ON THE MICHAEL ADDITION OF WATER TO C = C BONDS

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Proefschrift

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de RectorMagnificus Prof. ir. K. C. A. M. Luyben,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen op dinsdag 01 September 2015 om 10:00 uur

door

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Dit proefschrift is goedgekeurd door de promotoren:
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Samenstelling promotiecommissie:

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Keywords:	water, Michael hydratase, ene-reductase, Rhodococcus, biocatalysis, lipase, β-hydroxy carbonyl compound
Printed by:	Ipskamp Drukkers
Cover by:	Huayang Cai
ISBN:	978-94-6259-773-0

To my parents

Contents

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STEREOCHEMISTRY OF ENZYMATIC WATER ADDITION TO C = C BONDS

Water addition to carbon-carbon double bonds using hydratases is attracting great interest in biochemistry. Most of the known hydratases are involved in primary metabolism and to a lesser extent in the secondary metabolism. New hydratases have recently been added to the toolbox, both from natural sources or artificial metalloenzymes. In order to comprehensively understand how the hydratases are able to catalyse the water addition to carbon-carbon double bonds, this chapter will highlight the mechanistic and stereochemical studies of the enzymatic water addition to carbon-carbon double bonds, focusing on the *syn*/*anti* addition and stereochemistry of the reaction.

This chapter is based on

B.-S. Chen, L. G. Otten and U. Hanefeld,

Biotech. Adv., **2015**, *33*, 526-546

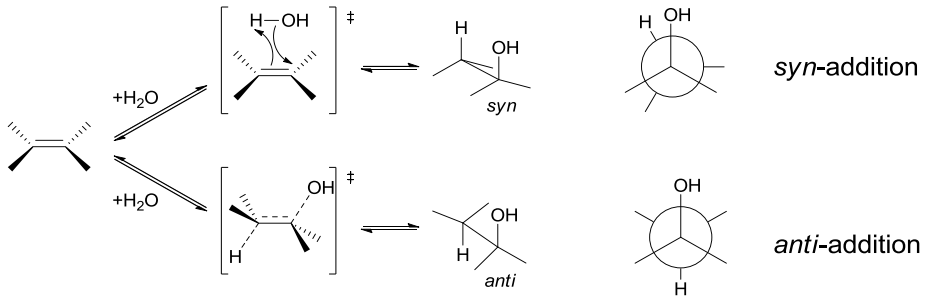
1.1 INTRODUCTION

1

The enzyme catalysed addition of water to C = C bonds, isolated or conjugated with a carbonyl group, is an essential reaction in nature.[1-5] The enzymes that catalyse this reaction are called hydratases or hydro-lyases. The best studied examples are part of our primary metabolism, for example the Michael addition of water to fumaric acid or to aconitic acid, both being part of the citric acid cycle.[6] Also in the secondary metabolism the water addition is essential, for instance to isolated C = C bonds in terpene formation and degradation.[7]

These reactions show a reliable and straightforward access towards primary (1°), secondary (2°) and tertiary (3°) alcohols thus opening up new possibilities for their synthesis.[8] This is all the more important as the water addition still represents a chemically very challenging reaction and few chemists ever apply the direct reaction since it is so complicated in the laboratory.[3,4,9] The enzymatic water addition to C = C bonds hence is of great interest to preparative organic chemistry.

The stereospecificity of enzyme-catalysed reactions has been a fruitful source of information about the mechanisms of enzyme catalysis and vice versa; the application of stereospecifically labelled substrates allows for studying the course of the reaction. It offers a very promising opportunity to comprehensively understand the precise mechanistic and kinetic details of even the most complex enzymatic reactions.[10] The water addition to C = C bonds is a prime example of the interplay between stereospecificity and reaction detail that has to be investigated. From a thermodynamic point of view, the addition of water to C = C bonds is an equilibrium reaction. In the case of water addition to the isolated, unpolarised C = C bonds, the equilibrium is slightly on the side of the alkene; in the case of the Michael addition of water, the alcohol is the favoured compound. It has to be emphasised that this general statement needs to be verified for each substrate as the thermodynamics of a reaction are always depending on the substrates. Both the addition and elimination of water can occur in *syn*- or *anti*-fashion depending on the enzymes (Scheme 1). This means either all atoms are added to the C = C bond approach from one side (*syn*) to the flat alkene or the electrophile is added from one side and the nucleophile from the other side (*anti*). Chemically *syn*-additions are very unusual. It was found that the *syn*- or



SCHEME 1. The addition of water can either occur exclusively from one side (*syn*) or from both sides (*anti*).

anti- stereochemistry plays a fundamental role in most metabolic pathways. The enzymes that catalyse the addition of water to conjugated carboxylate substrates or isolated C = C including oleate hydratase,[11-13] fumarase,[14] malease,[15] aconitase,[16] type II dehydroquinase[17,18] and six other enzymes,[19,20] add the water with *anti* stereospecificity. Those enzymes that catalyse the addition of water to α,β-unsaturated thioesters, including type I dehydroquinase,[21] enoyl-CoA hydratase,[22-24] the artificial hydratase[25,26] and seven other enzymes[10], catalyse *syn*-addition of water. It has been suggested that these stereospecificities exist because of mechanistic efficiency, i.e. the acidity of the proton attached to the α-carbon might influence what is the most efficient pathway.

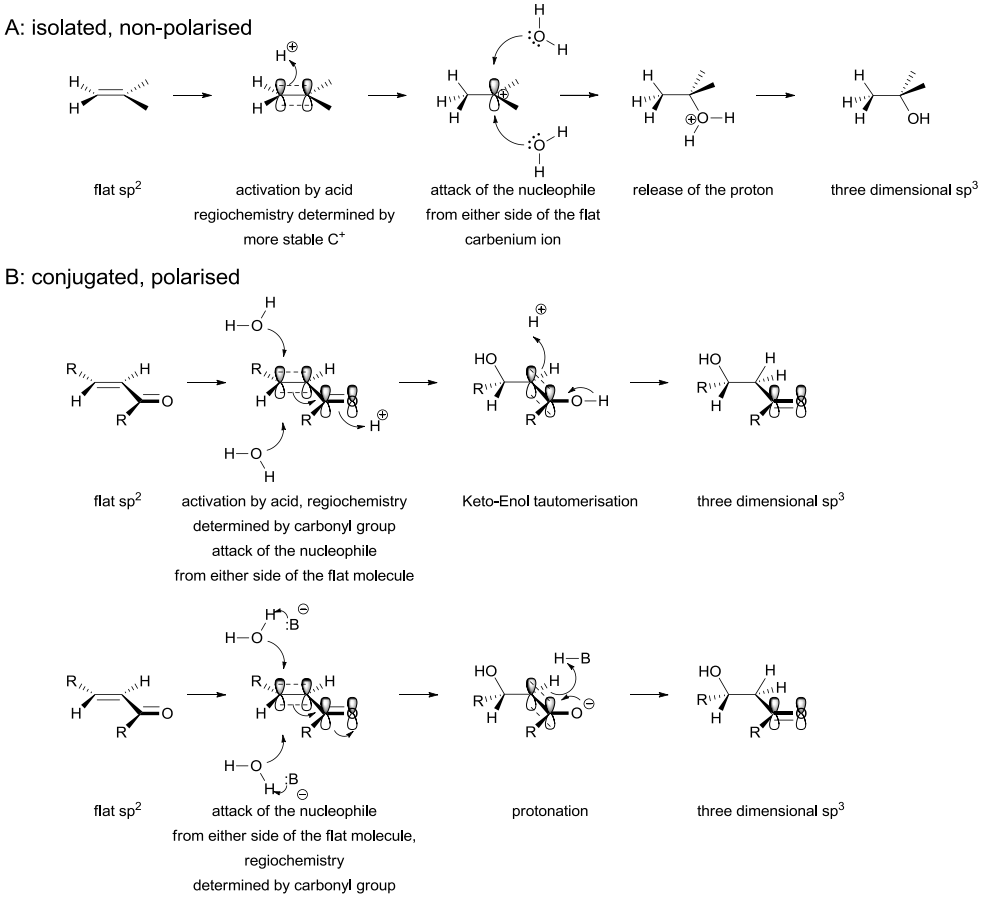
In recent years, new enzymatic water addition activities to C = C bonds have been developed, high enantio-, regio- and chemo-selectivity was achieved.[27,28] Herein, a summary of the stereochemistry of enzymatic water addition to C = C bonds is presented. The stereochemical course of an enzymatic reaction is highly important for the organisation of the active site and for details of a reaction mechanism. In this chapter the emphasis is on the stereochemistry of enzymatic water addition to C = C bonds concerning the diastereoselectivity (*anti*/*syn*-addition of water), enantioselectivity (*R*/*S*-selectivity) and stereospecificity towards substrates (*Z*/*E* conformations).

1.2 CLASSIFICATION OF HYDRATASES

1

Enzymes that catalyse the addition of water to C = C bonds are called hydratases (or hydro-lyases) and are classified as lyases (E.C. 4.2.1-). Mechanistically, these enzymes fall into two groups:

1) An electrophilic addition reaction, where after protonation of the isolated, unpolarised C = C bond, a H_2O as nucleophile is added (Scheme 2A). The acid-catalysed 1,2-addition of water to an alkene follows the rule of Markovnikov[29] thus the hydroxyl group binds to the carbon with the most carbon-carbon bonds whereas the proton prefers the carbon with the higher number of hydrogen atoms.



SCHEME 2. Water addition to A) isolated non-polarised C = C and B) conjugated, polarised C = C (Michael addition).

2) H_2O as a nucleophile is added to α,β-unsaturated (Michael) acceptors (conjugated, polarised C = C bonds) (Scheme 2B). This can occur as either acid or base catalysed. In this case, the carbon-carbon double bond is polarised by an electron withdrawing group such as ketones, lactones, aldehydes, carboxylic acids, thioesters or a phosphate group, making it more electrophilic, and thus susceptible for the nucleophilic addition of water. This polarisation also determines the regiochemistry of the addition (Scheme 2B).

To date a number of hydratases that catalyse the addition of water to isolated, unpolarised C = C bonds have been described. 1) Oleate hydratase (EC 4.2.1.53) catalyses the addition of water to oleic acid to form usually (*R*)-10-hydroxystearic acid;[30-32] 2) carotenoid 1,2-hydratase (EC 4.2.1.131) catalyses the addition of water to neurosporene to give 1-hydroxyneurosporene;[33] 3) kievitone (EC 4.2.1.95) and phaseollidin (EC 4.2.1.97) hydratase catalyses the addition of water to yield kievitone hydrate;[34] 4) limonene hydratase catalyses the addition of water to (*R*)-(+)-limonene to furnish (*R*)-(+)-α-terpineol;[35] 5) linalool dehydrogenase-isomerases catalyses the hydration and isomerization of myrcene (Table 1, entries 1-6).[36,37]

Also a number of hydratases that catalyse the addition of water to conjugated, polarised C = C bonds have been described. 1) Malease (EC 4.2.1.31) catalyses the addition of water to maleic acid to give (*R*)-malate;[38] 2) fumarase (EC 4.2.1.2) catalyses the addition of water to fumaric acid to form (*S*)-malate;[39] 3) aconitase (EC 4.2.1.3) catalyses the addition of water to *cis*-aconitate to yield isocitrate;[16] 4) urocanase (EC 4.2.1.49) catalyses the addition of water to urocanate to furnish 3-(4-hydroxy-1*H*-imidazol-5-yl)propanoate that can also be written as its tautomer imidazolone 5-propionate;[40] 5) enoyl-CoA hydratase (EC 4.2.1.17) catalyses the addition of water to *trans*-enoyl-CoA thioesters to form (*R*)- and (*S*)-3-hydroxy thioesters;[22,41,42] 6) 3-dehydroquinate dehydratase (EC 4.2.1.10) catalyses the addition of water to 3-hydroshikimate to obtain 3-dehydoquinate;[21] 7) 3-dehydroshikimate dehydratase (EC 4.2.1.118) catalyses the addition of water to protocatechuate to form 3-dehydroshikimate (Table 1, entries 7-13).[43]

Other member of the hydratases are enzymes that catalyse the addition of water to C≡C bonds such as 1) acetylene hydratase (EC 4.2.1.112) catalyses the addition of water to acetylene to obtain acetaldehyde tautomer.[44-46] 2)

hydratase-tautomerase catalyses the addition of water to acetylene dicarboxylate to obtain pyruvate (Table 1, entries 14-15).[47]

A promiscuous hydratase activity has been also reported for two enzymes. 1) Hydroxycinnamoyl-CoA hydratase-lyase (EC 4.2.1.101) catalyses the addition of water to feruloyl-CoA to give 3-hydroxy-3-(4-hydroxy-3-methoxyphenyl)propanoyl-CoA, which cannot be isolated, forming vanillin and acetyl-CoA in a subsequent step;[48,49] 2) phenolic acid decarboxylase catalyses the addition of water to hydroxystyrene-type substrates to furnish the desired alcohols (Table 1, entries 16-17).[27,28]

An artificial metalloenzyme with hydratase activity has been reported to catalyse the addition of water to α,β-unsaturated 2-acyl imidazoles to form the corresponding alcohols in moderate enantiomeric purities (Table 1, entries 18).[25,26,50]

1.3 ENZYMATIC *SYN*- OR *ANTI*-ADDITION OF WATER TO C = C BONDS

Many hydratases are known to date (Table 1). Here those hydratases for which mechanistic information is available, including how it was obtained, will be discussed.

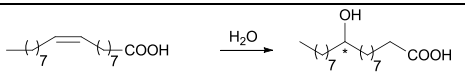
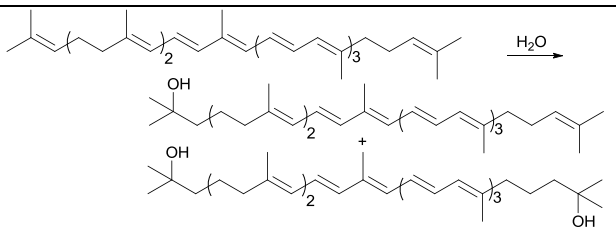
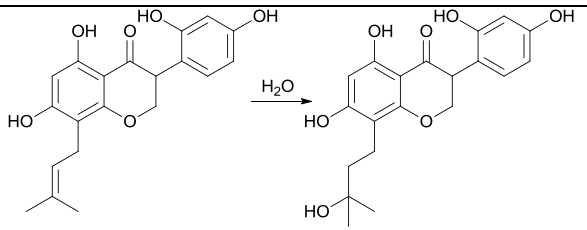
1.3.1 Examples of enzymatic *anti*-addition of water to C = C bonds

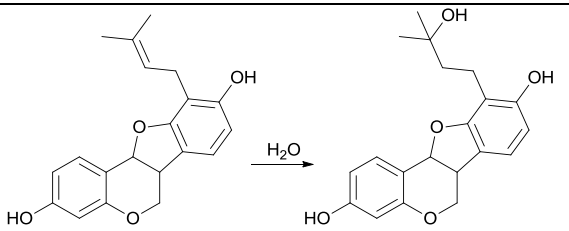
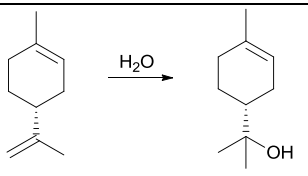
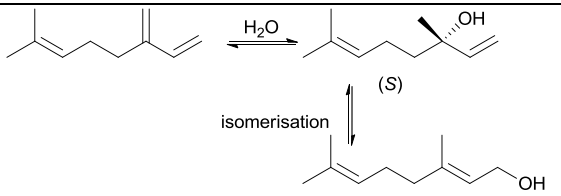
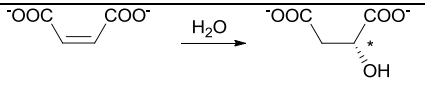
1.3.1.1 Oleate hydratase catalyses the anti-addition of water to C = C bonds

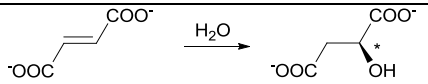
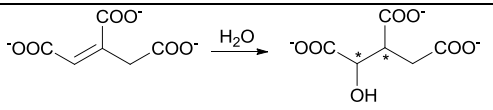
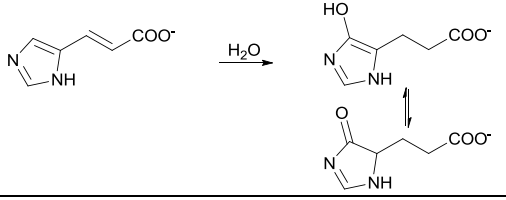
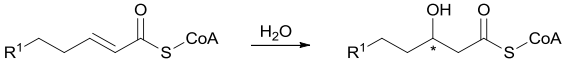
Oleate hydratase (EC 4.2.1.53) catalyses the addition of water to oleic acid yielding (*R*)-10-hydroxystearic acid (Scheme 3). The absolute configuration of the hydration product was established by measuring the optical rotation ([α] 546 = − 0.16)[51] and showing that the 10-hydroxyl had the (*R)*-configuration according to the fact that the optical rotation was found to be positive when the 10-hydroxyl was chemically converted to the opposite (*S*-) configuration.[11]

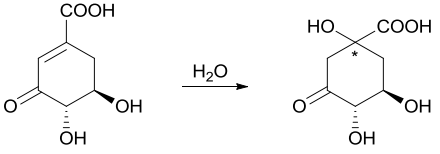
The first investigation on the enzymatically specific hydration of oleic acid dates back to the 1960s.[11,12,52] Following this early work, oleate hydratase has become a favourite topic to many researchers for a very long period; all of this work was performed with *Elizabethkingia meningoseptica* (former *Pseudomonas* sp. strain 3266) harbouring oleate hydratases or enzymes that were

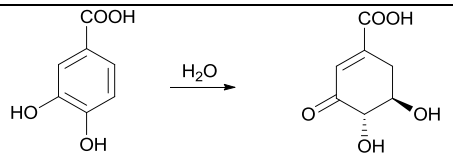
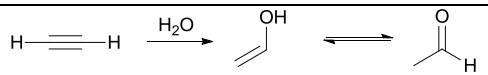
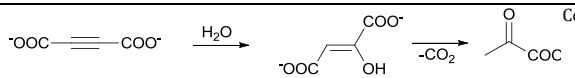
TABLE 1. Overview of enzymes that catalyse water addition to C = C bonds.[a]

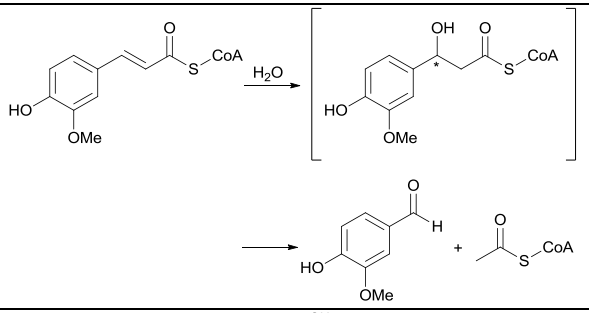
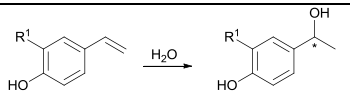
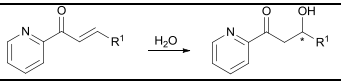
	Name (EC-number)	Sources (PDB-number)	Types of reaction	Cofactor	Regio- and stereoselectivity
1	Oleate hydratase (EC 4.2.1.53)	*Elizabethkingia meningoseptica (*former *Pseudomonas* sp. strain 3266) *Streptococcus pyogenes* *Bifidobacterium breve* *Lysinibacillus fusiformi* *Stenotrophomonas maltophilia* *Macrococcus caseolyticus* *Lactobacillus rhamnosus* LGG *Lactobacillus plantarum* ST-III *Lactobacillus acidophilus* NCFM (4IA6) *Bifidobacterium animalis* subsp. *lactis* BB12		Uncertain	*Z*-C = C bond at C-9 and/or C-12 position, *R*-selectivity, *anti*-addition
2	Carotenoid 1,2-hydratase (EC 4.2.1.131)	*Deinococcus radiodurans* R1 *Deinococcus geothermalis* DSM 11300 *Rubrivivax gelatinosus* *Thiocapsa roseopersicina* *Rhodobacter capsulatus* *Chlorobium tepidum*		Cofactor independent	C = C bond at C-2 position, Markovnikov's rule
3	Kievitone hydratases (EC 4.2.1.95)	*Fusarium solani* f. sp. *phaseoli*		Cofactor independent	Markovnikov's rule

4	Phaseollidin hydratase (EC 4.2.1.97)	*Fusarium solani* f. sp. *phaseoli*		Cofactor independent	Markovnikov's rule
5	Limonene hydratase	*Fusarium oxysporum* 152B *Pleurotus sapidus* *Aspergillus niger* ATCC 16404 *Aspergillus niger* ATCC 9642 *Aspergillus niger* ATCC 1004 *Penicillium* spp. *Pseudomonas gladioli* *Escherichia coli*		Cofactor independent	*R*-selectivity
6	Linalool dehydrogenase-isomerase	*Castellaniella (ex Alcaligenes) defragrans*		Cofactor independent	*S*-selective, Markovnikov's rule
7	Maleases (EC 4.2.1.31) (citraconases, EC 4.2.1.35)	*Methanocaldococcus jannaschii* (3VBA, 4KPL, 4NQY) *Pseudomonas pseudoalcaligenes* rabbit kidneys *Arthrobacter pascens*		Iron-sulfur or cofactor independent	*Z*-substrate, *R*-selectivity, *anti*-addition

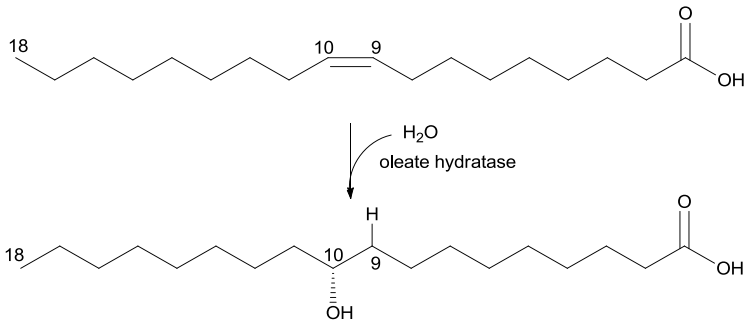
8	Fumarases (EC 4.2.1.2)	*Archaeoglobus fulgidus* dsm 4304 (1VPJ) *Saccharomyces cerevisiae* (1YFM) *Homo sapiens* (3E04) *Mycobacterium abscessus* (3RRP) *Sinorhizobium meliloti* (4HGV) *Thermus thermophilus* (1VDK) *Rickettsia prowazekii* (3GTD) *Mycobacterium tuberculosis* (3NO9, 4ADL, 4ADM, 4APA, 4APB) *Mycobacterium marinum* (3QBP) *Mycobacterium smegmatis* (3RD8) *Burkholderia pseudomallei* (3TV2) *Escherichia coli* (1YFE, 1FUO, 1FUP, 1FUQ, 1FUR, 1KQ7, 2FUS)		Iron-sulfur (fum A and B) or cofactor independent (fum C)	*E*-substrate, *S*-selectivity, *anti*-addition
9	Aconitases (EC 4.2.1.3)	*Bos Taurus* (1ACO, 1AMI, 1AMJ, 1C96, 1C97, 1FGH, 1NIS, 1NIT, 8ACN) *Sus scrofa* (1B0J, 1B0K, 1B0M, 5ACN, 6ACN, 7ACN) *Escherichia coli* (1L5J) *Homo sapiens* (2B3X, 2B3Y) *Oryctolagus cuniculus* (3SN2, 3SNP, 2IPY)		Iron-sulfur	*Anti*-addition
10	Urocanases (EC 4.2.1.49)	*Pseudomonas fluorescens* *Agrobacterium tumefaciens* *Pseudomonas putidn* (1UWK, 1UWL, 1W1U, 2V7G) *Geobacillus stearothermophilus* (1X87) *Bacillus subtilis* (2FKN)		NAD^+	2-position on the imidazole ring
11	Enoyl-CoA hydratases (EC 4.2.1.17)	*Thermus Thermophilus HB8* (1UIY, 3GOW) *Bacillus halodurans* (3LKE) (3MOY, 3MYB, 3NJB, 3NJD, 3OME, 4QFE) *Mycobacterium avium* (3OC7) *Rhodobacter sphaeroides* (4J2U)		CoA activated substrates	*R/S*-selectivtiy, *syn*-addition

