY AND OUTLOOK

Recognition that chirality plays a crucial role in nature has fostered much effort in enantioselective synthesis, and significant advances in asymmetric catalysis has been made in recent years. Major contributions to the development of organometallic asymmetric catalysts for the synthesis of optically active epoxides and diols were made by Prof. K. Barry Sharpless and Prof. Eric Jacobsen. Jacobsen's salen complexes are highly efficient, and less than 1 mol% catalyst is required. This greatly reduces the concern about environmental pollution with heavy metals, especially since these catalysts can be recovered and recycled with ease. However, reactions with metal based catalysts are, with a few exceptions, carried out in chlorinated solvents, which is a major environmental concern. Salt free technologies are becoming a necessity, and alternatives for the osmium catalyzed asymmetric dihydroxylation and halohydrin routes to epoxides must be found. The catalysts developed by Sharpless and Jacobsen are sold under license, and are relatively expensive. Futhermore, their synthesis require high skill. Synthetic chemists have also increasingly explored the potential of microbial enzymes, which are by design exquisite asymmetric catalysts.

Microbial enzymes, traditionally used in food processing, are currently used to produce ca. 1800 kilotonnes of intermediates per year for the pharmaceutical industry. An additional 1500 tonnes of aspartame are also produced enzymatically (Roberts, 1999). Advances in bioprocess engineering, immobilization techniques and membrane technology have removed many of the obstacles associated with the use of oxidoreduction enzymes in industrial processes. However, employing hydrolytic enzymes in organic synthesis require no special skills and they are increasingly considered by synthetic chemists in the same context as synthetic catalysts. Biocatalysis based on classic resolution techniques, as well as dynamic resolutions by *in situ* racemization and stereoinversions give synthetic organic chemists the opportunity to consider completely different strategies to manufacture enantiopure target molecules from inexpensive racemates. Epoxide hydrolases have become the focus of a number of research groups, and had been applied in the synthesis of various important bioactive compounds. Initial assessment of their potential identified these hydrolytic enzymes as very promising biocatalysts. It is essential however, that extensive screening

programmes should be undertaken to identify epoxide hydrolases with novel substrate specificities. The recent report of the first evaluation of epoxide hydrolases from yeast and fungi originating from the Brazilian rain forest (Cagnon, Porto *et al.*, 1999) is encouraging.

Enzyme mimetics such as catalytic antibodies and polyamino acids are still in an early phase of research, and their potential as generally useful catalysts remains to be established.

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Chapter 2

Enantioselective hydrolysis of unbranched aliphatic 1,2-epoxides by *Rhodotorula glutinis*

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Enantioselective hydrolysis of unbranched aliphatic 1,2-epoxides by *Rhodotorula glutinis*

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Abstract

Epoxide hydrolase catalysed resolution of aliphatic terminal epoxides has been demonstrated for the hydrolysis of a homologous range of unbranched 1,2-epoxyalkanes by the yeast *Rhodotorula glutinis*. Both enantioselectivity and reaction rate were strongly influenced by the chain length of the epoxide used. Enantioselectivity showed an optimum in the hydrolysis of 1,2-epoxyhexane (E = 84). Resolution of (±)-1,2-epoxyhexane resulted in (*S*)-1,2-epoxyhexane (e.e. > 98%, yield = 48%) and (*R*)-1,2-hexanediol (e.e. = 83%, yield = 47%).

1. Introduction

A great interest exists in the development of methods for the synthesis of enantiomerically pure epoxides, because they are important chiral building blocks in the preparation of more complex enantiopure bioactive compounds. Various chemical and biological production methods have been reviewed.[1,2] In biological production methods, special attention has been given to terminal aliphatic epoxides because of their relatively high chemical stability in water-containing reaction mixtures. Furthermore, the absence of reactive side groups other than the oxirane ring, in most cases excludes undesired side-reactions while using crude enzyme preparations or whole cell biocatalysts.

Most studies on the biological production of enantiopure aliphatic epoxides involved direct epoxidation of alkenes by mono-oxygenase containing bacterial cells.[3-5] In all cases, aliphatic 1,2-epoxides were produced either as a racemic mixture or with the (*R*)-configuration in excess. The method, however, was problematic due to product toxicity.[6] Resolution of several aliphatic 1,2-epoxides has been observed in a previous study for non-hydrolytic epoxide conversions by *Nocardia* H8.[7] However, because of insufficient enantioselectivities, the yields of hte obtained (*S*)-epoxides were low.

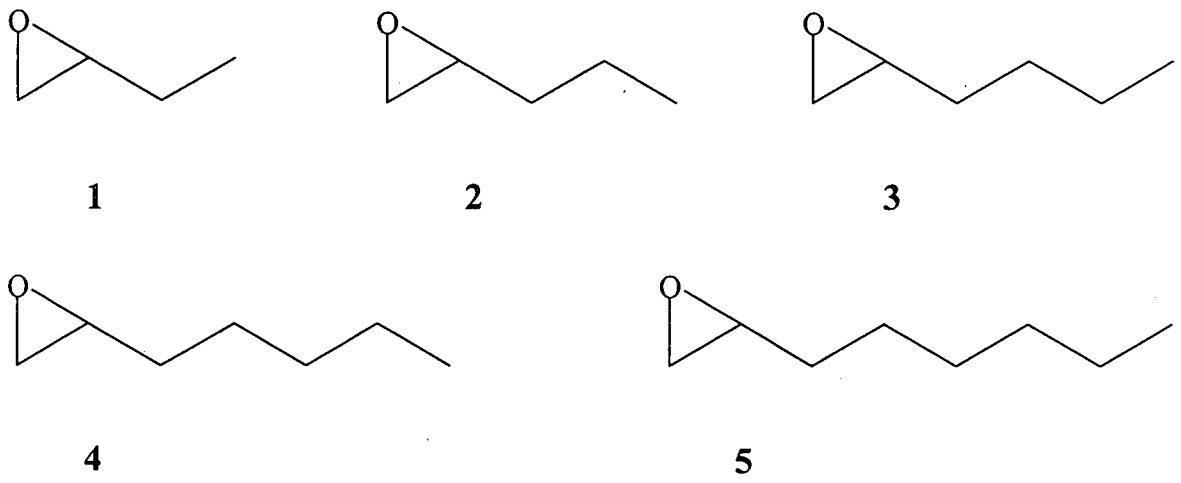
Epoxide hydrolase catalysed hydrolysis of aliphatic 1,2-epoxides has been studied with biocatalysts from different sources. Mammalian microsomal epoxide hydrolase (mEH) was used for the hydrolysis of 1,2-epoxyhexane and 1,2-epoxydecane.[8] However, very low enantioselectivities were observed and the formed diols were of low enantiomeric purity, even at very low conversions. Fungal epoxide hydrolase activities towards aliphatic 1,2-epoxides have been reported for the hydrolysis of C_6 to C_{14} 1,2-epoxyalkanes by crude cell extracts from *Ulocladium atrum* and *Zopfiella karachiensis*.[9] Activities were at a maximum for C_8 and C_{10} epoxides but, enantioselectivities were not given. Bacterial epoxide hydrolase activities were tested in a study for the resolution of 1,2-epoxyoctane with cell suspensions of the genera *Rhodococcus*, *Nocardia* and *Mycobacterium*.[10] From this screening it was concluded that the enantioselectivities of all biocatalysts tested were very low for the hydrolysis of this substrate ($E < 6$) .

Recently, we have investigated the presence of enantioselective epoxide hydrolases in yeasts. Enantioselective hydrolysis of various aryl, alicyclic and aliphatic epoxides by a strain of the yeast *Rhodotorula glutinis* has been demonstrated.[11] We have now extended our study by investigating substrate specificities and enantioselectivities for a homologous range of C_4 to C_8 aliphatic 1,2-epoxides.

Results and Discussion

Substrate specificity and enantioselectivity of the yeast epoxide hydrolase towards aliphatic 1,2-epoxides was investigated by incubating epoxides (±)-**1** to (±)-**5** with resting cell suspensions of glucose-grown *Rhodotorula glutinis* (Scheme 1). The reactions were monitored by periodic sampling and followed by GLC analysis using chiral columns. The present study was initiated by optimizing the reaction conditions

for epoxide **1**. In comparison with experiments performed in our previous study,[11] we have now raised the substrate concentration to a final concentration of 20 mM and lowered the biocatalyst concentration to maximal 0.5 grams dry weight per 10 ml of reaction mixture. Under these conditions the reaction rate as well as the enantioselectivity for epoxide **1** were improved (Table 1). The optimized reaction conditions were used in subsequent experiments for the hydrolysis of epoxides (±)-**2** to (±)-**5**.



Scheme 1: Aliphatic 1,2-epoxides used as substrates for hydrolysis by *Rhodotorula glutinis*

In the hydrolysis of racemic epoxides **1** to **5**, the reaction was terminated when the residual epoxide reached an e.e. of more than 98%. The reaction time, yield of the epoxide, e.e. and yield of the formed diol were then determined. Initial hydrolysis rates, absolute configurations, e.e. values and yields of the residual epoxides and of the formed diols are summarized in Table 1. Absolute configurations of the epoxides and diols were determined by co-injection of commercially available reference compounds on chiral GLC and by comparison of the specific rotation values with data from the literature.

Table 1.

Hydrolysis of linear-chain aliphatic 1,2-epoxides by *Rhodotorula glutinis*

	Epoxide (residual substrate)					Diol product			
	Reaction rate [a]	e.e.	Abs. Conf.	Yield	Reaction time (h)		e.e.	abs. conf.	Yield
1	2.3	> 98 %	(S)	21 %	3.0	**1a**	29 %	(R)	78 %
2	7.2	>98 %	(S)	40 %	0.7	**2a**	66 %	(R)	54 %
3	50.8	> 98 %	(S)	48 %	0.4	**3a**	83 %	(R)	47 %
4	106.1	> 98 %	(S)	44 %	0.3	**4a**	73 %	(R)	52 %
5	85.2	> 98 %	(S)	38 %	0.3	**5a**	55 %	(R)	60 %

a) Initial rate of epoxide hydrolysis in nmol/min.mg dw

Hydrolysis of 20 mM epoxide in 10 ml reaction mixture with glucose-grown cells of *Rhodotorula glutinis* (dry weights ranging from 0.1 to 0.5 g).

From the results summarized in Table 1 it is evident that the epoxide hydrolase from *Rhodotorula glutinis* has a preference for substrates with a chain length of six carbon atoms and more. The reaction rates for epoxides **3**, **4** and **5** are much higher than those for epoxides **1** and **2**. The high enantioselectivity observed for hydrolysis of epoxide (±)-**4** and, in particular, for epoxide (±)-**3** is remarkable. Resolution of unbranched aliphatic terminal epoxides with moderate or high selectivities has not been reported for epoxide hydrolases from other sources.

All tested epoxides were hydrolysed to (*R*) diols with *retention* of configuration at the more hindered carbon atom. The enantiomeric purities of the diols, obtained after complete resolution of the corresponding epoxides,were in all cases low. Enantiopure diols could only be obtained from the corresponding epoxides at low conversions.

For determination of the enantiomeric ratio *E*, ln [Ro]/[R] versus ln [So]/[S] was plotted. The slope of this curve represents the *E* value. The concentrations of the (*R*) enantiomer at times 0 and *t* are represented by [Ro] and [R], while [So] and [S] are the concentrations of the (*S*) enantiomer at times 0 and *t*, respectively. Concentrations at times *t* were determined from samples taken at two minute intervals, during the course of the reaction. This method for determining *E* has previously been used in a study on the hydrolysis of *p*-nitrostyrene oxide by an epoxide hydrolase preparation from *Aspergillus niger*.[12] The influence of the substrate chain length on the enantiomeric ratio

E in the resolution of epoxides (±)-**1** to (±)-**5** by *Rhodotorula glutinis*, is shown in Figure 1. In other relevant studies on the hydrolysis of aliphatic terminal epoxides, it was found that enantioselectivities were only significantly higher when substrates with branched alkyl chains were used.[8-10]

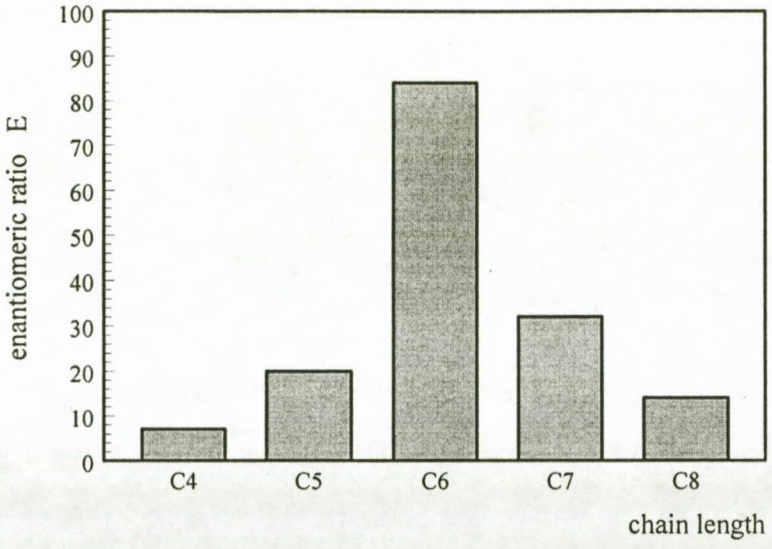


Fig. 1. Effect of the substrate chain length on the enantioselectivity in the hydrolysis of a range of unbranched aliphatic 1,2-epoxides by *Rhodotorula glutinis*.

In order to investigate the effect of increased substrate concentration on the biocatalyst, we raised the concentration of epoxide (±)-**3**. In this experiment 500 mg epoxide (±)-**3** was added to 1200 mg (dry weight) cells of *Rhodotorula glutinis* in a total volume of 10 ml. Such a substrate concentration, theoretically calculated to be 500 mM, exceeds the maximal solubility of epoxide-**3**. Under these conditions substantial amounts of epoxide will be separated from the aqueous phase during the reaction. Chemical hydrolysis of the epoxide will be minimized and larger quantities of substrate can thus be resolved. This high epoxide concentration had no significant adverse effect on the biocatalyst since there was only about 10 % decrease in reaction rate (rate r = 45 nmol/min,mg dw), compared with the rate for 20 mM epoxide (±)-**3**. The use of high substrate concentrations will greatly facilitate the application of this method on a preparative scale.

3. Conclusions

Epoxide hydrolysis with moderate to high enantioselectivities has, for the first time, been demonstrated for the resolution of a homologous range of unbranched aliphatic 1,2-epoxides by cells of the yeast *Rhodotorula glutinis*. (*R*) Epoxides were preferentially hydrolysed to (*R*) diols with *retention* of configuration at the more hindered carbon atom. Reaction rates as well as enantioselectivities were strongly influenced by the chain length of the epoxide used. High enantioselectivity (E = 84) in combination with relatively high activity was observed in the resolution of (±)-1,2-epoxyhexane.

4. Experimental Section

4.1 General

Gas chromatography (GLC) was performed on Chrompack CP9000 and Hewlett-Packard 6890 gas chromatographs equipped with FID detectors and using N_2 as carrier gas. Determination of the enantiomeric excesses was performed by GLC using fused sillica cyclodextrin capillary columns (30 m length, 0.25 mm ID and 0.25 μm film thickness). For epoxides **1** and **2** a ß-DEX 225 column (Supelco Inc.) was used at oven temperatures of 50°C and 55°C, respectively. Enantiomer analysis of epoxides **3**, **4** and **5** was done on a ß-DEX 120 column (Supelco Inc.) at oven temperatures of 45°C, 45°C and 55°C, respectively. Chiral GLC analysis for diols **1a**, **2a**, **3a**, **4a** and **5a** was performed on a ß-DEX 120 column at oven temperatures of 90°C, 100°C, 125°C, 125°C and 130°C. Concentrations of epoxides and diols were derived from calibration curves using heat-killed cells. Optical rotation values were measured on a Perkin-Elmer 241 polarimeter at 589 nm. ^{1}H-NMR spectra were recorded on a Brucker 300MHz spectrometer.

4.2 Epoxides 1 to 5.

The commercially available racemic substrates (±)-1,2-epoxybutane **1**, (±)-1,2-epoxypentane **2**, (±)-1,2-epoxyhexane **3**, and (±)-1,2-epoxyoctane **5** were all obtained from Aldrich Chemie. Epoxide **5** was available from Fluka in enantiomeric pure (*R*) and (*S*) form as well.

(±)-1,2-Epoxyheptane **4** was synthesized by direct epoxidation of the corresponding 1-heptene using *m*-chloroperoxybenzoic acid (*m*-CPBA) in dichloromethane at 0 ^{o}C. The structure of epoxide **4** was characterized by GC/MS analysis m/z 85(12, M^{+}-CHO), 71(100, $CH_3(CH_2)_4^{+}$), 55(43, $CH_3(CH_2)_2^{+}$) and by ^{1}H-NMR in $CDCl_3$ δ_H 0.91 (3H, t, J=7Hz, CH_3), 1.28-1.68 (8H, m, -$(CH_2)_4$-), 2.48 (1H, dd, J=5.1 and 2.8Hz, C$\underline{H_2}$(O)CH-), 2.76 (1H, dd, J=5.1 and 4 Hz, C$\underline{H_2}$(O)CH), 2.90-2.95 (1H, m, CH_2(O)C$\underline{H}$-).

4.3 Reference compounds 1a to 5a

For identification and determination of the yields of the formed diols from epoxides **1** to **5**, the corresponding racemic reference diol compounds **1a**, **2a**, **3a** and **5a** were purchased from Aldrich Chemie. Acid catalysed hydrolysis was used to prepare (±)-1,2-heptanediol **4a** from the corresponding epoxide (±)-**4**. GC/MS analysis m/z 101(27, M^{+}-CH_2OH), 83(77, M^{+}-(CH_2OH+H_2O)), 55(100, $CH_3(CH_2)_2^{+}$). ^{1}H-NMR of (±)-**4** in acetone-d_6 δ_H 0.89 (3H, t, J=7 Hz, CH_3), 1.18-1.42 (6H, m, -$(CH_2)_3$-), 1.42-1.60 (2H, m, -C$\underline{H_2}$CH(OH)-), 2.04-2.08 (1H, m, -CH(OH)-), 3.30-3.53 (2H, m, -CH_2OH).

4.4 Growth conditions for Rhodotorula glutinis

The yeast *Rhodotorula glutinis* strain CIMW 147 was from our own laboratory culture collection. A mineral medium supplemented with 0.2% ($^{w}/_{v}$) yeast extract and 1% ($^{w}/_{v}$) glucose was used for cultivation. *Rhodotorula glutinis* was routinely grown in a chemostat culture under aerobic conditions in a 2-l fermentor (with 1-l working volume) at 30^{o}C, with a dilution rate of 0.15 h^{-1}. The pH of the culture was maintained at 6.0. The cells were harvested by centrifugation at 16,000 *g*, washed twice with 50 mM potassium phosphate buffer pH 7.5, concentrated, and stored at - 20^{o}C.

4.5 Epoxide hydrolysis by Rhodotorula glutinis

Hydrolysis of epoxides was routinely performed in 100 ml screw-capped bottles sealed with rubber septa. The bottles contained 1 to 5 ml concentrated washed cell suspension of *Rhodotorula glutinis* (0.1 to 0.5 g dry weight) and 50 mM potassium phosphate buffer pH 7.5 to a total volume of 10 ml. The bottles were placed into a shaking waterbath at 35^{o}C and the reaction was started by addition of 0.20 mmol epoxide. The course of the epoxide hydrolysis was followed by periodic taking headspace sampling followed by

analysis with chiral GLC. Initial reaction rates were determined from the epoxide disappearrance and correlated to the dry weight of the used yeast suspension. In general, reactions were terminated when the residual epoxides reached e.e.'s of more than 98%. Subsequently, diols were extracted with ethylacetate from NaCl saturated supernatants, obtained after centrifugation of the reaction mixture. Analysis of the diols was by chiral GLC.

4.6 Absolute configuration of epoxides 1 to 5.

Absolute configurations of the residual epoxides obtained were determined after hydrolysis of 300 mg of the appropriate epoxide by 800 mg (dry weight) of *Rhodotorula glutinis* cells as described in this paper. The moment for terminating the reactions was determined by monitoring head space samples with chiral GLC. The reactions were stopped by removal of the yeast cells by centrifugation (20,000 *g*, 10 minutes, 4 ^{o}C). Subsequently, the supernatants were extracted twice with an equal volume of cold pentane. The combined organic layers were dried over $MgSO_4$ and concentrated by evaporation at 40 ^{o}C under atmospheric pressure. Because of the high volality of most epoxides, concentration was not further continued and measurement of the specific optical rotation values was performed in the concentrated pentane fraction. Chiral GLC was used for determination of e.e.'s and concentrations were derived from calibration curves. Data of chiral GLC analysis and specific optical rotation values of the residual epoxides are:

epoxide (*S*)-**1**: $[\alpha]^{24}_{D}$ = − 15.5 (c = 0.20, pentane; e.e. = 95%)
[Lit.[13]: (*R*)-**1**: $[\alpha]^{16}_{D}$ = + 12.4 (c = 5.98, dioxane; e.e. > 98%)]

epoxide (*S*)-**2**: $[\alpha]^{24}_{D}$ = − 16.8 (c = 0.28, pentane; e.e. > 98%)

epoxide (*S*)-**3**: $[\alpha]^{24}_{D}$ = − 18.7 (c = 0.93, pentane; e.e. > 98%)
[Lit.[4]: (*R*)-**3**: $[\alpha]^{25}_{D}$ = + 12.1 (neat; e.e. = 66%)]

epoxide (*S*)-**4**: $[\alpha]^{24}_{D}$ = − 12.3 (c = 0.31, pentane; e.e. = 78%)
[Lit.[4]: (*R*)-**4**: $[\alpha]^{25}_{D}$ = + 15.1 (neat; e.e. = 94%)].

Determination of the absolute configurations was by comparison of our results with the data reported in the literature.[4,13] Absolute configuration of the resolved residual epoxide (*S*)-**5** was determined by co-injection on chiral GLC with the enantiopure reference compounds (*R*)-**5** and (*S*)-**5** from Fluka.