		Thermoplasma volcanium (4JYL) *Polaromonas* sp. JS666 (4JFC) *Thermobifida fusca* (4JSB, 4JVT, 4OMR) *Rattus norvegicus* (1DUB, 1EY3, 1MJ3, 1ZCJ, 2DUB, 2X58, 3ZW8, 3ZW9, 3ZWA, 3ZWB, 3ZWC) *Pseudomonas fragi* (1WDK, 1WDL,1WDM, 2D3T) *Mycobacterium tuberculosis* (2C2I, 3H81, 3HE2, 3PZK, 3Q0G, 3Q0J, 4FJW, 4FN7, 4FN8, 4FNB, 4FND, 4HC8) *Homo sapiens* (2HW5) *Geobacillus kaustophilus* (2PPY) *Arabidopsis thaliana* (2WTB) *Legionella pneumophila* subsp (3I47) *Bacillus anthracis* (3KQF) *Escherichia coli* (4FZW) *Cupriavidus metallidurans* (4JCS) *Nvosphingobium aromaticivorans* (4JWV)			
12	3-Dehydroquinate dehydratase [b] (EC 4.2.1.10)	*Mycobacterium tuberculosis* (1H05, 1H0R, 1H0S, 2DHQ, 2XB8, 2Y71, 2Y76, 2Y77, 3N59, 3N76, 3N7A, 3N86, 3N87, 3N8K, 3N8N, 4B6O, 4B6P, 4B6Q, 4CIV, 4CIW, 4CIX, 4CIY, 4KI7, 4KIJ, 4KIU, 4KIW) *Streptomyces coelicolor* (1D0I, 1GTZ, 1GU0, 1GU1, 1V1J, 2BT4, 2CJF) *Emericella nidulans* (1DQS,1NR5, 1NRX, 1NUA, 1NVA, 1NVB, 1NVD, 1NVE, 1NVF, 1SG6) *Salmonella typhi* (1GQN, 1L9W, 1QFE) *Bacillus subtilis* (1GQO) *Helicobacter pylori* (1J2Y, 2C4V, 2C4W, 2C57, 2WKS, 2XB9, 2XD9, 2XDA, 4B6R, 4B6S) *Staphylococcus aureus* subsp (1SFJ, 1SFL) *Actinobacillus pleuropneumoniae* (1UQR)		Metal ion (Mn^{2+})	*Syn*-addition (type I) *Anti*-addition (type II)

		Aquifex aeolicus (2EGZ, 2YSW) *Arabidopsis thaliana* (2GPT, 2O7Q, 2O7S) *Streptococcus pyogenes* serotype m1 (2OCZ) *Archaeoglobus fulgidus* (2OX1) *Thermus thermophiles* (2UYG) *Geobacillus kaustophilus* (2YR1) *Clostridium difficile* (3JS3, 4H3D) *Candida albicans* (3KIP) *Salmonella enterica* subsp (3L2I, 3LB0, 3M7W, 3NNT, 3O1N, 3OEX, 3S42, 4CNO, 4GFS, 4GUF, 4GUG, 4GUH, 4GUI, 4GUJ, 4IUO) *Streptococcus mutans* (3L9C) *Yersinia pestis* (3LWZ) *Bifidobacterium longum* (3U80) *Pseudomonas aeruginosa* (4L8L) *Acinetobacter baumannii* (4RHC)		
13	3-Dehydroshikimate dehydratase (EC 4.2.1.118)	*Bacillus anthracis* (3DX5)		Mn^{2+}
14	Acetylene hydratases (EC 4.2.1.112)	*Pelobacter acetylenicus* (2E7Z) *Mycobacterium lacticola* *Nocardia rhodochrous* *Rhodococcus rhodochrous*		Iron-sulfur and Tungsten
15	Hydratase-tautomerase	*Pseudomonas fluorescens* *Pseudomonas putida*		Cofactor independent

16	Hydroxycinnamoyl-CoA hydratase-lyases[c] (EC 4.2.1.101)	*Pseudomonas fluorescens* (2J5I, 2VSS, 2VSU)		FAD	
17	Phenolic acid decarboxylases	*Lactobacillus plantarum* *Bacillus amyloliquefaciens* *Mycobacterium colombiense* *Methylobacterium* sp. *Pantoea* sp. *Bacillus pumilus* UI-670 (3NAD)		Cofactor independent	*S*-selectivity
18	Artificial hydratases	--		Cu^{II} complex	*Syn*-addition

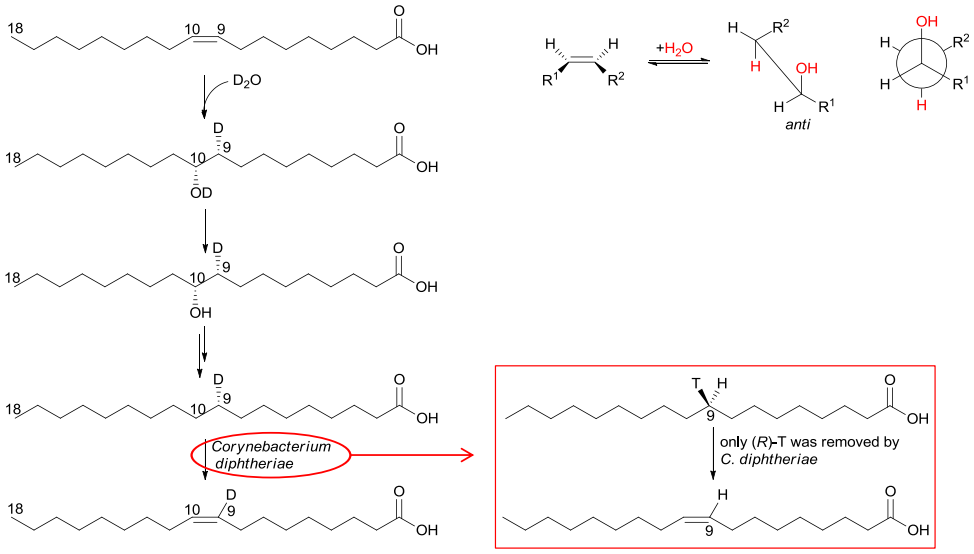
[a] until April 01, 2015; [b] formerly known as 5-dehydroquinate dehydratase; [c] formerly known as *trans*-feruloyl-CoA hydratase.



SCHEME 3. Oleate hydratase catalyses the water addition to oleic acid to form (*R*)-10-hydroxystearic acid.[11]

found to behave identically.[11,30] To date a number of putative enzymes have been described, biochemically characterized and identified as oleic acid hydratases or fatty acid hydratases. For example, the hydration activity towards unsaturated fatty acids has been found in *Streptococcus pyogenes*,[32] *Bifidobacterium breve*,[53] *Lysinibacillus fusiformi*,[54,55] *Stenotrophomonas maltophilia*,[56,57] *Macrococcus caseolyticus*,[58] *Lactobacillus rhamnosus* LGG, *Lactobacillus plantarum* ST-III, *Lactobacillus acidophilus* NCFM and *Bifidobacterium animalis* subsp. lactis BB12.[59]

As the 10-hydroxyl group in the hydration product (10-hydroxystearic acid) was established to have (*R*)-configuration, the absolute configuration of the hydrogen introduced at 9-position of the hydration product [(*R*)-10-hydroxystearic acid] should reveal whether the addition of water takes place with *syn*- or *anti*-orientation. Thus, the hydration of oleic acid catalysed by oleate hydratase yielding (*R*)-10-hydroxystearic acid was performed in a medium enriched in deuterium oxide (Scheme 4).[13] The reaction occurred with regiospecific incorporation of one deuterium at carbon atom 9 as proven by regioselective removal. The deuterium of the hydroxyl group of the deuterium-labelled product was replaced by hydrogen during extraction and purification with organic solvent (Scheme 4). Then the C-9 deuterium-labelled (*R*)-10-hydroxystearic acid was chemically converted into deuterium-labelled stearic acid, which was subsequently incubated with a growing culture of a strain of *Corynebacterium diphtheria*, a system which stereospecifically removes the (*R*)-hydrogen of carbon atom 9 of stearic acid yielding the Δ^9-double bond of oleic acid (Scheme 4, red box).[51,60] Complete retention of

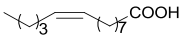
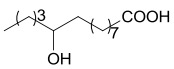
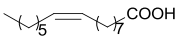
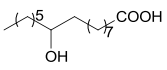
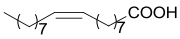
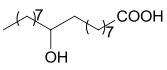
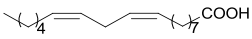
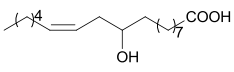
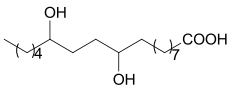
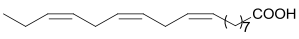
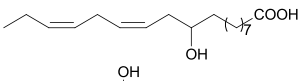
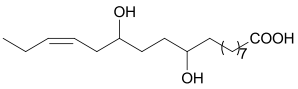
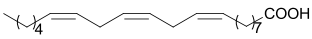
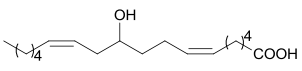
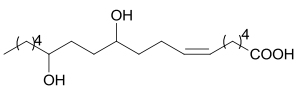
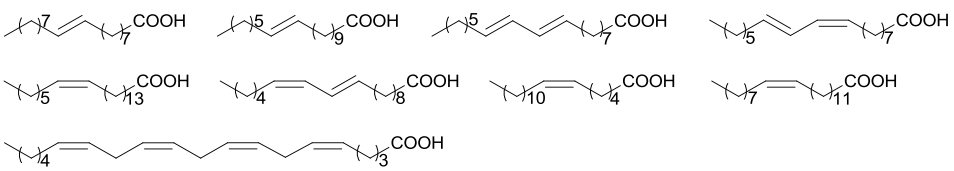


SCHEME 4. Stereochemical course of the enzymatic conversion of oleic acid to (*R*)-10-hydroxystearic acid in deuterated medium.[13] *Box*: The stereospecificity at carbon 9 atom of the enzymatic conversion of stearic acid to oleic acid by *C. diphtheria* was shown previously.[51,60]

deuterium observed by a combination of chemical and mass spectrometric evidence proved the deuterium to have remained in the molecule. Since the reactions used to prepare the [9-D] stearic acid from the 10-hydroxy-[9-D]-stearate should not affect the stereochemistry at carbon atom 9, the deuterium-labelled stearic acid thus must have (*R*)-configuration in the hydroxystearic acid. This corresponds to the (*S*)-configuration in the stearic acid. Therefore the oleate hydratase catalyses the addition of water to the C = C bond in *anti*-fashion.[11-13]

Since oleate hydratases are present in many different organisms, it has been suggested that oleate hydratases from different sources differ structurally and might have quite different substrate specificities. Their stereoselectivity however seems to always be (*R*) for the hydroxyl group. For instance, oleate hydratase from *Macrococcus caseolyticus* has been found to introduce a second hydroxyl-group at the C-12 position when linoleic acid is used as the substrate.[58] Table 2 shows an overview of all the tested substrates for fatty

TABLE 2. Hydration activity for *Z* and/or *E*-double bonds of unsaturated fatty acids using oleate hydratase from *Macrococcus caseolyticus*.[58]

Substrates		Product(s)
Name	Structure	structure
Myristoleic acid		
Palmitoleic acid		
Oleic acid		
Linoleic acid		 
α-Linolenic acid		 
γ-Linolenic acid		 
Unconverted substrates:		

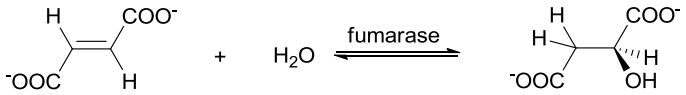
acid hydratases from *M. caseolyticus*.[58] In addition to oleic acid, which was considered as the specific substrate for oleate hydratase, the enzyme also accepts unsaturated fatty acids like: myristoleic acid (C14:1$^{\Delta 9Z}$), palmitoleic acid (C16:1$^{\Delta 9Z}$), linoleic acid (C18:2$^{\Delta 9Z,12Z}$), α-linolenic acid (C18:3$^{\Delta 9Z,12Z,15Z}$), and γ-linolenic acid (C18:3$^{\Delta 6Z,9Z,12Z}$), but had no activity towards petroselinic acid (C18:1$^{\Delta 6Z}$), elaidic acid (C18:1$^{\Delta 9E}$), vaccenic acid (C18:1$^{\Delta 11Z}$), conjugated linoleic acids (C18:2$^{\Delta 9E,11E}$, C18:2$^{\Delta 9Z,11E}$, C18:2$^{\Delta 10E,12Z}$), arachidonic acid (C20:4$^{\Delta 5Z,8Z,11Z,14Z}$), erucic acid (C22:1$^{\Delta 13Z}$), or nervonic acid (C22:1$^{\Delta 15Z}$). The

symbol Δ indicates the position and configuration of the double bonds; C16:1 indicates the chain length and the numbers of double bonds. These results indicate oleate hydratases exclusively catalyse water addition to *Z*-9 and/or *Z*-12-double bond of unsaturated fatty acids without double bonds of the *E*-configuration.

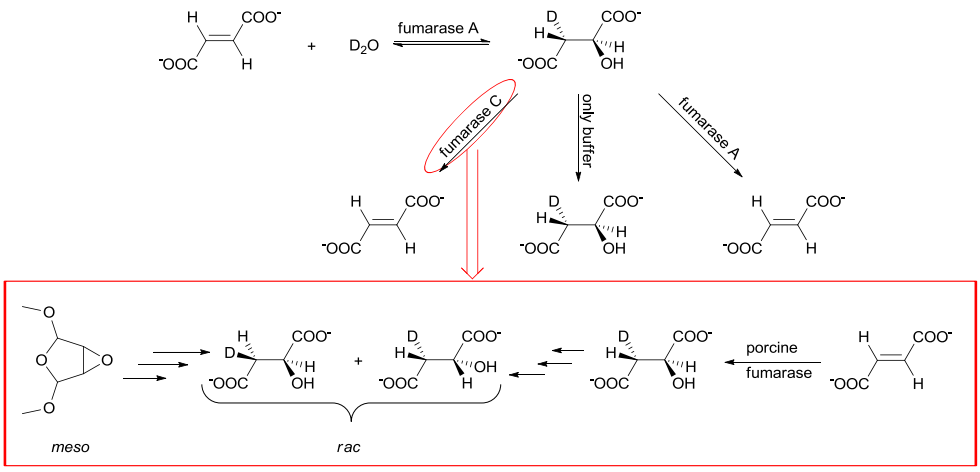
1.3.1.2 Fumarase catalyses the anti-addition of water to C = C bonds

Fumarase (EC 4.2.1.2) is the enzyme that catalyses the addition of water to fumaric acid (*E*-isomer) to form (*S*)-malate (Scheme 5). (*S*)-malate is commercially available with positive optical rotation ($[\alpha]_D^T$ = +111.6).[61] Fumarase is one of the best characterised and longest studied hydratase. The first report on its activity, the specific conversion of fumarate, dates back to 1941.[62] The fumarase-catalysed water addition reaction is a part of the citric acid cycle, representing an essential part of the primary metabolism. The fumarase-catalysed elimination reaction (from right to left in Scheme 5) also plays an additional role under anaerobic conditions in *Escherichia coli*. Due to its involvement in primary mebabolism, fumarase is widely distributed in nature including animals, plants, invertebrates, moulds, yeast and bacteria. So far there are three structurally different types of fumarases and they are categorized into two classes.[63] Fumarase A (fum A) and fumarase B (fum B) belong to the class I fumarases, which are dependent of iron (II), dimeric and sensitive to heat (incubation at 50 °C caused a rapid inactivation), while fumarase C belongs to the class II fumarases, which are heat-stable (70% of fum C activity remained after 80 min incubation at 50 °C), tetrameric and do not require a cofactor.[64,65] Due to their high stability, class II fumarases (fum C) are most commonly used.[66,67]

The stereochemical course of the reaction was investigated by the incubation of porcine fumarase with fumaric acid in D_2O. The initial results were wrongly



SCHEME 5. Fumarase catalyses the addition of water to fumaric acid to form (*S*)-malate.[39]



SCHEME 6. Stereochemistry of the hydration of fumaric acid to (*S*)-malate catalysed by fumarases A and C from *E. coli* in deuterium oxide.[14] *Box*: porcine fumarase that is very similar to fumarase C from *E. coli*, was earlier already shown to catalyse *anti*-addition of water.[68,70-72]

interpreted to indicate a *cis*-addition. The independent synthesis of the deuterated reference compound was essential to clarify the assignment. By *trans*-attack of a *meso* epoxide with deuteride the relation between OH and D could be established unequivocally (red box in Scheme 6). Thus the stereochemistry for cofactor free fumarase was established. When fumarase A was purified from *E. coli* strain JRG 1905[14] and shown to be an iron-sulphur dependent enzyme the question of the mechanism again arose. GC-MS analysis of the hydration mixture in D_2O showed that (*S*)-malate contained one deuterium, but no deuterium was present in the fumaric acid isolated after the equilibrium reaction (Scheme 6). These results indicate that 1) the addition of a proton at C-3 position is stereospecific in the fumaric acid conversion to (*S*)-malate catalysed by fumarase A; and that 2) a hydrogen atom from the identical position is removed in the reverse direction. This is in line with the older experiments with the porcine fumarase. Therefore, the stereospecificity was further ascertained by the incubation of the deuterium-labelled (2*S*)-[3-D]-malate obtained from the deuterium oxide addition experiments of initially unknown stereochemistry in buffered H_2O with fumarase A (iron-sulphur containing) and fumarase C from *E. coli* that is closely related to porcine

fumarase for which the mechanism had been proven. Additionally the (2*S*)-[3-D]-malate was incubated without enzyme as a control (Scheme 6). After the incubations, the deuterium content of (*S*)-malate and fumaric acid from all three experiments was determined by GC-MS analysis. No deuterium was present in the (*S*)-malate or fumaric acid in the reaction mixtures incubated with fumarase A or fumarase C, while the deuterium was still present in the (*S*)-malate in the fraction to which no enzyme was added. Since fumarase C is known to specifically eliminate water from (*S*)-malate in *anti*-fashion (Scheme 6, red box)[68,69] and the stereochemical course for fumarase A was identical it also had to be *anti*. Thus both fumarases catalyse the *anti*-addition of water to fumaric acid to form (*S*)-malate.[70-72]

Most structural and mechanistic work on fumarase C has been done using the *E. coli* enzyme. The important amino acid residues in the enzyme mechanism

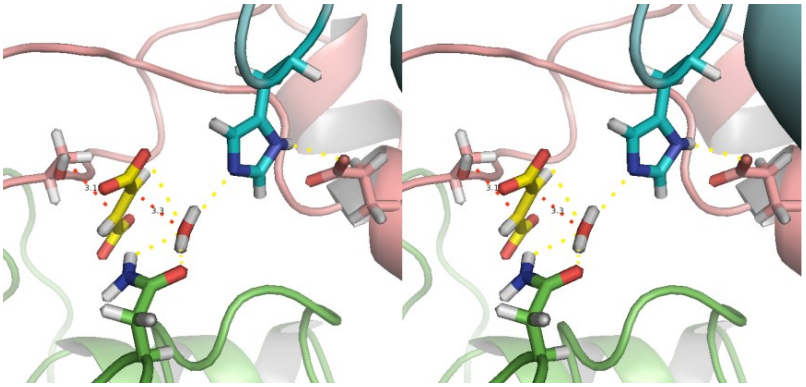


FIGURE 1. Stereo view of fumarate in fum C of *E. coli*. The active site of fum C is constituted from 3 different subunits (pink, green, blue). The residues important for catalysis are shown as sticks. The water molecule is strongly bound by a hydrogen network of residues HisB188 (blue), GluC331 (pink) and AsnA140 (green), which keeps it at the correct distance of the fumarate (3.3Å). The fumarate (yellow) is kept in place by positive charges and hydrogen bonds (not shown for clarity). HisB188 abstracts a proton from the water molecule, resulting in the OH^- attacking the fumarate. The H^+ of Ser318 that comes in on a loop from the other side probably quenches the carbanion, resulting in the formation of (*S*)-malate by an *anti*-addition.[3] Hydrogen bonds are yellow dots, the distance between the water molecule and C2 of fumarate is depicted in red. The picture was made from PDB structure 4ADL, using PyMol.[75]

are still not completely clear, but it is obvious that the reaction follows a general acid-base catalysis.[73] The nature of the first base has been well established and is the highly conserved His188 (*E. coli* numbering), which forms a charge relay pair with the equally conserved Glu331.[74] One water molecule is strongly bound by a hydrogen network of residues HisB188, GluC331 and AsnA141 (Figure 1). The fumarate is kept in place by positive charges and hydrogen bonds. The nature of the 2nd base is still under debate. It was thought to be a lysine residue (Lys324), but recent structures of *Mycobacterium tuberculosis* wild type and mutant fumarase shed light on the nature of the 2nd base.[3] When mutating a serine residue (Ser318 in *E. coli*), which is fully conserved over the fumarase/aspartase superfamily,[73] the mutant is inactive, and structures of this S318C mutant with fumarate bound show good positioning of the fumarate. This Ser was not considered before, since the loop in which this amino acid is situated is disordered in structures of apo enzyme. *E. coli* structures crystallised with citrate are not fully closed, since citrate is a larger substrate than fumarate. Furthermore, the water molecule and activating His-residue are on the opposite site of the substrate compared to the Ser (Figure 1), which is in agreement with the *anti*-addition of water in Fum C, making this hypothesis the most likely.

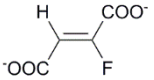
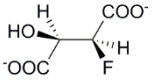
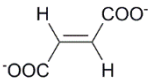
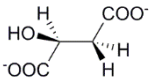
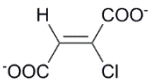
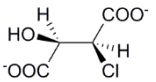
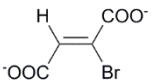
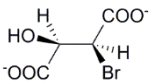
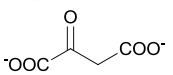
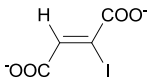
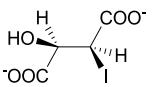
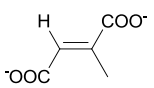
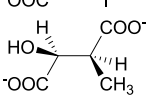
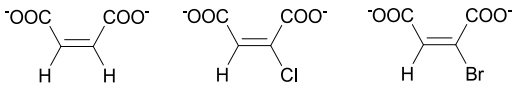
Fumarase was initially described to only perform the water addition/elimination of fumaric acid and (*S*)-malate, and therefore the enzyme was considered to have a very strict substrate spectrum.[39] Recent studies, however, have shown that fumarase also has the hydration activity towards the substrates in Table 3.[76,77] Obviously, fumarase only performs the water addition to the double bonds with the carbonyl groups *E* to each other in the fumaric acid and its derivatives. No fumarase activity was found to maleate, chloromaleate and bromomaleate showing a clear preference for the *E*-isomer (Table 3).