4.7 Absolute configuration of diols 1a to 5a.

Absolute configurations were determined of the diols formed from duplicate hydrolysis of 300 mg of the appropriate epoxide by 800 mg (dry weight) of *Rhodotorula glutinis* cells as described. Experiments were carried out in duplicate as described in this paper for resolution of the epoxides. However, in case of the diols, the reactions were already terminated at low conversions in order to obtain diols with higher enantiomeric purities. The residual epoxides are removed from the reaction mixture by extraction with pentane as described. The remaining aqueous reaction mixtures were subsequently saturated with NaCl and extracted twice with an equal amount of ethyl acetate. The combined organic layers were dried over $MgSO_4$ and evaporated under reduced pressure to give an oily residu of diols **1a** to **4a**, and a white solid in the case of diol **5a**. For measurement of the specific optical rotation values and enantiomeric purities, the diols were redissolved in methanol. Chiral GLC analysis was performed for determination of the e.e. values. Data of chiral GLC analysis and specific optical rotation values of the formed diols are:

diol (*R*)-**1a**: $[\alpha]^{24}_D$ = + 3.6 (c = 3.5, methanol; e.e. = 36%)
[Lit.[14]: (*S*)-**1a**: $[\alpha]^{20}_D$ = − 8.6 (c = 1.0, methanol; e.e. > 99%)]

diol (*R*)-**2a**: $[\alpha]^{24}_D$ = + 9.9 (c = 5.4, methanol; e.e. = 67%)
[Lit.[14]: (*S*)-**2a**: $[\alpha]^{20}_D$ = − 17.3 (c = 1.0, methanol; e.e. > 99%)]

diol (*R*)-**3a**: $[\alpha]^{24}_D$ = + 13.5 (c = 5.8, methanol; e.e. = 91%)
[Lit.[14]: (*S*)-**3a**: $[\alpha]^{20}_D$ = − 16.4 (c = 1.0, methanol; e.e. > 99%)]

diol (*R*)-**4a**: $[\alpha]^{24}_D$ = + 14.4 (c = 2.4, methanol; e.e. = 93%)
[Lit.[14]: (*S*)-**4a**: $[\alpha]^{20}_D$ = − 15.4 (c = 1.0, methanol; e.e. > 99%)]

diol (*R*)-**5a**: $[\alpha]^{24}_D$ = + 12.8 (c = 0.95, methanol; e.e. = 80%)
[Lit.[14]: (*S*)-**5a**: $[\alpha]^{20}_D$ = − 13.6 (c = 1.0, methanol; e.e. > 99%)]

Determination of the absolute configurations was by comparison of our results with the data reported in the literature.[14]

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Chapter 3

Biocatalytic resolution of 1,2-epoxyoctane using resting cells of different yeast strains with novel epoxide hydrolase activities

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Summary

Yeast strains (187) from 25 different genera were screened for the hydrolysis of 1,2-epoxyoctane. Epoxide hydrolase activity was found for 54 yeast strains. Asymmetric hydrolysis of 1,2-epoxyoctane was found for 8 yeast strains belonging to the genera *Trichosporon, Rhodotorula,* and *Rhodosporidium.* All these strain preferentially hydrolysed (*R*)-1,2-epoxyoctane to (*R*)-1,2-octanediol. Excellent enantioselectivity (E>200) for 1,2-epopxyoctane is reported for the first time. Substrate concentrations of 500 mM were used without any decrease in enzyme activity.

Introduction

The synthesis of enantiomerically pure compounds, especially pharmaceuticals in which biological activity resides in only one of the enantiomers, has become important research areas in organic chemistry. Epoxides, due to their high reactivity with a large number of reagents (Leak *et al.,* 1992), and vicinal diols, employed as their corresponding cyclic sulfates or sulfites as reactive intermediates (Lohray, 1992), are versatile chiral synthons in the synthesis of such bioactive compounds. Extensive research efforts have thus been directed towards the chemical synthesis of optically active epoxides (Katsuki, 1995; Larrow *et al.,* 1996; Jacobsen *et al.,* 1997; Tokunaga *et al.,* 1997) and vicinal diols (Kolb *et al.,* 1994). Various biocatalytic methods for the production of chiral epoxides have been evaluated as viable alternatives to these chemical catalysts (Archelas and Furstoss, 1997). Although epoxide hydrolases from mammalian origin are known to effect kinetic resolution of epoxides during the

metabolism of xenobiotic compounds, their usefulness as biocatalysts is impeded by their limited availability. The exploration of this biocatalytic route received a new impetus when highly enantioselective epoxide hydrolases from bacterial (Mischitz *et al.,* 1995; Archer *et al.,* 1996; Ospiran *et al.,* 1997) and fungal origin (Zhang *et al.,* 1995; Grogan *et al.,* 1996; Nellaiah *et al.,* 1996; Pedragosa-Moreau *et al.,* 1996) were recently identified.

Epoxide hydrolase activity from only one yeast, *Rhodotorula glutinis* strain CIMW 147 has thus far been reported (Weijers, 1997). This yeast was found to be enantioselective for a homologous range of C_4 to C_8 aliphatic 1,2-epoxides, with optimum enantioselectivity for 1,2-epoxyhexane (E=84) (Weijers *et al.,* 1997). The enantioselectivity for 1,2-epoxyoctane was however low (E=14). This yeast also displayed high reaction rates and could tolerate a substrate concentration of 500 mM 1,2-epoxyhexane without significant loss of activity. No significant biocatalytic resolution of racemic straight chain terminal epoxides has been achieved with any other biocatalyst studied to date. While many enzymes showed activity towards 1,2-epoxyoctane, selectivity for this substrate was very low (E<6) and no suitable biocatalyst for this substrate had been found from bacterial sources (Ospiran *et al.,* 1997) or filamentous fungi (Pedragosa-Moreau *et al.,* 1996). Furthermore, the application of bacterial and fungal epoxide hydrolases on a preparative scale, are hampered by low substrate concentrations (10 - 100 mM) and long reaction times (Mischitz *et al.,* 1995; Archer *et al.,* 1996; Pedragosa-Moreau *et al.,* 1996). Partial enzyme purification was required to facilitate the hydrolysis of 330 mM *para*-nitrostyrene oxide with a lyophilized epoxide hydrolase preparation from *Aspergillus niger* (Morisseau *et al.,* 1997).

In the current study, 187 yeast strains from 25 different genera were screened to establish the distribution of epoxide hydrolase activity within the yeast domain, and to determine whether any strains could hydrolyse 1,2-epoxyoctane enantioselectively. The effect of substrate concentrations of up to 500 mM on enzyme activity was also investigated.

Materials and Methods

General

Yeasts were obtained from the Yeast Culture Collection of the University of the Orange Free State. Reactions were monitored and optical purities were analyzed by GLC (equipped with FID) on a fused silica cyclodextrin column (β-DEX 120, 30 m x 0.25 mm, 0.25μm film), using N_2 as carrier gas at 60°C and 130°C for 1,2-epoxyoctane and 1,2-octanediol, respectively. R_t (60°C) = 36.2 and 36.7 min for (*R*)-and (*S*)-1,2-epoxyoctane, respectively. R_t (130°C) = 22.2 and 22.9 min for (*S*)- and (*R*)-1,2-octanediol respectively. The absolute configurations of the remaining epoxides and diol products were deduced from the previously established elution order on the β-DEX 120 column (Weijers *et al.,* 1997). Concentrations of the epoxide and diol were derived from calibration curves.

Screening for hydrolysis of 1,2-epoxyoctane

Yeasts were grown in 100 ml shake-flask cultures containing 20 ml of yeast extract/malt extract (YM) medium (3% yeast extract, 2% malt extract, 1% peptone w/v) supplemented with 1% (w/v) glucose or Tween. Aliquots (1 ml) were centrifuged in micro-centrifuge tubes, the supernatant discarded and the cells washed with phosphate buffer (50 mM, pH7.5). The cells (ca. 100μl) were resuspended in buffer (400 μl) and 1,2-epoxyoctane (50 μl of a 110 mM ethanolic stock solution) was added to give 10 mM. The reaction mixtures were incubated at room temperature for 4 h. Reactions were terminated by extracting with 250 μl ethyl acetate. Diol formation was evaluated by TLC (silica gel Merck 60 F_{254}). Compounds were visualized by spraying with vanillin/conc. H_2SO_4 (5g/l).

Preparation of frozen yeast cells

Yeasts which displayed epoxide hydrolase activity were grown at 30°C in 1 L shake-flask cultures containing 200 ml YM medium supplemented with 1% glucose (w/v). At late growth phase (48 - 72 h) the cells were harvested by centrifugation (10 000 *g*, 10 min, 4°C), washed with phosphate buffer (50 mM, pH7.5), centrifuged and frozen in glycerol (10%) at -20°C in micro-centrifuge tubes (1 ml cells per micro centrifuge tube). The cells were stored for several months without significant loss of activity.

Screening for asymmetric hydrolysis of 1,2-epoxyoctane

Frozen cells (1 ml) were *thawed*, washed with phosphate buffer (50 mM, pH7.5) and resuspended in 4 ml buffer in 20 ml glass bottles closed with screw caps fitted with septa. The substrate (5µl) was added neat to a final concentration of 10 mM. The mixture was agitated on a shaking water bath at 30°C. The course of the bioconversion was followed by withdrawing samples (200 µl) at appropriate time intervals. Samples were extracted with an equal volume of ethyl acetate. After centrifugation (3000 *g*, 2 min), the remaining epoxide and formed diol were quantified and the enantiomeric excesses determined by chiral GLC analysis at the appropriate isotherm temperature.

Kinetic resolution of 1,2-epoxyoctane

The most promising yeasts identified by the screening with 10 mM substrate were used in time course experiments. Hydrolysis of 1,2-epoxyoctane was performed as described above, but with 10 - 12 µl (18.7 -20 mM) 1,2-epoxyoctane.

Preparative scale hydrolysis

Frozen cells (5 ml, ca. 500 mg dry weight) were *thawed*, washed with phosphate buffer (50 mM, pH7.5) and resuspended in 20 ml buffer in 50 ml glass bottles with screw caps fitted with septa. The substrate (1200 µl) was added neat to a final concentration of 500 mM. The mixture was agitated on a shaking water bath at 30°C. The course of the bioconversion was followed by withdrawing samples (100 µl) and analysing ethyl acetate extracts at appropriate time intervals. Reactions were terminated by extraction with Et_2O (3 x 25 ml) when the residual epoxide reached an e.e.> 98%. The combined organic layers were dried ($MgSO_4$) and evaporated. The products were separated by TLC (Kieselgel 60, Merck) using chloroform:ethyl acetate (1:1 v/v) as mobile phase.

Results and Discussion

Screening

Our search for yeast epoxide hydrolases that are enantioselective for 1,2-epoxyoctane was initiated with a screening of 187 strains from more than 25 different genera. Yeasts were grown in YM media supplemented with glucose or Tween. For the first small scale screening reactions, hydrolysis of 1,2-epoxyoctane was monitored by TLC and the formation of 1,2-octanediol evaluated. Those yeasts that were able to hydrolyse 1,2-

epoxyoctane, did so irrespective of the carbon source used. Yeasts that were able to hydrolyse 1,2-epoxyoctane to the 1,2-diol, were grown in YM media with glucose as carbon source, harvested and frozen for later use. Frozen cells did not show any significant loss of activity even after several months. Yeasts strains that hydrolysed 1,2-epoxyoctane enantioselectively were identified by chiral GLC analysis of samples taken at appropriate time intervals. The results are summarized in Table 1.

Although epoxide hydrolase activity is fairly widespread (29% of yeasts screened), enantioselectivity for 1,2-epoxyoctane, which bear no directing functional groups, was found in very few yeasts (less than 4% of yeasts screened). This was expected, since no significant enantioselectivity for 1,2-epoxyoctane has yet been reported. Enantioselectivity is restricted to only a few basidiomycetes genera, which include *Trichosporon*, *Rhodotorula* and *Rhodosporidium*. The latter two are both red yeasts and *Rhodosporidium* is the teleomorph of *Rhodotorula*. Epoxide hydrolases were previously found to be constitutively expressed in dematiaceous fungi, coincident with secondary metabolite pigment production in stationary or iodophase (Grogan *et al.*, 1996).

Enantioselective hydrolysis

The time course of the hydrolysis of 1,2-epoxyoctane by the yeasts that showed enantioselectivity was followed by chiral GLC analysis and is given in Figure 1. Table 2 summarizes the results obtained. E-values, where applicable, were calculated using the equation given in the footnotes to Table 2 (Rakels *et al.*, 1993).

Strains for which E-values are not indicated, did not display a straight line when $\ln(R_0/R)$ was plotted against $\ln(S_0/S)$ (Morisseau *et al.*, 1997). This may be due to the presence of more than one epoxide hydrolase, with different activities and enantioselectivities (Nakamura *et al.*, 1994). Interactions between the substrate and the cell surface, and the formation of other products, observed in some cases, may also lead to a non-linear plot. Diol dehydratase and epoxide isomerase activities (Yamada *et al.*, 1992; Weijers *et al.*, 1995) would also influence the yield of the diol obtained. This may account for the lower yield of diol obtained with some yeasts and for the fact that the enantiomeric purity of the formed diol is lower than expected. This may be due to some inversion of configuration during the hydrolysis of the *(R)*-epoxide, or due to stereoinversion of the *(R)*-diol to the *(S)*-diol as observed for *Candida parapsilosis* (Hasegawa *et al.*, 1990).

Table 1: Distribution of epoxide hydrolase (EH) activity towards 1,2-epoxyoctane within the yeast domain.

Genera	*Number of Species screened*	*Number of strains screened*	*Number with EH activity*	*Number with enantioselective EH activity*
Bullera	1	1	1	
Candida	22	29	11	
Cryptococcus	3	3		
Debaryomyces	4	5	1	
Galactomyces	1	1		
Geotrichum	8	13	2	
Kloeckera	1	1		
Kluyveromyces	1	1		
Lipomyces	4	15	2	
Myxozyma	3	5	1	
Pachysolen	1	2		
Phaffia	1	2		
Pichia	28	37	8	
Rhodosporidium	4	5	4	2
Rhodotorula	8	9	3	2
Sporabdomyces	1	1		
Sporidiobolus	1	1	1	
Sporobolomyces	1	1		
Sporopachydermia	2	2		
Trichosporon	7	8	6	2
Wickerhamia	1	1		
Williopsis	2	3		
Wingea	1	1	1	
Yarrowia	4	4	3	
Zygozyma	2	3		
Unclassified		33	10	2[a]
Total	112	187	54	**8**

[a] The two unclassified strains (UOFS Y-2042 and UOFS Y- 2049) were subsequently classified by the CBS as a novel *Rhodotorula* spp. with some unusual characteristics.

Table 2: Hydrolysis of 1,2-epoxyoctane by yeasts[a]

Biocatalyst	*Epoxide (residual substrate)*			*Diol product*			*Reaction*		
	e.e. [%]	*Abs. Conf.*	*Yield [%]*	*e.e. [%]*	*Abs. Conf*	*yield [%]*	*Rate*[b]	*Time (h)*[c]	*E*[e]
Rhodotorula araucariae CBS 6031	>98	(*S*)	49	87	(*R*)	48	56	0.25	>200
Rhodosporidium toruloides CBS 0349	>98	(*S*)	47	91	(*R*)	49	180	0.05	>100
Trichosporon sp. UOFS Y-0118	>98	(*S*)	40	58	(*R*)	59	30	4.0	43
Trichosporon sp. UOFS Y-0119	>98	(*S*)	30	56	(*R*)	65	29	4.0	10
Rhodotorula rubra UOFS Y-0112	>98	(*S*)	n.d.[d]	n.d.	(*R*)	n.d.	6	n.d.	5
Rhodotorula sp. UOFS Y-2049	>98	(*S*)	41	66	(*R*)	48	31	2.5	n.d.
Rhodotorula sp. UOFS Y-2042	>98	(*S*)	40	77	(*R*)	44	28	1.5	n.d.
Rhodosporidium toruloides CBS 0014	>98	(*S*)	37	71	(*R*)	47	29	3.6	n.d.

[a] Hydrolysis of 20 mM 1,2-epoxyoctane in 5 ml reaction volume by 1 ml concentrated wet yeast cells. Dry weights ranged from 80 to 120 mg.

[b] Initial rate of epoxide hydrolysis in nmol/min.mg dry weight.

[c] Reaction time for complete hydrolysis of (*R*)-1,2-epoxyoctane.

[d] Not determined.

[e] Enantiomeric ratio (E) calculated as

$$E = \frac{\ln\{(1-c)(1-ees)\}}{\ln\{(1-c)(1+ees)\}}$$

Table 3. Preparative scale hydrolysis of 1,2-epoxyoctane

Biocatalyst	*Reaction*		*Epoxide (residual substrate)*			*Diol product*		
	Time (h)[a]	Rate[b]	e.e. [%]	Yield (mg)	Yield [%]	e.e. [%]	Yield (mg)	Yield [%]
Rhodotorula araucariae CBS 6031	6.3	48	>98	480	48	80	490	43
Rhodosporidium toruloides CBS 0349	3.2	150	>98	420	42	77	534	47

[a] Reaction time for complete hydrolysis of (*R*)-1,2-epoxyoctane.

[b] Initial rate of epode hydrolysis in nmol/min.mg dry weight.

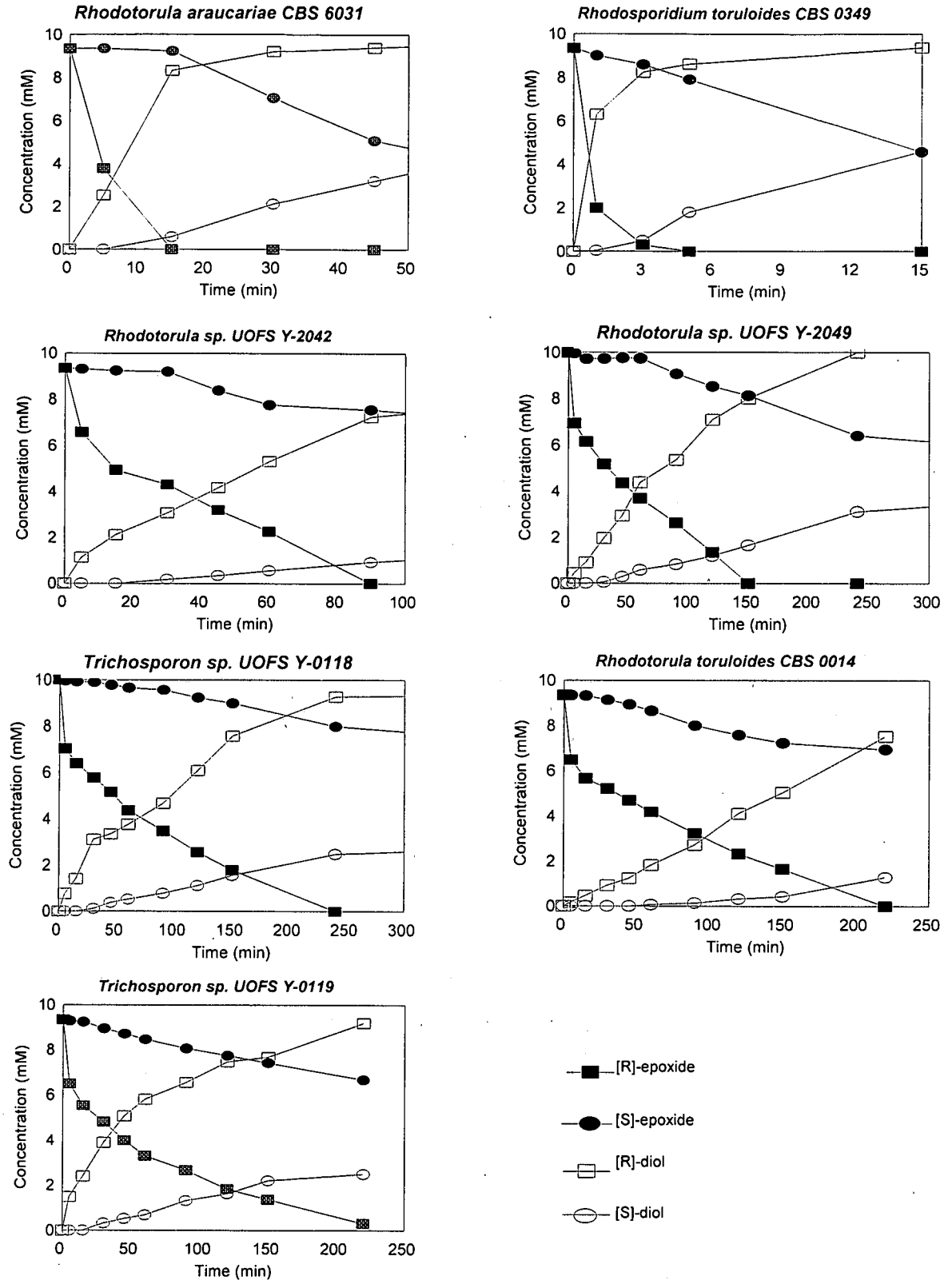

Figure 1. Changes in concentrations of enantiomers of substrate and product with time, during the hydrolysis of 1,2-epoxyoctane by different yeast strains

Initial reaction rates for all the yeasts were high, and for *Rhodosporidium toruloides* CBS 0349 the reaction had to be repeated with lower cell concentrations and samples taken at shorter time intervals to be able to determine initial rates and e.e. values at less than 50% conversion. All the yeasts showed the same enantio-preference, i.e. *(R)*-1,2-epoxyoctane was hydrolysed with retention of configuration at the stereogenic centre to *(R)*-1,2-octanediol at a much higher rate than the hydrolysis of *(S)*-1,2-epoxyoctane to the (*S*)-diol.

Preparative scale hydrolysis

Two strains, *Rhodotorula araucariae* CBS 6031 and *Rhodosporidium toruloides* CBS 0349 were selected for preparative scale hydrolysis. The results are summarized in Table 3.

The initial reaction rates were comparable to those obtained previously. No deleterious effect was thus observed in a two-phase system. The isolated yields of the remaining *(S)*-1,2-epoxyoctane and the short reaction time required, illustrates the potential of this biocatalytic method for application on a preparative scale as a viable alternative to the chiral Co(III)-based salen catalysts (Tokunaga *et al.,* 1997). The enantiomeric puritity of the isolated diols were, however, unsatisfactory, mainly because the reactions were stopped some time after the *(R)*-enantiomer was completely hydrolysed.

Conclusion

Excellent enantioselectivity for 1,2-epoxyoctane was demonstrated for the first time. Eight yeast strains possessing this unusual epoxide hydrolase activity were found. These yeasts can potentially be employed as biocatalysts for the preparative scale synthesis of optically pure 1,2-epoxyoctane and octanediol, since substrate concentrations as high as 500 mM can be used. It is remarkable that these yeasts are able to hydrolyse an epoxide bearing neither a directing group on the chiral C-2 carbon, as required for enantioselectivity in the case of bacteria (Mischitz *et al.,* 1995), nor a phenyl ring, required for enantioselectivity in the case of fungi (Pedragosa-Moreau *et al.,* 1996) or a branched alkyl chain, required for enantioselectivity in the case of rabbit liver microsomal epoxide hydrolase (Bellucci *et al.,* 1989; Bellucci *et al.,* 1995).