1.3.1.3 Malease catalyses the anti-addition of water to C = C bonds

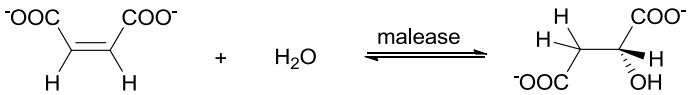
Maleic acid hydratase (malease) (EC 4.2.1.31) was first isolated as an iron-requiring enzyme that catalyses the water addition to maleic acid (*Z*-isomer) to form (*R*)-malate (Scheme 7). The first report of this activity specific hydration of maleate dates back to 1951, when this hydratase activity was discovered in corn kernels and then also rabbit kidneys.[38,78] A structurally

1

TABLE 3. Hydration activity for fumaric acid and its derivatives with *E*-double bonds using porcine fumarase.[76,77]

Substrates		Products
Name	Structure	(Structure)
Fluorofumarate		
Fumarate		
Chlorofumarate		
Bromofumarate		
Acetylenedicarboxylate		
Iodofumarate		
Mesaconate		
Unconverted substrates:		

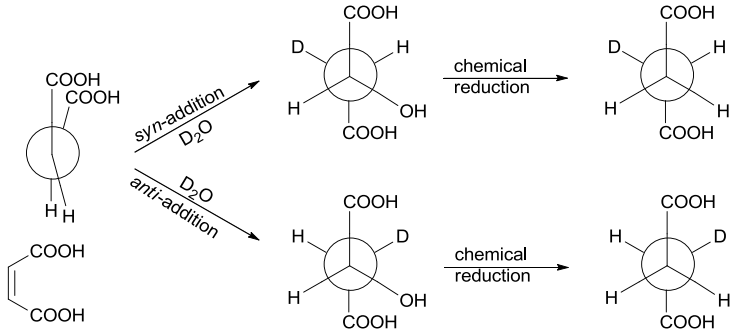
different malease, which is suggested to be common in microorganisms,[61] was isolated from *Pseudomonas pseudoalcaligenes*,[79] biochemically characterized and identified as a heterodimer consisting of two subunits (24 and 57 KDa, respectively). This malease does not require a cofactor. In contrast to malease from rabbit kidney, malease from *P. pseudoalcaligenes* also accepts citraconic acid as a substrate albeit with lower rates (Table 4). A detailed characterization has recently been described showing that citraconic



SCHEME 7. Malease catalyses the water addition of maleic acid to form (*R*)-malate.[38]

acid is capable of inducing the expression of this enzyme during the conversion into the corresponding citramalate but maleate not. Therefore, the enzyme catalysing the water addition to citraconic acid to form citramalate has been suggested to be named citraconase (EC 4.2.1.35).[80]

The stereochemical course of the malease catalysed reaction was studied by incubating maleic acid with a partially purified preparation of malease in a medium enriched with deuterium oxide (Scheme 8).[15] The reaction mixture was analysed by GC-MS to show that the (*R*)-malate formed contained one deuterium, but no deuterium was detected in maleic acid. These results indicate that the addition of a proton at C-3 position is stereospecific in the conversion of maleic acid to (*R*)-malate catalysed by malease and that a hydrogen atom from the identical position is removed in the reverse direction.[15] In theory, there are two possible isomeric forms of (2*R*)-[3-D]-malate, depending on the stereochemistry of the malease catalysed water addition to maleic acid. Since the absolute configuration for C-2 is already known to be (*R*), the two isomers differ only with respect to the C-3 configuration.[81] A *syn*-addition of D_2O to maleic acid results in a product (2*R*)-[3-D]-malate in which the two protons are opposite to each other (Scheme 8, top), whereas the same two protons are gauche to one another in the isomer arising from an *anti*-addition of D_2O (Scheme 8, bottom). The experimentally obtained hydration product (2*R*)-[3-D]-malate was chemically reduced to the corresponding mono-deuterated succinic acid. Comparing with the known optical rotation of mono-deuterated succinic acid established the

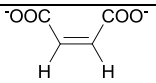
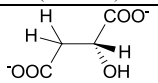
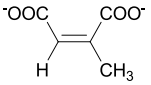
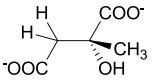
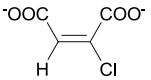
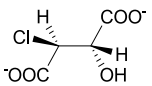
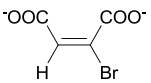
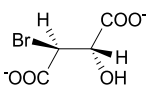
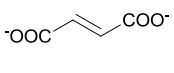
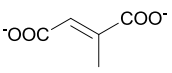
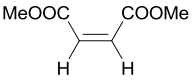


SCHEME 8. Stereochemistry of the hydration of maleic acid to (*R*)-malate catalysed by malease: (2*R*)-[3-D]-malate, which on chemical reduction to yield (2*R*)-[2-D]-succinic acid, is configurationally compatible only with an *anti*-addition of water.[15]

configuration of the deuterated succinic acid derived from the hydration product as (2*R*)-[2-D]-succinic acid.[82] With this configurations in mind, it is now seen that the (2*R*)-[3-D]-malate, which on chemical reduction yields (2*R*)-[2-D]-succinic acid, is configurationally in line with the *anti*-addition (Scheme 8, bottom).[15,78]

The maleate hydratase activity is found in both homoaconitase of methanogens and isopropylmalate (IPM) isomerase, which are part of the aconitase family. The malease reaction is the hydration half of the normal activity of these 2 enzyme classes. Although recently crystal structures have been released from both the small and large subunit of these enzymes,[83,84] the [4Fe-4S]-cluster was not co-crystalised. From these structures it is obvious that the active site is very similar to aconitase (see Aconitase catalyses the *anti*-addition of water to C = C bonds), but the cysteines in the IPM isomerase that should bind the [4Fe-4S] cluster, do form disulphide bonds instead, resulting in a huge conformational change of the active site. Reducing the enzyme before crystallisation breaks the disulphide bonds, resulting in a more open structure of the enzyme, which is more similar to aconitase.[84] Since the important positively charged active site residues and the catalytic His101 and Ser264 are conserved in all enzymes, it is likely that the substrates will

TABLE 4. Hydration activity for maleate and its derivatives with *Z*-double bonds using malease from *Arthrobacter* sp. strain MCI2612.[85]

Substrates		Products
Name	Structure	(Structure)
Maleate		
Citraconate		
Chloromaleate		
Bromomaleate		
Unconverted substrates:		
		 

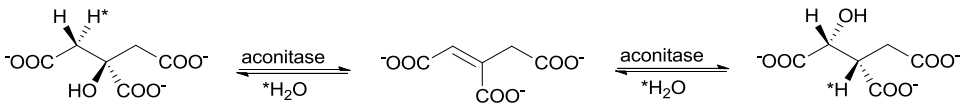
bind in a similar way. Therefore an *anti*-addition is the most probable reaction mechanism for this enzyme, as was confirmed by the experiments described above.

Although malease was described[78] to strictly perform water addition/elimination of maleic acid, later studies[85] observed hydration activity also for chloromaleate and bromomaleate. Notably, malease has higher activity towards chloromaleate than maleate (rates of hydration of these substrates followed: chloromaleate > maleate > citraconate > bromomaleate). The enzyme displays no activity towards dimethyl maleate, acetylenedicarboxylate, fumarate, mesaconate, *Z*-epoxysuccinate, or *E*-epoxysuccinate (Table 4). These results indicate maleases exclusively catalyse water addition to *Z*-double bonds in maleic acid and its derivatives.

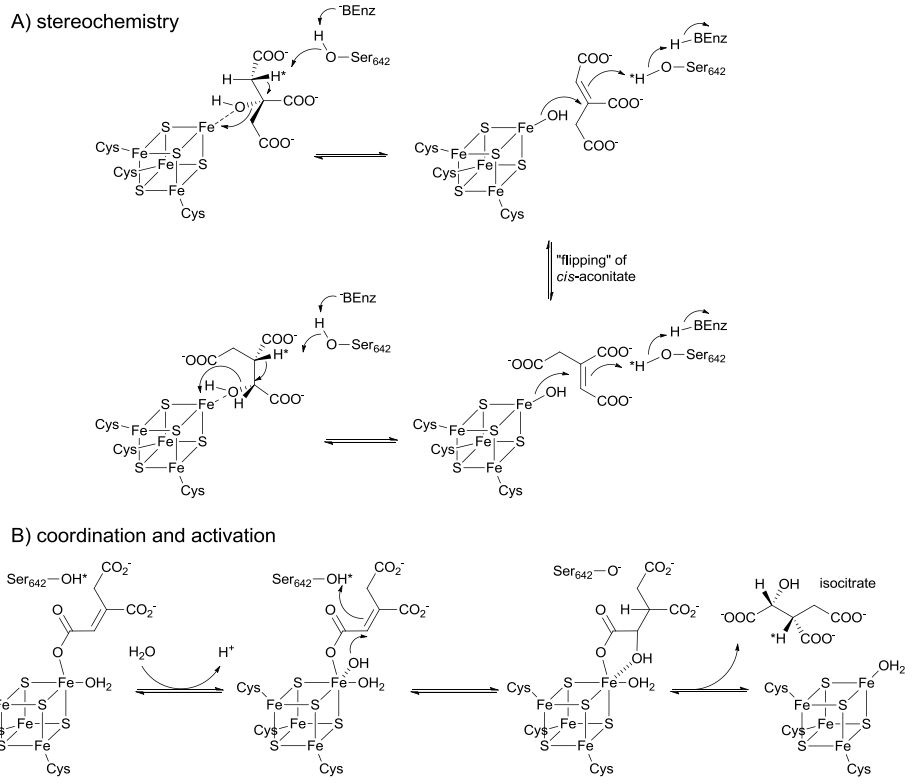
1.3.1.4 Aconitase catalyses the anti-addition of water to C = C bonds

Aconitase (EC 4.2.1.3) is an iron-sulfur dependent enzyme that converts symmetric citric acid into isocitric acid (Scheme 9), at first glance an isomerisation. This is an essential step in the citric acid cycle of the aerobic metabolism. The overall isomerisation proceeds *via* an elimination addition process. The enzyme catalyses the water elimination of citrate to form *cis*-aconitate. In addition to converting citrate and *cis*-aconitate, it also converts *cis*-aconitate to isocitrate. Hence, aconitase converts citrate and isocitrate (Scheme 9).[16] Overall, aconitase catalyses four stereospecific reactions: the dehydration of citrate or isocitrate respectively to *cis*-aconitate, and the rehydration of *cis*-aconitate to isocitrate or citrate. The equilibrium mixture of the overall reaction is 91% citrate, 3% *cis*-aconitate and 6% isocitrate. *Cis*-aconitate is the *Z*-prop-1-ene-1,2,3-tricarboxylate isomer.

Aconitases are monomeric proteins containing [4Fe-4S] iron-sulfur clusters in their active sites. One of the iron atoms acts as Lewis acid in the activation of



SCHEME 9. Aconitase catalyses the water addition of *cis*-aconitate to form citrate or isocitrate.[16]



SCHEME 10. Mechanism for aconitase indicates A) the *anti* elimination/addition of water to *cis*-aconitase; B) it proceeds due to activation and coordination of the substrates to one iron atom of the iron-sulfur cluster.

water and it brings the two reactants of the addition reaction in close proximity of each other. The overall three step process —dehydration, flip, rehydration— and the stereochemical consequences in the action of aconitase are depicted in Scheme 10A.[86] The [4Fe-4S] cluster has as usual a cube-like structure that is bound to the protein backbone by three cysteine residues. The iron with vacant coordination positions binds the substrate by coordination of the carboxylate and one hydroxyl group. It also facilitates the departure of the hydroxyl group in the dehydration step by serving as a Lewis acid (Scheme 10B). Although the hydroxyl group removed is exchanged, the same proton atom abstracted by Ser642 (acts as a general base) is returned upon hydration of *cis*-aconitate. This phenomenon implies that Ser642 is shielded from the solvent water and that the *cis*-aconitate is then flipped over

in the active site of the enzyme before rehydration, blocking the active site for solvent water. The flip over then allows the addition of water at C-3 of *cis*-aconitate to form isocitrate. Thus, the model accounts for the stereospecificity of the reactions and for the *anti*-elimination/addition of a hydroxyl group and a proton in the dehydration and rehydration steps.[16] It should be noted that when comparing to the usual *anti* addition/elimination of aconitase with methylcitrate dehydratase (PrpD) it was suggested[87] that the latter catalyses the *syn* elimination of (2*S*, 3*S*)-2-methylcitrate to form *cis*-2-methylaconitate. No further evidence was provided and a misunderstanding on the rotational freedom in the addition product might be at the basis of this suggestion.

The *anti* stereochemical course of aconitase was also investigated by incubation of *cis*-aconitate in D_2O with aconitase.[88] Experimental data showed the formed citrate and isocitrate contained one deuterium and no deuterium was found in *cis*-aconitate. These results illustrated that the reaction catalysed by aconitase is stereospecific. That is, the same hydrogen that was added to *cis*-aconitase must be removed to form the methylene group in the subsequent dehydration so that the reaction in D_2O can never lead to labelled *cis*-aconitate. No doubly labelled citrate or isocitrate molecules were obtained in the reaction further indicating the stereospecificity of the hydration and dehydration steps. The *anti*-stereochemistry was established by comparison the formed citrate with the chemically synthesized citrate.[71,72,89]

The [4Fe-4S]-cluster in the active site of aconitase is bound by three cysteines to the protein, Cys358, Cys421 and Cys424.[90] The cluster is hydrogen bound with the substrate, and the not fully coordinated iron can act as a Lewis acid. In the hydration half of the aconitase reaction the free iron coordinates an OH^- molecule when no substrate is bound, and a water molecule upon binding of the substrate. This water is also hydrogen bound to Asp165 and attacks the double bond at the C2 position of *cis*-aconitate. Apart from the hydrogen bonds to the [4Fe-4S]-cluster, the substrates is very tightly bound by hydrogen bonds and many positive charges from Asp165, Ser166, Arg447, Arg452, Arg580, Ser643 and Arg644. After the addition of OH to the substrate, a proton from Ser642 (pink), which is situated on the other side of the substrate, completes the (re)hydration. As can be seen in Figure 2, the relative position of the water molecule is on the opposite side of the substrate

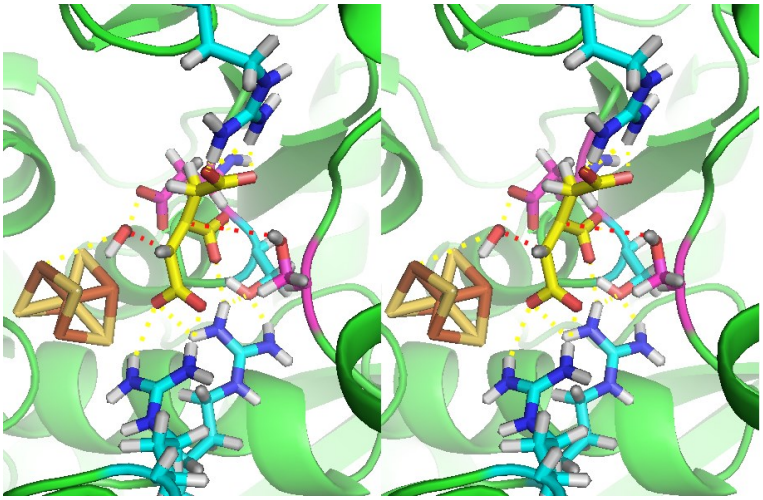
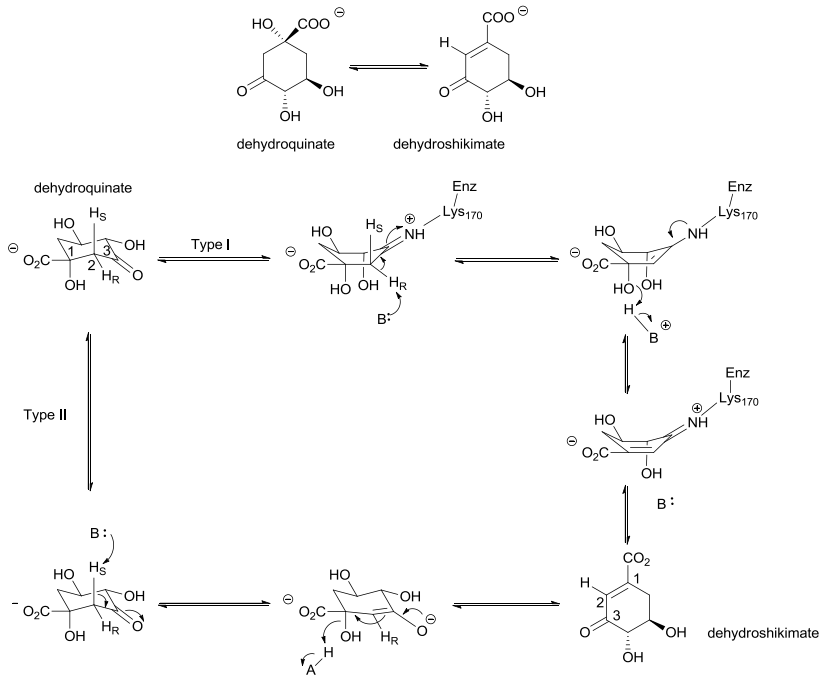


FIGURE 2. Stereo view of *cis*-aconitate in porcine mitochondrial aconitase. The aconitase active site consists of a cube-like [4Fe-4S]-cluster (brown-beige) which is bound by 3 Cys residues (not shown for clarity). The iron at the top left corner is not fully liganded and can act as a Lewis acid. In the hydration half of the aconitase reaction the free iron coordinates the water molecule that will attack the double bond at the C2 position. This water is also hydrogen bound to Asp165 (pink, in the back). The substrate *cis*-aconitate (yellow) is positioned by many positive charges from several different residues (blue). After the addition of OH to the substrate, a proton from Ser642 (pink), which is situated on the other side of the substrate, completes the (re)hydration. As can be seen in the picture, the relative positions of the water molecule and the serine residue result in an *anti*-addition, as was also shown by other methods. Hydrogen bonds are yellow dots, the distance between the water molecule and the C2, and between the H from Ser642 and C2 of the substrate is depicted in red.[90] The picture was made starting from PDB-file 1B0J using PyMol.[75]

compared to the serine residue. This explains the *anti*-addition of water in this enzyme.

1.3.1.5 Type II dehydroquinase catalyses the anti-addition of water to C = C bonds



SCHEME 11. Type I and II dehydroquinase catalyses the water addition of 3-dehydroshikimate to 3-dehydroquinate having very different mechanisms.[87,95]

The enzyme dehydroquinase (3-dehydroquinate dehydratase, EC 4.2.1.10) catalyses the reversible hydration of 3-dehydroshikimate to 3-dehydroquinate (Scheme 11).[91] An equilibrium mixture of the substrate (3-dehydroquinate) and product (3-dehydroshikimate) (Keq. = 15) exists.[92] Both 3-dehydroshikimate and 3-dehydroquinate are intermediates in the biosynthesis of a wide range of aromatic compounds, in primary and secondary metabolism. The absolute configuration of the hydration product was established by converting it into citric acid of known absolute configuration (Scheme 12).[21] The dehydroquinase catalysed reaction is part of two metabolic pathways, the biosynthetic shikimate pathway and the catabolic quinate pathway.[93,94] Two structurally completely different dehydroquinases (types I and types II) are responsible for catalysing this transformation, by different mechanisms (Scheme 11).[87,95] The type I enzymes are exclusively biosynthetic, whereas type II enzymes have both biosynthetic and catabolic roles. The mechanism of type I enzyme has been

shown to act *via* an enamine intermediate between the substrate and a conserved lysine residue, and catalyses a *syn* addition/elimination of water and will be discussed below (chapter 1.3.2.1). In contrast, the type II enzyme catalyses an *anti*-addition/elimination. The mechanism proceeds *via* an enolate intermediate and the enzymes are usually dodecameric of subunit Mr around 16000.[17,18]

In type II 3-dehydroquinase the substrate dehydroshikimate is bound by hydrogen bonds from several positive and polar amino acids. Residues Pro15, Asn16 and Ala82 stabilise a water molecule that donates a proton to the C3 carbonyl, to produce an enolate and shifting of the double bond. A second

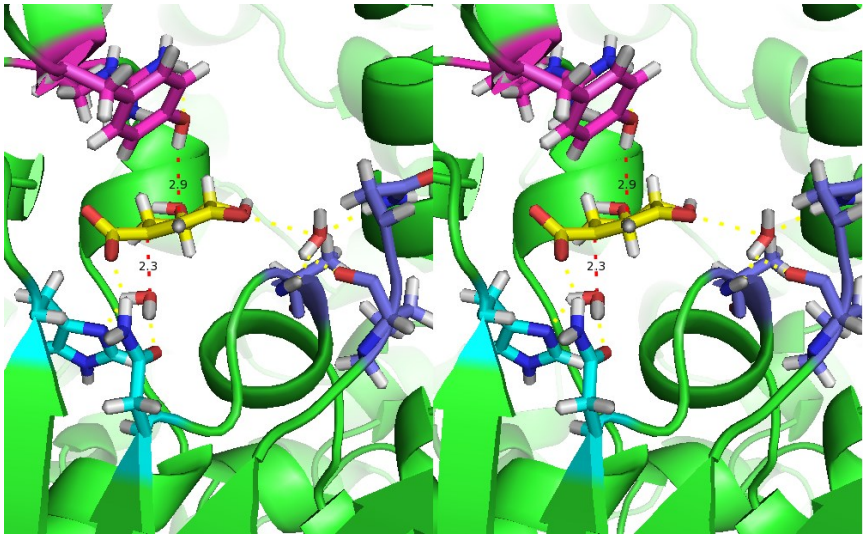


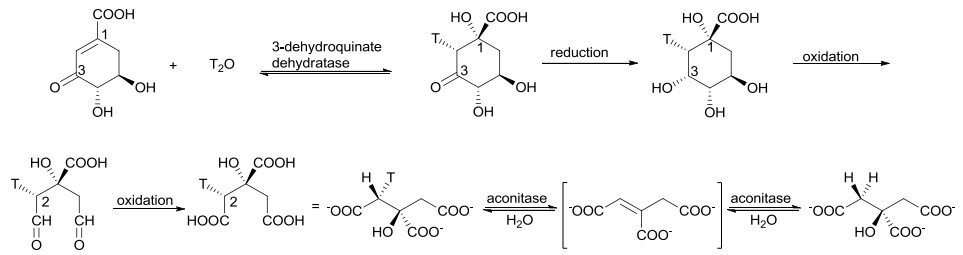
FIGURE 3. Stereoview of 3-dehydroquinate dehydratase Type II in complex with dehydroshikimate. In type II 3-dehydroquinase the substrate dehydroshikimate (yellow) is bound by many hydrogen bonds (not shown for clarity). Residues Pro15, Asn16 and Ala82 (purple) stabilise a water molecule that attacks the C3 carbonyl, which results in the shifting of the double bond. A second water molecule is activated by residues Asn79 and His106 (blue) and attacks the double bond from the bottom. The hydrogen of Tyr28 (pink), stabilised by the positive charge of Arg113 (pink), quenches the carbanion from the top, resulting in *anti*-addition.[95] Hydrogen bonds are yellow dots, the distance between the water molecule or H-Tyr and the substrate is depicted with red dots. The picture was made starting from PBD-file 1GTZ using PyMol.[75]

water molecule is activated by residues Asn79 and His106 and attacks the double bond. The hydrogen of Tyr28, which is stabilised by the positive charge of Arg113, quenches the carbanion from the other side, resulting in *anti*-addition (Figure 3).

1.3.2 Examples of enzymatic *syn*-addition of water to C = C bonds

1.3.2.1 Type I dehydroquinase catalyses the syn-addition of water to C = C bonds

The type I dehydroquinase is the first reported enzyme that catalyses the *syn*-addition of water to a C = C double bond. These enzymes are dimers of subunit Mr around 27000. The stereochemical course of the type I dehydroquinase reaction was studied by performing the hydration reaction in tritium-labelled water using 3-dehydroquinate dehydratase from Aerobacter aerogenes A170-143 (Scheme 12).[21] GC-MS analysis showed that the hydration product 3-dehydroquinate contained one tritium at carbon-2 position as expected. The labelled hydration product [2-T]-3-dehydroquinate was reduced to give [2-T]-quinic acid, which was oxidized into [2-T]-citric acid according to a known method.[96] Previous studies showed that aconitase (EC 4.2.1.3) is capable to selectively exchange the (*R*)-hydrogen attached to C-2 of citric acid with the hydrogen of the medium *via anti*-elimination and addition.[72] Therefore the results of the incubation of labelled [2-T]-citric acid with aconitate hydratase can be used to distinguish the labelled hydrogen added to 3-dehydroshikimate at carbon-2 position. When the labelled [2-T]-citric acid, prepared from [2-T]-



SCHEME 12. Stereochemistry of the hydration of 3-dehydroshikimate to 3-dehydroquinate catalysed by 3-dehydroquinate dehydratase type I. i) hydration; ii) reduction ($NaBH_4$); iii) oxidation (HIO_4); iv) oxidation (Br_2/H_2O); v) treatment with aconitase in water. This led to removal of the tritium label C-2 in the (*R*)-position. [21]

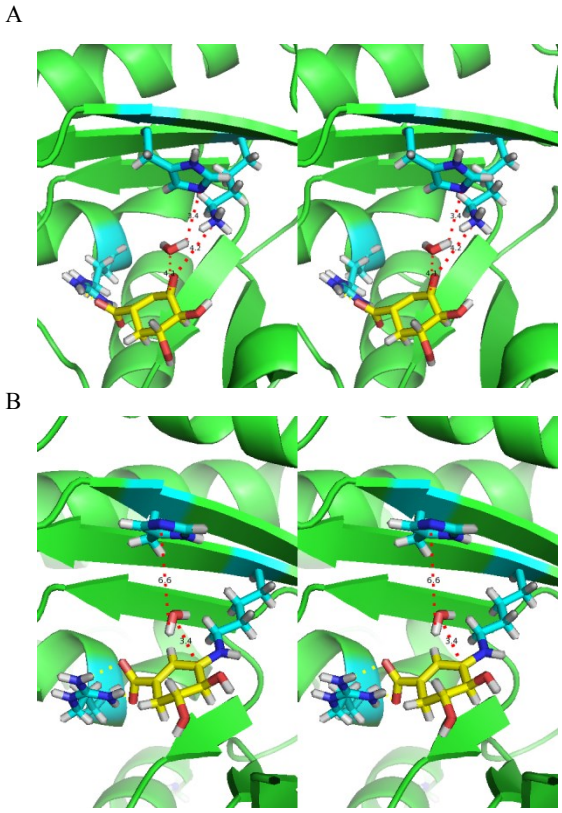


FIGURE 4. Stereoview of 3-dehydroquinate dehydratase Type I in complex with dehydroshikimate. A. In 3-dehydroquinase Type I the substrate dehydroshikimate (yellow) is bound by many hydrogen bonds (not shown for clarity) among which Arg213. The substrate is then activated by the formation of a Schiff base with residue Lys170 (blue).[97] B. When the Schiff base is formed the substrate and the protein take another conformation and a water molecule can attack the double bond. The proton for the other carbon originates from the same water molecule, resulting in *syn*-addition. Release of the Schiff base is facilitated by a water molecule activated by His143.[100] Hydrogen bonds are yellow dots, the distance between the water molecule and the substrate is depicted with red dots. The pictures were made starting from PDB-file 4CNO (A) and 4GUG (B) using PyMol.[75]

3-dehydroquinate, was treated with aconitase, it was found that nearly all of the tritium was released into the water of the reaction medium. Since the reactions used to prepare the [2-T]-citric acid from [2-T]-3-dehydroquinate

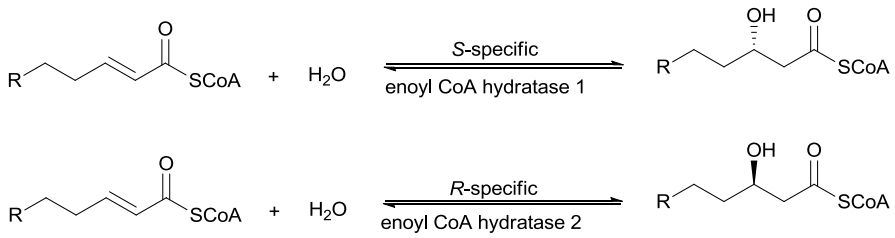
should not affect the stereochemistry, it follows that the tritium at carbon atom 2 of [2-T]-3-dehydroquinate was also in the (*R*)-configuration. This demonstrated that the 3-dehydroquinate dehydratase catalysed reaction is a *syn*-addition.[21]

The type I 3-dehydroquinase also binds the substrate dehydroshikimate by an extensive hydrogen network, among which Arg213 (Figure 4A).[97] The reaction is however catalysed by the formation of a Schiff base of Lys170 with the substrate (Figure 4B). The OH^- for the first step of the hydration originates from water, and the remaining proton comes from the same water molecule, resulting in *syn*-addition. Release of the Schiff base is facilitated by His143, in which the H bound to the His is given to Lys and not the free proton as formerly thought.[98,99]

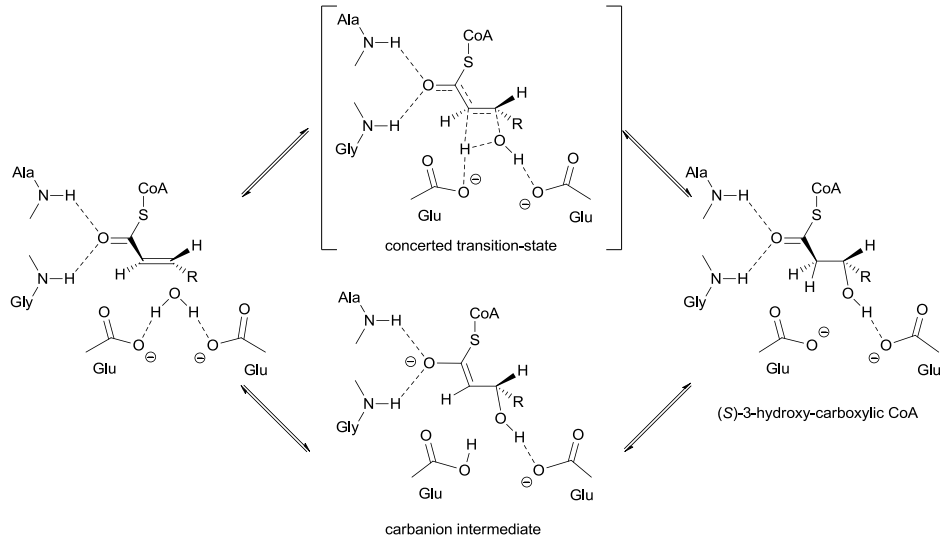
1.3.2.2 Enoyl-CoA hydratase catalyses the syn-addition of water to C = C bonds

Enoyl-CoA hydratase (EC 4.2.1.17) catalyses the second step in the physiologically important β-oxidation pathway of fatty acid metabolism. It accelerates the addition of water to the double bond of a *trans*-2-enoyl-CoA thioester, yielding α,β-hydroxyacyl-CoA thioester (Scheme 13).[41]

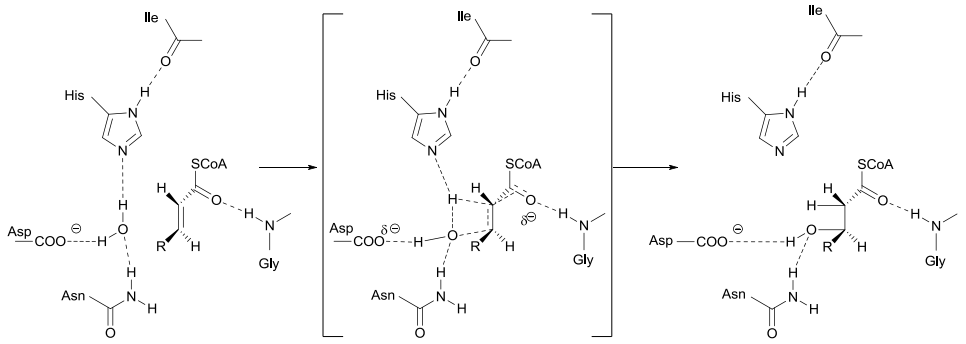
Concerning the enantioselectivity, there are two different types of enoyl CoA hydratases.[22,41,42] The (*S*)-specific enoyl CoA hydratase 1 (also known as crotonase) from mammalian mitochondria has a molecular mass of 161 kDa with six subunits. This enzyme is able to perform the addition of water to α,β-hydroxyacyl-CoA thioesters with a chain length varying between 4 and 20



SCHEME 13. Enoyl CoA hydratase catalyses the addition of water to *trans*-2-enoyl-CoA thioesters to form the corresponding alcohols with different enantioselectivity depending on the enzyme used.[41]



SCHEME 14. Enoyl CoA hydratase type 1 catalyses the (*S*)-specific addition of water to *trans*-2-enoyl-CoA thioesters.



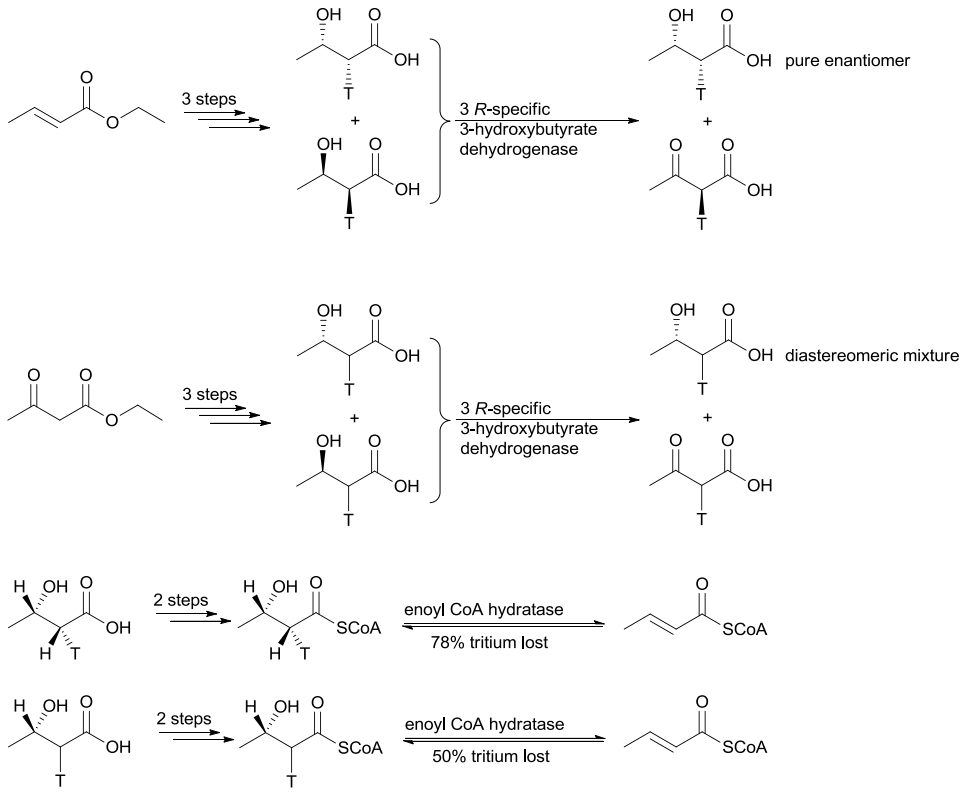
SCHEME 15. Enoyl CoA hydratase type 2 catalyses the (*R*)-specific addition of water to *trans*-2-enoyl-CoA thioesters.

carbon atoms;[19] essentially the fatty acid chain that is degraded step by step releasing acetate. This rather broad substrate spectrum in terms of chain length is achieved by a flexible loop separating the active site pocket from the surroundings, giving space for the larger substrate.[101] The hydration/dehydration reactions have been suggested to proceed *via* a concerted or a more likely sequential mechanism (Scheme 14). Also a very

recently described hydratase-isomerase from the crotonase family follows this type of mechanism.[102]

The (*R*)-specific enoyl CoA hydratase 2 (EC 4.2.1.119), from humans and other mammals, *Candida tropicalis* and the fruit fly[103] has a hot-dog fold. This enzyme shows a preference for straight medium-length substrates, but is also capable of catalysing the hydration of long-chain substrates, depending on the enzyme sources.[104] The hydration /dehydration reactions have been suggested to proceed *via* a concerted mechanism (Scheme 15). The geometry of the active site (in a mirror image fashion) can easily explain the difference in enantioselectivity between of the enoyl CoA hydratase 1 and 2.[104,105]

To date there is no evidence that the catalytic mechanism of enoyl CoA hydratases require any cofactors or metal ion. The stereochemical investigation showed that the enzymatic reaction occurs *via* the *syn* addition/elimination of water (Scheme 16).[22-24,106] Stereospecifically labelled (2*R*, 3*S*)-3-hydroxy-[2-T]butyric acid and (2*S*, 3*R*)-3-hydroxy-[2-T]butyric acid were synthesized by *syn*-specific hydroboration[62] with tritiated sodium borohydride, subsequent oxidation (hydrogen peroxide) and hydrolysis (sodium hydroxide). This racemic mixture of the diastereoisomers was then submitted to an enantioselective (3*R*)-specific 3-hydroxybutyrate dehydrogenase. Kinetic resolution yielded (2*R*, 3*S*)-3-hydroxy-[2-T]butyric acid enantiomerically pure. In parallel (3*R*)-3-hydroxy-[2-T]butyric acid and (3*S*)-3-hydroxy-[2-T]butyric acid (tritiated at C-2 with one T but racemic) were also prepared by isotopic exchange of ethyl acetoacetate in tritiated water, followed by reduction and hydrolysis. At the second stage kinetic resolution with (3*R*)-specific 3-hydroxybutyrate dehydrogenase yielded (3*S*, rac2)-3-hydroxy-[2-T]butyric acid. This was isolated as diastereomeric mixture due to the undefined stereochemistry for the tritium at C-2. With these tritiated compounds the investigation of the stereochemical course of the hydratase was performed. (2*R*, 3*S*)-3-hydroxy-[2-T]butyric acid and (3*S*, rac2)-3-hydroxy-[2-T]butyric acid were chemically converted to the corresponding acyl-CoA derivatives, followed by incubation with commercially available enoyl CoA hydratase. After incubation for 10 min to reach the equilibrum, racemically labelled (3*S*, rac2)-3-hydroxy-[3-T]butyryl-CoA lost nearly 50 % of its tritium label, while (2*R*, 3*S*)-3-hydroxy-[3-T]butyryl-CoA lost about 78%, slightly less than 100% loss expected for *syn*-elimination/addition. The somewhat lower than expected loss was probably



SCHEME 16. Stereochemistry of the hydration of *E*-crotonyl-CoA catalysed by enoyl-CoA hydratase 18).[22-24,106] *Top*: preparation of (3*S*)-3-hydroxy-[2-T]butyric acid; *Bottom*: reversible water eliminaton and addition between (3*S*)-3-hydroxy-[2-T]butyral-CoA and *E*-crotonyl-CoA incubation by enoyl-CoA hydratase.[23]

due to impurities of the enzyme preparation. As shown in Scheme 16, in the elimination system of enoyl CoA hydratase, the hydrogen in (*R*)-configuration at C-2 and the (*S*)-hydroxyl group at C-3 are expelled from the (3*S*)-enantiomers to yield *E*-crotonyl-CoA. In the reverse direction, hydrogen is added to the *re*-face of C-2 (*R*-configuration) and the hydroxyl group is added to the *re*-face of C-3 (*S*-configuration) of the *E*-crotonyl-CoA. These results clearly indicate that the addition/elimination of water by enoyl CoA hydratase occurs stereospecifically in a *syn* fashion.[22-24,106]

In the both (*R*)- and (*S*)-specific enoyl-CoA hydratase the C1-carboxygroup of the substrate is kept in place by an H-bond to position the substrate correctly.

In the *C. tropicalis* enzyme this H-bond is formed by the backbone of Gly205 (Figure 5A), in the rat enzyme by Ala98 and Gly141 (Figure 5B). In both enzymes several residues form a tight hydrogen network to assist the OH^-

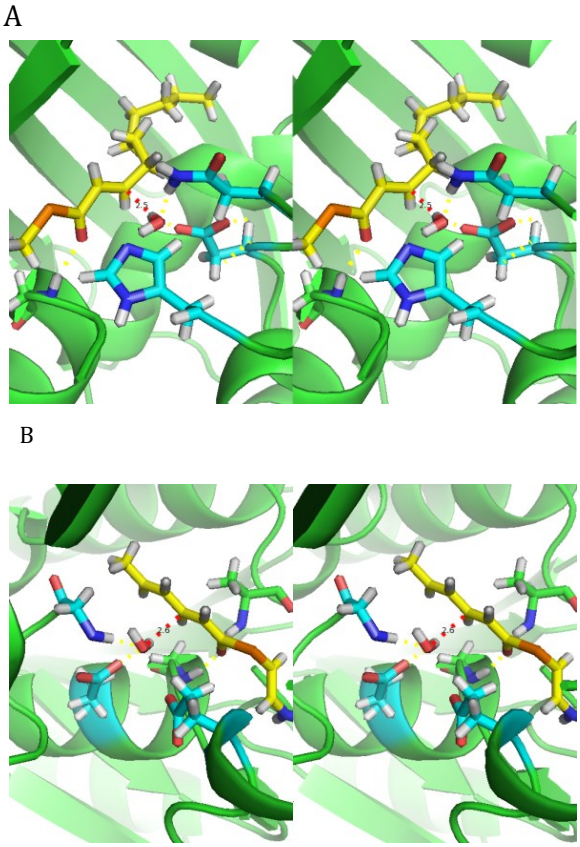


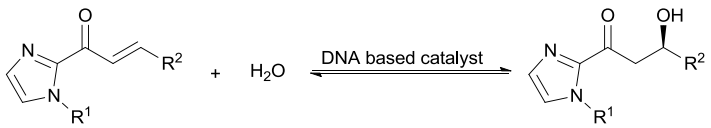
FIGURE 5. Stereo view of substrate bound to Enoyl-CoA hydratase. A. In the (*R*)-specific enoyl-CoA hydratase of *Candida tropicalis* (PDB 1PN4) the C1-carbonyl group to the substrate is kept in place by an H-bond to the backbone of Gly205 (green sticks). The residues Asp182, Asn184 and His 187 (blue) position the water molecule at the right distance (2.5Å) of the substrate (yellow) in order to hydrate the double bond in a *syn*-fashion. B. In the (*S*)-specific enoyl-CoA hydratase of the rat (PDB 1MJ3) the carbonyl group of the substrate is kept in place by Ala98 and Gly141 (green sticks). The residues Glu144, Glu164 and Gly172 (blue) position the water molecule at the right distance (2.6Å) to the substrate (yellow) in order to hydrate the double bond in a *syn*-fashion.[104] Hydrogen bonds are yellow dots, the distance between the water molecule and the C3 of the substrate is depicted in red. The picture was made using PyMol.[75]

transfer to the substrate by keeping it at the right distance to the substrate and accommodating the remaining proton before this adds to C2 of the substrate. Since both the OH and H originate from the same water molecule that is on one side of the substrate, the hydration of the double bond is in a *syn*-fashion .[104]

1.3.2.3 Artificial hydratase catalyses the syn-addition of water to C = C bonds

Recently an artificial hydratase activity was created. This metalloenzyme catalyses the water addition to α,β-unsaturated 2-acyl imidazole to produce (*R*)-β-hydroxy ketones (Scheme 17). The reaction proceeds in an enantioselective manner and several substrates with varied functionality at β-position of the enone substituent have been tested.[25] The hydration activity was observed using DNA-based catalyst as a second coordination sphere.[25,107]

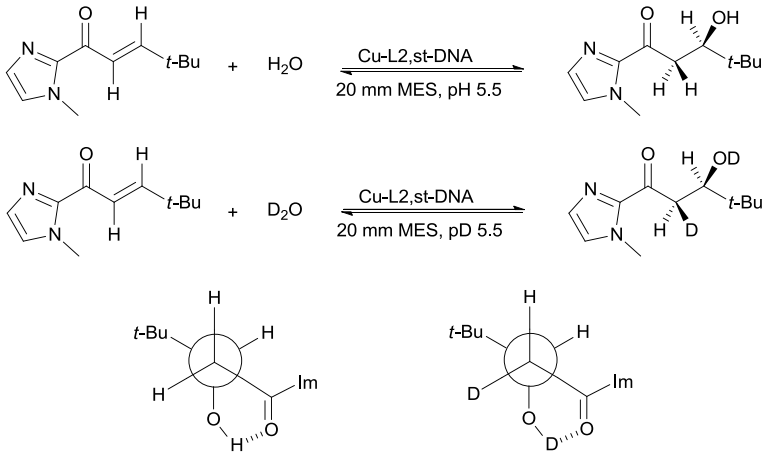
The stereochemical course of the hydration reaction was investigated by performing the biotransformation in H_2O (Scheme 18, the first reaction) and D_2O (Scheme 18, the second reaction) as a medium, respectively.[25] The hydration reaction in D_2O requires more time to reach the isotopic equilibrium conversion than that in H_2O. The ^{1}H-NMR spectral analysis of the water hydration product (Scheme 18, left Newman projection) and labelled hydration product (Scheme 18, right Newman projection) showed for the H_2O hydration product a geminal coupling constant between the two α-protons of 15.7 Hz. The two vicinal coupling constants of 9.4 and 2.3 Hz between the α-



R^1=Me, R^2=*t*-Bu
R^1=Me, R^2=Me
R^1=Me, R^2=*n*-Pentyl
R^1=Me, R^2=*i*-Pr
R^1=*i*-Pr, R^2=Me

R^1=Me, R^2=*t*-Bu
R^1=Me, R^2=Me
R^1=Me, R^2=*n*-Pentyl
R^1=Me, R^2=*i*-Pr
R^1=*i*-Pr, R^2=Me

SCHEME 17. Enantioselective hydration of α,β-unsaturated ketones catalysed by a copper (II)-containing artificial hydratase.[25]



SCHEME 18. Stereochemistry of the *syn* hydration of α,β-unsaturated 2-acyl imidazole to (*R*)-β-hydroxy ketones catalysed by an artificial hydratase.[25]

protons and the β-proton involve the *anti* and the gauche protons, respectively. In the spectrum of the D_2O hydration product a single diastereoisomer with a vicinal coupling constant of 2.0 Hz was identified. These results demonstrate the *syn*-addition of D_2O.[25] Since *syn* diastereospecificity until now was reported only for hydratase enzymes, such as 3-dehydroquinate dehydratase (Type I dehydroquinase catalyses the *syn*-addition of water to C = C bonds) and enoyl-CoA hydratase (Enoyl-CoA hydratase catalyses the *syn*-addition of water to C = C bonds) it was entirely unexpected to find this in a chemo-catalyst based artificial enzyme.

1.4 THESIS AIMS

The chemistry of hydratases is a treasure-trove that has been opened, but very limited chemical application exist, often due to a lack of understanding in the past. Based on understanding the enzymatic reaction mechanism (This Chapter), new enzymes or artificial hydratases can be developed. Very few examples of enantioselective Michael additions of water have been described and most of them are enzyme catalyzed. These enzymes, hydratases, however suffer in most cases from a very narrow substrate scope. Indeed some only accept one single substrate. Therefore, the aim of this thesis was dedicated to the search for a Michael hydratase with a

more relaxed substrate specificity (Chapter 2). This concept can greatly expand the number of hydratase-catalysed reactions available within the toolbox. This also opens up an entirely new approach to the synthesis of chiral β-hydroxy carbonyl compounds, which represent an important class of compounds that is often found as structural motif in natural products.

Generally, gene annotation offers an excellent opportunity for discovering novel enzymes. With the developing of genome sequencing technology, more hydratases should come into the picture from different organisms. Therefore looking for annotated hydratases in the sequenced genomes of the organism used will be one important part in this thesis (Chapter 3).

During the work presented in Chapter 2, we found that most β-hydroxy ketones are not commercially available or expensive, which makes the determination of the stereoselectivity of water addition difficult. Although the molecules themselves look rather simple, their synthesis can be challenging, in particular so if they are optically active. Therefore, it is important to establish a straightforward approach to prepare enantiomerically enriched β-hydroxy carbonyl compounds (Chapter 4).

Additionally, an initially unexpected stereoselective reduction of conjugated C = C bonds was discovered during studies on the enantioselective Michael addition of water. It is of high interest to probe whether the system is really a one-step water addition reaction or a stepwise (reduction-hydroxylation) reaction, since the hydroxylation was literally unknown. Nevertheless, mechanistic studies will demonstrate how the reaction works. On the other hand, ene reductase activity should also be investigated (Chapter 5) in order to rule out the stepwise reaction, further confirming the proposed one-step water addition.

1.4.1 Outline of this thesis

CHAPTER 2 will discuss the direct Michael addition of water to a series of substrates using whole-cells of six *Rhodococcus* strains, including the mechanistic studies. CHAPTER 3 will describe the genome sequence and annotation of strain *Rhodococcus rhodochrous* ATCC 17895. CHAPTER 4 will discuss the enantioselective preparation of (*R*)- and (*S*)-3-hydroxycyclopentanone by kinetic resolution. CHAPTER 5 will discuss the NADH dependent ene-reductases isolated from *R. rhodochrous* ATCC 17895 for asymmetric reduction of conjugated C = C bonds. CHAPTER 6 will

summarize the main results from this thesis and elaborate on the possible future research.

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2

ENANTIOSELECTIVE MICHAEL ADDITION OF WATER

The enantioselective Michael addition using water as both nucleophile and solvent has to date proved beyond the ability of synthetic chemists. In this chapter, the direct, enantioselective Michael addition of water in water to prepare important β-hydroxy carbonyl compounds using whole-cells of *Rhodococcus* strains is described. Good yields and excellent enantioselectivities were achieved with this method. Deuterium labelling studies demonstrate that a Michael hydratase catalyzes the water addition exclusively with *anti*-stereochemistry.

This chapter is based on

B.-S. Chen, V. Resch, L. G. Otten and U. Hanefeld,

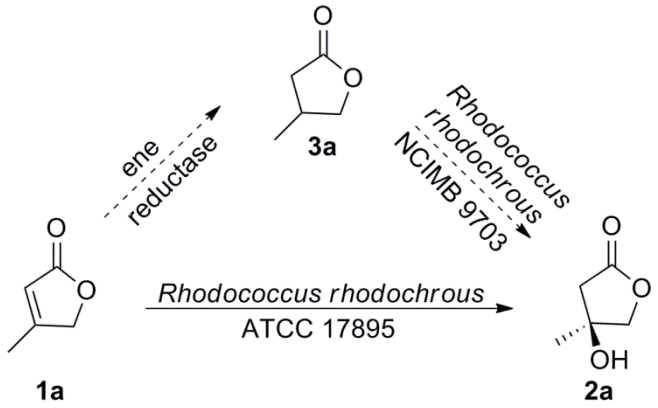
Chem. Eur. J., **2015**, *21*, 3020-3030.