These results demonstrate that enantioselective hydrolysis of unsubstituted terminal epoxides is not restricted to one *Rhodotorula* strain or specie. It is evident that the substrate specificity and enantioslectivity of epoxide hydrolases in basidiomycetes yeasts are strain dependent, since some of the yeasts contain enzymes which are enantioselective for 1,2-epoxyhexane (Weijers *et al.,* 1997), while others contain enzymes selective for 1,2-epoxyoctane. It should thus be possible to obtain, with further screening, yeasts with enantioselective epoxide hydrolase activity towards other unsubstituted terminal epoxides.

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Chapter 4

Epoxide hydrolase activity of *Chryseomonas luteola* for the asymmetric hydrolysis of aliphatic mono-substituted epoxides

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Epoxide hydrolase activity of *Chryseomonas luteola* for the asymmetric hydrolysis of aliphatic mono-substituted epoxides

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Summary

Asymmetric hydrolysis of a homologous range of straight chain 1,2-epoxyalkanes was achieved using whole cells of *Chryseomonas luteola*. Depending on the chain length, hydrolyses of the racemic epoxides afforded optically active epoxides and diols with varying degrees of optical purity. In the case of 1,2-epoxyoctane, the enantiomeric excess of the remaining (*S*)-epoxide and formed (*R*)-diol was excellent (ee_s>98% and ee_p=86%). This is the first report of a bacterial epoxide hydrolase with such unusual enantioselectivity for terminal mono-substituted epoxides bearing no directing group on the chiral C-2 carbon. Benzyl glycidyl ether and the 2,2-disubstituted epoxide, 2-methyl-1,2-epoxyheptane, were hydrolysed, but no enantioselectivity was observed.

Introduction

Our recent finding of novel yeasts epoxide hydrolases which are highly enantioselective for straight chain 1,2-epoxyalkanes (Weijers *et al.*, 1997; Botes *et al.*, 1998) prompted us to examine an unclassified, yellow pigmented bacterium in our collection, for similar enantioselectivity. No suitable biocatalysts for the biocatalytic resolution of straight-chain epoxides were previously found amongst bacteria. Enantiomeric excess (ee) reported for residual (*S*)-1,2-epoxyoctane varied between 26-57%, and for the formed (*R*)-diol between 3-54%. The best values reported were for *Nocardia*, (Ospiran *et al.*, 1997) which can utilize alkenes via an alkene monooxygenase. All bacteria for which epoxide hydrolase activity had been reported, i.e. *Rhodococcus, Mycobacterium paraffinicum* and *Nocardia* (Hechtberger *et al.*, 1993; Mischitz *et al.*, 1995; Faber *et al.*, 1996; Ospiran *et al.*, 1997) produce red or yellow carotinoids as secondary metabolites. Since the

bacterium in our collection, which was subsequently identified, as a strain of *Chryseomonas luteola*, was also yellow pigmented, we were optimistic that epoxide hydrolase activity might be present. We had previously noted the possible relationship between pigment production by microorganisms and epoxide hydrolase activity (Botes *et al.*, 1998). A 2,2-disubstituted epoxide, 2-methyl-1,2-epoxyheptane, for which excellent enantioselectivity was obtained using *Rhodococcus* and *Nocardia* (Ospiran *et al.*, 1997) was included, for comparison. Benzyl glycidyl ether, a potentially useful compound in the synthesis of chiral amino alcohols (Kamal *et al.*, 1992) and bioactive compounds such as β-blockers (Kloosterman *et al.*, 1988), were also subjected to hydrolysis.

Materials and Methods

General

Reactions were monitored and optical purities were analysed by GLC on fused silica cyclodextrin columns (β-DEX 120 and β-DEX 225, 30 m x 0.25 mm, 0.25μm film) using N_2 as carrier gas). Absolute configurations of the formed aliphatic 1,2-diols were established by GLC analysis after deracemisation of the racemic 1,2-diols to the *S*-enantiomer by *Candida parapsilosis* CBS 0604 (Hasegawa *et al.*, 1990). Absolute configurations of the epoxides were deduced from the established elution order on the β-DEX 120 and β-DEX 225 columns (Weijers *et al.*, 1997). Concentrations of the epoxides and diols were derived from calibration curves. Preparative chromatography was performed on silica gel (Merck 60; 40-63 μm). ^{1}H-NMR spectra were recorded in $CDCl_3$ or acetone-d_6 on a Bruker MSL 300 (300Mhz) spectrometer. Chemical shifts (δ) are reported in p.p.m. relative to TMS.

Epoxides 1 to 8

(±)1,2-Epoxypentane (**1**), (±)1,2-epoxyhexane (**2**), (±)1,2-epoxyoctane (**4**), and (±)1,2-epoxydodecane (**6**) were commercially available from Fluka. (±)1,2-Epoxyheptane (**3**) was synthesised as previously described (Weijers *et al.*, 1997), (±)1,2-epoxydecane (**5**) and (±)2-methyl-1,2-epoxyheptane (**7**) were synthesised by direct epoxidation of the corresponding terminal alkenes with 3-chloroperbenzoic acid in dry CH_2Cl_2. (±)1,2-Epoxydecane: Yield 60%, ^{1}H-NMR ($CDCl_3$): δ = 0.80 - 0.92 (t, 3H, J=7 Hz, ω-CH_3), 1.28 - 1.52 (m, 14H, 7 x CH2), 2.45 - 2.49 (dd, 1H, J=5.1 and 2.8 Hz, -CHO-), 2.73 - 2.78 (dd,

1H, J=5.1 and 4 Hz, -CH_2O-), 2.88 - 2.94 (m, 1H, -CH_2O-). (±)2-Methyl-1,2-epoxyheptane (**7**): Yield 50%, ^{1}H-NMR (CDCl3): δ = 0.8 - 1.0 (t, 3H, J=7 Hz, ω-CH_3), 1.25 - 1.30 (s, 3H, -CH_3), 1.30 - 1.70 (m, 8H, 4 x CH_2), 2.57 - 2.65 (dd, 2H, J = 6.8 and 5.5 Hz, -CH_2O-). (±)Benzyl glycidyl ether (**8**) was synthesised by BF_3·Et_2O-catalyzed addition of benzyl alcohol onto epichlorohydrin and subsequent treatment of the intermediate halogenhydrin with NaOH as described by Takano *et al.* (1990). The product **8** was obtained in 40% yield after chromatography on silica (hexane/ether 6:1) and had the same GLC retention times as commercially available (+)benzyl (S)-glycidyl ether and (-)benzyl (*R*)-glycidyl ether.

Reference compounds

The corresponding diols of the epoxide substrates **1**, **2**, **4**, **6** and **8** were purchased from Fluka or Sigma. 1,2-Heptanediol and 1,2-decanediol, the corresponding diols of epoxides **3** and **5**, respectively, were synthesized by OsO_4 catalyzed hydroxylation of 1-heptene and 1-decene (Schröder, 1980) and purified by bulb to bulb distillation. 1,2-Heptanediol ^{1}H-NMR (acetone-d_6): δ = 0.86 - 0.93 (t, 3H, J=7 Hz, ω-CH_3), 1.18 - 1.42 (m, 6H, 3 x CH_2), 1.42 - 1.60 (m, 2H, -CH_2CHOH-), 2.04-2.08 (m, 1H, -CHOH-), 3.30 - 3.53 (m, 2H, -CH_2OH). 1,2-Decanediol ^{1}H-NMR ($CDCl_3$): δ = 0.82 - 0.98 (t, 3H, J=7 Hz, ω-CH_3), 1.25 - 1.50 (m, 14H, 7 x CH2), 2.05 - 2.20 (m, 2H, -CH_2CHOH-), 3.36 - 3.52 (m, 1H, CHOH), 3.62 - 3.78 (m, 2H, 2 x CH_2OH). 2-Methyl-1,2-heptanediol was synthesized by acid-catalyzed hydrolysis of **7** in THF-H_2O (Pedragosa-Moreau *et al.*, 1996). The crude product was purified by silica-gel chromatography ($CHCl_3$/ETOAc 1:1).

Preparation of frozen cells cells

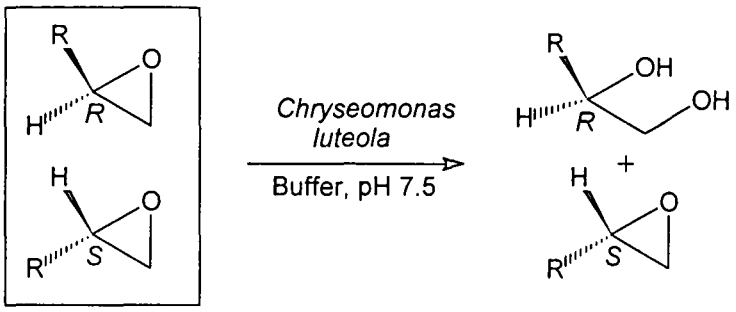
Chryseomonas luteola was grown at 30°C in 1 L shake-flask cultures containing 200 ml yeast extract / malt extract (YM) medium (3% yeast extract, 2% malt extract, 1% peptone w/v) supplemented with 1% glucose (w/v). At late exponential growth phase (48 - 72 h) the cells were harvested by centrifugation (10 000 *g*, 10 min, 4°C), washed with phosphate buffer (50mM, pH7.5), centrifuged and frozen in glycerol (10%) at -20°C in micro-centrifuge tubes (1 ml cells per microcentrifuge tube). The cells were stored for several months without significant loss of activity.

Asymmetric hydrolysis of epoxides

Frozen cells (1 ml) were *thawed*, washed with phosphate buffer (50mM, pH7.5) and resuspended in 4 ml buffer in 20 ml glass bottles closed with screw caps fitted with septa. The substrates were added neat to a final concentration of 13 to 23 mM. The mixtures were agitated on a shaking water bath at 30°C. The course of the bioconversions were followed by withdrawing samples (200 µl) at appropriate time intervals. Samples were extracted with an equal volume of ethyl actetate or ether. Samples from the reactions with 1,2-epoxypentane and 1,2-epoxyhexane were saturated with NaCl prior to extraction. After separation of the two phases by centrifugation (3000 *g*, 2 min), the remaining epoxide and formed diol in the organic phase were quantified and the enantiomeric excesses determined by chiral GLC analysis at the appropriate isotherm temperature.

Results and Discussion

The epoxides (±)-**1** - (±)-**6** were subjected to hydrolysis employing whole cells of *Chryseomonas luteola* in buffer at pH 7.5 (Scheme 1). The results are summarised in Table 1. *Chryseomonas luteola* displayed an enantio-preference for (*R*)-epoxides **1-6**, which were mainly hydrolysed with retention of configuration at the chiral centre, to (*R*)-diols. 1,2-Epoxyoctane (±)-**4** was hydrolysed with the best activity and enantioselectivity. The time course of the hydrolysis of the epoxide and the formation of the diol product is shown in Figure 1. The change in the concentrations and ee of the substrate and product, with time, is shown in Figure 2. The ee of the remaining epoxide increased from 0 to 98% after 45 minutes, while the ee of the diol decreased slightly from 100 to 86%. After 45 minutes, the residual epoxide concentration was 4.85 mM (analytical yield, 38%; ee, 98%) and the diol 5.76 mM (analytical yield, 44%; ee, 86%). Some of the initial substrate was thus unaccounted for since the initial epoxide concentration was 13 mM. This phenomenon was also observes with the other substrates. Varying amounts of, as yet, unidentified products from the 1,2-epoxyalkanes was observed, which indicates the presence of epoxide isomerase and/or diol dehydratase activities (de Bont *et al.*, 1982; Yamada *et al.*, 1992; Weijers *et al.*, 1995; Allen and Ensign, 1996) in this bacterium. Both the 2,2-disubstituted epoxide (±)-**7** and benzyl glycidyl ether (±)-**8** (Scheme 2) were hydrolysed, but no marked enantioselectivity was observed.



(±)-**1** R = *n*-C_3H_7
(±)-**2** R = *n*-C_4H_9
(±)-**3** R = *n*-C_5H_{11}
(±)-**4** R = *n*-C_6H_{13}
(±)-**5** R = *n*-C_8H_{17}
(±)-**6** R = *n*-$C_{10}H_{21}$

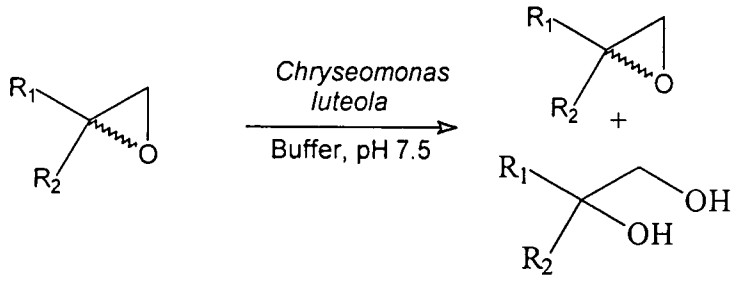
Scheme 1. Resolution of monosubstituted terminal epoxides (±)-**1** - (±)-**6** by *Chryseomonas luteola.*

Table 1: Optical purity and yield of products from the kinetic resolution of substrates (±)**1** - (±)**8** by *Chryseomonas luteola* [a].

Substrate	*Reaction*		*Epoxide (residual substrate)*			*Diol product*		
	Rate[b]	*Time (min)*	*Abs. Conf.*	*ee (%)*	*Yield (%)*	*abs. conf.*	*ee (%)*	*Yield (%)*
(±)-**1**	2.0		(S)	42	70	(R)	67	25
(±)-**2**	10.9	240	(S)	44	52	(R)	69	32
(±)-**3**	33.0	60	(S)	61	45	(R)	76	51
(±)-**4**	31.5	45	(S)	98	38	(R)	86	44
(±)-**5**	11.7	300	(S)	n.d.	50	(R)	61	49
(±)-**6**	3.1	390	(S)	n.d.	39	(R)	54	60
(±)-**7**	5.4	375	n.d.	<1	51	n.d.	<1	48
(±)-**8**	5.1	270	n.d.	<1	48	n.d.	n.d.	n.d.

[a] Hydrolysis of 13-23 mM epoxide in 5 ml reaction volume. Concentrated cells (1 ml) were used. Dry weights ranged between 95 and 110 mg.

[b] Initial reaction rate in nmol/min/mg dry wt.



(±)-7 R^1 = -CH_3 and R_2 = *n*-C_6H_{13}
(±)-8 R^1 = -H and R_2 = -CH_2-O-Bn

Scheme 2. Hydrolysis of 2-methyl-1,2-epoxyheptane (±)-**7** and benzyl glycidyl ether (±)-**8** by *Chryseomonas luteola.*

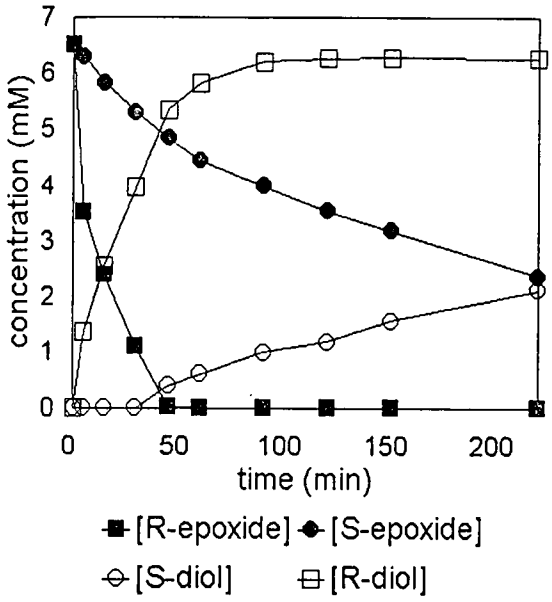


Figure 1 Time course of the hydrolysis of 1,2-epoxyoctane.

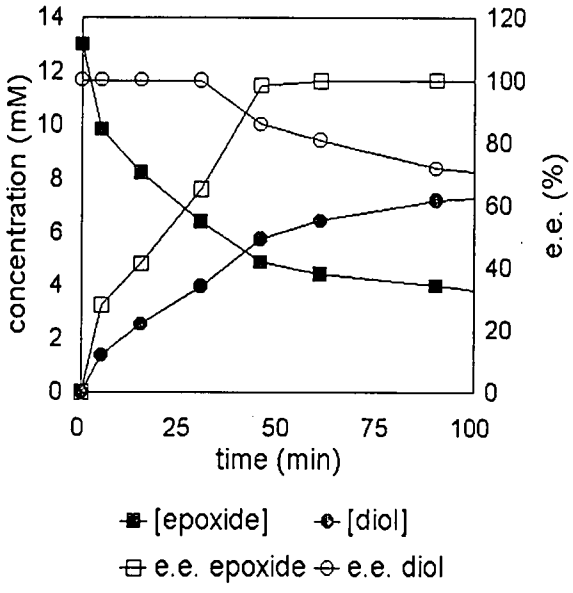


Figure 2 Change in concentration and e.e. of epoxide and formed diol with time.

Conclusions

The strain *Chryseomonas luteola* displayed a unique enantioselectivity for terminal aliphatic epoxides when compared with other bacterial epoxide hydrolases, which only show enantioselectivity when the substrate bears a directing group on the chiral C-2 carbon. It is evident from these results that epoxide hydrolases, which show enantioselectivity towards unsubstituted terminal epoxides, are not restricted to yeasts

(Weijers *et al.* 1997, Botes *et al.*, 1998). It will be informative to compare the properties and structure of this bacterial epoxide hydrolase, with those of previously described bacterial epoxide hydrolases, which showed no enantioselectivity towards unsubstituted terminal epoxides.

Acknowledgements

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Chapter 5

Enantioselectivities of yeast epoxide hydrolases for 1,2-epoxides

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Tetrahedron Asymmetry (accepted)

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Abstract

Kinetic resolution of homologous ranges of unbranched 1,2-epoxyalkanes (C-4 to C-12), and 1,2-epoxyalkenes (C-4, C-6 and C-8), a 2,2-dialkylsubstituted epoxide (2-methyl-1,2-epoxyheptane) and a benzyloxy-substituted epoxide (benzyl glycidyl ether) was investigated using resting cells of 10 different yeast strains. Biocatalysts with excellent enantioselectivity (E>100) and high initial reaction rates (>300nmol/min.mg dry weight) were found for the 2-monosubstituted aliphatic epoxides C-6 to C-8. Yeast strains belonging to the genera *Rhodotorula*, *Rhodosporidium* and *Trichosporon* all preferentially hydrolysed (R)-1,2-epoxides with retention of configuration. The epoxide hydrolases of all the yeast strains are membrane-associated.

1. Introduction

Hydrolytic kinetic resolution of racemic epoxides offers a convenient route to obtain single enantiomer synthons for enantiopure fine chemicals. Since both the remaining epoxide and the diol product (employed as cyclic sulfates or sulfites) are useful as reactive intermediates, high product recovery can be achieved. Terminal epoxides are arguably the most important subclass of epoxides that serve as building blocks for organic chemistry. Asymmetric hydrolytic catalysts able to resolve these inexpensive racemates into optically active epoxides and vicinal diols have thus become an important focus of research. An efficient (salen)Co(III)(OAc) catalyst for the hydrolytic kinetic resolution of terminal C-3 to C-8 epoxides was recently developed.[1] Resolution of long-chain alkyl epoxides (C-10 to C-20) was also achieved (ee's >95% and isolated yields

>43%) with this catalyst.[2] Biocatalytic hydrolytic resolution of epoxides as an alternative to heavy metal catalysts, has also been explored in recent years. The synthetic potential of bacterial[3,4] and filamentous fungal[5] epoxide hydrolases as chiral biocatalysts was extensively investigated. Epoxide hydrolase activity was found to be ubiquitous in these microorganisms, and the structural requirements of their epoxide substrates for chiral recognition are now well established.[6]

In contrast, the occurrence of these enzymes in yeasts and their enantioselectivities for epoxides of different structural classes had not been investigated until very recently. Pioneering work done by Weijers on the yeast strain *Rhodotorula glutinis* CIMW 147[7] and subsequent screening of yeasts from many different genera for epoxide hydrolase activity,[8] revealed unusual enantioselectivities,[9] which differed significantly from those found in bacteria and filamentous fungi. The following results obtained with yeast epoxide hydrolases are particularly noteworthy: (1) *Rhodotorula glutinis* displayed epoxide hydrolase activity and enantioselectivity with an exceptionally broad range of structurally different substrates, which included aryl, alicyclic, *meso* and aliphatic epoxides with terminal, subterminal, *trans* and *cis* location of the epoxide moiety. In contrast, epoxide hydrolases of individual bacterial and filamentous fungal strains have stringent structural requirements for enantioselective hydrolysis of substrates, which severely limits the range of substrates that, can be resolved with single strains. (2) Kinetic resolution of 1,2-epoxyhexane and 1,2-epoxyoctane had been achieved with exquisite enantioselectivity using yeast epoxide hydrolases. No similar enantioselectivity for unbranched 1,2-epoxyalkanes had been achieved with bacterial epoxide hydrolases.[10] However, the results obtained with *Chryseomonas luteola*[11] (safety class II, which is risky to work with) suggests that this unusual enantioselectivity for 2-monosubstituted 1,2-epoxyalkanes may not be restricted to yeasts. (3) Hydrolysis of most of the aliphatic substrates proceeds with unusually high initial reaction rates. Direct comparison of specific activities obtained with bacterial and fungal biocatalysts cannot be made from the available literature, but careful examination of experimental conditions published[12-15] indicate that hydrolysis of epoxides with bacterial or filamentous fungal epoxide hydrolases using similar substrate to catalyst ratios would require significantly longer reaction times. (4) High substrate concentrations (500 mM) of 1,2-epoxyhexane[8] and 1,2-epoxyoctane[9] can be used without any adverse effects on the reaction rate or

enantioselectivity of yeast epoxide hydrolases. This should facilitate efficient synthesis of enantiopure epoxides on a preparative scale.

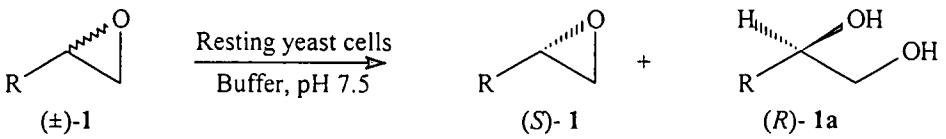
From the limited data available on yeast epoxide hydrolases, it is clear that these enzymes have promising synthetic potential. In the present study, the biocatalytic potential of yeast epoxide hydrolases is further elucidated.

2. Results and discussion

Asymmetric hydrolysis of the terminal epoxides (±)-**1** to (±)-**4** was achieved by employing resting cells of the different yeast strains in buffer at pH 7.5. All the strains preferentially hydrolysed the (*R*)-1,2-epoxide to the corresponding (*R*)-*vic* diol. From the fact that the formed diol and the remaining non-hydrolysed epoxide represent opposite enantiomeric forms, it can be assumed that the hydrolysis is proceeding with retention of configuration to a large extend. However, more detailed studies (^{18}O-labelling) have to be done to elucidate the reaction mechanism. Although all the substrates were hydrolysed, both the enantioselectivities and the reaction rates were strongly influenced by both the chain length and substitution pattern of the substrates. Enantiomeric ratios given in Table 2 and 3 were calculated based on the enantiomeric excess of the substrates (ees), since epoxide hydrolysis does not necessarily proceed with complete regioselectivity[16] in which case Sih's equation[17] involving the enantiomeric purity of the product (eep) is not applicable. E-values that were calculated using eep indicated that hydrolysis did not proceed with complete regioselectivity.

*Hydrolysis of 1,2-epoxyalkanes (±)-**1** (Scheme 1).* Excellent enantioselectivities were displayed by several strains for C-6 to C-8 1,2-epoxyalkanes. The efficiency of these biocatalysts, expressed as specific activity and catalytic activity (turnover frequency) is given in Table 1. A decrease in enantioselectivity and reaction rate was displayed with both an increase and decrease in the chain length (Table 2, Figure 1). Strains (1), (3) and (5) retained useful (E >20) enantioselectivity for the C-5 epoxide. Only strain (6) retained enantioselectivity for the C-10 epoxide, while almost no enantioselectivity was displayed by any of the catalysts for the C-12 epoxide. It should be noted however, that the C-10 and C-12 epoxides are highly hydrophobic, and that the low reaction rates obtained is probably due to low availability of the substrate to the catalyst in the aqueous

buffer, since no co-solvents were used.[18] Secondly, low effective substrate concentration have been noted to decrease the enantioselectivity of hydrolysis by epoxide hydrolases.[19,8]



(±)-1 (*S*)- 1 (*R*)- 1a

R = n-C_nH_{2n+1}

C-4: $n = 2$; C-5: $n = 3$; C-6: $n = 4$; C-7: $n = 5$; C-8: $n = 6$; C-10: $n = 8$; C-12: $n = 1$

Scheme 1. Hydrolytic kinetic resolution of a homologous range (C-4 to C-12) 1,2-epoxyalkanes

Table 1

Efficiency of purified yeast epoxide hydrolase enzymes

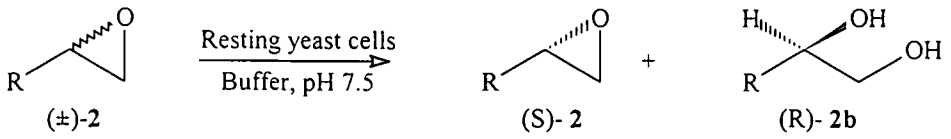
Catalyst	***Rhodosporidium toruloides*** **UOFS Y-0471**[a]	***Rhodotorula glutinis*** **CIMW 147**[b]
Substrate	1,2-epoxyoctane	1,2-epoxyhexane
Specific activity (umol.min^{-1}mg catalyst^{-1})	172	67
Turnover frequency (mol substrate processes / mol catalyst. min^{-1})	9440	3015

a) Based on a Mr 54 000,[20] and linear reaction rate.

b) Based on a Mr 45000,[21] and linear reaction rate

*Hydrolysis of 1,2-epoxyalkenes (±)-**2** (Scheme 2).* A decrease in enantioselectivity (Table 3) was observed for most strains with the introduction of a ω-double bond. As an exception, strain (1) displayed similar excellent enantioselectivity and reaction rates (Figure 2) for 1,2-epoxyoctene as for 1,2-epoxyoctane. From the results for the 1,2-epoxyalkenes it can be observed that the enantioselectivity pattern has moved to longer chainlenghts in relation to the results for 1,2-epoxyalkanes. This might be caused by the small decrease in actual chainlength of epoxyalkenes in comparison with the corresponding epoxyalkanes. Of possible commercial interest will be the hydrolysis of the simplest allylic epoxide, 1,2-epoxy-3-butene. Enantiopure forms of this epoxide and

its corresponding diol are small polyfunctional building blocks for the synthesis of a number of natural products.[22]

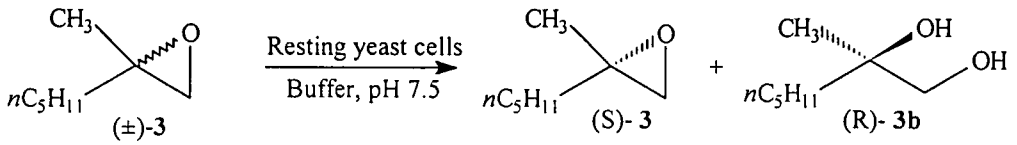


(±)-2 (S)- 2 (R)- 2b

C-4: R = $CH{=}CH_2$; C-6: R = $(CH_2)_2$-$CH{=}CH_2$; C-8: R = $(CH_2)_4$-$CH{=}CH_2$

Scheme 2. Hydrolytic kinetic resolution of a homologous range 1,2-epoxyalkenes

*Hydrolysis of 2-methyl-1,2-epoxyheptane (±)-**3** (Scheme 3).* A 2-alkyl-substituent resulted in a dramatic decrease in the enantioselectivity (Table 3) of all strains except for strain (8), for which the methyl substituent improved enantioselctivity. These results contrast sharply with the results obtained for several bacterial epoxide hydrolases,[10] which require a directing methyl group in the 2-position of terminal aliphatic epoxides for enantioselectivity. The effect of the methyl group on C-2 was less pronounced for strains (6) and (8), for which moderate enantioselectivity (E = 10 and 18) (Figure 2A) was found. Reaction rates (Figure 2B) were comparable to that of the unsubstituted C-6 – C-8 epoxides.



(±)-3 (S)- 3 (R)- 3b

Scheme 3. Hydrolytic kinetic resolution of a 2,2-disubstituted 1,2-epoxyalkane, 2-methyl-1,2-epoxyheptane

*Hydrolysis of benzyl glycidyl ether (±)-**4** (Scheme 4).* Asymmetric hydrolysis of benzyl glycidyl ether was achieved using strain (6) and (7). Although the enantioselectivity (Table 3, Graph 2) was too low (E<10) to be of practical use, this is the first report of kinetic resolution of this important building block of β-blocker drugs by epoxide hydrolases. All the strains, which were moderate (E >10) to excellent (E >30) biocatalysts for straight chain epoxides, displayed a decrease in enantioselectivity and activity upon the incorporation of a benzyloxy substituent. It is noteworthy that the effect of both a 2-methyl and a benzyloxy substituent on strain (6) was less pronounced than for the other strains.

Table 2

Enantiomeric ratios (E)[a] and initial reaction rates (V_0)[b] of yeast strains towards a homologous range of 1,2-epoxyalkanes (20 mM)

	Epoxide (±)-1	**C-4**		**C-5**		**C-6**		**C-7**		**C-8**		**C-10**		**C-12**	
	Biocatalyst	E	V_0	E	V_0	E	V_0	E	V_0	E	V_0	E	V_0	E	V_0
1	*Rhodosporidium toruloides* UOFS Y-0471	8.0	2.9	23	10	100	35	100	366	100	134	2.3	27	1.1	50
2	*Rhodosporidium toruloides* UOFS Y-0472	2.5	0.3	12	0.6	65	6.3	7.0	12	15	31	1.0	0.2	1.0	0.8
3	*Rhodotorula glutinis* CIMW 147	8.0	2.3	20	7.2	85	51	32	106	16	85	1.0	40	1.0	4.6
4	*Rhodotorula araucariae* UOFS Y-0473	4.5	4.7	4.0	10	97	18	13	37	200	55	2.5	8.3	1.2	2.3
5	*Rhodotorula glutinis* UOFS Y-0123	6.0	2.3	20	20	103	67	22	143	4.0	250	1.7	17	1.0	9.0
6	*Rhodotorula aurantiaca* UOFS Y-2049	2.5	0.8	4.5	1.0	12	2.1	30	15	20	3.8	12.0	11	1.2	3.2
7	*Rhodotorula rubra* UOFS Y-0112	3.5	1.1	5.0	0.9	10	2.3	4.0	12	5.0	3.3	3.5	0.4	1.5	1.3
8	*Rhodotorula glutinis* UOFS Y-2042	3.0	0.8	3.0	1.3	7.0	9.4	7.5	15	14	47	3.5	6.7	1.2	2.9
9	*Trichosporon mucoides* UOFS Y-0118	2.0	1.3	2.0	1.8	6.5	2.2	6.0	15	24	16	3.0	2.2	1.2	4.0
10	*Trichosporon jirovecii* UOFS Y-0119	4.0	0.8	2.0	3.5	3.6	12	7.5	3.3	10	19	3.0	2.9	1.2	4.0

[a] $E = \frac{\ln\{(1-c)(1-ees)\}}{\ln\{(1-c)(1+ees)\}}$

[b] Initial rate of epoxide hydrolysis in $nmol.min^{-1}$. mg dry $weight^{-1}$.

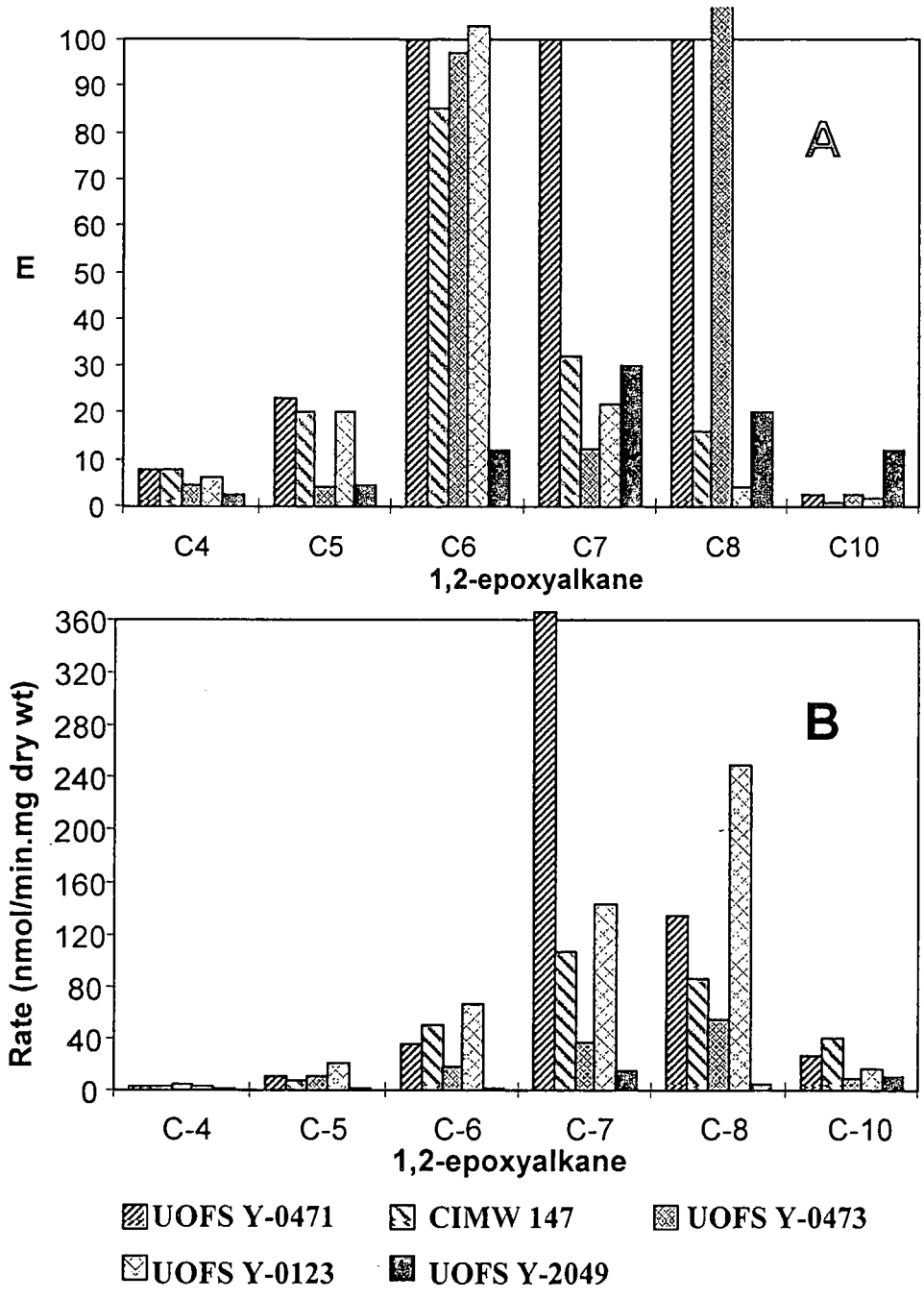
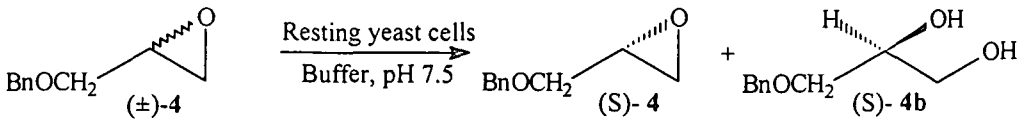


Figure 1. Enantiomeric ratio (A) and initial reaction rate (B) of the five most enantioselective yeasts towards a homologous range of 1,2-epoxyalkanes



Scheme 4. Hydrolytic kinetic resolution of a benzyloxy-substituted 1,2-epoxide, benzyl glycidyl ether. The absolute configuration of the formed diol is (*S*) due to a switch in CIP-nomenclature.

Table 3

Enantiomeric ratios (E)[a] and initial reaction rates (V_0)[b] of yeasts towards a homologous range of 1,2-epoxyalkenes, 2-methyl-1,2-epoxyheptane and benzyl glycidyl ether (20 mM)

	Epoxide (±)- **2**	**C-4**		**C-6**		**C-8**		(±)- **3**		(±)- **4**	
	Biocatalyst	E	V_0	E	V_0	E	V_0	E	V_0	E	V_0
1	*Rhodosporidium toruloides* UOFS Y-0471	2.0	2.0	14	32	100	168	5.4	65	2.6	7.0
2	*Rhodosporidium toruloides* UOFS Y-0472	4.0	0.8	7.0	0.9	8.0	2.3	2.0	28	1.0	1.0
3	*Rhodotorula glutinis* CIMW 147	4.0	2.6	23	37	18	104	5.6	39	2.5	9.0
4	*Rhodotorula araucariae* UOFS Y-0473	1.0	0.4	5.0	1.6	22	9.7	2.5	35	2.0	4.0
5	*Rhodotorula glutinis* UOFS Y-0123	2.0	2.1	21	30	11	66	3.0	67	3.0	30
6	*Rhodotorula aurantiaca* UOFS Y-2049	1.0	0.6	5.0	0.9	20	7.3	10	11	8.5	14
7	*Rhodotorula rubra* UOFS Y-0112	2.0	0.2	4.0	0.7	4.0	1.4	1.5	2.0	6.4	18
8	*Rhodotorula glutinis* UOFS Y-2042	2.0	0.4	6.0	0.9	4.0	5.3	18	3.2	4.0	15
9	*Trichosporon mucoides* UOFS Y-0118	1.0	1.1	1.0	1.2	2.0	1.9	2.0	1.0	2.0	1.0
10	*Trichosporon jirovecii* UOFS Y-0119	1.0	1.2	7.0	5.0	12	4.0	1.0	3.1	3.0	1.0

[a] $E = \frac{\ln\{(1-c)(1-ees)\}}{\ln\{(1-c)(1+ees)\}}$

[b] Initial rate of epoxide hydrolysis in nmol.min^{-1}. mg dry weight^{-1}.

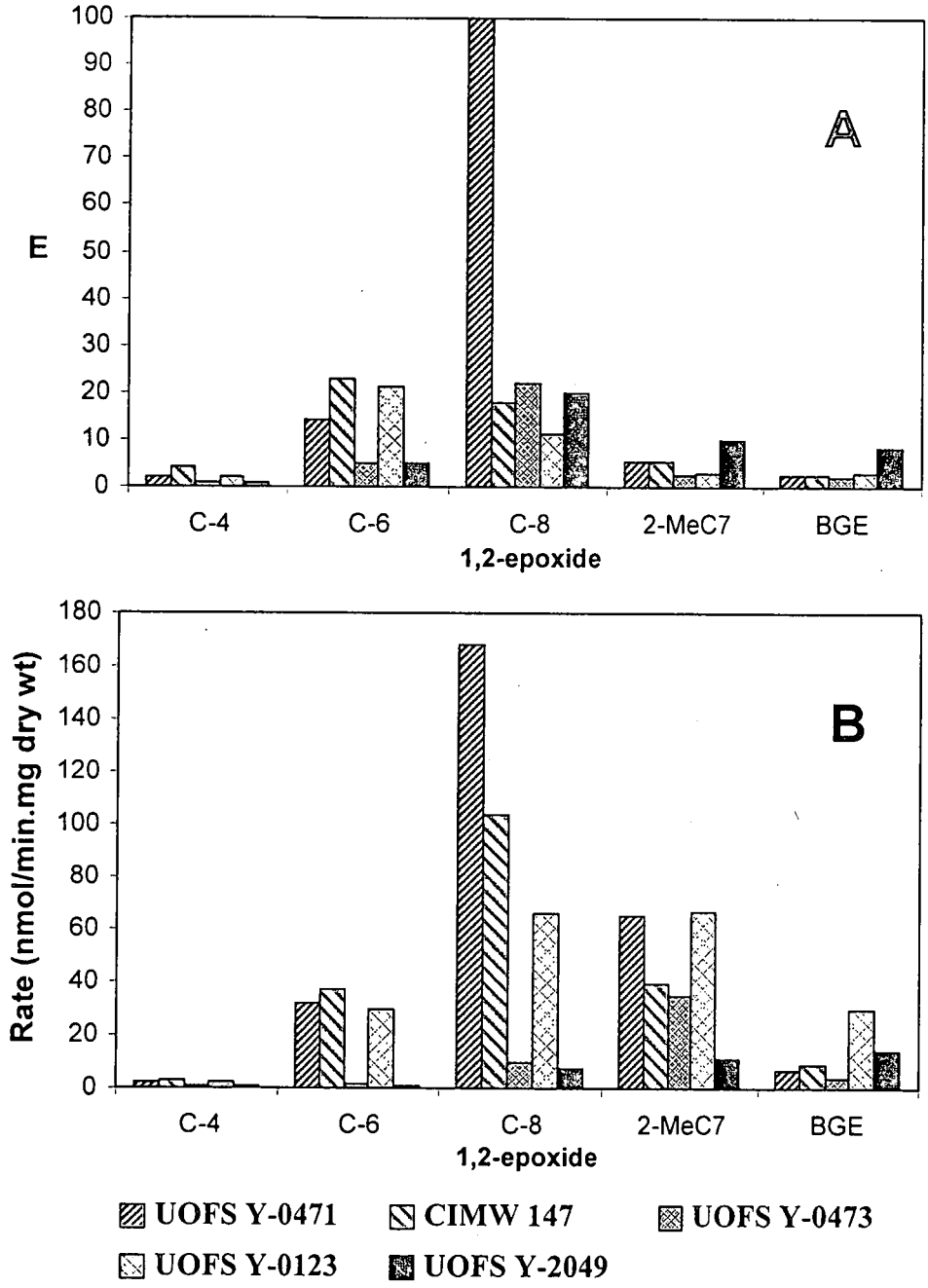


Figure 2. Enantiomeric ratio (A) and initial reaction rate (B) of the five most enantioselective yeasts towards a homologous range of 1,2-epoxyalkenes, 2-methyl-1,2-epoxyheptane and benzyl glycidyl ether

Cellular localization of yeast epoxide hydrolases.

The epoxide hydrolases of strain (1)[20] and (3)[21] were previously shown to be membrane-associated. No epoxide hydrolase activity could be detected in the 13,000 x *g* supernatants of any of the yeast lysates after extraction of soluble enzymes with Y-PER™ yeast protein extraction reagent. Total enzyme activity was retained in the cell debris. The epoxide hydrolases of all the tested yeast strains are thus membrane-associated.

3. Conclusion

Kinetic resolution of a homologous range of C-4 to C-12 epoxides by various yeast epoxide hydrolases, albeit with different enantioselectivity and reaction rates, had been demonstrated. Excellent E-values and extremely high reaction rates were displayed for unbranched C-6 to C-8 epoxides by several yeast strains belonging to the genera *Rhodotorula* and *Rhodosporidium*. Hydrolytic kinetic resolution of these substrates using yeast biocatalysts, may be a viable alternative to the (salen)Co(III)(OAc) catalysts developed by Jacobsen. In contrast to bacterial epoxide hydrolases, the presence of a directing group on C-2 destroys chiral recognition in most cases. A benzyloxy group resulted in loss of enantioselectivity by the strains displaying high enantio-preference for straight-chain epoxides. Asymmetric hydrolysis of benzyl glycidyl ether was however achieved with yeast strains that displayed lower enantio-preference for straight-chain epoxides. Studies using various other aryl, alicyclic and *meso*-epoxides are in progress to assess whether these strains share with *Rhodotorula glutinis* CIMW 147 the unusually broad range of substrates[7] that can be hydrolysed enantioselectively.

1. Experimental

General

All yeasts were obtained from the Yeast Culture Collection of the University of the Orange Free State, except *Rhodotorula glutinis* CIMW 147, which was from the laboratory culture collection of Industrial Microbiology, AU Wageningen. Reactions were monitored and enantiomeric purities were analyzed by GLC (Hewlett Packard 4890 equipped with FID) on fused silica cyclodextrin capillary columns (β-DEX 225 and β-DEX 120, Supelco Inc., and Chiraldex A-TA, Astec, 30 m x 0.25 mm, 0.25 μm film) using N_2 as carrier gas. Concentrations of epoxides and diols were derived from calibration curves with heat-killed cells. Optical rotation values were measured on a Perkin-Elmer 241 polarimeter at 589 nm.