2.1 INTRODUCTION

The direct addition of water to C = C bonds is a highly attractive transformation, yielding (chiral) alcohols.[1-4] However, the enantioselective addition of water to α,β-unsaturated carbonyl (Michael) acceptors still represents a chemically very challenging reaction,[5-6] due to the poor nucleophilicity of water and its small size, which make regio- and stereo- inductions difficult. Equally, the often unfavourable equilibrium of water-addition reactions remains to be solved. Although this reaction benefits from its simplicity and excellent atom economy, no protocol with broad applicability has been developed. Indirect methods[7,8] using complex catalysts[9-11] or strong alternative nucleophiles[12,13] have been employed. Some of the described methods require either cumbersome catalyst preparation or reductive/oxidative follow-up chemistry. Selective direct methods have been reported by Roelfes and co-workers, applying DNA based Cu^{II} catalysts[14-16] or the use of a protein as chiral ligand.[17] However, they are limited to α,β-unsaturated 2-acyl imidazoles as substrates and yield the corresponding alcohols in moderate enantiomeric purities. The only chemo-catalytic process run on industrial scale was the addition of water to acrolein.[4] Nevertheless, due to its poor selectivity and productivity, even this seemingly straightforward reaction has been replaced by a fermentative process.[4,18]

In contrast, enzymes such as fumarase, malease, citraconase, aconitase and enoyl-CoA hydratase have been successfully used on industrial scale, and their excellent (enantio-) selectivities are highly valued.[4,19] Unfortunately, most hydratases are part of the primary metabolism where perfect substrate specificity is required. This very high substrate selectivity severely limits their practical applicability in organic synthesis.[5] A recent report on an asymmetric hydration of hydroxystyrene-type substrates catalyzed by phenolic acid decarboxylases showed that a broader flexibility in the substrate spectrum for hydratases is possible.[20,21] In order to broaden the biocatalytic toolbox of hydratases, the work represented herein is dedicated to the search for a Michael hydratase with a more relaxed substrate specificity.

In our search for a straightforward approach for the preparation of β-hydroxy carbonyl compounds via the direct Michael addition of water, it was noted that whole-cells of *Rhodococcus rhodochrous* ATCC 17895 convert 3-methylfuran-



SCHEME 1. Biotransformation of **1a** to **2a** by *R. rhodochrous* by Michael addition of water or alternatively by a reduction-oxidation stepwise approach.[22,26]

2(5*H*)-one **1a** into (*S*)-3-hydroxy-3-methylfuranone **2a**; as briefly described[22] in 1998. Neither substrate **1a** nor product **2a** are part of the primary metabolism indicating the involvement of a putative Michael hydratase with possibly a broader substrate scope. Since whole cells were used in this transformation, the hydratase activity needed to be critically evaluated.[22-25] Instead of a direct addition of water, the conversion of **1a** to **2a** could also occur *via* a two-step approach (Scheme 1). Indeed, the enantioselective hydroxylation of a range of THF and THP derivatives was reported for *R. rhodochrous* strains.[26] Therefore, it is highly interesting to probe whether the conversion of **1a** to **2a** is actually a Michael addition of water and how broadly it is applicable.

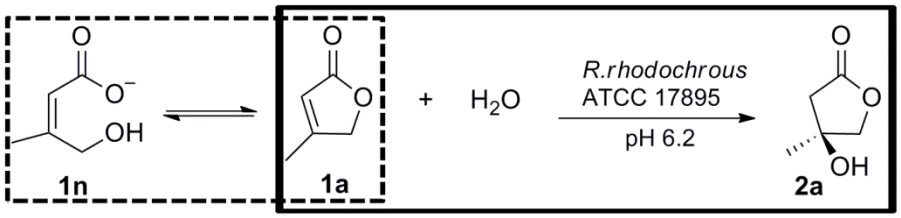
Herein we report the results of screening several *Rhodococcus* strains as promising biocatalysts for the enantioselective Michael addition of water to a variety of α,β-unsaturated carbonyl compounds.

2.2 RESULTS AND DISCUSSION

2.2.1 Optimization

To fully assess the potential of the putative Michael addition of water, the previously reported conversion of 3-methylfuran-2(5*H*)-one (**1a**)[22] was

TABLE 1. Influence of the catalyst concentration on the conversion.



	Catalyst	Catalyst conc. (wet cells)	Substrate	Conversion [a] of **1a** [%]	Yield [a] of **2a** [b] [%]	*ee* [a] of **2a** [%]
this study	resting cells	100 mg mL^{-1}	**1a**	69	57	91
	resting cells	330 mg mL^{-1}	**1a**	99	87	90
ref.[22]	resting cells	100 mg mL^{-1}	**1a**	55	55	95
control 1	resting cells [c]	330 mg mL^{-1}	**1a**	-	<3	n.d. [f]
control 2	denatured cells [d]	330 mg mL^{-1}	**1a**	12 [e]	<3	n.d. [f]

[a] Conversion, yield and *ee* values were determined by GC; [b] absolute configuration of **2a** has been established by converting **2a** into the corresponding methyl ester [methyl *S*-(-)-3,4-dihydroxy-3-methylbutanoate];[23,24] [c] reaction with **3a** was carried out to rule out possible oxidation; [d] reaction with heat-denaturated cells was carried out to ensure no background reaction is taking place; [e] conversion is caused by the ring opening of lactone **1a**, no water addition product (**2a**) was detected. [f] n.d. = not determined.

repeated and optimized. **1a** was synthesized using a modified literature procedure.[27] Whole-cells of *R. rhodochrous* ATCC 17895 were used in two different concentrations (100 mg mL^{-1} and 330 mg mL^{-1} of wet cells; Table 1). The reaction with 100 mg mL^{-1} cells gave a maximum conversion of 69% after 17 h and, even after a prolonged reaction time (4 days), no further increase in conversion was observed. Furthermore, an *ee* of 91% was determined, which is in agreement with the previously reported study.[22] An increase of the cell concentration to 330 mg mL^{-1} of wet cells resulted in full conversion after 17 h while *ee*-values remained unchanged (90%). When using **3a** as substrate under aerobic conditions (Table 1, control 1), no conversion to **2a** was detected, indicating that no oxidation occurs. In previous studies[6,25] we were able to show that a chemically catalyzed addition reaction occurs when 2-cyclohexenone (**1h**) is used as a substrate. Therefore any undesired background reaction needed to be ruled out. Heat-denaturated cell preparations in control experiments (Table 1, control 2) clearly show that

there is no chemically catalyzed reaction taking place; thus the reaction is effected by the active enzyme.

Encouraged by the complete conversion after 17 h, we evaluated the rate of the reaction with 330 mg mL^{-1} of wet cells. This revealed an almost linear increase in product formation during the first 6 h of the reaction and 75% of **2a** was formed in 75% yield (Figure 1A). Complete conversion based on the consumption of **1a** was obtained after 9 h. No significant changes of the product *ee* within the first 9 hours were observed. It should be noted that, since the desired Michael addition products (**2a**) are highly soluble in water, the choice of the organic solvent for extraction is crucial. For example, using ethyl acetate or dichloromethane as the extraction solvent only allowed recovery of 30% of the product (data not shown). In extraction studies isoamyl alcohol gave the best result for extraction of (*S*)-3-hydroxy-3-methylfuranone (**2a**). However, due to the similar polarity of isoamyl alcohol and water addition product **2a**, severe problems, such as separation issues during purification, arose. Therefore, for preparative scale experiments, reaction mixtures were always continuously extracted overnight using a liquid-liquid extractor and ethyl acetate as the organic solvent. This procedure had no influence on the *ee* values of the product (data not shown).

The temperature profile of the reaction was evaluated as well. Temperatures ranging from 18 °C to 48 °C were tested. Conversions and *ee* values at different temperatures are summarized in Figure 1B. When increasing the temperature above 28 °C a decrease in enzyme activity was observed. At 48 °C, a yield of only 5% was detected (an additional 12% was brought about by ring open of lactone **1a**). Due to the low amount of product at 48 °C, no reliable *ee* determination was possible. Taking both the conversion and enantioselectivity into account, the best results were achieved at 28 °C. These results are in agreement with the reported optimal growth temperature of 26 °C for *R. rhodochrous*.[28]

Since water serves not only as the reaction media but also as a substrate, the pH needs to be considered as a very important parameter. To quantify this effect, the reaction system was tested at different pH values using potassium phosphate buffer (pH 5.2-8.2) and citrate/phosphate buffer (pH 4.2) to control the pH of the reaction medium. The results from this study clearly show the dependence on the pH. The conversion increased with increasing pH

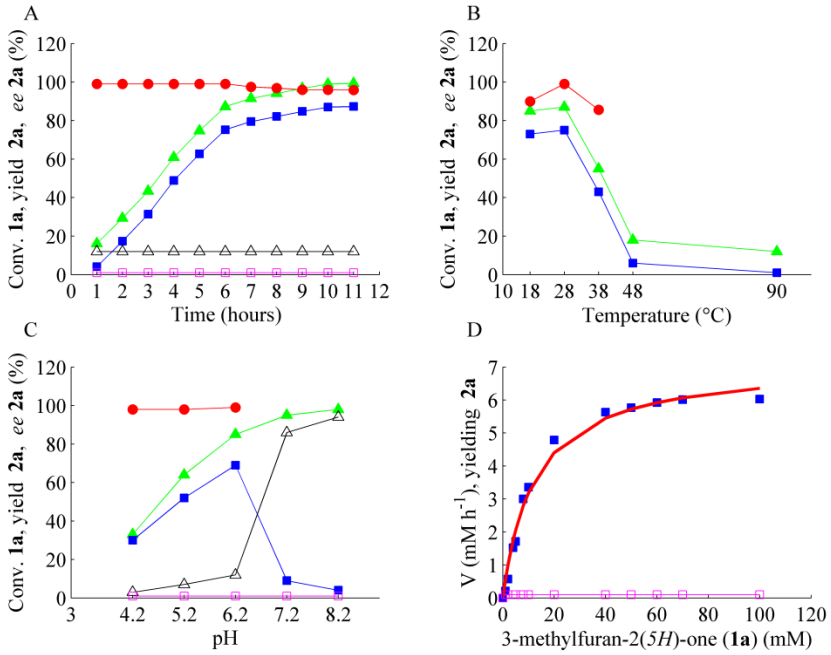


FIGURE 1. Time course (A), temperature profile at reaction 6h (B), pH profile at reaction 6h (C) and Michaelis-Menten kinetics (D, based on the yield of **2a**) of the putative Michael addition catalyzed using whole cells of *R. rhodochrous* ATCC 17895. For reaction conditions see Experimental Section. Conversion, yield and *ee* values were determined by GC. Filled circles represent *ee* of **2a**. Filled triangles represent consumption of **1a**. Filled squares represent yield of **2a**. Empty triangles represent consumption of **1a** in blank reactions. Empty squares represent yield of **2a** in blank reactions (in A and D, blank reaction was carried out with heat-denaturated cells; in C, blank reaction was carried out without the addition of cells).

(Figure 1C, filled triangles) as expected from our previous study[6] demonstrating that the hydration reaction is in general base-catalyzed. However, at neutral and slightly basic conditions (pH 7.2 and pH 8.2), significant ring opening of lactone **1a** took place (Figure 1C, empty triangles), which explains the rather poor product yield (Figure 1C, filled squares). This effect can be explained by the spontaneous hydrolysis of the lactone in basic aqueous medium, which is an often observed phenomenon.[29] To confirm this, the blank reaction mixtures at pH 7.2 and 8.2 were acidified with conc. HCl to pH 1. This leads to complete recovery of the substrate **1a**, validating our hypothesis. No desired enantioselective Michael addition product **2a** was

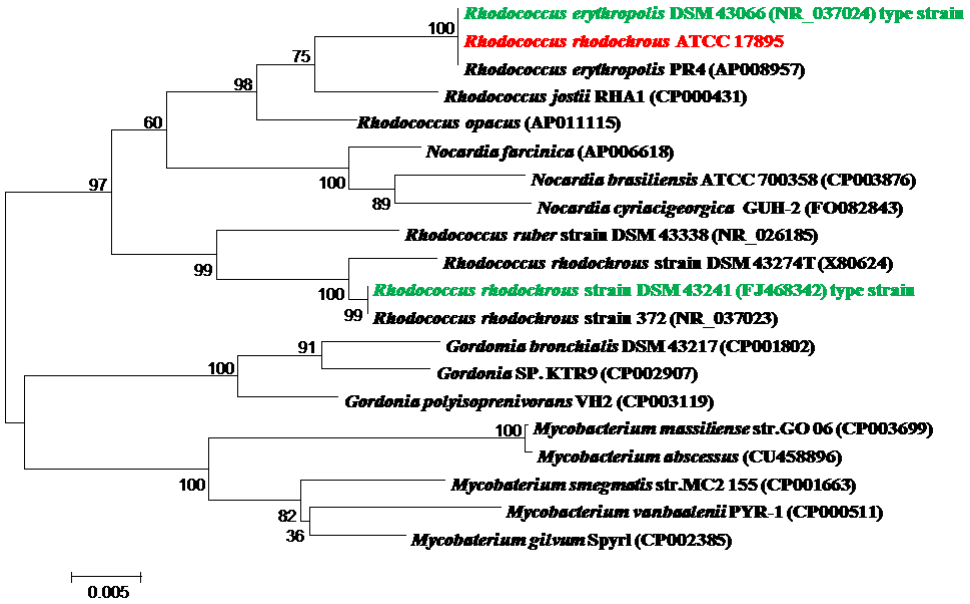
detected in the blank reactions (Figure 1C, empty squares) indicating that chemical/base-catalyzed Michael addition does not occur in the measured pH ranges. Therefore, the conversion in the blank reactions (which is based on the decrease in amount of substrate) is caused by the hydrolysis of **1a**. Moreover, product **2a** showed good stability under strongly acidic conditions and only 10% yield was lost overnight. Comparing the mass balance and reaction rate, slightly acidic condition (pH 6.2) represented the optimal pH for this substrate.

Control experiments (Table 1 and Figure 1A-C) confirmed that the formation of **2a** is based on an enzymatic reaction with high enantioselectivity and that no chemical background reaction occurred. Therefore, the kinetic parameters K_m, V_{max}, V_{max}/K_m were determined with the optimized conditions. The Michaelis-Menten Plot (Figure 1D) allows to calculate the affinity constant (K_m) as 1.7 x 10^{-2} M and V_{max} as 6.9 nmol • s^{-1} • g^{-1} (wet cells), providing further support for one enzymatic reaction, rather than a sequence of reactions (Scheme 1).

In order to establish the distribution of the enzymatic activity over different organisms, we proceeded with testing different closely related *Rhodococcus* strains. The selection was based on phylogenetic analysis (Table 2). The previously reported strain *R. rhodochrous* ATCC 17895 was shown to be much more closely related to *R. erythropolis* than to *R. rhodochrous.*[17] For this reason, strains *R. erythropolis* DSM 43296, *R. erythropolis* CCM 2595, *R. erythropolis* NBRC 100887 and *R. erythropolis* DSM 43066 were evaluated (Table 2). Experiments for comparing the different organisms were carried out under conditions optimized for *R. rhodochrous* ATCC 17895. Gratifyingly, in each case, 3-methylfuran-2(5*H*)-one (**1a**) was converted into (*S*)-3-hydroxy-3-methylfuranone (**2a**) with good yields and excellent enantioselectivities. Encouraged by these results, the less closely related strain *R. rhodochrous* DSM 43241 was also tested for water addition activity. Interestingly, the enantiomerically enriched water-addition product (*S*)-3-hydroxy-3-methylfuranone **2a** was also obtained in 75% yield and with 86% *ee*, which was slightly lower than that with *R. erythropolis* strains. All the results suggest that this promising hydratase activity is not limited to *R. rhodochrous* ATCC 17895 but may be a general feature in several *Rhodococcus* strains. Taking the conversion, enantioselectivity, and available genome

TABLE 2. Comparison of closely related *Rhodococcus* strains.

Phylogenetic tree based on 16 rRNA



Entry[a]	Catalysts	Conversion[b] of **1a** [%]	Yield[b] of **2a** [%]	*ee*[b] of **2a** [%]	Genome sequence
1	*R. rhodochrous* ATCC 17895	87	75	95	+
2	*R. erythropolis* DSM 43296	82	70	93	–
3	*R. erythropolis* CCM 2595	88	76	95	+
4	*R. erythropolis* NBRC100887	80	68	93	+
5	*R. erythropolis* DSM 43066	90	78	95	–
6	*R. rhodochrous* DSM 43241	87	75	86	–
7	90 °C heat-denaturated cells of *R. rhodochrous* ATCC 17895	12	<3	n.d.	

[a] list of entries comparing activities using different organisms. [b] Conversion, yield, and *ee* values were determined by GC.

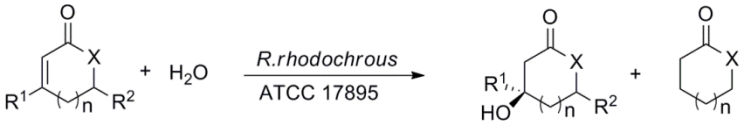
sequence into account, we decided to continue to use strain *R. rhodochrous* ATCC 17895 for all further studies.

2.2.2 Substrate scope and limitations

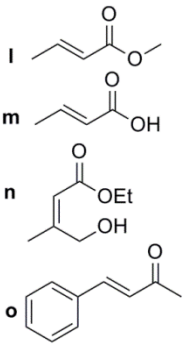
Since the very limited substrate scope of the known hydratases is one of the challenging factors for their broad application, we were interested in the scope of the Michael hydratase from *R. rhodochrous* ATCC 17895. Neither substrate **1a** nor product **2a** are known to be part of primary metabolic pathways, therefore the substrate scope of the hydratase from *R. rhodochrous* ATCC 17895 might be more relaxed than that for other known hydratases. Hence we tested a set of different substrates to evaluate the limitations of the enzyme (Table 3). For α,β-unsaturated lactones (X=O; Table 3, entries 1-3), with substituents in the β-positions, the reaction proceeded smoothly in all cases to yield the corresponding hydration products, whereas for R^1=H (X=O; Table 3, entries 4 and 5), no water addition product was obtained. This result is surprising, as the tertiary alcohols obtained are sterically much more demanding than the secondary alcohols, and suggests that substituents in the β-position might play an important role for proper orientation of the lactones in the enzyme's active site. However, the enzyme did not accept substrates with substituents in both β- and γ-positions, such as **1f** (Table 3, entry 6), which is probably due to its more bulky structure. Products **2a** and **2b** are tertiary alcohols, representing a class of compounds that are difficult to prepare by chemical methods, to date only accessible *via* this route.[22] The enantioselectivity was measured using a chiral Ivadex 7 / PS086 GC column and, in parallel, the *ee* was confirmed by analysis of ^{1}H and ^{19}F NMR of their corresponding Mosher esters. In both cases, results from ^{1}H and ^{19}F NMR of the Mosher esters and chiral GC analysis of alcohols were comparable, showing excellent enantioselectivities. The absolute configuration of the product was established by converting **2a** into the corresponding methyl ester [methyl (*S*)-(-)-3,4-dihydroxy-3-methylbutanoate].[23,24]

Interestingly, the hydration of substrate **1c** (Table 3, entry 3) gives access to the natural product mevalonolactone **2c**, the salt form of which represents an intermediate in the pathway leading to terpenoids.[30] Absolute configuration of (*R*)-**2c** was determined by comparison with previously reported optical rotation data.[31] Mevalonate is a product of acetate metabolism and thus a key building block in the secondary metabolism.[32,33] To identify whether the putative Michael hydratase is a promiscuous enzyme of the mevalonate pathway, bioinformatics studies were performed. We have sequenced and annotated the genome of *R. rhodochrous* ATCC 17895 in another study[28]

TABLE 3. Substrate scope for the enantioselective Michael addition of water.



a $R^1 = CH_3$, $R^2 = H$, X = O, n = 0
b $R^1 = CH_2CH_3$, $R^2 = H$, X = O, n = 0
c $R^1 = CH_3$, $R^2 = H$, X = O, n = 1
d $R^1 = H$, $R^2 = H$, X = O, n = 0
e $R^1 = H$, $R^2 = H$, X = O, n = 1
f $R^1 = CH_3$, $R^2 = CH_3$, X = O, n = 0
g $R^1 = H$, $R^2 = H$, X = C, n = 0
h $R^1 = H$, $R^2 = H$, X = C, n = 1
i $R^1 = H$, $R^2 = H$, X = C, n = 2
j $R^1 = CH_3$, $R^2 = H$, X = C, n = 0
k $R^1 = CH_3$, $R^2 = H$, X = C, n = 1

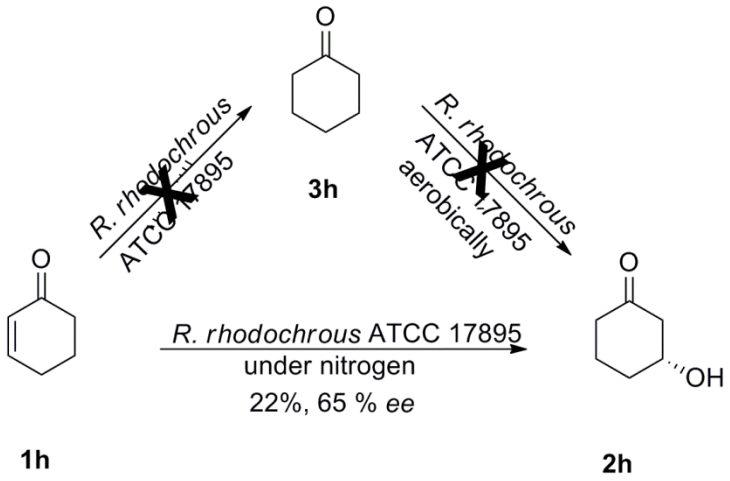


Entry	Substrate	Product	Conversion[a] of **1** [%]	Yield[a] of **2** [%]	*ee* of **2** [%]	Enantio-preference	Equilibrium yield of **2** [%]
1	**1a**	**2a**	87	75	95[c,d]	*S*[e]	>97[i]
2	**1b**	**2b**	80	68	94[d]	*S*	—[j]
3[b]	**1c**	**2c**	75	62	73[c]	*R*[f]	—[j]
4	**1d**	**2d**	12	<3	n.d.	n.d.	—[j]
5	**1e**	**2e**	32	<3	n.d.	n.d.	—[j]
6	***rac*-1f**	**2f**	12	<3	n.d.	n.d.	—[j]
7	**1g**	**2g**	93	18	22[c]	*R*[g]	—[j]
8	**1h**	**2h**	98	22	65[c]	*R*[g]	25[k]
9	**1i**	**2i**	95	15	20[c]	*R*[h]	—[j]
10	**1j**	**2j**	<3	<3	n.d.	n.d.	—[j]
11	**1k**	**2k**	<3	<3	n.d.	n.d.	—[j]
12	**1l**	**2l**	42	40	48[c]	*R*[f]	—[j]
13	**1m**	**2m**	<3	<3	n.d.	n.d.	—[j]
14	**1n**	**2n**	<3	<3	n.d.	n.d.	—[j]
15	**1o**	**2o**	<3	<3	n.d.	n.d.	—[j]

[a] Conversion and yield were determined by GC; [b] reaction was performed at pH 5.2 to suppress ring open of lactone **1c** at pH 6.2; [c] *ee* was determined by GC; [d] *ee* was determined by ^1H and ^{19}F NMR of the corresponding Mosher ester; [e] changing CIP priorities; [f] (*R*)-enantiomers commercially available. [g] absolute stereochemistry was determined by converting them into literature known derivatives, following a procedure established earlier in our lab;[36,37] [h] absolute configuration was determined by comparison the retention times using the same GC column with a reported method;[38] [i] reverse reaction with **2a** as substrate was performed, analysis of this sample showed no dehydration and no decrease of the *ee*; [j] no literature values available; [k] see references. [6,35]

(Chapter 3). Looking for annotated hydratases in this genome only showed known hydratases with their narrow substrate specificity, emphasizing the hydratase of this study has not been described before. This enzyme could therefore be one of the many unknown gene functions in the genome, or a promiscuous activity of a known enzyme. Screening through all 3 sequenced Rhodococci genomes (Table 2, entry 1, 3 and 4), we unexpectedly found that most of the typical enzymes from the mevalonate pathway are missing. Instead the full deoxyxylulose phosphate (mevalonate-independent) pathway for terpenoid biosynthesis was present.