*Epoxides **1** and formed diols **1a***

C-4, C-5, C-6, C-8 and C-12 racemic epoxides were obtained from Fluka. C-7 and C-10 epoxides were synthesized as previously described.[8,11] Chiral analysis of C-4 and C-5 epoxides was performed on β-DEX 225 (40°C isotherm), C-6 and C-7 epoxides on β-DEX 120 (45°C and 50°C isotherm), C-8 on either β-DEX 225 or Chiraldex A-TA (55°C and 52°C) and C-10 and C-12 epoxides on Chiraldex A-TA (90°C and 95°C isotherm). Absolute configurations of C-4 to C-8 epoxides were established from the previously reported data.[8] Determination of the absolute configurations of the resolved residual C-10 and C-12 epoxides was by co-injection on chiral GLC with the enantiopure (*R*) reference compounds from Aldrich. C-4, C-5, C-6, C-8 and C-12 diols were obtained from Fluka. C-7 and C-10 diols were synthesized as previously described.[11] Chiral analysis of all the diols was performed on β-DEX 120. Absolute configurations of the C4 to C8 diols were deduced from the previously reported data[8] and of C10 and C12 diols by co-injection on chiral GLC with the enantiopure reference compounds from Aldrich.

*Epoxides **2** and formed diols **2a***

The C-4, C-6 and C-8 racemic 1,2-epoxyalkenes were obtained from Aldrich. Chiral analysis of C-4 and C-6 epoxyalkenes was performed on β-DEX 225 (50°C and 60°C isotherm) and C-8 on β-DEX 120 (60°C isotherm). The elution order of the C-6 and C-8 enantiomers was first (*R*) and second (*S*). Surprisingly, however, a reversed elution order of the C-4 enantiomers on the β-DEX 225 column was observed; first (*S*) and second (*R*)-1,2-epoxy-3-butene was eluted from this column.

*Absolute configurations of residual 1,2-epoxyalkenes **2***

Absolute configurations were determined of the residual 1,2-epoxyalkenes obtained after hydrolysis of 500 mg of the appropriate epoxide by 1 g (dry weight) of *Rhodotorula glutinis* CIMW 147 cells as described before for corresponding (*S*)-1,2-epoxyalkanes.[8] Chiral GLC was used for determination of ee's and concentrations were derived from calibration curves. Data of chiral GLC analysis and specific optical rotation values of the residual epoxides are:

epoxide (*S*)-**2**, C-4: $[\alpha]^{22}_D = +\ 9.1$ (c = 0.11, pentane; ee = 37%)

[Lit.[24]: (*R*)-**2**, C-4: $[\alpha]^{23}_D = -\ 0.68$ (c = 4.44, 2-propanol; ee = 9%)]

epoxide (*S*)-**2**, C-6: $[\alpha]^{22}_D = -\ 12.4$ (c = 1.61, pentane; ee = 95%)

epoxide (*S*)-**2**, C-8: $[\alpha]^{22}_D = -\ 14.0$ (c = 1.45, pentane; ee = 90%)

[Lit.[25]: (*R*)-2, C-8: $[\alpha]^{23}_D = +\ 12.2$ (neat; ee > 80%)]

Determination of the absolute configurations was by comparison of our results with the data reported in the literature.

*Absolute configurations of formed alkene-1,2-diols **2a***

Absolute configurations were determined for the diols formed from hydrolysis of 500 mg of the appropriate epoxide by 1 g (dry weight) of *Rhodotorula glutinis* CIMW 147 cells as described before for corresponding (*R*)-alkane-1,2-diols.[8] Chiral GLC analysis was performed for determination of the ee values. Data of chiral GLC analysis and specific optical rotation values of the formed diols are:

diol (*R*)-2a, C-4: $[\alpha]^{22}_D = +\ 7.5$ (c = 4.8, methanol; ee = 32%)

[Lit.[24]: (*S*)-**2a**, C-4: $[\alpha]^{20}_D$ = - 43.6 (c = 4.6, 2-propanol; ee > 99%)]

diol (*R*)-**2a**, C-6: $[\alpha]^{22}_D$ = + 8.3 (c = 8.8, methanol; ee = 70%)

diol (*R*)-**2a**, C-8: $[\alpha]^{22}_D$ = + 12.2 (c = 8.3, methanol; ee = 95%)

Determination of the absolute configurations was by comparison of our results with the data reported in the literature.

*Epoxide **3** and formed diol **3a***

(±)-2-Methyl-1,2-epoxyheptane **3** was synthesized by direct epoxidation of the corresponding alkene and the diol (±)-**3a** by acid hydrolysis of (±)-**3** as previously described.[11] Chiral analysis was performed for the diol (±)-**3a** only, on β-DEX 120 (55°C). Absolute configuration of the diol was deduced from reported data.[22]

*Epoxide **4** and formed diol **4a***

(±)-Benzyl glycidyl ether **4** was synthesized by $BF_3 \cdot Et_2O$-catalyzed addition of benzyl alcohol onto epichlorohydrin followed by NaOH. Chiral analysis of epoxide (±)-**4** and the acetonide derivative of diol (±)-**4a** was performed on β-DEX 120 (110°C and 145°C). Absolute configurations were established by co-injection of the commercially available enantiomerically pure epoxides and diols (Fluka).

Cultivation and preparation of yeast cells

Yeasts which displayed epoxide hydrolase activity were selected and grown at 30°C in 1 L shake-flask cultures containing 200 ml YM medium supplemented with 1% glucose (w/v). At late exponential phase (48 - 72 h) the cells were harvested by centrifugation (10 000 g, 10 min, 4°C), washed with phosphate buffer (50mM, pH7.5), centrifuged again and frozen in glycerol (10%) at -20°C in micro centrifuge tubes (1 ml cells per micro centrifuge tube). The cells could be stored for several months without significant loss of activity.

General procedure for hydrolysis of epoxides

Frozen cells (1 ml) were thawed, washed with phosphate buffer (50mM, pH7.5) and resuspended in 4 ml buffer in 20 ml glass bottles with screw caps fitted with septa. The substrates were added neat to final concentrations of 20mM. The mixtures were agitated on a shaking water bath at 30°C. The course of the bioconversions of epoxides was followed by withdrawing 50 µl headspace samples with a gas-tight syringe and/or by withdrawing samples (200 µl) at appropriate time intervals for longer chain epoxides. Samples were saturated with NaCl if necessary to facilitate improved extraction of the short chain diols and extracted with an equal volume of ethyl acetate. After centrifugation (3000 x *g*, 2 min), the remaining epoxides and formed diols were analyzed by chiral GLC.

Determination of cellular localization of epoxide hydrolases

Pelleted cells (1ml) were resuspended in 2 ml Y-PER™ Yeast Protein Extraction Reagent (Pierce), pH 7.4, gently vortexed until the mixture was homogeneous and allowed to stand at room temperature for 1 hr. Cell debris and lysates were separated by centrifugation (13,000 x *g*, 10 min). The cell debris pellets were resuspended in 50 mM phosphate buffer, pH 7.4 (2ml). 1,2-Epoxyoctane (20µl) was added to the cell pellets and cell lysates, and the reaction mixtures were incubated at 30°C for 6 hr. The reactions were terminated by extraction with 1 ml ethyl acetate, and epoxide hydrolase activities were assayed by GC analysis of the formed diols. The effect of Y-PER™ on epoxide hydrolase activity was evaluated by incubating 1 ml pelleted cells treated with 2 ml Y-PER™ as above with 20µl 1,2-epoxyoctane as a control.

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Chapter 6

Affinity purification and characterization of a yeast epoxide hydrolase

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Summary

Purification of the membrane-associated epoxide hydrolase from the yeast *Rhodosporidium toruloides* CBS 0349 to electrophoretic homogeneity was achieved in a single chromatographic step employing the affinity ligand adsorbent Mimetic Green. More than 68% of the total epoxide hydrolase activity present in the whole cells was recovered from the membrane fraction. The enzyme was purified 26-fold with respect to the solubilized membrane proteins and was obtained in a 90% yield. The purified epoxide hydrolase has an apparent monomeric molecular weight of ~54 kDa, and a pI of 7.3. The enzyme was optimally active at 30 - 40°C, and pH 7.3 – 8.5. The enzyme is highly glycosylated with a carbohydrate content >42%. The specific activity of the purified enzyme for (±)-1,2-epoxyoctane is 172 μmol.min^{-1}.mg protein^{-1}. The amino acid composition of the protein was determined. This is the first report of a yeast epoxide hydrolase purified to homogeneity in milligram amounts.

Keywords: epoxide hydrolase; *Rhodosporidium toruloides*; yeast; purification

Introduction

Epoxide hydrolases are ubiquitous, stable, co-factor-independent enzymes that catalyze the hydrolysis of epoxides to the corresponding vicinal diols, often with high enantio- and regioselectivity. Microbial epoxide hydrolases, due to their abundant availability, have thus been identified as highly versatile tools for the synthesis of single enantiomer bioactive compounds derived from enantiopure epoxides and vicinal diol intermediates (Archelas & Furstoss, 1998).

Epoxide hydrolases from various mammals, insects and plants had been purified and characterized. Purification of epoxide hydrolases from microbial sources however, had been achieved for only a few bacteria. Neither filamentous (Archelas 1998) nor unicellular fungal epoxide hydrolases (Weijers & de Bont, 1999) had been purified to date.

Bacterial epoxide hydrolases from *Agrobacterium radiobacter* AD1 (Rink *et al.*, 1997) and *Corynebacterium* sp. C12 (Misawa *et al.*, 1998) showed significant similarity to plant and mammalian soluble epoxide hydrolases which belong to the α/β-hydrolase fold family. The monomeric molecular mass of these enzymes ranged from 32 to 34 kDa. With the exception of the epoxide hydrolases of *Rhodococcus* (Mischitz *et al.*, 1995) and *Nocardia* sp. Eh1 (Kroutil *et al.*, 1998), bacterial epoxide hydrolases do not display significant ($E > 20$) enantioselectivity. Furthermore, the specific activities for their preferred substrates are generally low.

Filamentous fungi, while displaying excellent enantioselectivity for various epoxides (Archelas 1998), have low epoxide hydrolase activity (Cleij *et al.*, 1998). A 7-fold purified lyophilized enzyme extract with a specific activity of 0.174 $\mu mol.min^{-1}mg^{-1}$ for *para*-nitrostyrene oxide was prepared from a cytosolic fraction of *Aspergillus niger* (Morisseau *et al.*, 1997). Epoxide hydrolase activity of the fungus *Cunninghamella elegans* (Wackett & Gibson, 1982) was shown to be associated with the 100,000 *g* pellet obtained from disrupted mycelia. Although this indicates the occurrence of microsomal as well as cytosolic forms of epoxide hydrolases in fungi, like in other eukaryotes, no purification and thus biochemical characterization of fungal epoxide hydrolases has been reported to date.

Yeast epoxide hydrolases have recently emerged as perhaps the most promising of these microbial biocatalysts, due to their exceptional substrate ranges (Weijers 1997), excellent enantioselectivity for many of these substrates, and extremely high reaction rates (Botes *et al.*, 1998). In addition, substrates in concentrations in excess of 500 mM had been hydrolysed by whole cell systems without any inhibitory effect, implying that the hydrolysis may be carried out in a biphasic system. Various strains from the genera *Rhodotorula*, *Rhodosporidium* and *Trichosporon* were found to display slightly different

chiral recognition patterns in terms of enantioselectivity and reaction rates for 1,2-epoxyalkanes of different chain lengths (unpublished results). Thus, they probably represent a series of natural mutants of the epoxide hydrolase gene in basidiomycetous yeasts. Purification of one such enzyme and amino acid sequencing could facilitate the design of primers for the sequencing, cloning and over expression of a number of these yeast epoxide hydrolase genes with the ultimate goal of enzyme modeling for tailor-made substrate specificity and enantioselectivity.

We describe here the purification of a yeast epoxide hydrolase, using an elegant one step affinity chromatography protocol.

Materials and methods

General

The Mimetic ligand adsorbent Mimetic Green was obtained from ACL (Affinity Chromatography Ltd.) (England). *Rhodosporidium toruloides* CBS 0349 was cultivated in shake flasks and biomass harvested as previously described (Botes *et al.*, 1998). All procedures with enzyme preparations were carried out at 4°C.

Enzyme assays

EH activity was assayed by monitoring the formation of 1,2-octanediol from 1,2-epoxyoctane by GC (Hewlett Packard 4890 equipped with FID) using a capillary column (Chrompack 7542, 10 m x 530 μm x 0.17μm film) with N_2 as carrier gas at 160°C and by chiral GLC analysis of 1,2-epoxyoctane (Hewlett Packard 5890 equipped with FID) using a capillary column (Chiraldex A-TA, 30 m x 0.25 mm x 0.25μm film) with N_2 as carrier gas at 52°C. Aliquots of cell fractions or column fractions were incubated with 10 – 100 mM 1,2-epoxyoctane in 50 mM phosphate buffer pH 7.5 containing 150 mM KCl, 15% (v/v) glycerol and 2.5 mM EDTA (buffer A) at 30°C for 10 min. The reaction mixtures were extracted with half volume ethyl acetate and dried over $MgSO_4$.

1U enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmol 1,2-octanediol min^{-1}.

Protein concentrations were determined using the BCA Protein Assay (Pierce) with BSA as standard.

Localization and stabilization of the enzyme

Harvested cells were disrupted with glass beads with and without the addition of 5 mM PMSF and DTT to buffer A and centrifuged to remove unbroken cells and cell debris. Membrane fractions were precipitated from the cell free extract by the addition of 16 mM $CaCl_2$ (Käppeli *et. al.*, 1982) and centrifuged (20,000 *g*, 20 min), and also by ultracentrifugation (105,000 *g*, 1 h). The pellets containing membrane proteins and the supernatants containing cytosolic enzymes were assayed for epoxide hydrolase activity.

Cellular distribution and solubilization of epoxide hydrolase

Harvested cells were suspended in buffer A, and homogenized on a bead beater. Unbroken cells and cell debris was removed by centrifugation (5000 *g*, 10 min). The supernatant (cell free extract) was centrifuged (18,000 *g*, 20 min) to sediment the mitochondria and microbodies. The post-mitochondrial supernatant was ultracentrifuged (105,000 *g*, 2 h) to sediment the microsomes. Membrane containing fractions were incubated with anionic (sodium deoxycholate), zwitterionic (CHAPS) and non-ionic (n-octyl-β-D-glucopyranoside) detergents at different concentrations. The various cell fractions were assayed to determine the distribution of epoxide hydrolase activity and the effectiveness of solubilization.

Purification of epoxide hydrolase

Biomass from 2 l medium was harvested after 72 h by centrifugation (5000 *g*, 10 min). The pellet (80 g) was suspended in buffer A (60 ml) and homogenized on a Bead Beater (Biospec) in a 200 ml stainless steel chamber (glass bead diameter 425 – 600 μm, 10 cycles of 2 min). Unbroken cells and cell debris were removed by centrifugation (5000 *g*, 10 min). The supernatant (cell free extract) was ultracentrifuged (105,000 *g*, 2h). The pellet was suspended to a concentration of 200 $mg.ml^{-1}$ in buffer A containing 10 mM CHAPS and incubated for 6 hours at room temperature on a horizontal shaker. After

solubilization, the suspension was ultracentrifuged (105,000 *g*, 2 h) to remove the residual membranes.

The supernatant containing the solubilized membrane proteins (30 ml) was concentrated by ultrafiltration (NMWCO 30000, Amicon) and the buffer was exchanged to 25 mM phosphate buffer, pH 7.2 containing 15% (v/v) glycerol, 2.5 mM EDTA and 10 mM CHAPS (buffer B) by ultrafiltration and dilution. The solubilized membrane proteins (50 ml, 2.64 mg protein ml^{-1}) was applied to a Mimetic Green column (2.5 cm x 10 cm) pre-equilibrated with buffer B. The column was washed with 300 ml buffer B, and elution was carried out with a stepwise gradient of potassium chloride (100 ml 0.1 M KCl, 220 ml 0.15 M KCl, 230 ml 0.5 M KCl). The pH of the buffer was adjusted to 7.5 after elution with 100 ml 0.15M KCl. The flow rate was maintained at 60 $ml.h^{-1}$ throughout, and 10 ml fractions were collected. The enzyme eluted at a potassium chloride concentration of 0.5 M. The active fractions were pooled, concentrated and desalted by ultrafiltration and dilution prior to polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis

SDS/PAGE was performed in a vertical mini-gel apparatus with 10% (w/v) polyacrylamide slab gels. The gels were stained for protein with Gelcode Blue (Pierce). Marker proteins were β-galactosidase (116.4 kDa), fructose-6-phosphate kinase (85.2 kDa), glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa), triose phosphate isomerase (26.6 kDa) and trypsin inhibitor (20.1 kDa) (Combithek, Boehringer Mannheim).

Denaturing isoelectric focussing on 6.5% (w/v) polyacrylamide gels containing 2% (v/v) ampholytes was carried out in the same vertical minigel system as SDS/PAGE (Roberts *et al.*, 1987) with carrier ampholytes in the pH range 3 – 10 and a mixture of marker proteins (Sigma IEF-MIX 3.5-9.3).

Glycoprotein analysis

Carbohydrate content of the purified epoxide hydrolase was estimated with the glycoprotein carbohydrate estimation kit (Pierce).

Temperature optimum

Aliquots of the purified enzyme (100 µl) were diluted to 1 ml with buffer A containing 10 mM CHAPS and 20 mM 1,2-epoxyoctane and incubated at different temperatures for 10 minutes. The experiment was repeated in Tris/HCl buffer (pH 7.5).

pH optimum

The pH optimum was determined at 35°C in buffer A, adjusted to different pH values, by using aliquots of the purified enzyme as above. The experiment was repeated in Tris/HCl buffer.

Amino acid analysis

An aliquot of the enzyme (80µg) was exhaustively dialyzed against water and lyophilized. Acid hydrolysis was performed, and the amino acids in the hydrosylate quantified by ion exchange chromatography using post-column *ortho*-phthalaldehyde (OPA) methodology (Dong & Grant, 1985).

Results and discussion

Localization and stabilization of epoxide hydrolase activity

When membranes of *Rhodosporidium toruloides* CBS 0349 were harvested by precipitation with 16 mM $CaCl_2$, no epoxide hydrolase activity was observed in the supernatant, while some activity was observed in the membrane pellet. Activity could not be restored by the addition of EDTA. This indicated that epoxide hydrolase activity was associated with the membranes and that 16 mM $CaCl_2$ caused irreversible inhibition of the enzyme. EDTA (2.5 mM) was thus added to all buffers to chelate divalent cations and to inhibit the Ca^{2+}- and Mg^{2+}-dependent proteases associated with membranes. Activity assays of the membrane-associated and cytosolic enzyme fractions obtained by ultracentrifugation indicated that epoxide hydrolase activity was mainly associated with the membrane fraction. The addition of 5 mM PMSF caused more than 80% loss of

epoxide hydrolase activity in both fractions. No PMSF was thus added during the purification protocol. The enzyme was resistant to degradation by proteases, except during gel filtration, when SDS/Page analysis showed an increase in the number of bands. DTT had no effect on the stability of the protein and was not included in buffers. Epoxide hydrolase activity in all enzyme preparations prior to solubilization remained stable for several months when stored at -20°C in buffer A.

Cellular distribution and solubilization of epoxide hydrolase

The distribution of epoxide hydrolase in the various cell fractions after optimization of the fractionation protocol is shown in Table 1. 76% of the total activity was present in the membrane fractions (mitochondria, microbodies and microsomes). The epoxide hydrolase could be solubilized most effectively from the membrane suspension (200 $mgml^{-1}$) with 10 mM CHAPS, allowing the recovery of 90% of the epoxide hydrolase activity present in the membranes. Preparations of the solubilized enzyme could be stored without loss of activity under the same conditions as above, with the addition of 10 mM CHAPS to the storage buffer.

Table 1. Distribution of epoxide hydrolase activity in the various cell fractions.

Cell fraction	% EH activity
Cell debris	12
Supernatant	12
Mitochondria	25
Microsomes	51

Purification of epoxide hydrolase

The difficulty experienced by other laboratories in the purification of both filamentous and unicellular fungal epoxide hydrolases, as evident from the fact that these enzymes had not been purified yet, warrants a discussion of the behavior of this enzyme during chromatography.

Various chromatography media were screened for binding of the epoxide hydrolase. Of the different anion exchangers, only Super Q (TosoHaas) retained the epoxide hydrolase, which eluted with 0.1 M KCl and resulted in a 4-fold purification. The epoxide hydrolase did not bind to any cation exchangers, and was inactive at the pH range inductive to binding on these supports (pH <7). Hydrophobic interaction chromatography media (PIKSI H screening kit, Affinity Chromatography Ltd.) were screened. No purification was achieved with any of the 10 hydrophobic interaction media. Gel filtration on Toyopearl HW-55F resulted in a significant loss of activity. Hydroxyapatite was not screened due to the inactivation of the enzyme by Ca^{2+}-ions. Active fractions could not be recovered by reversed-phase HPLC on a C-18 column.

The enzyme could clearly not be efficiently purified by conventional chromatography procedures. Cytosolic epoxide hydrolase from mouse liver (Wixtrom *et al.*, 1988) and the bacterium *Rhodococcus* (Mischitz *et al.*, 1995) had previously been purified with affinity/hydrophobic interaction chromatography on Benzylthio-Sepharose. This affinity matrix was synthesized as described (Wixtrom *et al.*, 1988) by epoxy-activation of Sepharose CL-6B with 1,4-butanediol diglycidyl ether to provide a 13 atom spacer arm, followed by coupling of the ligand benzyl mercaptan and extensive washing with diluted hydrochloric acid to hydrolyse unreacted epoxy groups. Half of this matrix was also treated with 2-mercaptoethanol to block any remaining free epoxide groups. The epoxide hydrolase was completely retained on the unblocked matrix, but could not be eluted even with epoxides. However, the enzyme was not retained at all on the blocked matrix. It was concluded that retention on the unblocked matrix was due to binding of the enzyme to remaining free epoxide groups that were not hydrolysed by acid treatment, and that the enzyme did not bind to the ligand.

Lectin affinity chromatography was not attempted, even after it had been established that the epoxide hydrolase is highly glycosylated, since chelators like EDTA must generally be avoided because Ca^{2+} and Mn^{2+} are part of the binding site of mannose-binding lectins. Also, the epoxide hydrolase was inactive at the optimum pH (< 6) for binding.

In the search for other affinity media, we screened the PIKSI M screening kit (Affinity Chromatography Ltd.). No epoxide hydrolase activity was present in the flow-through

fraction of the Mimetic Green adsorbent. Subsequent preparative scale chromatography on 40 ml of this affinity adsorbent (Fig. 1) with careful manipulation of the pH and salt concentration, allowed purification of the solubilized epoxide hydrolase to electrophoretic homogeneity (Fig. 2) in a single step. The results of a typical purification are represented in Table 2.