For α,β-unsaturated ketones (X=C), substrates without substituent in the β-position were surprisingly accepted by the putative Michael hydratase (Table 3, entries 7-9) but no activity towards the β-substituted 3-methylcyclohex-2-enone and 3-methylcyclopent-2-enone was found (Table 3, entry 10 and 11). This might lead to the conclusion that substituent in β-position in α,β-unsaturated ketones may be challenging for Michael addition of water using *R. rhodochrous*, although the opposite is true for α,β-unsaturated lactones. The α,β-unsaturated ketones **1g-i**, were mostly reduced into ketones **3g-i** (75%, 76% and 80%, respectively), which explains the rather poor yield of the water-addition reaction (Table 3, entry 7-9). Experiments to rule out the reduction-oxidation as a possible reaction pathway were performed for these cyclic ketones (Scheme 2). Reaction using **1h** as substrate was performed



SCHEME 2. Control experiments to confirm that **2h** was formed by enzymatic water addition, rather than a reduction-oxidation sequence.

under a nitrogen atmosphere to exclude air as a potential oxygen source. Even so, 22% yield of **2h** was obtained with 65% *ee*, ruling out that the involvement of O_2 as an active species in the reaction. Furthermore, when **3h** was used as a substrate directly under aerobic condition, no product **2h** was detected. These two control experiments demonstrate that the alcohol **2h** was the result of the enantioselective Michael addition of water to **1h**. The competing reduction reaction to **3g**, **3h** and **3i** is most likely due to an ene reductase also present in the *Rhodococcus* cells.

To further probe whether the putative Michael hydratase also accepts acyclic α,β-unsaturated carbonyl compounds, methyl crotonate (**1l**), crotonic acid (**1m**), (*E*)-ethyl-4-hydroxy-3-methylbut-2-enoate (**1n**) and benzylideneacetone (**1o**) were subjected to the resting cell suspensions (Table 3, entries 12-15). Gratifyingly, the enzyme readily accepted acyclic α,β-unsaturated ester (**1l**), although no activity was observed for acyclic α,β-unsaturated carboxylic acid **1m**, ester **1n**, or ketone **1o**. Notably, in many water-addition reactions to carbon-carbon double bonds, the equilibrium can be on the side of the starting material although the reaction is performed in water.[4,16,34] The unfavourable equilibrium might impede the Michael addition of water; for example, the equilibrium yield of 3-hydroxycyclohexanone (**2h**) was determined to be 25% (Table 3, entry 8),[6,35] corresponding with the measured yield of 22%.

Finally we tested the scalability of the developed reaction system. Therefore the reaction was scaled to gram scale using **1a** (2 gram, 20 mmol, 200 gram of wet cells) to give **2a** in 69% isolated yield after column chromatography and an ee of 90% was determined.

2.2.3 Recyclability and enzyme investigation

One of the most important characteristics of a catalyst is its operational stability and reusability over an extended period of time, to ensure a practical application.[39,40] From the viewpoint of process economics, the higher the number of cycles that an enzyme remains stable, the more efficiently a process can be run. Experiments were performed to examine this recyclability of the whole-cells of strain *R. rhodochrous* ATCC 17895 for the Michael addition of water to 3-methylfuran-2(5*H*)-one (**1a**). Based on the results summarized in Table 1, every reaction was carried out in a 50 mL Erlenmeyer flask at 28 °C

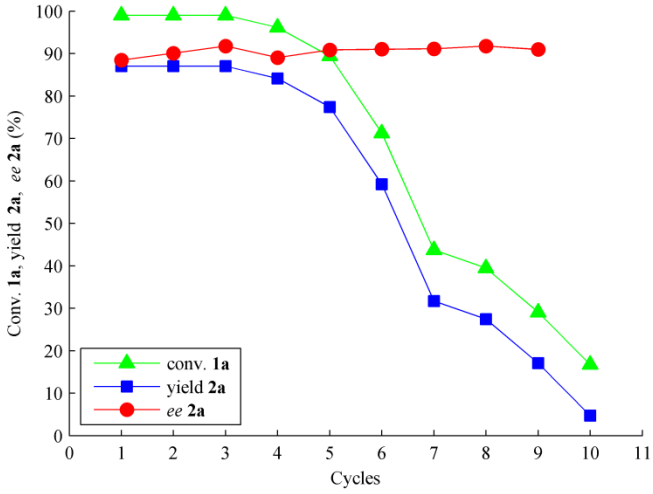


FIGURE 2. Repeated water-addition reactions catalyzed by whole cells of *R. rhodochrous* ATCC 17895. Conversion, yield and *ee* values were determined by GC.

with 180 rpm for 23 hours. At the end of the reaction, the cells were centrifuged, washed twice with potassium phosphate buffer (100 mM, pH 6.2), and then reused for the next cycle. Whole cells showed high activity and complete conversion for 4 cycles (Figure 2). Only a slight decrease was observed in cycle 5, whereas 27% lower conversion was detected in the cycle 6. However, even after 9 consecutive cycles, the whole cells retained 20% of the initial activity. Notably, no significant changes in enantioselectivities of the water-addition reactions were detected during the 9 cycles.

To isolate the putative Michael hydratase for further investigation, we first broke the whole cells of *R. rhodochrous* ATCC 17895. The desired hydratase activity (yielding **2a**) was only found in the cell pellets, rather than in the cell-free extract, when **1a** was used as a substrate (Figure 3). Furthermore, no significant difference was found between the initial rate of whole cells and pelleted cell debris (Figure 3). Additionally, with **1h** as a substrate, most of the ene reductase activity (Table 3, entries 7-9) was retained in the cell-free extract, whereas only minor activity was still detectable in the cell pellets. These results indicate that the putative Michael hydratase is not a soluble protein but bound to either the membrane or cell wall, whereas the reductase activity resides apparently within another enzyme that is soluble. This natural

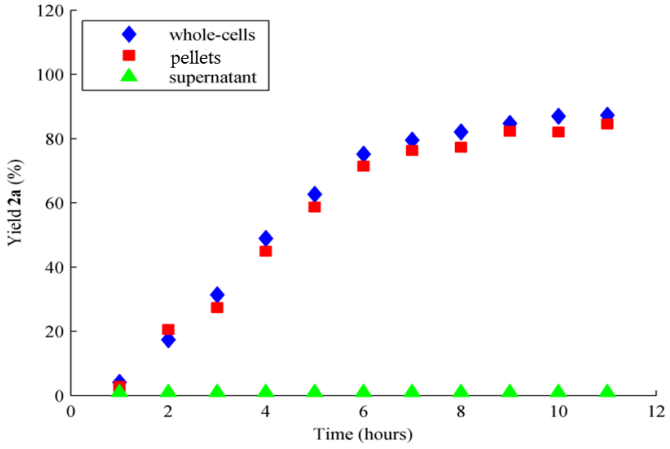
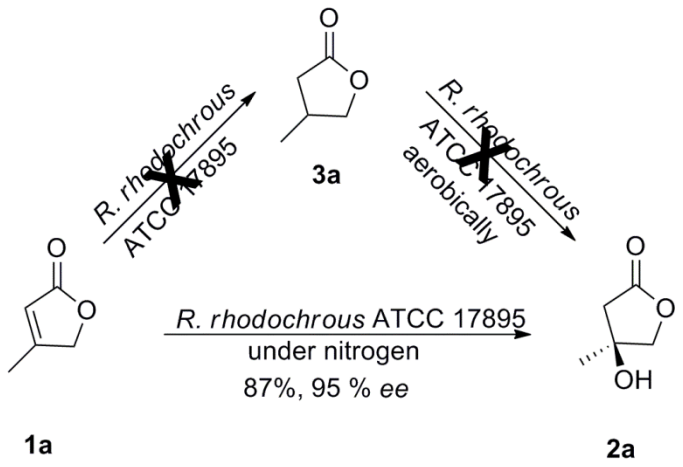


FIGURE 3. Michael hydratase activities in different biocatalyst preparations (whole cells, pelleted cell debris and cell-free extract). Conversion was determined by GC.

immobilization of the putative Michael hydratase explains the high reusability of the whole cells (Figure 2).

2.2.4 Mechanistic studies

As mentioned above, *Rhodococci* have been shown to mediate the hydroxylation of unactivated C-H bonds on selected THF and THP derivatives.[26] To clearly rule out the possibility of detecting a hydroxylation reaction instead of a hydration, we turned our attention to the mechanism, including the stereochemical course of the reaction. The reaction was performed under a nitrogen atmosphere to exclude air as a potential oxygen source. Under these conditions, 87% conversion with 95% enantioselectivity was still obtained, ruling out that O_2 is involved as an active species in the reaction. The second approach included the use of substrate **3a**, which might be formed by reduction of **1a**. Therefore **3a** was synthesized according to a standard procedure.[41] When using **3a** with a resting cell suspension, no corresponding oxidation product was detected under aerobic conditions (Scheme 3). These two control experiments demonstrated that the enantiomerically enriched tertiary alcohol **2a** was the result of the direct enantioselective Michael addition of water to **1a**.



SCHEME 3. Control experiments to confirm that **2a** was formed by enzymatic water addition, rather than a reduction-oxidation sequence.

The stereochemical course of this water-addition reaction was further evaluated by carrying out the biotransformation in D_2O using lyophilized cells as catalyst. The reaction in D_2O was found to be slower than that in H_2O, which might be due to activity loss caused by the lyophilization or an isotope effect. However, upon elongation of the reaction time to 24 hours, deuterium oxide-addition product **4a** was found at a conversion of 90%. After extraction with ethyl acetate and column purification, compound **4a**, containing the optically active OD group was exchanged back into an OH group, which is an often observed phenomenon.[43-46] NMR and GC-MS measurements showed that the compound obtained (**4b**) contained one deuterium at the α-carbon (Figure 4A). In the ^{1}H NMR spectrum of **2a**, the geminal coupling constant between the two α-protons is 17.6 Hz, whereas the ^{1}H NMR spectrum of **4b** showed only one α-proton, which indicates one deuterium at the α-carbon. Comparing with the singlet signal of **2a** in the ^{13}C NMR spectrum, the triplet signal (coupling constant of 19.75 Hz) of **4b** indicates one deuterium at the α-carbon. GC-MS spectra also show **4b** to be one unit heavier than **2a**. A control experiment was performed by shaking pure 3-hydroxy-3-methylfuranone (**2a**) in D_2O. Analysis of this sample showed that no deuterium was incorporated into the α-position; hence, the ^{2}H-labelled product **4b** must have resulted from enzymatic water addition. This further supports a one-step hydration mechanism, *via* a Michael addition reaction.

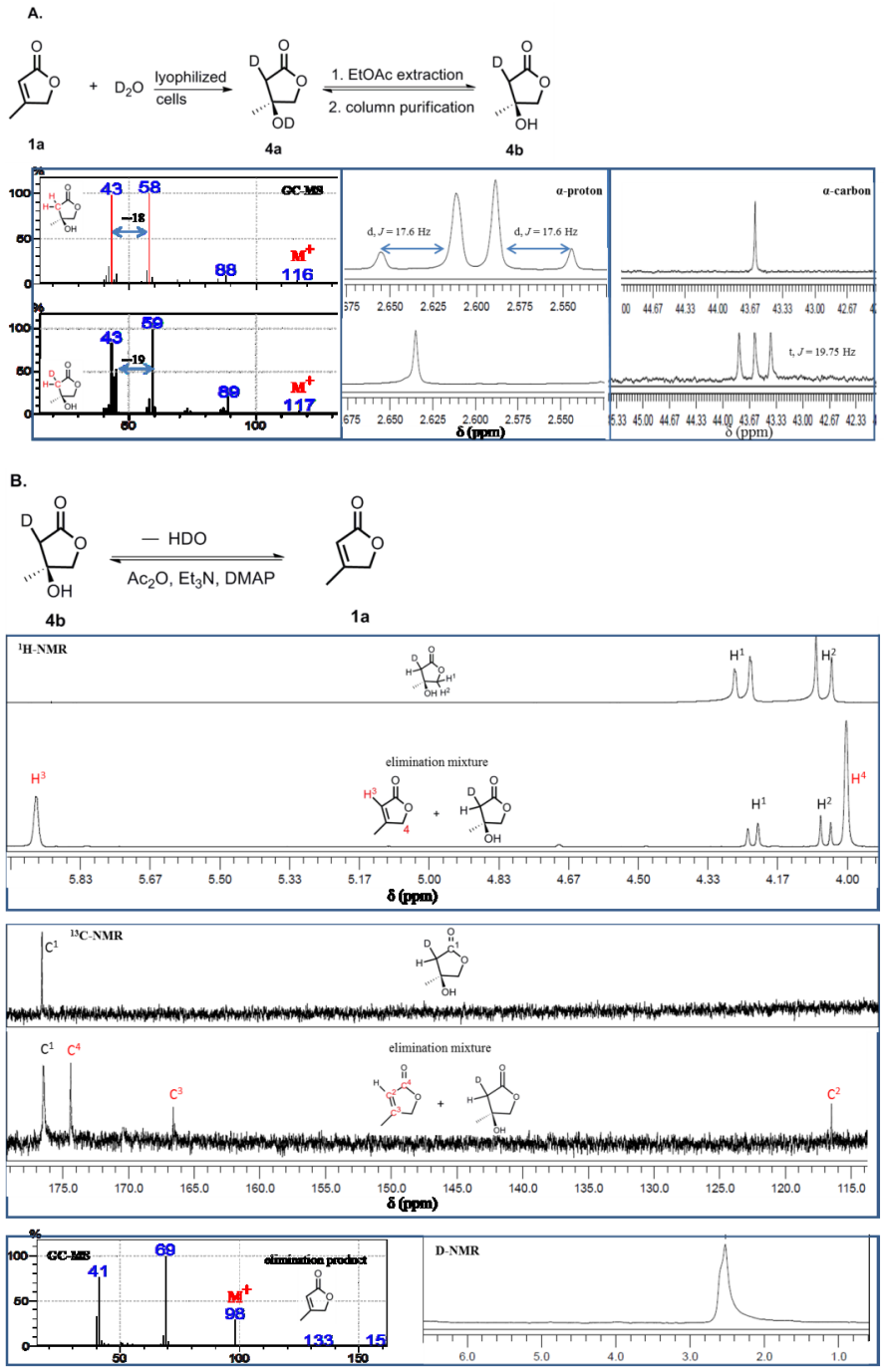


FIGURE 4. Diastereoselective Michael addition of water catalysed by lyophilized cells of *R. rhodochrous* ATCC 17895.

According to the NMR measurements, the reaction of substrate **1a** and lyophilized *R. rhodochrous* ATCC 17895 cells in D_2O yielded monodeuterated **4b** as a sole diastereoisomer. The observation brought us to investigate the diastereospecificity of the Michael addition of water, which until now was reported to show, depending on the enzyme, either *syn* or *anti* preference.[47,48] For example in the case of enoyl-CoA hydratase, selectivity towards *syn*-addition was observed,[49] whereas enzymes belonging to the aspartase/fumarase superfamily, such as fumarase, aconitase, or enolase, show *anti*-preference.[50,51]

Nuclear Overhauser Effect (NOE) experiments unfortunately did not give conclusive results on the stereochemical course of the water addition. To further probe the stereoselectivity of the addition of water, an *anti* E2 elimination of the deuterium oxide addition product **4b** was performed.[52,53] The reaction was accomplished with acetic anhydride/triethylamine in the presence of a catalytic amount of DMAP and the corresponding product was measured directly by NMR and GC-MS without any further purification. The results showed **1a** as the only elimination product, HDO being expelled during the elimination process (Figure 4B). ^{1}H NMR spectroscopy showed the appearance of signal for α proton at 5.91 ppm, indicating that HDO was eliminated *via* an *anti* E2. The appearance of singlet signal (which was visible as a triplet at 43.6 ppm previously in **4b**) at 116.2 ppm for the α carbon in the ^{13}C-NMR spectrum also proved the loss of molecular HDO. The D-NMR showing only one peak at 2.60 ppm, which belongs to the unconverted **4b**, but no peak at 5.91 ppm, again proving that HDO was eliminated. This elimination was further confirmed by GC-MS analysis. Since the E2 elimination always occurs exclusively in *anti* fashion, and removed α-D and the β-OH groups, the enzymatic D_2O addition must have proceeded with exclusive *anti* stereochemistry. Our results are supported by the findings of Mohrig et al.,[47,51] who described that the stereopreference of water addition depends on the position of the abstracted proton: if the proton is in α-position to the carboxylate group, as in the case of our studies, *anti*-selectivity is observed; abstracted protons that are in the α-position to the carbonyl group of the thioester lead to *syn*-selectivity.[47,51] In summary, the new Michael hydratase belongs to the family members of hydratases: oleate hydratase, fumarase, malease, aconitase and type II dehydroquinase with a preference for the *anti*-addition; whereas, type I dehydroquinase,

enoyl-CoA hydratase and artificial hydratase exclusive prefer for the *syn*-addition as discussed in Chapter 1.

2.3 CONCLUSIONS

β-Hydroxy carbonyl compounds represent an important class of compounds that is often found as a structural motif in natural products. Although the molecules themselves look rather simple, their synthesis can be challenging. A straightforward route for the preparation of chiral β-hydroxy carbonyl compounds was established employing whole cells from several *Rhodococcus* strains harboring a Michael hydratase. They catalyzed the enantioselective Michael addition of water in water with good yields and excellent enantioselectivities. Compared to the very narrow substrate scope of known hydratases, the particularly intriguing feature and advantage of this new hydratase is its broad substrate range; α,β-unsaturated lactones with substituent in β-position (**1a**, **1b** and **1c**), α,β-unsaturated cyclic ketones with no substituent in β-position (**1g**, **1h** and **1i**), and an α,β-unsaturated ester (**1l**). A series of control experiments and deuterium labelling studies demonstrate that the reaction is diasterospecific, with only *anti* hydration product formed. The biocatalytic reaction system was carefully optimized for gram-scale synthesis, resulting in good conversions and excellent enantioselectivities. Under the optimized conditions, whole cells could be reused for 4 cycles without significant loss of activity while maintaining up to 90% *ee*. Our study suggests that this promising Michael hydratase is not soluble but membrane-bound or cell wall-associated. In summary, whole cells from *Rhodococcus* strains are able to catalyze the enantioselective Michael addition of water to several different substrates using water as both solvent and substrate under mild conditions. This opens up an entirely new approach to the synthesis of chiral 3-hydroxy carbonyl compounds.

2.4 EXPERIMENTAL

2.4.1 Material and Method

All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) and were used without further purification unless otherwise specified. The culture

media components were obtained from BD (Becton, Dickinson and Company, Germany).

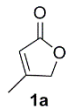
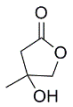
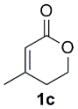
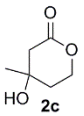
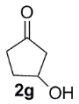
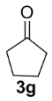
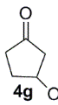
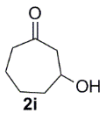
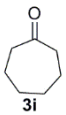
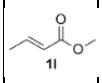
^{1}H, ^{2}H, ^{13}C and ^{19}F NMR spectra were recorded with Bruker Advance 400 or Varian 300 (400 MHz, 61.4 MHz, 100 MHz and 376.33 MHz, respectively) instrument and were internally referenced to residual solvent signals. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration, coupling constant (Hz) and assignment. Data for ^{13}C NMR and ^{19}F NMR are reported in terms of chemical shift. Optical rotations were obtained at 20 °C with a Perkin-Elmer 241 polarimeter (sodium D line). Column chromatography was carried out with silica gel (0.060-0.200 mm, pore diameter ca. 6 nm) and with mixtures of petroleum ether (PE) and ethyl acetate (EtOAc) as solvents. Thin-layer chromatography (TLC) was performed on 0.20 mm silica gel 60-F plates. Organic solutions were concentrated under reduced pressure with a rotary evaporator.

Conversion of substrates and yield of products were quantified by GC using calibration lines with dodecane as an internal standard (specifications and temperature programs given in Table 4) and the optical purity of the products [excepted for **2b**] were determined using chiral GC (specifications and temperature programs given in Table 4). The enantiomeric excess (*ee*) of **2b** was determined by ^{1}H and ^{19}F of the corresponding Mosher ester.

2.4.2 Microorganisms and culture conditions

Rhodococcus rhodochrous ATCC 17895 was purchased from ATCC (American Type Culture Collection, Manassas, USA). *Rhodococcus erythropolis* DSM 43296, *Rhodococcus erythropolis* DSM 43060, *Rhodococcus erythropolis* DSM 43066, *Rhodococcus rhodochrous* DSM 43241 were purchased from DSMZ (Germany). *Rhodococcus erythropolis* PR4 NBRC 100887 was purchased from NBRC (Biological Resource Centre, Chiba, Japan). The organisms were maintained on agar plates at 4 °C and these were subcultured at regular intervals. The medium used for cultivation[22] contained Solution A (980 mL) with potassium dihydrogen phosphate (0.4 g), dipotassium hydrogen phosphate (1.2 g), peptone (5 g), yeast extract (1 g), glucose (15 g), final pH = 7.2, sterilized at 110 °C in an autoclave; Solution B (10 mL) with magnesium

TABLE 4. GC methods for all compounds
Column A: CP-Wax 52 CB, 50 m x 0.53 mm x 2 μm
Column B: Chiral Ivadex7/PS086, 25 m x 0.25 mm x 0.25 μm
Column C: Chiraldex GTA, 50 m x 0.53 mm x 0.12 μm

Sub-strate	Product	Analysis Column	Temperature program and t_R [min] (for conversion) (using nonchiral column)	Temperature program and t_R [min] (for *ee*) (using chiral column)
 1a	 2a	A B	50/0.25/70/127/0.10/20/250/5.0 **1a** = 9.5; **2a** = 7.6	75/5.00/10/140/15.0/60/225/1.08 **2a** (*S*) = 18.5; **2a** (*R*) = 18.9
 1c	 2c	A B	50/0.25/70/127/0.10/20/250/5.00 **1c** = 9.6; **2c** = 8.6	100/3.00/35/165/13.14/60/225/1.00 **2c** (*R*) = 10.9; **2c** (*S*) = 11.3
 1g	 2g  3g  4g	A C	50/0.25/70/127/10.0/60/250/2.00 **1g** = 3.3 **2g** = 13.4 **3g** = 2.3	150/10.0 /25/170/1.20 **4g** (*R*) = 4.7 **4g** (*S*) = 4.9
 1i	 2i  3i	A B	50/0.25/50/155/4.00/60/250/2.00 **1i** = 4.2; **2i** = 9.1; **3i** = 3.3	80/3.00/5/100/1.00/1/160/1 **2i** (*R*) = 36.7; **2i** (*S*) = 37.5
 1l	 2l	A C	50/0.25/10/127/3.00 **1l** = 7.0; **2l** = 8.4	50/2.00/10/60/14.00/40/170/1.00 **2l** (*S*) = 13.8; **2l** (*R*) = 14.5

sulphate (0.5 g), filter sterilized; Solution C (10 mL) with iron(II) sulphate (0.3 g), filter sterilized. Solutions were mixed before inoculation to make 1 L medium with a buffer concentration of 3 mM. A loop of bacteria was used to inoculate 1 L medium in a 2 L Erlenmeyer flask. This culture was shaken reciprocally at 28 °C for about 72 h to an optical density (OD_{600}) of around 6.3. The cells of *Rhodococcus* strains were harvested by centrifugation at 10000 rpm and at 4 °C for 20 min. The supernatant was removed and the cells were rinsed with potassium phosphate buffer (100 mM, pH 6.2) and centrifuged again. The supernatant was discarded and the pellets were stored at -20 °C. When needed, the wet pellets were freeze dried overnight and collected as lyophilized cells.

2.4.3 Biotransformation

2.4.3.1. General biotransformation procedure for catalyst concentration study

Cells in the culture age of OD_{600} = 6.3 were harvested by centrifugation, washed twice with 100 mM of potassium phosphate buffer (pH 6.2). Around 100 mg mL^{-1} or 330 mg mL^{-1} were resuspended in the same buffer (15 mL) containing 33 mM of 3-methylfuran-2(5*H*)-one (**1a**; 50 mg, 0.51 mmol). The resting cell reactions were carried out in screw-capped Erlenmeyer flasks. Reactions were shaken at 28 °C overnight (17 h). For the blank reaction the setup was the same but heat-denaturated cells (90 °C, 30 min) were used. For work-up, the cells were removed by centrifugation and 1 mL of the supernatant was saturated with NaCl followed by extraction with 2 x 0.5 mL of isoamyl alcohol (containing internal standard) by shaking for 5 min. The combined organic layer was dried over Na_2SO_4 and measured by GC for conversion, yield and *ee* (Table 1).