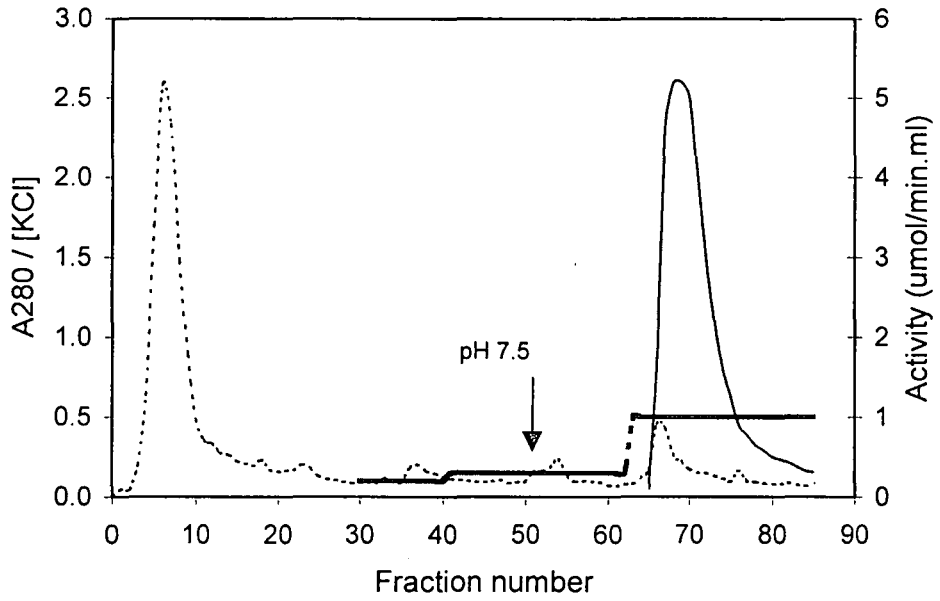

Fig. 1. Mimetic Green affinity chromatography of the solubilized membrane proteins. (.....) A_{280}; (—) Activity ($\mu molmin^{-1}ml^{-1}$); (–) [KCl] (M). Fractions of 10 ml were collected. There was no detectable epoxide hydrolase activity in fractions eluting before fraction 65.

Table 2. Purification of *Rhodosporidium toruloides* CBS 0349 epoxide hydrolase. 1U enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmol 1,2-octanediol min^{-1}.

Step	Total Protein (mg)	Total Activity (U)	Specific Activity (Umg^{-1})	Purification Factor (-fold)	Yield (%)
Solubilized proteins	130	885	6.3	1	100
Mimetic Green	4.95	850	171.7	26	96

Fig. 2. SDS-Page analysis of the purified epoxide hydrolase from *Rhodosporidium toruloides* CBS 0349. Lanes: 1, markers; 2, solubilized membrane proteins; 3, pooled fractions after Mimetic Green chromatography.

Characterization

SDS-PAGE indicated that the protein had a single polypeptide subunit of approximately 54 kDa (Fig. 2), which is similar to that reported for vertebrate microsomal epoxide hydrolase (Lu *et al.*, 1979) and the related membrane-associated juvenile hormone epoxide hydrolase of insects (Wojtasek & Prestwich, 1996). The enzyme exhibited a pI of 7.3 during denaturing IEF, which differs markedly from those reported for soluble epoxide hydrolases from bacteria (4.7 and 5.5) (Mischitz *et al.*, 1995; Jacobs *et al.*, 1991;) and plants (5.4) (Blèe & Schuber, 1992). The carbohydrate content of the enzyme exceeded that of the highest standard (α_1-acid glycoprotein, 41.4%). More than 90% activity was retained at pH 7.3 - 8.5 and temperatures of 30 - 40°C (Fig. 3).

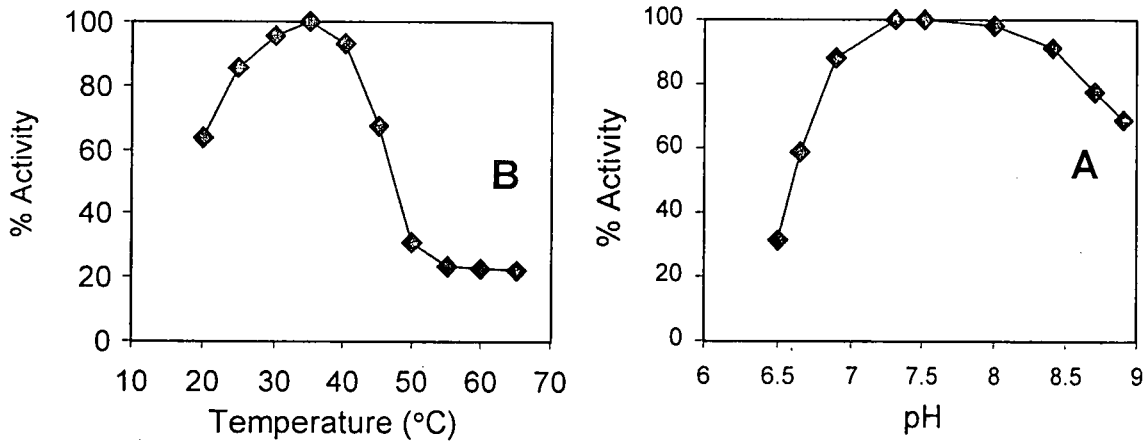

Fig. 3. Effect of temperature and pH on enzyme activity. (A) Effect of temperature, measured at pH 7.5. (B) Effect of pH, measured at 35°C.

Amino acid composition

Hydrophobic amino acids (Ala, Val, Met, Ile, Leu, Tyr, Phe, Trp) account for 37% of the total residues (Table 3). The high ratio of Asp/Asn and Glu/Gln residues relative to basic residues (95 *vs.* 56) compared to that of vertebrate microsomal epoxide hydrolases (Porter *et al.*, 1986) is notable, as well as the low number of His residues. Initial attempts to determine the molecular mass by MALDI mass spectrometry failed, probably due to heterogeneous glycosylation. Peptide sequencing is in progress and will be reported in due course.

Table 3. Amino acid composition of the epoxide hydrolase. The number of residues was calculated on the basis of a molecular mass of 54 kDa.

Amino acid	Amino acid Content (mol %)	Residues/ Enzyme (mol/mol)
Asp/Asn	10.8	53
Thr	6.6	32
Ser	7.7	37
Glu/Gln	12.0	58
Pro	0	0
Gly	9.8	48
Ala	10.3	50
Cys	0	0
Val	7.5	36
Met	1.9	9
Ile	5.2	25
Leu	9.3	45
Tyr	3.2	16
Phe	4.1	20
Trp	n.d.[a]	n.d.
His	2.0	10
Lys	5.8	28
Arg	5.8	28
Total	100	496

[a] Trp was not determined.

Conclusion

An elegant one step protocol for the affinity purification of the membrane-associated epoxide hydrolase from the basidiomycetous yeast *Rhodosporidium toruloides* CBS 0349 was developed. Several milligrams of purified enzyme was obtained for amino acid sequencing. This is the first report a fungal epoxide hydrolase purified to homogeneity in milligram amounts.

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Chapter 7

Physico-chemical properties of the epoxide hydrolase from the yeast *Rhodosporidium toruloides*

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Biotechnology Letters (submitted)

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Summary

The physico-chemical properties of the microsomal epoxide hydrolase from the yeast *Rhodosporidium toruloides* UOFS Y-0471 were determined. Residue-specific chemical modification of amino acid residues revealed that the enzyme is inactivated through modification of Asp/Glu and His residues, as well as through modification of Ser. Since Asp acts as the nucleophile, and Asp/Glu and His as charge relay partners in the catalytic triad of microsomal and soluble epoxide hydrolases during epoxide hydrolysis, inactivation of the enzyme by modification of the Asp/Glu and His residues is in agreement with the established reaction mechanism of these enzymes. However, the total inactivation of the enzyme through modification of Ser residues is an unexpected result. It is proposed that a Ser in the catalytic site is indispensable for substrate binding by analogy of the role of Ser residues in the related L-2-haloacid dehalogenases, as well as the ATPase and phosphatase enzymes. The effect of different metal ions and other inhibitors on the enzyme activity was determined. The activation energy for inactivation of the enzyme was determined from temperature stability experiments as 167 $kJ.mol^{-1}$. Kinetic constants for the enzyme could not be determined, since unusual behaviour was displayed during hydrolysis of 1,2-epoxyoctane by the purified enzyme. Enantioselectivity was strongly dependent on substrate concentration. When the substrate was added in concentrations ensuring two-phase conditions, the enantioselectivity was greatly enhanced. On the basis of these results, it is proposed that this enzyme acts at the interface, analogous to lipases.

Introduction

Epoxide hydrolases (EH) are functionally related enzymes that catalyse the addition of water to epoxides, thereby generating vicinal *trans*-diols. In mammals, five different types of epoxide hydrolases have been purified, sequenced and characterised. Mammalian EH are grouped on the basis of their enzymatic activity and biochemical characteristics, and include soluble EH (sEH) (Wixtrom *et al.*, 1988; Beetham *et al.*, 1993), microsomal EH (mEH) (Heinemann and Ozols, 1984; Falany *et al.*, 1987; Skoda *et al.*, 1988), leukotriene A_4 EH (LTA_4H) (Andberg *et al.*, 1997), cholesterol EH (Watabe *et al.*, 1981) and hepoxilin EH (Pace-Asciak and Lee, 1989). In plants, both sEH and mEH have been identified, and the soluble forms from various plants have been sequenced (Blèe and Schuber, 1992; Guo *et al.*, 1998). The juvenile hormone-metabolising EH (JHEH) from insects are mainly membrane-associated, and several of these EH have been sequenced (Harris *et al.*, 1999). In fungi, both sEH and mEH have been found (Wackett and Gibson, 1982; Grogan *et al.*, 1996), but none of these enzymes had been purified to homogeneity, or sequenced. Only mEH have been found in yeasts so far, and have been purified from two related strains, *Rhodotorula glutinis* (Kronenburg *et al.*, 1999) and *Rhodosporidium toruloides* (Botes, 1999). However, very little sequence data is available yet. Bacterial sEH from various strains have been characterised and sequenced (Rink *et al.*, 1997; Misawa *et al.*, 1998).

The analysis of amino acid sequence relationships of mEH and sEH with haloacid dehalogenases (Arand *et al.*, 1994) and haloalkane dehalogenases, enzymes with known three-dimensional structures (Li *et al.*, 1998; Verschueren *et al.*, 1993), revealed that all these hydrolytic enzymes belong to the α,β-hydrolase fold group of enzymes. The topology of this class of enzymes shows two domains: a main domain that consists of a central β-sheet surrounded by α-helices, which provide the framework for the catalytic triad residues excursing on top of the sheet at loops, and a cap domain that consists predominantly of α-helices and is involved in substrate binding. Mammalian EHs contain an additional N-terminal domain, which in the case of mEH, serves as a membrane anchor that spans the membrane once in a type I (N_{exo}/C_{cyt}) orientation (Holler *et al.*, 1997). This membrane anchor, consisting of 20 amino acids, is dispensible for catalytic activity, but serves to stabilise the protein (Friedberg *et al.*, 1994).

The catalytic triad of the α,β-hydrolase fold family consist of nucleophile, base and acid residues. The catalytic nucleophile may be Ser, Asp or Cys, although the latter is less common (Dodson and Wlodawer, 1998). The acidic residue may be either Asp or Glu, which functions as a charge relay partner with His. This basic His residue is conserved among all proteins of this family.

A proposed phylogenetic tree representing the evolutionary relationship of epoxide hydrolases and dehalogenase-related α,β-hydrolase fold enzymes has recently been established by M. Arand (Archelas, 1998). In the HYL family (HYL is the root name which has been proposed to designate a superfamily of enzymes that are evolutionary related to the α,β-hydrolase fold family), the HYL 1 (mEHs), HYL 2 (sEH) and HYL 3 (dehalogenases) subfamilies have an Asp residue as catalytic nucleophile, while the HYL 4 subfamily (lipases, esterases, peroxidases and various other hydrolases), have Ser as catalytic nucleophile (Figure 1).

The acidic residue of the catalytic triad of mEH is Glu, while sEH and haloalkane dehalogenases have Asp as catalytic acid residue (Figure 2). Interestingly, replacement of the acidic Glu^{404} residue with Asp in the catalytic triad of rat mEH lead to a strongly increased turnover rate, and increased enzymatic activity 20 – 40 fold (Arand *et al.*, 1999).

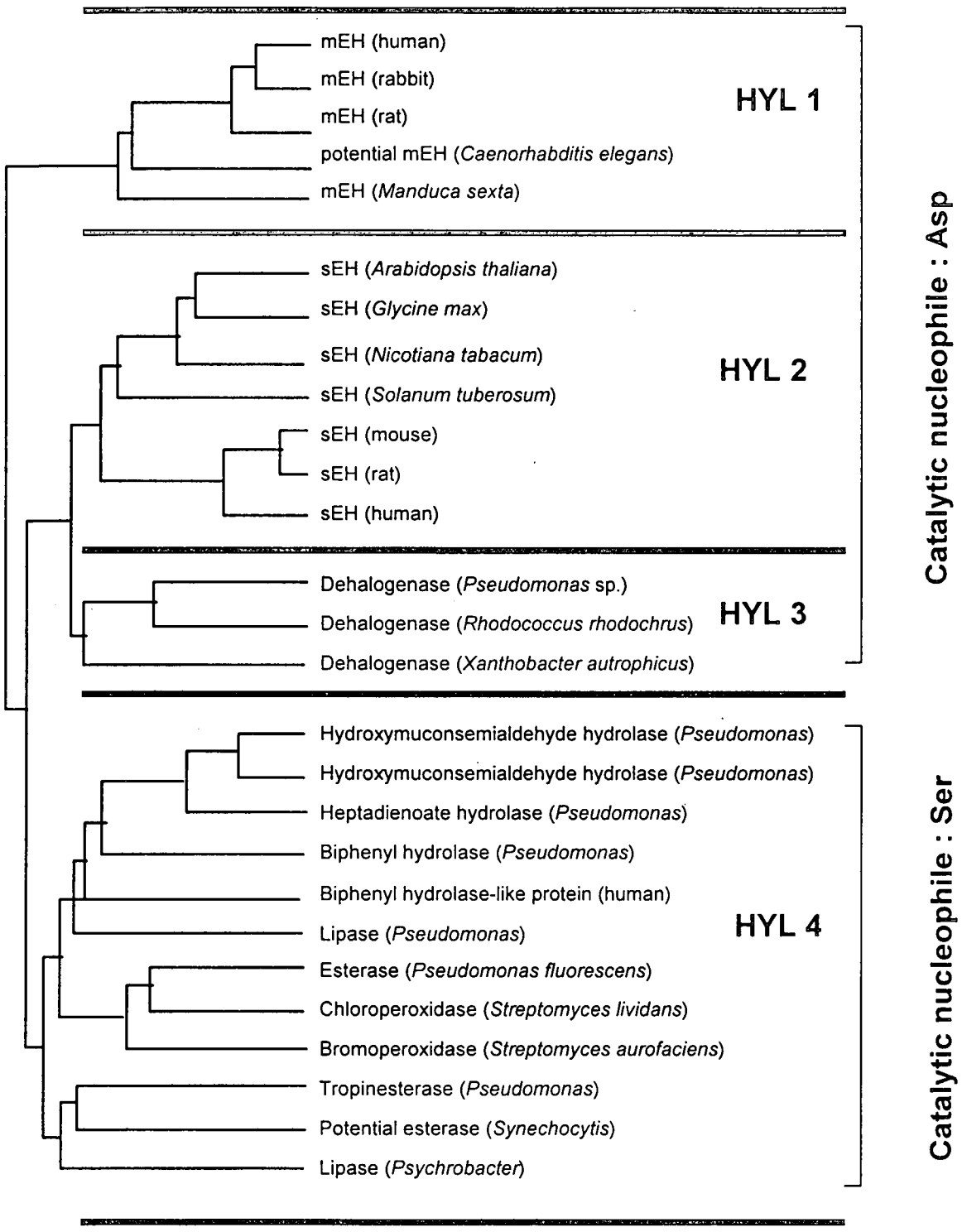

Figure 1. Proposed phylogenetic tree for epoxide hydrolase, haloalkane dehalogenase-related α,β-hydrolase fold enzymes (adapted from Archelas, 1998).

	N	A	B
(a)			
RhmEH			PEGGHFAALK
hmEH	221 YIQGGDWGSL	400 AFPFELLHTP	427 VRGGHFAAFE
bmEH	221 YIQGGDWGAL	400 AFPCEIMHVP	427 PRGGHFAAFE
rmEH	221 YIQGGDWGSL	400 AFPSELLHAP	427 ERGGHFAAFE
MmEH	222 YIQAQDWGSQ	397 NFKYEVLYQP	424 DFGGHFAALH
TmEH-1	221 FVQGGDWGSV	399 QGKYEIAYQP	426 DDGGHFFAFE
(b)			
hsEH	329 VFIGHDWGGM	491 TAEKDFVLVP	519 EDCGHWTQMD
msEH	327 VFIGHDWAGV	490 TAEKDIVLRP	518 EDCGHWTQIE
rsEH	328 VFIGHDWAGV	491 TAEKDIVLRP	519 EDCGHWTQIE
tsEH	95 FLVGKDFGAR	251 MGEKDYALK	285 EGS.HFVQEQ
asEH	98 FVVGHDWGAL	261 IGELDLVYYM	296 EGVAHFINQE
psEH	100 FVVAHDWGAL	261 VGEFDLVYHI	296 EGAAHFVSQE
(c)			
ECHA	102 YVVGHDFAAI	242 WGLGDTCVPY	271 EDCGHFLMVE
CEH	94 GVIGHDWGAL	(UNKNOWN)	260 ENCGHFVPEE
HLD1	119 TLVVQDWGGF	256 IGMKDKLLGP	285 ADAGHFVQEF

Figure 2. Comparison of the amino acid sequences of epoxide hydrolases and haloalkane dehalogenase in the regions of the catalytic triad. The nucleophile (N) of the catalytic triad (Asp) and the base (B) of the catalytic triad (His) are conserved among all members. However, microsomal forms of EH have Glu as acidic residue, while soluble forms of EH and haloalkane dehalogenase have Asp as acidic residue of the catalytic triad. (a) Microsomal epoxide hydrolases; RhmEH, *Rhodotorula glutinis* (Kronenburg et al., 1999); hmEH, human; bmEH, rabbit; rmEH, rat; MmEH, *Manduca sexta*; TmEH-1, JHEH from *Trichoplusia ni*. (b) Soluble epoxide hydrolases; hsEH, human; msEH, mouse; rsEH, rat; tsEH, tobacco (Guo et al., 1998); asEH, *Arabidopsis thaliana*; psEH, potato. (c) Bacterial epoxide hydrolases and haloalkane dehalogenase; ECHA, *Agrobacterium radiobacter* ADI EH (Rink et al., 1997); CEH. *Corynebacterium* C12 EH (Misawa et al., 1998); HLD1, *Xanthobacter autrophicus* haloalkane dehalogenase. Scheme adapted from Harris et al., 1999 (Harris et al., 1999), with expansion from the cited references.

All members of the HYL family hydrolyse their substrates by a similar reaction mechanism through the catalytic triad. The first step of the enzymatic reaction involves the formation of a covalent enzyme-substrate ester by nucleophilic attack on the substrate by an Asp. The formation of this covalent intermediate has been unequivocally demonstrated for mEH (Lacourciere and Armstrong, 1993; Muller *et al.*, 1997), sEH (Hammock *et al.*, 1994; Borhan *et al.*, 1995) and haloalkane dehalogenases (Verschueren *et al.*, 1993) and haloacid dehalogenases (Li *et al.*, 1998). In the second step of the reaction, the formed ester intermediate is hydrolysed by a water molecule that has been activated via proton abstraction by a His-Asp/Glu pair (Figure 3).

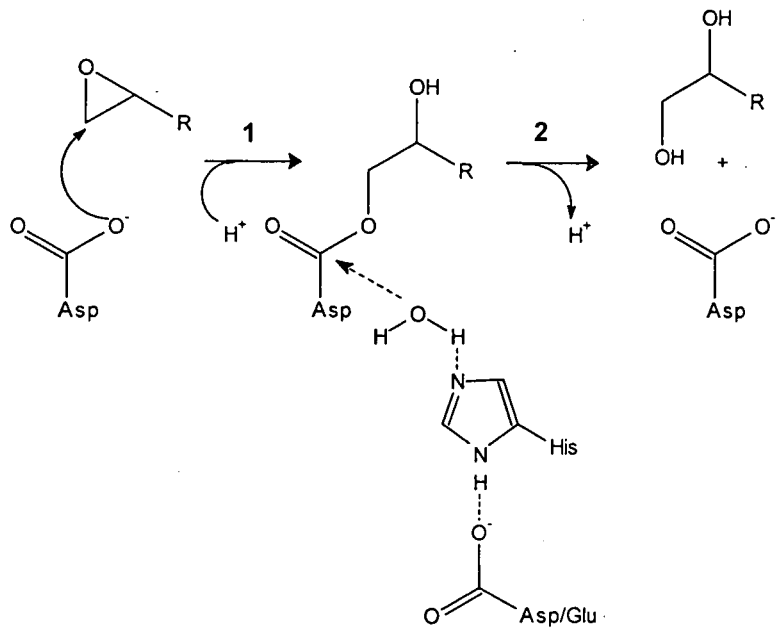

Figure 3. Enzymatic mechanism of epoxide hydrolase. In the first step of the reaction, the catalytic Asp residue opens the epoxide ring in a *trans* nucleophilic attack at the least hindered of the oxirane carbon atoms, and forms a covalent substrate-enzyme ester intermediate. During step 2, a water molecule, activated through proton abstraction by a His-acidic residue charge relay system, hydrolyses the ester bond between the enzyme and product. Taken from Arand *et al.*, 1999

The results obtained during the physico-chemical characterisation of the microsomal epoxide hydrolase from the yeast *Rhodosporidium toruloides* UOFS Y-0471 are interpreted against the background provided above.

Experimental

General

The epoxide hydrolase was purified and enzyme activity was assayed as previously described (Botes,1999).

Residue-directed inhibition

Modification of histidine

A stock solution of diethyl pyrocarbonate (DEPC) in anhydrous ethanol (50 – 200 mM) was freshly prepared and immediately added to a solution of purified epoxide hydrolase in phosphate buffer (pH 7) to a final concentration of 4 - 20 mM. The absorbance at 242 nm was monitored at 30 s intervals. Samples (100 μl) were withdrawn at appropriate time intervals, and assayed for enzyme activity. Hydroxylamine (0.2 vol; 1M) and Tris-base (0.2 vol; 1M), respectively, was added to the remaining reaction mixture to reverse histidine modification through decarboxyethylation, and enzyme activity was again assayed.