2.4.3.2 General biotransformation procedure for rate measurement

The reaction setup for rate determination was the same as for the catalyst concentration study. Duplicate experiments were performed respectively in potassium phosphate buffer (100 mM, 90 mL, pH 6.2) containing 33 mM of 3-methylfuran-2(5*H*)-one (**1a**; 300 mg, 3.06 mmol) and resting cells (330 mg mL^{-1}). For the blank reaction the setup was the same but heat-denaturated cells (90 °C, 30 min) were used. Reactions were allowed to proceed at 28 °C. Every 1 hour a 1.5 mL sample was taken from the reaction mixture. Cells were

removed by centrifugation and then 1 mL of the supernatant was saturated with NaCl followed by extraction with 2 x 0.5 mL of isoamyl alcohol (containing internal standard) by shaking for 5 min. The combined organic layer was dried over Na_2SO_4 and measured by GC for conversion, yield and *ee* (Figure 1A).

2.4.3.3. Reaction temperature study

The reaction setup for the temperature study was the same as for the rate determination. Reactions were performed in potassium phosphate buffer (100 mM, 15 mL, pH 6.2) containing **1a** (50 mg, 0.51 mmol) and wet cells (330 mg mL^{-1}) at given temperatures for 6 h. Workup and analysis were as described above in General biotransformation procedure for rate measurement (Figure 1B).

2.4.3.4. pH study

The reaction setup for the pH profile was the same as for the rate determination. Reactions were performed buffer (15 mL) containing **1a** (50 mg, 0.51 mmol) and wet cells (330 mg mL^{-1}) at given pH values (pH 5.2-8.2 were prepared as potassium phosphate buffers and pH 4.2 was prepared as citrate/phosphate buffer, all at a buffer strength of 100 mM) at 28 °C for 6 h. For the blank reactions the setup was the same but without the addition of whole cells. For substrate recovery studies, experiments were performed by dissolving 3-methylfuran-2(5*H*)-one (**1a**; 6.5 mg, 0.07 mmol) in buffer (pH 7.2 or pH 8.2, 2mL, 100 mM) and shaken at 28 °C for 6 h (the same condition as for the reaction), then 1 mL of the mixture was extracted directly while the remaining 1 mL was acidified with HCl to pH 1.0 before extraction. Workup and analysis were as described in General biotransformation procedure for rate measurement (Figure 1C).

2.4.3.5. Enzyme kinetic study

The reaction setup for the enzyme kinetic study was the same as for the rate determination. Reactions were performed in potassium phosphate buffer (100 mM, pH 6.2) at 28 °C for 2 h with various substrate concentrations (1, 2, 4, 5, 8, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100 mM) of **1a** and with wet cells (330 mg mL^{-1}). For the blank reaction the setup was the same but heat-denaturated cells (90 °C, 30 min) were used. Workup and analysis were as

described in General biotransformation procedure for rate measurement (Figure 1D).

2.4.3.6. General procedure for organisms activity screening

The reaction setup for organisms activity screening was the same as for the rate determination. Reactions were performed potassium phosphate buffer (100 mM, 30 mL, pH 6.2) containing **1a** (100 mg, 1.02 mmol) with whole cells of given organisms at 28 °C for 6 h. For the blank reaction the setup was the same but heat-denaturated cells (90 °C, 30 min) were used. Workup and analysis were as described in General biotransformation procedure for rate measurement (Table 2).

2.4.3.7. General procedure for substrate screening

Reactions were carried out as described in the General biotransformation procedure for rate measurement using the same concentration for each substrate. After extraction with isoamyl alcohol (2 x 0.5 mL) samples were dried over Na_2SO_4 and crude samples were analysed by GC when product reference material was available or GC-MS (Varian FactorFour VF-1ms column [25 m x 0.25 mm x 0.4 μm] and He as carrier gas) when product reference material were not commercially available (Table 3).

2.4.3.8. General procedure for recyclability

Reactions were carried out with substrate **1a** (50 mg, 0.51 mmol) in potassium phosphate buffer (100 mM, 15 mL, pH 6.2) and wet cells (330 mg mL^{-1}), shaken at 28 °C for 23 h. At the end of the reaction, cells were centrifuged at 4000 rpm for 20 min to be separated from the reaction mixture, then washed by potassium phosphate buffer (100 mM, pH 6.2), and resuspended in 15 mL of the same buffer containing the same substrates. The reaction mixture (1 mL of supernatant separated from cells) was saturated with NaCl and then extracted with 2 x 0.5 mL of isoamyl alcohol (containing internal standard) by shaking for 5 min. The combined organic phase were dried over Na_2SO_4 and crude samples were analysed by GC (Figure 2).

2.4.3.9. Activities comparison using pelleted cell debris and cell free extract

15 g of cells in the culture age of OD_{600} = 6.3 were harvested by centrifugation, washed twice with 100 mM of potassium phosphate buffer (pH 6.2) and resuspended in the same buffer (45 mL). The cells were incubated first with lysozyme (1 mg mL^{-1}, 4 °C, 1h) and subsequently disrupted using a French

press (2.05 kBar, 2 shots). Cell-free extract and cell debris were separated by centrifugation for 40 min at 10000 rpm at 4 °C. Substrate **1a** (150 mg, 1.53 mmol) was added to the supernatant (cell-free extract) and shaken at 28 °C (reaction A). Cell debris were resuspended in potassium phosphate buffer (100 mM, 45 mL, pH 6.2) containing the same concentration of substrates (reaction B). Every 1 h, a 1.5 mL sample was taken from both reaction A and B. For workup, the cells were removed by centrifugation and 1 mL of the supernatant was saturated with NaCl followed by extraction with 2 x 0.5 mL of isoamyl alcohol (containing internal standard) by shaking for 5 min. The combined organic layer was dried over Na_2SO_4 and measured by GC for conversion, yield and *ee* (Figure 3).

2.4.3.10. General acetylation procedure for ee determination

Biotransformation reaction was stopped by centrifugation to remove cells followed by extraction with isoamyl alcohol (see General biotransformation procedure for initial rate measurement). Organic phases were combined, dried with Na_2SO_4, and then were acetylated using DMAP and acetic anhydride. DMAP (5 mg) was dissolved in 5 mL of ethyl acetate. From this mixture 100 µL were added to the samples followed by addition of 100 µL acetic anhydride. The reaction was allowed to proceed for 1h at room temperature and was stopped by the addition of 0.5 mL water. After shaking for an additional hour, phases were separated and the organic layer was dried over Na_2SO_4. Samples were then measured on chiral GC for *ee* determination (for method see Materials and methods).

2.4.4 Synthesis

*2.4.4.1. (S)-3-hydroxy-3-methylfuranone (**2a**; preparative scale)*

For isolation and characterization of the Michael addition product, the reaction was carried out on preparative scale. Pelleted cells from 20 L medium were resuspended potassium phosphate buffer (100 mM, 600 mL, pH 6.2), and substrate 3-methylfuran-2(5*H*)-one (**1a**; 2 g, 20.38 mmol) was added. Reaction was incubated at 28 °C and shaken at 180 rpm for 24 h. Then the cells were removed by centrifugation and the supernatant was saturated with NaCl. Due to the high solubility of the resulting alcohols in water, continuous extraction with ethyl acetate was performed overnight. The extract was then

concentrated under reduced pressure and purified by flash column chromatography on silica gel (eluent: PE/EtOAc 1:1) to yield **2a** (1.63 g, 14.06 mmol, 69%) as a colourless oil; $[\alpha]_D^{20}$ +46.6 (*c* 0.96 in $CHCl_3$) [lit[22] $[\alpha]_D$ +53.92 (*c* 0.96 in $CHCl_3$)]; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.48 (s, 3H), 2.57 (d, *J* = 17.6 Hz, 1H), 2.63 (d, *J* = 17.6 Hz, 1H), 3.71 (s, 1H), 4.14 (d, *J* = 9.6 Hz, 1H), 4.27 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 24.94, 43.06, 74.70, 79.82, 176.27 (in accordance with literature[22]).

*2.4.4.2. (S)-3-hydroxy-3-ethylfuranone (**2b**; preparative scale)*

For isolation and characterization of the Michael addition product, the reaction was carried out on preparative scale. Using the biotransformation procedure described for **2a**, reaction of 3-ethylfuran-2(5*H*)-one (**1b**; 1 g, 8.92 mmol) gave **2b** (0.75 g, 5.80 mmol, 65%) as a colorless oil. $[\alpha]_D^{20}$ +49.6 (*c* 0.75 in $CHCl_3$), [lit[22] $[\alpha]_D$ +48.9 (*c* 0.72 in $CHCl_3$)]; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.98 (t, *J* = 7.6 Hz, 3H), 1.72 (q, *J* = 7.4 Hz, 2H), 2.49 (s, 1H), 2.53 (s, 2H), 4.13 (d, *J* = 9.6 Hz, 1H), 4.21 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 8.70, 31.69, 42.36, 78.05, 79.26, 176.82 (in accordance with literature[22]).

*2.4.4.3. [2-D]-3-hydroxy-3-methylfuranone (**4b**)*

3 g lyophilized cells were resuspended in 100 mL of D_2O containing 4 drops of potassium hydroxide solution (100 mM, final pD 6.5, corresponds to pH 6.1). **1a** (330 mg, 3.40 mmol) was added. The reaction mixture was shaken at 180 rpm, 28 °C for 24h, then centrifuged and the supernatant was saturated with NaCl and continuously extracted with ethyl acetate (200 mL) overnight. The extract was dried over Na_2SO_4 and evaporated under reduced pressure. The crude product mixture was purified using flash chromatography on silica gel (eluent: PE/EtOAc 1:1) yielding deuterium oxide addition product (*S*)-[2-^{2}H]-3-hydroxy-3-methylfuranone **4b** (265 mg, 2.28 mmol, 67%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.51 (s, 3H), 2.64 (s, 1H), 2.96 (s, 1H), 4.15 (d, *J* = 9.6 Hz, 1H), 4.28 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 24.96, 43.29 (t, $^1J_{C,D}$ = 19.8 Hz, CD), 74.61, 79.80, 176.49. m/z: 117 (M^+, 2), 89 (5), 86 (4), 74 (3), 60 (9), 59 (100), 58 (20), 57 (4), 44 (36), 43 (87), 42 (10), 41 (4), 40 (7).

*2.4.4.4. Dehydration of deuterium oxide addition product (**4b**)*

To a solution of alcohol **4b** (30 mg, 0.26 mmol) in EtOAc (1 mL) was slowly added acetic anhydride (35 µL) and triethylamine (60 µL), followed by 4-dimethylaminopydridine (50 µL of 3 mg mL^{-1} solution in EtOAc). The reaction

was allowed to proceed for 30 min at room temperature and was stopped by the addition of 0.5 mL of water. The phases were separated and the organic layer was dried over Na_2SO_4 and evaporated. The crude product was measured by NMR and GC-MS which showed the elimination product is 3-methylfuran-2(5*H*)-one (**1a**). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.96 (s, 3H), 4.00 (s, 2H), 5.94 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.02, 73.86, 116.23, 166.32, 174.1 (in accordance with literature[41,42]). m/z: 98 (M^+, 26), 71 (16), 70 (62), 69 (100), 68 (13), 67 (3), 55 (3), 54 (3), 53 (6), 52 (3), 51 (3), 50 (6), 45(10), 44 (5), 43 (19), 42 (56), 41 (98), 40 (71).

*2.4.4.5. Synthesis of 3-methylfuran-2(5H)-one (**1a**)*

(Carbethoxymethylene)triphenylphosphorane **6** (8 g, 22.9 mmol) was dissolved in 50 mL of toluene. Hydroxyacetone **5** (2 g, 27.02 mmol) was added slowly and the green-yellow solution was refluxed for 4h. The solvent was then evaporated under reduced pressure and the crude product mixture was purified by flash column chromatography on silica gel (eluent: PE/EtOAc 1:1) to give a colorless oil **1n** (3.16 g, 21.98 mmol, 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.21 (t, *J* = 7.2 Hz, 3H), 2.00 (s, 3H), 3.08 (s, 1H), 4.04 (s, 2H), 4.09 (q, *J* = 7.2, 2H), 5.90 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.38, 15.71, 59.95, 66.95, 113.59, 157.80, 167.26. This material (3.16 g) was then dissolved in 20 mL of methanol and the solution treated with 5% aqueous NaOH (20 mL) at 0 °C. The reaction mixture was allowed to stir overnight at room temperature. The mixture was then diluted with water (40 mL) and washed with ether (20 mL x 2). The aqueous layer was then acidified to pH 1 (conc. HCl), saturated with NaCl and extracted with ethyl acetate (3 x 50 mL). The combined organic phases were dried over Na_2SO_4, then the solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography on silica gel (eluent: PE/EtOAc 1:2) to give **1a** (1.92 g, 19.53 mmol, 85%) as a colorless solid. M.p. 109-110 °C {lit[22]. M.p. 109-110 °C}; ^{1}H NMR (400 MHz, DMSO-d6) δ: 1.95 (s, 3H), 3.92 (s, 2H), 5.84 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d6) δ: 15.25, 65.31, 113.12, 158.45, 167.69 (in accordance with literature[41,42]).

*2.4.4.6. Synthesis of 3-ethylfuran-2(5H)-one (**1b**)*

Using the procedure described for the synthesis of 3-methylfuran-2(5*H*)-one (**1a**), reaction of 1-hydroxy-2-butanone (2 g, 22.72 mmol) gave **1b** (2.16 g, 19.27 mmol, 85%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.05 (t, *J* = 7.4 Hz, 3H), 2.50

(q, *J* = 7.6 Hz, 2H), 4.18 (s, 2H), 5.93 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.94, 22.58, 64.71, 112.25, 165.69, 171.76 (in accordance with literature[22]).

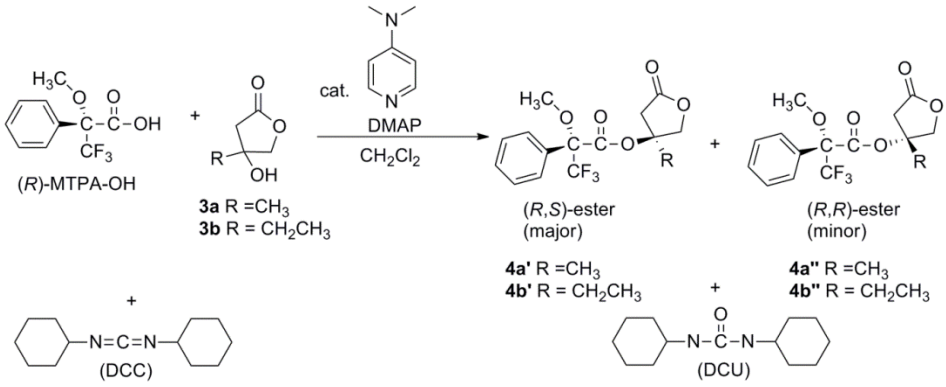
*2.4.4.7. Synthesis of 4-methyl-5,6-dihydro-2H-pyran-2-one (**1c**)*

Using the procedure described for the synthesis of 3-methyl-2-butenolide (**1a**), reaction of 4-hydroxy-2-butanone (2 g, 22.72 mmol) gave **1c** (2.16 g, 19.32 mmol, 85%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.16 (s, 3H), 2.39 (t, *J* = 6.2 Hz, 2H), 3.77 (t, *J* = 6.4 Hz, 2H), 5.72 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 18.27, 44.49, 59.59, 116.73, 159.06, 170.15 (in accordance with literature[41,42]).

*2.4.4.8. Synthesis of 4,5-dimethyl-2(5H)-furanone (rac-**1f**)*

Using the procedure described for the synthesis of 3-methylfuran-2(5*H*)-one (**1a**), reaction of 3-hydroxy-2-butanone (2 g, 22.72 mmol) gave *rac*-**1f** (2.16 g, 19.27 mmol, 85%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.14 (d, *J* = 6.4 Hz, 3H), 1.99 (s, 3H), 4.06 (q, *J* = 6.4 Hz, 1H), 5.83 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.70, 22.05, 70.48, 113.63, 162.08, 167.93 (in accordance with literature[53]).

2.4.4.9. Mosher esters preparation for ee determination



SCHEME 4. Synthesis of Mosher esters for *ee* determination.

*2.4.4.9.1. Mosher ester (**7a**) for ee determination of (S)-3-hydroxy-3-methyfuranone (**2a**)*

Solution A containing (*R*)-α-methyl-α-trifluoromethylphenylacetic acid [(*R*)-MTPA-OH] in anhydrous dichloromethane (50 mg/mL = 0.214 mol/L) (20 mL) and solution B containing 4-dimethylaminopyridine (DMAP) in anhydrous dichloromethane (3.4 mg/mL = 0.028 mol/L) (5 mL) were prepared separately in screw cap drum vials. (*S*)-3-hydroxy-3-methylbutanolide (**2a**) from preparative scale reaction (70 mg, 0.60 mmol) was placed into a 25 mL screw cap drum vial. Dicyclohexylcarbodiimide (DCC) (247 mg, 1.19 mmol, 2 equiv.) and pyridine (72 µL, 1.0 equiv.) were added. Solution A (6.44 mL, 1.5 equiv.) was added *via* a syringe followed by the addition of solution B (1.08 mL, 0.05 equiv.). The vials were closed tightly, mixed briefly and left standing in the dark at room temperature under an atmosphere of nitrogen for around 2 days. Upon complete conversion of the alcohols to the corresponding Mosher ester product (checked by TLC analysis), the reaction mixture was diluted with ethyl acetate and filtered through a cotton plug in a Pasteur pipet to remove dicyclohexylurea (DCU). Solvents were removed under reduced pressure and the dilution, filtration and evaporation cycle was repeated twice to remove traces of DCU. The filtrate was again concentrated under reduced pressure for ^{1}H NMR and ^{19}F NMR measurement. Yields were quantitative and no further purification was carried out. 90% *ee* was calculated by integration of the proton peak areas of group –OCH_3 and fluorine peak areas of group –CF_3; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.75 (s, 3H), 2.70 (d, J = 18 Hz, 1H), 2.98 (d, J = 18 Hz, 1H), 3.50 (dd, J_{HF} = 1.2 Hz, J_{HF} = 2.3 Hz, 3H), 4.28 (d, J = 10.8 Hz, 1H), 4.65 (d, J = 10.8 Hz, 1H), 7.37-7.47 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.71, 41.28, 55.58 (d, J_{CF} = 1.4 Hz), 76.06, 84.73, 123.32 (d, J_{CF} = 287 Hz), 127.24, 128.87, 130.09, 131.81, 166.04, 173.27. ^{19}F NMR (376 MHz, $CDCl_3$) δ: –71.54, –72.14.

*2.4.4.9.2. Mosher ester (**7b**) for ee determination of 3-hydroxy-3-ethylbutanolide (**2b**)*

Using the procedure described for the synthesis of 3-methyl-5-oxotetrahydrofuran-3-yl 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate (**7a**), reaction of 3-hydroxy-3-ethylbutanolide (**2b**) from preparative scale reaction (70 mg, 0.54 mmol) gave mosher ester in quantitative yield. 94% *ee* was calculated by integration of the proton peak areas of group –OCH_3 and fluorine peak areas of group –CF_3; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.54 (dd, J_{HF} = 1.2 Hz, J_{HF} = 2.3 Hz OMe); ^{19}F NMR (376.58 MHz, $CDCl_3$) δ: –71.31, –72.59.

*2.4.4.10. Synthesis of Dihydro-4-methylfuran-2(3H)-one (**3a**)*

A stirred solution of lactone **1a** (250 mg, 2.70 mmol) and 10% Pd/C (10% wt/wt) in MeOH (6 mL) was subjected to H_2 (1 atm, 25 °C) for 3h. The reaction was passed through celite and washed with MeOH (2 x 10mL), The solvent was evaporated under reduced pressure and the residue purified by flash column chromatography on silica gel (eluent: PE/EtOAc 4:1) to afford the product **3a** as a pale yellow oil (250 mg, 2.5 mmol, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.17 (d, *J* = 6.6 Hz, 3H), 2.25-2.33 (m, 1H), 2.37-2.44 (m, 1H), 2.65-2.70 (m, 1H), 3.63-3.68 (m, 1H), 3.77-3.81 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 16.84, 32.90, 38.51, 67.53, 179.57 (in accordance with literature[41,55]).

2.4.4.11. Synthesis of methyl (S)-(-)-3,4-dihydroxy-3-methylbutanoate

To a flask charged with **2a** (102 mg, 0.88 mmol) in MeOH (6 mL) concentrated sulphuric acid (0.05 mL, 0.09 mmol) was added, and the reaction mixture was stirred at room temperature for 3h.[56] Then the solution was neutralized to pH 7.0 with solide $NaHCO_3$, and filtered. 3mL of water was added, and the mixture was extracted with ethyl acetate (3 x 6 mL). The combined organic phases were dried over Na_2SO_4, then the solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography on silica gel (eluent: PE/EtOAc 1:1) to give the desired methyl ester (104 mg, 0.70 mmol, 80%). $[\alpha]_D^{20}$ −9.3 (*c* 2.15, $CHCl_3$), [lit[24] $[\alpha]_D$ +10.26 (c 1.93)]; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.50 (s, 3H), 2.56 (d, *J* = 17.6 Hz, 1H), 2.67 (d, *J* = 17.6 Hz, 1H), 3.26 (s, 3H), 3.47 (s, 1H), 3.73 (s, 1H), 4.12 (d, *J* = 9.6 Hz, 1H), 4.25 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 24.47, 43.53, 50.86, 73.92, 79.55, 175.58 (in accordance with literature[24]).

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In this study, the absolute stereochemistry was assigned as (*R*), based on the conversion of **2a** into the corresponding triol. However, the optical rotation {$[\alpha]D^{20}$ +0.96 (*c* 5 in EtOH)} of this triol is very small, therefore, impurities can easily lead to errors. Moreover, no experimental details and reaction conditions were given. Since our experiments with all the other substrates gave the opposite orientation of the hydroxyl group, we converted **2a** into its literature known methyl ester,[23] which gave an optical rotation of $[\alpha]D^{20}$ -9.3 (c 2.15 in $CHCl_3$; Ref.[24]: $[\alpha]D^{20}$ +10.26 for the (*R*)-enantiomer}. Therefore we reassigned the absolute stereochemistry of **2a** to be (*S*).
[23] See Part 2.4.4.10 for experimental details.
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3

DRAFT SEQUENCE OF RHODOCOCCUS RHODOCHROUS ATCC 17895

In this chapter the features of *Rhodococcus rhodochrous* ATCC 17895, together with the draft genome sequence and annotation will be described. This organism possesses an array of mono- and dioxygenases, as well as hydratases, which makes it an interesting organism for biocatalysis. *R. rhodochrous* is a Gram-positive aerobic bacterium with a rod-like morphology. The 6,869,887 bp long genome contains 6609 protein-coding genes and 53 RNA genes. Based on small subunit rRNA analysis, the strain is more likely to be a strain of *Rhodococcus erythropolis* rather than *Rhodococcus rhodochrous*.

This chapter is based on

B.-S. Chen, L. G. Otten, V. Resch, G. Muyzer and U. Hanefeld,

Stand. Genomic Sci., **2013**, *9*, 175-184.

3.1 INTRODUCTION

The genus *Rhodococcus* comprises genetically and physiologically diverse bacteria, known to have a broad metabolic versatility, which is represented in its clinical, industrial and environmental significance. Their large number of enzymatic activities, unique cell wall structure and suitable biotechnological properties make *Rhodococcus* strains well equipped for industrial uses, such as biotransformations and the biodegradation of many organic compounds. In the environmental field, the ability of *Rhodococcus* to degrade trichloroethene,[1] haloalkanes,[2-4] and dibenzothiophene (DBT)[5] is reported. Furthermore its potential for petroleum desulfurization[5] is known.