Modification of carboxylic acid side chains

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) was added to a solution of purified epoxide hydrolase in phosphate buffer, pH 5.5, to a final concentration of 500 mM. Samples (100 μl) were withdrawn at appropriate time intervals, the pH was adjusted to 7.5 and enzyme activity was assayed. Hydroxylamine (0.5 M) and Tris-base (1M), respectively, was added to the remaining reaction mixture to reverse tyrosine modification, as a control that inactivation was not due to tyrosine modification. Enzyme activity was again assayed.

Modification of cysteine

5,5'-Dithio-bis(2-nitrobenzoic acid) (Ellman's reagent, DTNB) was added to purified epoxide hydrolase in phosphate buffer, pH 7.5 to a final concentration of 25 mM. The absorbance at 410 nm was monitored over a period of 20 minutes, and enzyme activity was assayed. Dithiothreitol (Cleland's reagent, DTT) was added to reverse the modification of cysteine, and enzyme activity was again assayed.

Modification of serine

Phenylmethylsulfonyl fluoride (PMSF) (200 mM in ethanol) was added to purified epoxide hydrolase in phosphate buffer, pH 7.5, to a final concentration of 5 – 40 mM. The reaction mixtures were incubated for 30 minutes at room temperature and the enzyme activity was assayed. The effect of diisopropyl fluorophosphate (DFP) (20 mM) on enzyme activity was similarly evaluated.

Effect of metal ions

The chlorides of Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Co^{2+}, and Hg^{2+}, and the nitrate of Ag^{+} in phosphate buffer (pH 7.5) was added to a crude extract of epoxide hydrolase to a final concentration of 50 mM. The reaction mixtures were incubated for 5 minutes at room temperature, and the remaining enzyme activities were assayed.

Effect of EDTA

EDTA (30 mM) was added to purified epoxide hydrolase in phosphate buffer (pH 7.5) that already contained 5 mM EDTA to ensure that the effect observed would not be due to divalent ions present in the buffer. Activity of the enzyme with and without the additional EDTA was assayed. EDTA was also used to determine if inhibition by Ca^{2+} was reversible. Purified enzyme was incubated with Ca^{2+} (2.5 – 50 mM) for 5 minutes, and enzyme activity was assayed with and without the addition of an equivalent molar amount of EDTA.

Temperature stability

Aliquots of a crude extract of epoxide hydrolase were incubated at temperatures between 35 and 70°C, and enzyme activity assayed at appropriate time intervals.

Enzyme kinetics

Enzyme extract (5 ml) in phosphate buffer (pH 7.5) was incubated with different concentrations of 1,2-epoxyoctane (10 – 500 mM). The hydrolysis was followed by taking headspace samples at appropriate time intervals as previously described (Weijers *et al.*, 1998).

Results and discussion

Residue-directed inhibition

Modification of histidine

Histidine modification with DEPC caused irreversible inhibition of enzyme activity (Figure 4). Enzyme activity could not be restored by the addition of either hydroxylamine or Tris-base. This irreversibility of His modification has been observed for many enzymes (Lundland, 1995). A catalytic histidine responsible for activating water has been unequivocally demonstrated in microsomal (Arand *et al.*, 1999) and soluble epoxide hydrolases (Arand *et al.*, 1996), as well as in juvenile hormone epoxide hydrolase (Debernard *et al.*, 1998). Furthermore, a conserved catalytic histidine, functioning as a general base, is uniformly present in most known members of the α/β-hydrolases fold family of enzymes.

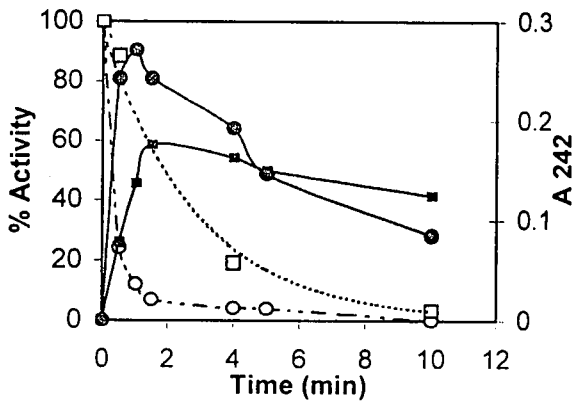

Figure 4. Effect of DEPC (4 mM and 20 mM, respectively), a modification agent of histidine, on enzyme activity. Carboxyethylation of His residues results in an increase in absorbancy at 242 nm. ● A242 (20 mM DEPC), ○ % activity (20 mM DEPC), ■ A242 (4 mm DEPC), □ % activity (4 mM DEPC).

Modification of carboxylic acid side chains

Incubation of the enzyme with EDAC caused irreversible inhibition of enzyme activity (Figure 5). Enzyme activity could not be recovered by addition of either hydroxylamine or Tris-base, indicating that inactivation was not due to Tyr modification. A catalytic Asp or

Glu functioning as a charge relay partner with His had been demonstrated for several epoxide hydrolases. For most microsomal epoxide hydrolases, the Glu^{404}- $His^{431(428)}$ pair is responsible for proton abstraction from a water molecule, while Asp-His activates the water molecule in soluble epoxide hydrolases (Arand *et al.*, 1996) and juvenile hormone epoxide hydrolases (Harris *et al.*, 1999). Furthermore, the Asp/Glu-His pair is also present in the haloalkane dehalogenases, another member of the α,β-hydrolase fold family, which shares significant sequence similarity with epoxide hydrolases (Arand *et al.*, 1994).

Asp also acts as the nucleophile in the catalytic triad of all known microsomal and soluble epoxide hydrolases, juvenile hormone epoxide hydrolase and haloalkane dehalogenases (Beetham *et al.*, 1995; Harris *et al.*, 1999). All other members of the haloacid dehalogenase (HAD) superfamily, comprised of epoxide hydrolases, P-type ATPases, phosphatases, and L-2-haloacid dehalogenases, have a conserved Asp that acts as nucleophile.

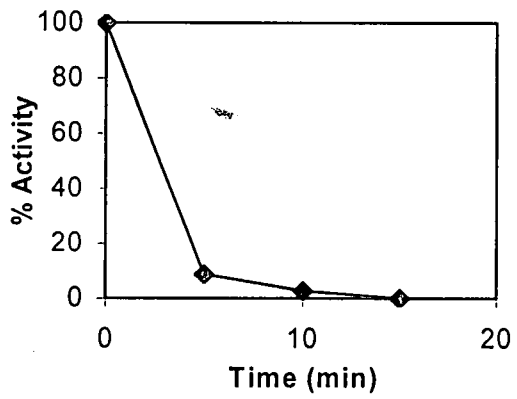

Figure 5. Effect of 500 mM EDAC, a modification agent of carboxylic acid side chains, on epoxide hydrolase activity

Modification of cysteine

No increase in A_{410} was observed over a period of 20 minutes during incubation of the purified enzyme with Ellman's reagent. The sulfhydryl reagent did not have a significant effect on enzyme activity. Enzyme activity measured after addition of DTT was slightly higher (Figure 6). DTNB was previously found to have a slightly inhibitory effect on mammalian mEH and JHEH, while another sulfhydryl-modifying reagent, iodoacetamide,

did not have any significant effect (Debernard *et al.*, 1998). These results indicate that Cys does not function as the catalytic nucleophile, and confirm the absence of Cys residues found when the amino acid composition of this enzyme was determined (Botes, 1999)

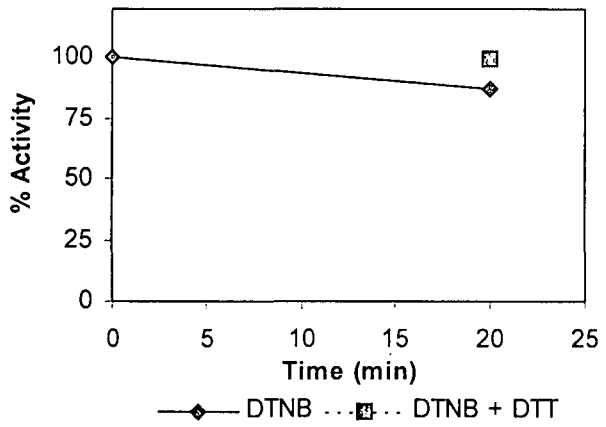

Figure 6. Effect of 25 mM Ellman's reagent, a modification agent of Cys residues, on epoxide hydrolase activity. DTT was added to the reaction mixture, and enzyme activity was again measured.

Modification of serine

PMSF at a concentration of 20 mM caused almost complete inhibition of enzyme activity. This result was confirmed by using DFP, which also caused inactivation of the enzyme at a concentration of 20 mM (Figure 7). These results suggest that Ser is either catalytically or structarally important. This is in contrast with results obtained for JHEH, which is not inhibited by PMSF (Debernard et al., 1998). No similar inhibition studies were done on any other epoxide hydrolases or related enzymes. However, it is noticeable that PMSF was not added during the purification of mEH, sEH or bacterial epoxide hydrolases. This may indicate that similar inhibitory effects were observed, or may reflect on the resistance of epoxide hydrolases to proteolysis by serine proteases (Craft et al., 1990; Holler et al., 1997).

The role of Ser in the catalytic site may be that of nucleophile, in place of Asp of other epoxide hydrolases. Ser acts as nucleophile in several other members of the α,β-hydrolase fold family of enzymes, e.g. serine proteases, esterases and lipases, and functions similarly to Asp to form a covalent enzyme-substrate intermediate. However, Asp acts as the nucleophile in all known mEH, sEH and JHEH, as well as haloalkane

dehalogenases and other members of the HAD family (haloacid dehalogenases ATPases, phosphatases) (Collet *et al.*, 1998). It would thus be surprising if yeast epoxide hydrolases acts through a different nucleophile. Furthermore, the resulting acyl intermediate formed with Ser as nucleophile would be too stable for release from the enzyme. Possibly, if Ser was involved in the mechanism, it could act to displace the acyl intermediate instead of –OH, and is then subsequently displaced itself to restore the enzyme to resting state. A more likely role for Ser would be involvement in substrate binding. A Ser in the active site of L-2-haloacid dehalogenases was found to be an essential residue for binding the carboxyl group of the substrate through hydrogen bonds (Figure 8A) (Li *et al.*, 1998). A similar role of a conserved Ser residue in the active site of the P-type ATPase and phosphatase members of the HAD superfamily was suggested. The proposed mechanism of ATPase and phosphatase activity involved substrate binding mediated by hydrogen bond formation between the phosphate group and a Ser in the catalytic site, followed by nucleophilic attack of Asp on the phosphate group and formation of an acyl-phosphate intermediate (Figure 8B) (Ridder and Dijkstra, 1999).

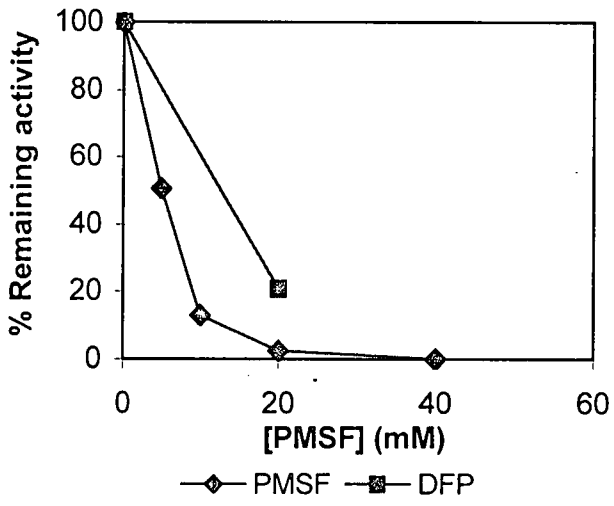

Figure 7. Effect of PMSF (◆) and DFP (■), modification agents of Ser, on epoxide hydrolase activity.

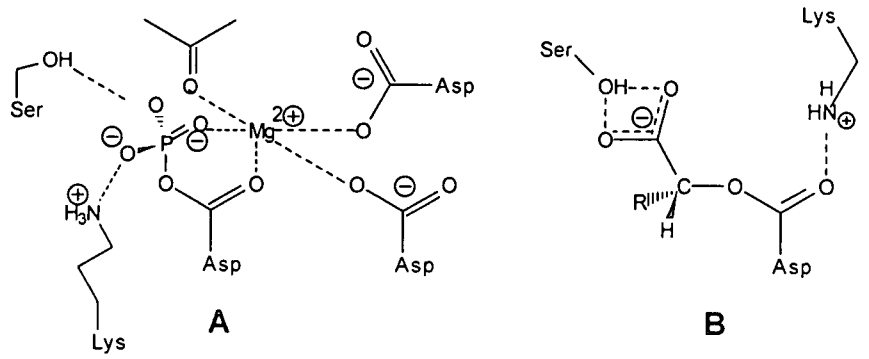

Figure 8. The role of conserved active site Ser residues in the catalytic mechanism of (A) ATPase and phosphatase activity, and (B) L-2-haloacid dehalogenase. After substrate binding, mediated by Ser, Asp performs a nucleophilic attack, and an acyl intermediate is formed

Effect of metal ions

Group 2 metal ions inhibited enzyme activity, and an increase in the size of the metal ions displayed a decrease in inhibitory effect. The transition metal ions, Co^{2+}, Hg^{2+} and Ag^{+}, caused nearly total loss of enzyme activity (Figure 9). A similar effect of Hg^{2+} was previously observed for JHEH (Debernard *et al.*, 1998).

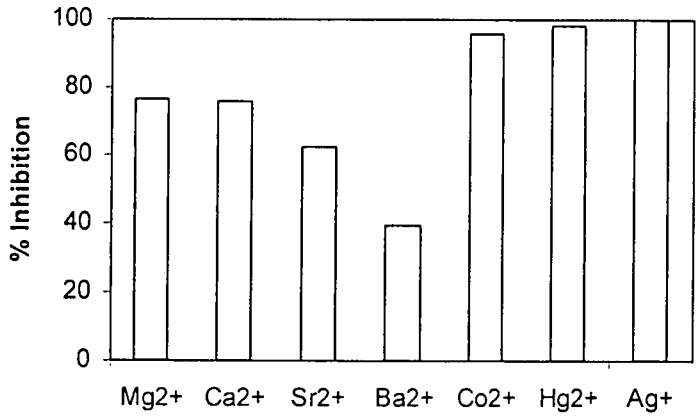

Figure 9. Effect of 50 mM metal ions on enzyme activity

Effect of EDTA

EDTA, an inhibitor of metalloenzymes, did not inhibit epoxide hydrolase activity. Indeed, an increase in enzyme activity was observed. Inhibition of enzyme activity by Ca^{2+} ions at concentrations below 30 mM could be reversed by the addition of EDTA (Figure 10).

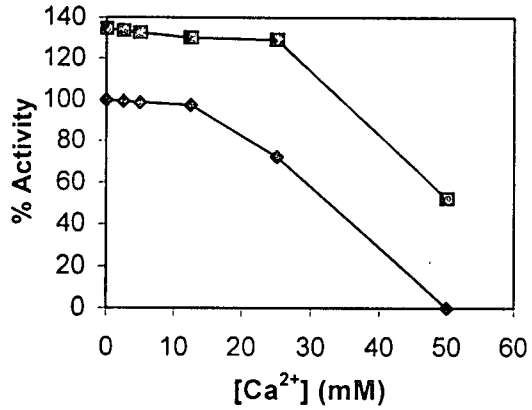

Figure 10. Effect of EDTA on enzyme activity, and the effect of Ca^{2+} ions in the presence (■) and absence (◆) of EDTA.

Temperature stability

The rate constant k_{obs} (s^{-1}) for thermal inactivation at different temperatures were obtained from a graph of log (%remaining activity) vs. time according to equation (1). The value of the activation energy E_a were obtained from the slope of the graph in Figure 11, according to equation (3), derived from the empirical Arrhenius equation (2). Half life times at the different temperatures were calculated using equation (4), after calculated rate constants (k_{calc}) were determined by substitution of the values of A and E_a in equation (2) (Table 1).

$$\log (\%Act) = \frac{-kt}{2.303} \qquad (1)$$

$$k = A\, e^{-E_a / RT} \qquad (2)$$

$$\log k = \frac{-E_a}{2.303\, R}\,\frac{1}{T} + \log A \qquad (3)$$

$$k = \frac{0.693}{t_{1/2}} \qquad (4)$$

From the graph in Figure 8:

E_a = 9737 x 2.303 x 8.314 J.mol^{-1}

= 167 kJ.mol^{-1}

According to the value obtained for E_a (167 kJmol^{-1}), the energy for thermal inactivation of the enzyme, and the half-life times of the enzyme at different temperatures (Table 1), the purified enzyme is not particularly stable. The value obtained for E_a is in the same order as those obtained for lipases from *Aspergillus niger* (215 kJ.mol^{-1}) (Van Heerden, 1999) and *Candida cylindriaceae* (201 kJ.mol^{-1}) (Pronk *et al.*, 1992).

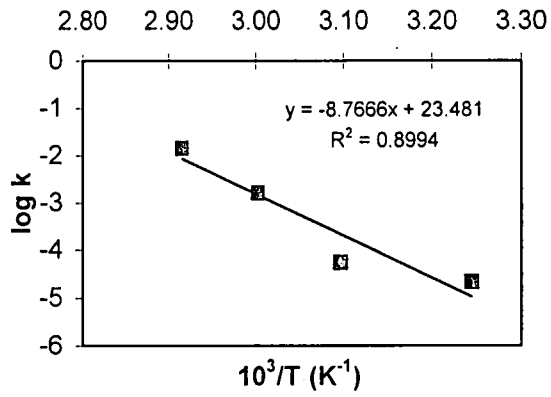

Figure 11. Graph of log k_{obs} vs. 1/T. Thermal inactivation energy E_a is obtained from the slope, and A from the Y-intercept

Table 1. Dependence of rate constants on temperature, and half-life times

Temp (°C)	T (K)	10^3/T (K^{-1})	k_{obs} x 10^5 (s^{-1})	k_{calc} x 10^5 (s^{-1})	$T_{1/2}$
70	343	2.915	1428	816	1.42 min
60	333	3.003	161	141	8.20 min
50	323	3.096	5.5	22	52.92 min
35	308	3.245	2.2	1.1	18.16 hr

Enzyme kinetics

The enantioselectivity of the hydrolysis of 1,2-epoxyoctane (Figure 12) was strongly dependent on the substrate concentration. Reactions with substrate concentrationsexceeding the solubility of the substrates in the aqueous phase proceeded with excellent enantioselectivity, while low concentrations of substrate lead to a dramatic decrease in enantioselectivity. E-values, based on normal Michaelis-Menten kinetics, are independent of substrate concentrations. The kinetic behaviour of the purified epoxide hydrolase from *Rhodosporidium toruloides* obviously deviates from the Michaelis-Menten model for uni-uni reactions. Kinetic analysis would thus require an in depth study. It is possible that each enantiomer is bound with different affinities, but is also a competitive inhibitor of the other enantiomer. Therefore at high substrate concentrations the more favoured enantiomer completely displaces the less favoured enantiomer. Another possible explanation for the observed behaviour may be that the enzyme acts at the interface, thus requiring the substrate to form a second (lipophylic) phase for enantioselective hydrolysis. The catalytic properties of lipases are also activated only when they come into contact with a lipophylic phase. Lipases and epoxide hydrolases have striking similarities; both belong to the α,β-hydrolase fold family which have a highly conserved domain that provides the framework for the catalytic triad, and both have a cap domain. A pronounced increase in enantioselectivity (13-fold) with increase in substrate concentration was also observed for the epoxide hydrolase from *Aspergillus niger*, but no explanation for this behaviour was presented (Cleij *et al.*, 1998).

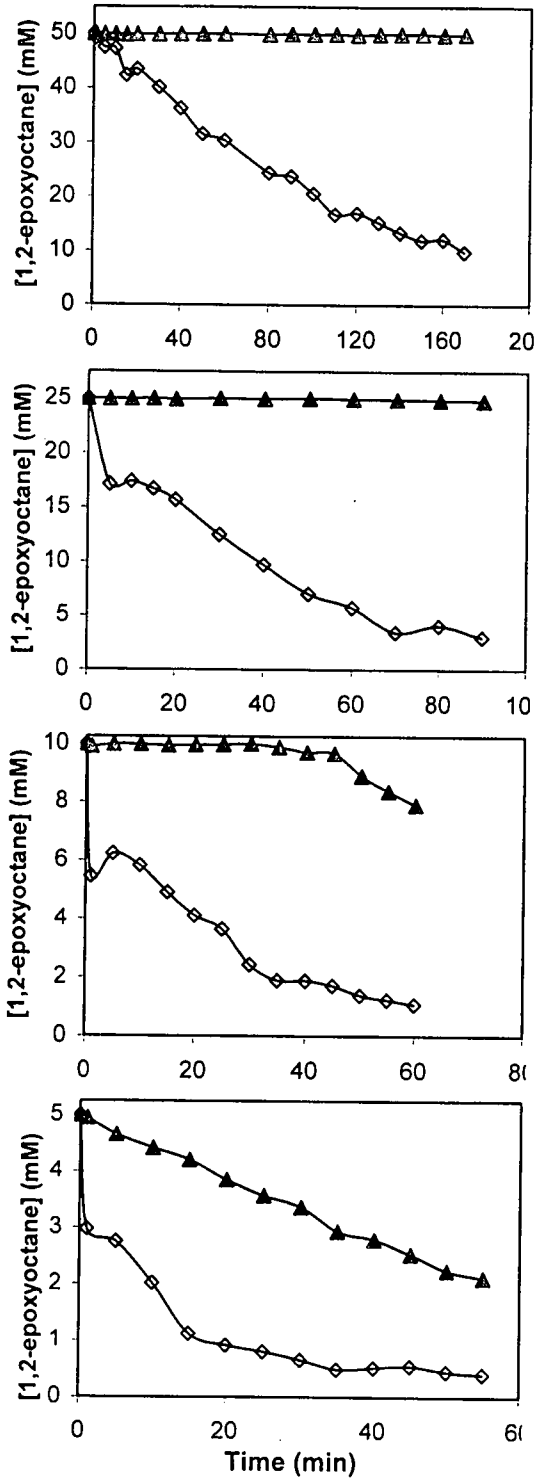

Figure 12. Enantioselective hydrolysis of 1,2-epoxyoctane by epoxide hydrolase. ▲ [S]-epoxide, ◊ [R]-epoxide

Conclusion

Some insight in the physico-chemical characteristics of the microsomal epoxide hydrolase from Rhodosporidium toruloides was obtained in the absence of sequence data. However, unequivocal determination of the catalytic mechanism can only be obtained by site directed mutagenesis.

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Chapter 8

Concluding Remarks

Concluding remarks

Enantiopure epoxides and *vic* diols are versatile chiral building blocks in the synthesis of numerous bioactive pharmaceuticals and agrochemicals. Furthermore, they also have important biological functions in plants, insects and microorganisms. Epoxides, generated during oxidative metabolism of unsaturated hydrocarbons by cytochrome P-450 enzymes, are highly reactive and their toxic nature allows them to interact with and damage biological macromolecules, including DNA, RNA and proteins. The synthesis of enantiopure epoxides as intermediates for drug synthesis, and their biological activities and metabolism in living systems, have thus become important areas of research.

Both chemical and biological catalysts that may be employed to obtain enantiopure epoxides from relatively inexpensive racemic substrates had been reviewed **(Chapter 1)**. The potential use of microbial epoxide hydrolases, including those from yeasts as elucidated during this study, was emphasised in this review.

The chemical catalysts developed by Sharpless and Jacobsen that are employed in the asymmetric synthesis of epoxides and *vic* diols, are based on toxic heavy metals. These metal-based catalysts are environmentally unfriendly, and require safe handling and disposal practices. The use of toxic reaction solvents and the generation of salt waste also have an impact on the environment. Furthermore, their synthesis involves multi-step procedures, which require high skill. Since these catalysts are patented, their applications in commercial processes have financial as well as legal implications.

The use of enzymes, the ultimate chiral catalysts, are thus increasingly considered by synthetic organic chemists as alternatives to metal catalysts for the synthesis of enantiopure epoxides and *vic* diols. Their usefulness has been established in many applications, and their use in a synthetic sequence provides unique advantages of efficiency, stereoselectivity and environmental friendliness. Epoxide hydrolases, originally studied for their role in detoxification of xenobiotics in mammals, have recently emerged as perhaps one of the most promising strategies to obtain enantiopure epoxides and *vic* diols.

Mammalian epoxide hydrolases are involved in the metabolism of many drugs xenobiotics, and endogenous compounds. Soluble epoxide hydrolase, present in the peroxisomes, and microsomal epoxide hydrolase, located on the cytoplasmic face of the endoplasmatic reticulum, are primarily involved in the metabolism of xenobiotics. Microsomal and soluble epoxide hydrolases play a critical role in detoxifying these reactive metabolites. As a consequence, these two enzymes have been studied in great detail. Although microsomal and soluble epoxide hydrolases differ considerably in their physical characteristics and substrate preferences, they share a common ancestry and catalytic mechanism with each other and other α,β-hydrolase fold family enzymes (Muller *et al.*, 1997). The kinetic and chemical mechanism of soluble and microsomal epoxide hydrolases has been reviewed recently (Armstrong, 1999). However, due to their limited availability, they have not been applied to the large- scale synthesis of enantiopure epoxides.

Epoxide hydrolases also play an important role in xenobiotic metabolism in insects. Juvenile hormone epoxide hydrolases, together with juvenile hormone esterases, regulate the concentration of juvenile hormones. These juvenile hormones play a critical regulatory role in insect cellular differentiation and metamorphosis, and much attention had been focussed recently on the role of epoxide hydrolases in endocrine regulation in insects. The juvenile hormone-metabolising epoxide hydrolases are mainly associated with the microsomes, and they share a common ancestry and catalytic mechanism with mammalian microsomal epoxide hydrolases (Harris *et al.*, 1999). Their application in the synthesis of enantiopure epoxides is also prohibited by limited availability.

Little is known about the actual function of epoxide hydrolases in plants, although they appear to be involved in the biosynthesis of cutin, a structural component of the cuticle that protects plants against pathogen attack. Epoxide hydrolases are also implicated in responses of plants to environmental stresses, and in the development of resistance to pathogens (Guo *et al.*, 1998). Epoxide hydrolases have also been found to play a crucial role in the enantioselective biosynthesis of γ-dodecanolactone, an important flavour compound in many fruits, from precursor saturated epoxyfatty acids (Schöttler and Boland, 1996). Although both microsomal and soluble forms of epoxide hydrolases have been identified in plants, only the soluble epoxide hydrolases of various plants have been characterised in detail. They share a common ancestry and catalytic mechanism

with mammalian soluble epoxide hydrolases. However, plant epoxide hydrolases have not been applied to the large-scale synthesis of enantiopure epoxides.

Microbial metabolism of epoxides had initially been studied in the context of the biodegradation of xenobiotic compounds. Curiously, microorganisms degrading alkenes via an epoxide intermediate have developed a complex epoxide-degrading system different from the effective and relatively simple epoxide hydrolases. This complex epoxyalkane-degrading system is probably linked to an alkene-monooxygenases (Swaving and De Bont, 1998). Bacterial epoxide hydrolases have been known for 30 years, and a bacterial epoxide hydrolase had been used for the industrial synthesis of L- and *meso*-tartaric acid from the precursor epoxide (Allen and Jacoby, 1969; Sato, 1975). However, it was only recently that the potential of microbial epoxide hydrolases as asymmetric catalysts for the production of enantiopure epoxides has captured the attention of synthetic chemists. The use of microbial epoxide hydrolases as chiral catalysts is particularly lucrative, because of the almost limitless availabilty at low cost.

The use of bacteria for preparative biotransformations of epoxides has received a lot of attention. Interestingly, epoxide hydrolases of certain strains are inducible, while other strains express these enzymes constitutively. Bacterial epoxide hydrolases from strains such as *Corynebacterium* C12 (Misawa *et al.*, 1998), *Pseudomonas putida* (Allen and Jacoby, 1969), *Agrobacterium radiobacter* (Jacobs *et al.*, 1991), and *Rhodococcus erythropolis* (Barbirato *et al.*, 1998) are induced by growth on epoxyalkanes, indicating a specific function in the catabolism of epoxides. In contrast, strains of *Rhodococcus*, *Nocardia*, *Arthrobacter* and *Mycobacterium* express these enzymes constitutively (Orru *et al.*, 1998). The substrate specificities of bacterial epoxide hydrolases have been elucidated and the primary structures of two bacterial epoxide hydrolases, *Corynebacterium* C12 (Carter and Leak, 1995) and *Agrobacterium radiobacter* AD1 (Rink and Janssen, 1998), have been determined. Although lacking the N-terminal domain of mammalian soluble epoxide hydrolases, the catalytic triad residues of *Agrobacterium radiobacter* (Asp^{107} as nucleophile, and Asp^{246}/His^{275} as charge relay partners) are identical, and a similar catalytic mechanism had been proposed. In the case of *Corynebacteriam*, the nucleophile Asp^{99} and the base His^{264} had been assigned, but not the position of the acidic residue. Bacterial epoxide hydrolases have been

applied to the synthesis of a number of enantiopure epoxides as well as *vic* diols. Their ease of handling in bioprocesses makes them extremely usefull as chiral catalysts.

The epoxide hydrolases of filamentous fungi have also received much attention, and preparative (Archelas, 1998) as well as large-scale synthesis (Chartrain *et al.*, 1998) of enantiopure epoxides were accomplished with these biocatalysts. Interestingly, fungi with opposite enantioselectivities were found (Pedragosa-Moreau *et al.*, 1993; Zhang *et al.*, 1995), and enantioconvergent hydrolysis of epoxides was accomplished. Most fungal epoxide hydrolases are soluble enzymes, although a microsomal epoxide hydrolase was found to be present in *Cunninghamella elegans* (Wackett and Gibson, 1982). Little is known about the physiological significance of epoxide hydrolases in fungi, and it had been suggested that they might be important in plant-fungus interactions. In the plant pathogen *Fusarium solani pisi*, diols of fatty acids induce a cutinase that facilitates the penetration of the fungus in the plant (Woloshuk and Kolattokudy, 1986). In the pathogen *Alternaria alternata lycopersici*, the AAL toxins that elicit disease in plants contain two pairs of vicinal diols. AAL toxin formation was found to be concomitant with high levels of epoxide hydrolase activity in this fungus (Pinot *et al.*, 1998). No epoxide hydrolase had been purified from filamentous fungi to date, and the catalytic mechanism remains to be established. Since fungal mycelia cause difficulties in bioprocesses, the use of enzyme extracts are indicated in the case of filamentous fungi, which does have a cost implication for large-scale applications.

In contrast to epoxide hydrolases from other microbial sources, yeast epoxide hydrolases have not received any attention until very recently (1997). At the onset of this study, epoxide hydrolase activity had been identified in only one yeast, *Rhodotorula glutinis* CIMW 147. The broad range of substrates that were hydrolysed with excellent enantioselectivity by this yeast, indicated that yeast epoxide hydrolases might be very interesting catalysts. This had indeed been found to be true during the course of this study. Enantioselective hydrolysis of a homologous range of aliphatic 1,2-epoxyalkanes (C-4 – C-12) was accomplished in collaboration with the group of Jan de Bont (Division Industrial Microbiology, Wageningen AU, the Netherlands). No other microbial epoxide hydrolases have been found that display this unique enantioselectivity for epoxides lacking other substituents **(Chapter 2)**.

Extensive screening of yeasts from the renowned UOFS Culture Collection revealed that epoxide hydrolase activity was constitutively present in about 20% of the yeasts screened, and that other basidiomycetous yeasts from the genera *Rhodotorula*, *Rhodosporidium* and *Trichosporon* shared this unique enantioselectivity for 1,2-epoxyoctane with *Rhodotorula glutinis* CIMW 147. Furthermore, it was demonstrated that yeast epoxide hydrolases were not affected by high concentrations of the substrate, and that two-phase biotransformations could be carried out. The epoxide hydrolase activity of several strains was extremely high, which indicated that the enzyme was not only expressed in high levels, but also highly active **(Chapter 3)**.

The apparent association between carotinoid production and epoxide hydrolase activity in bacteria as well as in the red yeasts *Rhodotorula* and *Rhodosporidium*, prompted us to investigate the epoxide hydrolase activity of the yellow pigmented bacterium *Chryseomonas luteola* in our collection. Indeed, this bacterium displayed epoxide hydrolase activity, and moderate enantioselectivity for 1,2-epoxyalkanes (E=20) by a bacterial epoxide hydrolase was found for the first time **(Chapter 4)**.

A survey of the enantioselectivities of yeasts for a homologous range of 1,2-epoxyalkanes, 1,2-epoxyalkenes as well as the 2,2-disubstituted 2-methyl-1,2-epoxyheptane (a model substrate for bacterial epoxide hydrolases) and the benzyloxy-substituted epoxide, benzyl glycidyl ether was conducted. Excellent biocatalysts for C-5 to C-8 epoxyalkanes and the C-8 epoxyalkene were found, while lower enantioselectivities were observed for the 2,2-disubstituted and benzyloxy-substituted epoxides. The epoxide hydrolases from all the enantioselective yeasts were found to be membrane-associated **(Chapter 5)**.

The epoxide hydrolase from the yeast *Rhodosporidium toruloides* was purified in an elegant one-step protocol from the microsomal fraction, using affinity chromatography. The enzyme was found to be expressed in high levels (about 4% of the solubilized microsomal proteins), and to have exceptionally high activity (specific activity 172 umol.min^{-1}mg catalyst^{-1}) and catalytic efficiency (turnover frequency 9440 mol substrate processed.mol catalyst^{-1}min^{-1}) **(Chapter 6)**. However, initial attempts to obtain amino-acid sequences failed. The epoxide hydrolase from the yeast *Rhodotorula glutinis* CIMW was purified at the same time, albeit not to electrophoretic homogeneity. Five peptides,

obtained by in-gel digestion, were sequenced. One of the peptides showed homology with other microsomal epoxide hydrolases around the catalytic histidine region (Kronenburg *et al.*, 1999).

In lieu of information about the primary structure of yeast epoxide hydrolases, inactivation of the enzyme by modification of specific amino acids was studied. Asp/Glu and His residues were found to be essential for catalytic activity. In addition, it was found that one or more Ser residues are indispensible for catalytic activity. These results indicate that yeast epoxide hydrolases probably belong to the same subfamily of α,β-hydrolase fold enzymes as the microsomal epoxide hydrolases from other eukaryotes. However, the complete primary strucure of yeast epoxide hydrolases will have to be determined before a catalytic mechanism can be proposed by analogy to other epoxide hydrolases. Elucidation of the kinetic mechanism will also require in depth study, since unusual kinetic behaviour was observed during the hydrolysis of 1,2-epoxyalkanes by purified epoxide hydrolase. Hydrolysis was characterised by a strong dependence of enantioselectivity on the presence of the substrate as a second (lypophilic) phase. The purified epoxide hydrolase was not exceptionally stable, with a half-life time at 35 °C of 18 hours **(Chapter 7)**.

Yeasts epoxide hydrolases are thus very promising biocatalysts, due to the exceptional enantioselectivities for a wide range of substrates, stability in whole cells as well as enzyme extracts, and the unusually high level of expression and activity. Yeasts are easier to handle in bioprocesses than filamentous fungi, since they are unicellular. Furthermore, yeast epoxide hydrolases may be employed as naturally immobilized enzymes in resting whole cells in large-scale processes. This eliminates the need to prepare enzyme extracts at additional cost.

The next phase of the exploration of the synthetic potential of microbial epoxide hydrolases will require extensive screening programmes to find epoxide hydrolases with novel substrate specificities and enantioselectivities. In our laboratory, screening of yeasts, using different substrates than 1,2-epoxides, are already in progress. Similar screening programmes had been reported for fungi (Moussou *et al.*, 1998), and microorganisms from the Brazilian rain forest (Cagnon *et al.*, 1999). The kinetic and chemical mechanism of yeast and fungal epoxide hydrolases remains to be established.

Despite the widespread occurrence of constitutive epoxide hydrolases in yeasts, the role of epoxide hydrolases in the metabolism of yeasts is not yet clear. Further studies in our laboratory promise to shed more light on the biological significance of epoxide hydrolases in yeasts and will undoubtedly open new avenues for the application of enzymes in organic synthetic chemistry.

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Summary

The synthesis of chiral pharmaceuticals in an enantiopure form had become increasingly important in the last few years. This same trend is now found in the synthesis of agrochemicals. Epoxides, due to their high reactivity with a large number of reagents, and *vic* diols, employed as their corresponding cyclic sulfates or sulfites as reactive intermediates, are versatile chiral synthons in the synthesis of many bioactive compounds. Extensive research efforts have thus been directed towards the synthesis of optically active epoxides and *vic*l diols. Kinetic resolution of racemic epoxides by epoxide hydrolases has recently emerged as a very attractive strategy for the synthesis of enantiopure epoxides.

Both chemical and biological catalysts that may be employed to obtain enantiopure epoxides from relatively inexpensive racemic substrates had been reviewed **(Chapter 1)**. The potential use of microbial epoxide hydrolases, including those from yeasts as elucidated during this study, was emphasised in this review.

At the onset of this study, epoxide hydrolase activity had been identified in only one yeast, *Rhodotorula glutinis*. The broad range of substrates that were hydrolyzed with excellent enantioselectivity by this yeast, indicated that yeast epoxide hydrolases might be very interesting catalysts. This had indeed been found to be true during the course of this study. Enantioselective hydrolysis of a homologous range of aliphatic 1,2-epoxyalkanes was accomplished in collaboration with the group of Jan de Bont (Division Industrial Microbiology, Wageningen AU, The Netherlands). No other microbial epoxide hydrolases have been found that display this unique enantioselectivity for epoxides lacking other substituents **(Chapter 2)**.

Extensive screening of yeasts from the renowned UOFS Yeast Culture Collection revealed that epoxide hydrolase activity was constitutively present in about 20% of the yeasts screened, and that other basidiomycetous yeasts from the genera *Rhodotorula*, *Rhodosporidium* and *Trichosporon* shared this unique enantioselectivity for 1,2-epoxyoctane with *Rhodotorula glutinis* **(Chapter 3)**.

The apparent association between carotinoid production and epoxide hydrolase activity in bacteria as well as the red yeasts *Rhodotorula* and *Rhodosporidium*, prompted us to investigate the epoxide hydrolase activity of the yellow pigmented bacterium *Chryseomonas luteola* in our collection. Indeed, this bacterium displayed epoxide hydrolase activity, and moderate enantioselectivity for 1,2-epoxyalkanes (E =20) by a bacterial epoxide hydrolase was found for the first time **(Chapter 4)**.

A survey of the enantioselectivities of yeasts for a homologous range of 1,2-epoxyalkanes, 1,2-epoxyalkenes as well as the 2,2-disubstituted 2-methyl-1,2-epoxyheptane and benzyl glycidyl ether was conducted. Excellent biocatalysts for C-5 to C-8 epoxyalkanes and the C-8 epoxyalkene were found. The epoxide hydrolases from all the enantioselective yeasts were found to be membrane-associated **(Chapter 5)**.

The epoxide hydrolase from the yeast *Rhodosporidium toruloides* was purified in an elegant one-step protocol from the microsomal fraction, using affinity chromatography **(Chapter 6)**. However, initial attempts to obtain amino-acid sequences failed. In lieu of information about the primary structure of yeast epoxide hydrolases, inactivation of the enzyme by modification of specific amino acids was studied. Asp/Glu and His residues were found to be essential for catalytic activity. In addition, it was found that one or more Ser residues in the catalytic site are indispensible for catalytic activity. These results indicate that yeast epoxide hydrolases probably belong to the same subfamily of α,β-hydrolase fold enzymes as the microsomal epoxide hydrolases from other eukaryotes. Unusual kinetic behaviour was observed during the hydrolysis of 1,2-epoxyalkanes by purified epoxide hydrolase. Hydrolysis was characterised by a strong dependence of enantioselectivity on the presence of the substrate as a second (lypophilic) phase. The purified epoxide hydrolase was not very stable, with a half-life time at 35 °C of 18 hours **(Chapter 7)**.

Samevatting

Die sintese van farmaseutiese middels in enantiomeries suiwer vorm het die afgelope paar jaar toenemend belangrik geword. Dieselfde neiging word nou in die sintese van landbou chemikalië waargeneem. Epoksiede, as gevolg van hul reaktiwiteit met talle ander molekules, en visinale diole, in die vorm van hul sikliese sulfate of sulfiete as reaktiewe intermediêre, is veelsydige chirale sintone in die sintese van talle bioaktiewe verbindings. Intensiewe navorsing, gerig op die sintese van opties aktiewe epoksiede en visinale diole, is dus die afgelope tyd gedoen. Kinetiese resolusie van rasemiese epoksiede is 'n aantreklike strategie vir die sintese van enantiomeries suiwer epoksiede.

In **Hoofstuk 1** is 'n oorsig van beide chemiese en biologiese kataliste wat gebruik kan word om enantiomeries suiwer epoksiede vanaf relatiewe goedkoop rasemiese substrate te verkry, aangebied. Die moontlike toepassings van mikrobiese epoksied hidrolases, ingeslote dié van giste, soos uitgebeeld tydens hierdie studie, is beklemtooon in die oorsig.

Met die aanvang van hierdie studie, is epoksied hidrolase aktiwiteit in slegs een gis, *Rhodotorula glutinis*, geïdentifiseer. Die wye reeks substrate wat met uitstekende enantioselektiwiteit deur hierdie gis gehidroliseer is, het daarop gedui dat gis epoksied hidrolases uiters interessante biokataliste mag blyk te wees. Dit is inderdaad bevind dat dit die geval is tydens hierdie studie. Enantioselektiewe hidrolise van 'n homoloë reeks alifatiese 1,2-epoksiealkane is verkry, in samewerking met die groep van Jan de Bont (Afdeling Industriële Mikrobiologie, Wageningen LU, Nederland). Geen ander mikrobiese epoksied hidrolases met hierdie unieke enantioselektiwiteit vir epoksiede sonder ander substituente, is voorheen gevind nie **(Hoofstuk 2)**.

Uitgebreide sifting van giste uit die welbekende UOVS Giskultuur Versameling het getoon dat epoksied hidrolase aktiwiteit in sowat 20% van die giste voorkom, en dat ander basidiomysete giste van die genera *Rhodotorula*, *Rhodosporidium* en *Trichosporon* ook die unieke enantioselektiwiteit van *Rhodotorula glutinis* vir 1,2-epoksieoktaan toon. Die klaarblyklike assosiasie tussen karotenoid produksie en epoksied hidrolase aktiwiteit in bakterieë en in die rooi giste *Rhodotorula* en *Rhodospordium*, het gelei tot die ondersoek van die epoksied hidrolase aktiwitiet van die

geel gepigmenteerde bakterium *Chryseomonas luteola* in ons versameling. Hierdie bakterium het inderdaad epoksied hidrolase aktiwiteit getoon, en matige enantioselektiwiteit (E =20) vir 1,2-epoksiealkane deur 'n bakteriese epoksied hidrolase is vir die eerste keer gevind **(Hoofstuk 4)**.

'n Opname van die enantioselektiwiteite van giste vir 'n homoloë reeks 1,2-epoksiealkane, 1,2-epoksiealkene, sowel as die 2,2-digesubstitueerde 2-metiel-1,2-epoksieheptaan en bensiel glisidiel eter, is gedoen. Uitstekende biokataliste vir C-5 tot C-8 epoksiealkane en die C-8 epoksiealkeen is gevind. Die epoksied hidrolases van al die enantioselektiewe giste was membraan-geassosieerd **(Hoofstuk 5)**.

Die epoksied hidrolase van die gis *Rhodosporidium toruloides* is in 'n elegante een-stap protokol uit die mikrosomale fraksie gesuiwer deur middel van affiniteitschromatografie **(Hoofstuk 6)**. Aanvanklike pogings om die aminosuur volgorde te bepaal, was egter onsuksesvol. By gebrek aan inligting oor die primêre struktuur van gis epoksied hidrolases, is die deaktivering van die ensiem deur modifikasie van spesifieke aminosure bestudeer. Asp/Glu en His residue was noodsaaklik vir katalitiese aktiwiteit. Verder is daar ook gevind dat een of meer Ser residue onontbeerlik vir katalitiese aktiwiteit is. Hierdie resultate dui daarop dat gis epoksied hidrolases waarskynlik tot dieselfde subfamilie van α,β-hidrolase vou ensieme behoort as die mikrosomale epoksied hidrolases van ander eukariote. Ongewone kinetiese gedrag is waargeneem tydens die hidrolise van 1,2-epoksiealkane deur gesuiwerde epoksied hidrolase. Hidrolise is gekenmerk deur 'n sterk afhanklikheid van enantioselektiwiteit van die teenwoordigheid van die substraat as 'n tweede (lipofiliese) fase. Die suiwer epoksied hidrolase was nie baie stabiel nie, met 'n halfleeftyd van 18 uur by 35°C **(Hoofstuk 7)**.

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