Rhodococcus rhodochrous strains are ubiquitous in nature. They possess an array of mono- and dioxygenases, as well as hydratases, which make them an interesting organism for biocatalysis.[6] One example would be the recently reported regio-, diastereo- and enantioselective hydroxylation of unactivated C-H bonds[7] which remains a challenge for synthetic chemists, who often rely on differences in the steric and electronic properties of bonds to achieve regioselectivity.[8] Furthermore most *Rhodococcus* strains harbour nitrile hydratases,[9-11] a class of enzymes used in the industrial production of acrylamide and nicotinamide[12] while other strains are capable of transforming indene to 1,2-indandiol, a key precursor of the AIDS drug Crixivan.[13] In another recent example, *R. rhodochrous* ATCC BAA-870 was used for the biocatalytic hydrolysis of β-aminonitriles to β-amino-amides.[14] One example for a rather rarely investigated reaction would be the biocatalytic hydration of 3-methyl- or 3-ethyl-2-butenolide giving the corresponding (*R*)-3-hydroxy-3-alkylbutanolide, a phenomenon observed in resting resting cells of *Rhodococcus rhodochrous* strain ATCC 17895.[15]

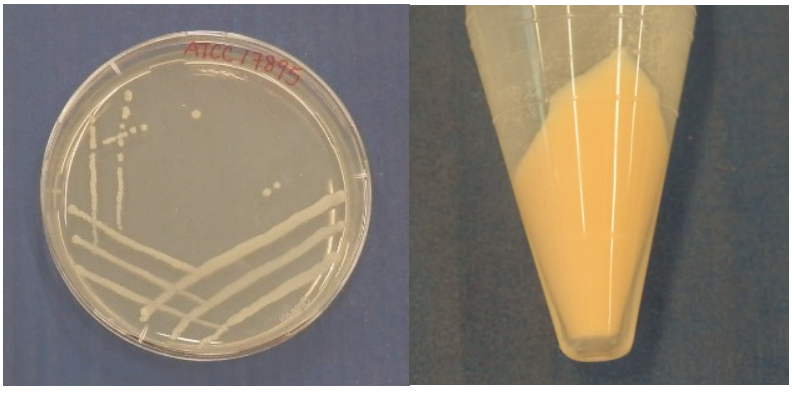
In order to obtain a comprehensive understanding of its high ability for biodegradation and biotransformation,[16] the genome of *R. rhodochrous* strain ATCC 17895 was sequenced. To the best of our knowledge, no complete genome sequence of this organism can be found in literature. Here we present a summary, classification and a set of features for *R. rhodochrous* strain ATCC 17895 together with the description of the genomic sequencing and annotation.

3.2 CLASSIFICATION AND FEATURES

Bacteria from the *Rhodochrous* group are taxonomically related to the genera *Nocardia* and *Mycobacterium*. In 1977 Goodfellow and Alderson proposed the genus *Rhodococcus* to be assigned to this group.[17] This assignment is due to the overlapping characteristics with *Nocardia* and *Mycobacterium* that were studied in morphological, biochemical, genetic, and immunological studies.[18] *R. rhodochrous* strain ATCC 17895 was previously deposited as *Nocardia erythropolis*[19] and *Rhodococcus erythropolis*.[17]

When incubated with fresh nutrient medium, *R. rhodochrous* grows as rod-shaped cells.[20] Furthermore cells are described to be Gram-positive actinomycetes with a pleomorphic behaviour often forming a primary mycelium that soon fragments into irregular elements.[21,22] It is known to be a facultative aerobe, non-motile and may be partially acid-fast. Production of endospores or conidia has not been reported, but for some strains a few feeble aerial hyphae are observed.[23,24] The optimal growth temperature reported is 26 °C on standard culture media. After initially growing sparsely, *R. rhodochrous* strain ATCC 17895 forms organized lumps on the agar surface, leading to the growth of dry opaque, pale orange, concentrically ringed colonies (Figure 1A and 1B). Usually growth is observed within 3 to 4 days.

R. rhodochrous strains are known to produce acid from glycerol, sorbitol, sucrose and trehalose, but not from adonitol, arabinose, cellobiose, galactose, glycogen, melezitose, rhamnose or xylose. The cell wall peptidoglycan incorporates *meso*-diaminopimelic acid, arabinose and galactose (wall type



A B

FIGURE 1. A: Characteristic of strain ATCC 17895 on nutrient agar plate after 72h; B: Harvested pale orange cells incubated with fresh nutrient medium after 72h.

TABLE 1. Classification and general features of *Rhodococcus rhodochrous* ATCC 17895 according to the MIGS recommendations[26]

MIGS ID	Property	Term	Evidence code
	Current classification	Domain *Bacteria*	TAS[27]
		Phylum *Actinobacteria*	TAS[28]
		Class *Actinobacteria*	TAS[29]
		Subclass *Actinobacteridae*	TAS[29,30]
		Order *Actinomycetales*	TAS[39-32]
		Suborder *Corynebacterineae*	TAS[29,30]
		Family *Nocardiaceae*	TAS[29,30,32,33]
		Genus *Rhodococcus*	TAS[234]
		Species *Rhododoccus rhodochrous*	TAS[32,35,6
		Strain ATCC17895	
	Gram stain	Positive	TAS[17]
	Cell shape	Rod-shaped	TAS[20]
	Motility	Non-motile	TAS[17]
	Sporulation	Non-sporulating	TAS[17]
	Temperature range	Mesophile	TAS[17]
	Optimum temperature	26 ^{o}C	TAS[19]
MIGS-6.3	Salinity	Not reported	NAS
MIGS-22	Oxygen requirement	Aerobe	TAS[17]
	Carbon source	fructose, glucose, mannose, sucrose	TAS[17]
	Energy source	butyrate, fumarate, propionate	TAS[17]
MIGS-6	Habitat	Marine, Aquatic	TAS[17]
MIGS-15	Biotic relationship	Free-living	TAS[37]
MIGS-14	Pathogenicity	Not reported	NAS
	Biosafety level	1	TAS[19]
	Isolation	Pacific Ocean seawater	TAS[37]
MIGS-4	Geographic location	Canada	TAS[37]
MIGS-5	Sample collection time	Not reported	NAS
MIGS-4.1	Latitude	Not reported	NAS
MIGS-4.2	Longitude	Not reported	NAS
MIGS-4.3	Depth	Not reported	NAS
MIGS-4.4	Altitude	Not reported	NAS

Evidence codes – IDA: Inferred from Direct Assay (first time in publication); TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from of the Gene Ontology project. If the evidence code is IDA, then the property was directly observed by one of the authors or an expert mentioned in the acknowledgments.

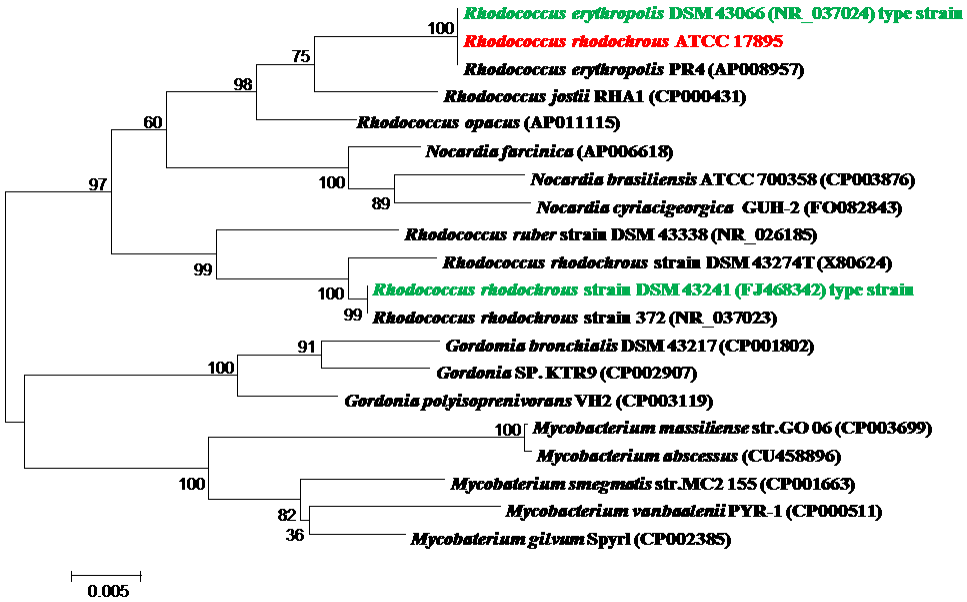


FIGURE 2. Phylogenetic tree based on the 16S rRNA sequence highlighting the phylogenetic position of *Rhodococcus rhodochrous* strain ATCC 17895 relative to other type strains within the genus *Rhodococcus*. Genbank accession numbers are indicated in parentheses. Sequences were aligned using CLUSTALW, and phylogenetic inferences obtained using the neighbour-jointing method within the MEGA v5 software.[38] Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1,000 times to generate a majority consensus tree. The scale bar indicates 0.005 nucleotide change per nucleotide position.

IV).[25] The bacterium is urease and phosphatase positive. The important characteristics of the strain based on literature descriptions are summarized in Table 1. On the basis of 16S rRNA gene sequencing the strain belongs to the genus *Rhodococcus* within class *Actinobacteria*, *Rhodococcus erythropolis* PR4 and *Rhodococcus erythropolis* strain N11 are its closest described phylogenetic neighbours (Figure 2).

3.3 GENOME SEQUENCING INFORMATION

3.3.1 Genome project history

This organism was selected for sequencing on the basis of its common use for a wide range of biotransformation, such as steroid modification, enantioselective synthesis, the production of amides from nitriles,[6,39,40] and its interesting hydration capabilities.[15] The complete genome obtained in this study was sequenced in October 2012 and has been deposited at GenBank under accession number ASJJ00000000 consisting of 423 contigs (≥300 bp) and 376 scaffold (≥300 bp). The version described in this paper is version ASJJ01000000. Sequencing was performed by BaseClear BV (Leiden, the Netherlands) and initial automatic annotation by Institute for Biodiversity and Ecosystem Dynamics (Amsterdam). A summary of the project information is shown in Table 2.

3.3.2 Growth conditions and DNA isolation

Rhodococcus rhodochrous ATCC 17895 was grown on nutrient medium [8.0 g nutrient broth (BD cat. 234000) in 1000 mL demi water] at pH 6.8 and 26 °C with orbital shaking at 180 rpm as recommended by ATCC. Extraction of chromosomal DNA was performed by using 50 mL of overnight culture, centrifuged at 4 °C and 4000 rpm for 20 min and purified using the following method.[41] 100 mg wet cells were transferred to a microcentrifuge tube and washed three times with 0.5 mL potassium phosphate buffer (0.1 M, pH 6.2). The resulting cell pellet was resuspended in 564 µL Tris-HCl buffer (10 mM) containing 1 mM EDTA (pH 8.0) and 10 µg lysozyme and incubated at 37 oC for 2 h. Next, Proteinase K (3 µL of 20 mg/mL stock), DNase-free RNase (2 µL

TABLE 2. Genome sequencing project information

MIGS ID	Characteristic	Details
MIGS-28	Libraries used	One Illumina paired-end library, 50 cycles
MIGS-29	Sequencing platform	Illumina HiSeq 2000
MIGS-31.2	Sequencing coverage	50 x
MIGS-31	Finishing quality	Permanent draft
MIGS-30	Assemblers	CLCbio Genomics Workbench version 5.5.1
MIGS-32	Gene calling method	RAST
	BioProject	PRJNA201088
	GenBank ID	ASJJ00000000
	GenBank date of release	23-9-2013
	Source material identifier	ATCC 17895
	Project relevance	Biotechnology

of 10 mg/mL stock), SDS (50 µL of 20% w/v stock) were added and the cell suspension was incubated at 50 °C for 3 h followed by the addition of 5 M NaCl (100 µL) and incubation at 65 °C for 2 min. After addition of 80 µL of CTAB/NaCl solution (10% w/v hexadecyl trimethyl ammonium bromide in 0.7 M NaCl) incubation at 65 °C for 10 min was performed. The cell lysate was twice extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and the aqueous layer was separated after centrifugation at 14000 rpm for 15 min. The DNA was precipitated with 0.7 volumes isopropanol and dissolved in sterile water for genome sequencing. The quality and quantity of the extracted DNA was evaluated by 0.8% (w/v) agarose gel electrophoresis to obtain good quality DNA, with an $OD_{260:280}$ ratio of 1.8-2, and as intact as possible.

3.3.3 Genome sequencing and assembly

Genomic DNA libraries for the Illumina platform were generated and sequenced at BaseClear BV (Leiden, The Netherlands). High-molecular weight genomic DNA was used as input for library preparation using the Illumina TruSeq DNA library preparation kit (Illumina). Briefly, the gDNA was fragmented and subjected to end-repair, A-tailing, ligation of adaptors including sample-specific barcodes and size-selection to obtain a library with median insert-size around 300 bp. After PCR enrichment, the resultant library was checked on a Bioanalyzer (Agilent) and quantified. The libraries were multiplexed, clustered, and sequenced on an Illumina HiSeq 2000 with paired-end 50 cycles protocol. The sequencing run was analysed with the Illumina CASAVA pipeline (v1.8.2). The raw sequencing data produced was processed removing the sequence reads which were of too low quality (only "passing filter" reads were selected) and discarding reads containing adaptor sequences or PhiX control with an in-house filtering protocol. The quality of the FASTQ sequences was enhanced by trimming off low-quality bases using the “Trim sequences” option of the CLC Genomics Workbench version 5.5.1. The quality filtered sequence reads were puzzled into a number of contig sequences using the “De novo assembly” option of the CLC Genomics Workbench version 5.5.1. Subsequently the contigs were linked and placed into scaffolds or supercontigs with SSPACE premium software v2.3.[42] The orientation, order and distance between the contigs was estimated using the insert size between the paired-end reads. Finally the gapped regions within

the scaffolds were (partially) closed in an automated manner using GapFiller v 1.10.[43]

3.3.4 Genome annotation

Genes were identified and annotated using RAST (Rapid Annotations based on Subsystem Technology).[44] The translated CDSs were used to search the National Center for Biotechnology Information (NCBI) nonredundant (nr) database, Pfam, KEGG, and COG databases. Additional gene prediction analysis and functional annotation were performed within the Integrated Microbial Genomes Expert Review (IMG-ER) platform.[45]

3

3.4 GENOME PROPERTIES

The genome size is around 6,869,887 bp. The G+C percentage determined from the genome sequence is 62.29%, which is similar to the value of its closest sequenced neighbour *R. erythropolis* PR4, determined by Sekine M.[46] The genomic information of strain PR4 was deposited to GenBank, but was not publically available until very recent. From the genome sequence of strain ATCC 17895, there are 6662 predicted genes of which 6609 are protein-coding genes, 53 are RNA genes. A total of 5186 genes (77.8%) are assigned a putative function. The remaining genes are annotated as either hypothetical proteins or proteins of unknown functions. The properties and statistics of the genome are summarized in Table 3 and the distribution of genes into COGs functional categories is presented in Table 4. The number and percentage of genes in different COG categories is equivalent to the closely related *R. erythropolis* PR4 and *R. jostii* RHA1, showing that most genes have been annotated, even though the genome was not fully closed.

As is obvious from Figure 2, the 16S rRNA of this *R. rhodochrous* strain is much closer to *R. erythropolis* than to *R. rhodochrous*. Also *R. erythropolis* PR4 is the closest neighbour of the currently sequenced organism. Furthermore, certain genes mentioned by Gürtler et al. to be part of *R. erythropolis* strains, but not to be present in *R. rhodochrous*,[47] are all present in the genome. Therefore, as recommended by Gürtler et al., we propose that this organism should be renamed to *Rhodococcus erythropolis* ATCC 17895.

TABLE 3. Genome statistics

Attribute	Value	% of Total
Genome size (bp)	6,869,887	100.00
DNA coding region (bp)	6,017,668	87.63
DNA G + C content (bp)	4,279,255	62.29
Number of replicons	1	
Extrachromosomal elements (plasmid)	0	
Total genes	6662	100.00
RNA genes	53	0.80
rRNA operons	3	0.05
Protein-coding genes	6609	99.20
Pseudogenes	0	
Genes in paralog clusters	5469	82.09
Genes assigned to COGs	4751	71.31
Genes assigned Pfam domains	5132	77.03
Genes with signal peptides	305	4.58
CRISPR repeats	0	

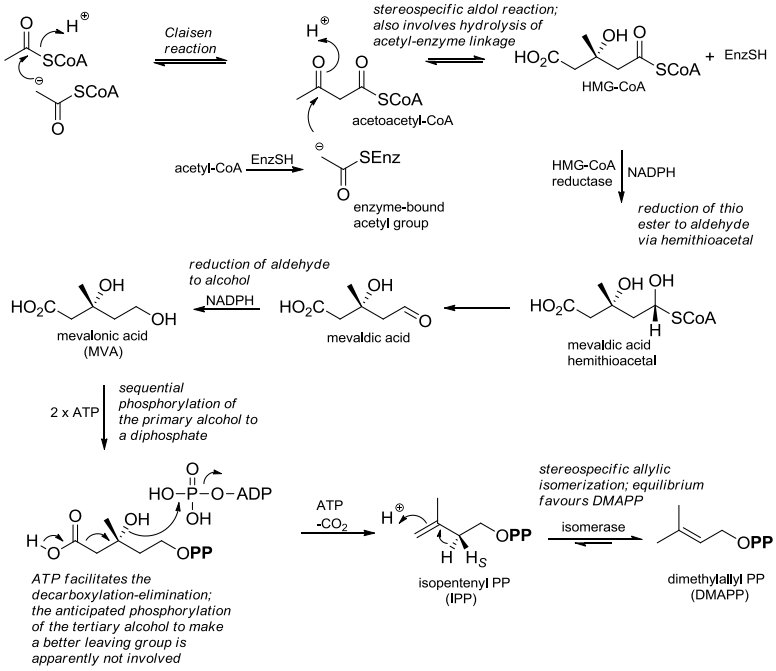
TABLE 4. Number of genes associated with the general COG functional categories.

Code	Value	% age	Description
J	194	3.63	Translation, ribosomal structure and biogenesis
A	5	0.09	RNA processing and modification
K	597	11.16	Transcription
L	155	2.90	Replication, recombination and repair
B	1	0.02	Chromatin structure and dynamics
D	42	0.79	Cell cycle control, mitosis and meiosis
V	88	1.64	Defense mechanisms
T	241	4.50	Signal transduction mechanisms
M	198	3.70	Cell wall/membrane biogenesis
N	4	0.07	Cell motility
U	37	0.69	Intracellular trafficking and secretion
O	143	2.67	Posttranslational modification, protein turnover, chaperones
C	364	6.80	Energy production and conversion
G	339	6.34	Carbohydrate transport and metabolism
E	460	8.60	Amino acid transport and metabolism
F	103	1.93	Nucleotide transport and metabolism
H	187	3.5	Coenzyme transport and metabolism
I	427	7.98	Lipid transport and metabolism
P	323	6.04	Inorganic ion transport and metabolism
Q	327	6.11	Secondary metabolites biosynthesis, transport and catabolism
R	711	13.29	General function prediction only
S	404	7.55	Function unknown
-	1911	28.69	Not in COGs

3.5 BIOCATALYTIC PROPERTIES

Since we are interested in the biocatalytic properties of this organism, we looked at enzymes known to be abundant in *Rhodococcus* strains. There are 27 different mono- and dioxygenases annotated in the genome, which is similar to the number in the closely related *R. erythropolis* PR4. And, as expected, there are 2 ureases and more than 10 phosphatases in the genome. Furthermore, there is a full nitrile metabolising operon present, comprising nitrile hydratase, regulators, amidase and aldoxime dehydratase. Although this organism is not a catabolic powerhouse like *Rhodococcus* sp. RHA1,[48] which was isolated from a polluted soil, there are numerous genes coding for proteins involved in producing amino acids, cofactors and lipids. For many of these proteins there are several copies of genes with similar function. This shows the versatility of this organism, like most members of its species. The various enzymes found by this genomic annotation can be used as a starting point to exploit this organism for biocatalytic operation, for instance, the rarely investigated biocatalytic hydration,[15,49] and the hydroxylation of unactivated C-H bonds,[7] which still is a major challenge for synthetic chemists.

As described in Chapter 2 (Enantioselective Michael addition of water), the hydration of substrate 4-methyl-5,6-dihydro-2*H*-pyran-2-one (Chapter 2, Table 3, entry 3) gives access to the natural product mevalonolactone, an intermediate in the pathway leading to terpenoids. The first part of the cholesterol pathway is also called the terpenoid backbone synthesis and can go *via* 2 routes: the Mevalonate pathway (Scheme 1)[50] and the non-mevalonate pathway. In order to identify whether the Michael hydratase discussed in Chapter 2 is a promiscuous enzyme of the mevalonate pathway, we carefully looked at the annotated hydratases in this genome, which only showed known hydratases with their narrow substrate specificity, and most of the typical enzymes from the mevalonate pathway (Figure 1)[51] are missing, both emphasizing the Michael hydratase of Chapter 2 has not been described before. Instead we did find that three candidates were annotated as Old Yellow Enzyme family NADH flavin oxidoreductases, which are further classified as ene reductases and discussed in details in Chapter 5, explaining the initially unexpected reduction of C = C bounds in Chapter 2 as a side reaction for the desired Michael addition of water.



SCHEME 1. Terpenoid backbone synthesis by Mevalonate pathway.

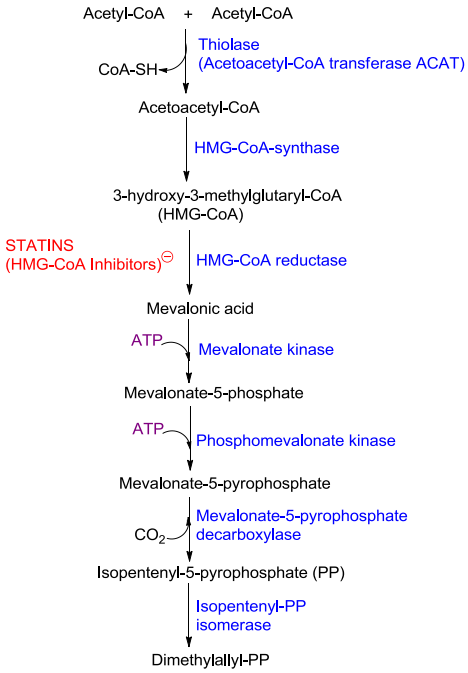


FIGURE 1. Enzymes involved in the Mevalonate pathway.

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4

ENANTIOSELECTIVE PREPARATION OF (*R*) AND (*S*)-3-HYDROXYCYCLOPENTANONE BY KINETIC RESOLUTION

In this chapter a straightforward approach to enantiomerically enriched (*R*) and (*S*)-3-hydroxycyclopentanone will be described. The key step involves a kinetic resolution of racemic 3-hydroxycyclopentanone using commercial *Pseudomonas cepacia* lipase immobilized on diatomite (Amano lipase PS-DI). The absolute stereochemistry of the product was determined by derivatization into (*R*)-3-(benzyloxy)cyclopentanone.

This chapter is based on

B.-S. Chen and U. Hanefeld,

J. Mol. Catal. B: Enzym., **2013**, *85*, 239-242.

4.1 INTRODUCTION

Cyclic and acyclic β-hydroxy ketones are important structural motifs in various biologically active organic compounds, as they provide two different functionalities in the same molecule ready for manipulation.[1-3] However, most β-hydroxy ketones are not commercially available. Indeed, many seemingly simple molecules have to be prepared via multi-step syntheses, in particular so if they are optically active. One such molecule is 3-hydroxycyclopentanone. At first glance it seems a quite straightforward molecule to chemists. However, in its simplicity it also lacks any handles for stereo- and regio-control during its synthesis and side reactions such as the intermolecular aldol reactions, and follow-up reactions (e.g. dehydration)[4,5] of the desired product can easily occur. In this reactivity the power of this building block is hidden, which can readily be converted into chiral diols,[6,7] and amino alcohols.[2,3,8] Here a straightforward approach to this valuable building block for amongst others the Prostaglandins' is described.[9,10] Moreover 3-hydroxycyclopentanone is a valuable compound for the study of enantioselective Michael addition reactions of water.[11-13]

To date, there are only two methods describing the preparation of enantiomerically enriched 3-hydroxycyclopentanone. In the chemical kinetic resolution of racemic 4-hydroxycyclopent-2-enone by BINAP-Ruthenium (II)-catalyzed hydrogenation (*R*)-enriched 3-hydroxycyclopentanone was obtained as a side product.[14] Due to the sensitivity of the desired product, the unconverted enantiomer of the starting material, (*R*)-3-hydroxycyclopentanone was not purified. In an enzymatic biohydroxylation it was first attempted to convert cyclopentanone into the 3-hydroxycyclopentanone but no reaction occurred. However, when the ketone was protected the hydroxylation proceeded. Nonetheless, enantioselectivity (40 % *ee*) and yields after deprotection were modest.[15]

Enzymatic kinetic resolutions are today recognized as a key tool for obtaining optically active compounds.[16,17] For secondary alcohols the outcome of the kinetic resolution is normally highly predictable and it follows Kazlauskas' rule.[17-19] It is however not straightforward to apply this rule to cyclic compounds[20] like the target molecule (Figure 1). Therefore a full screening

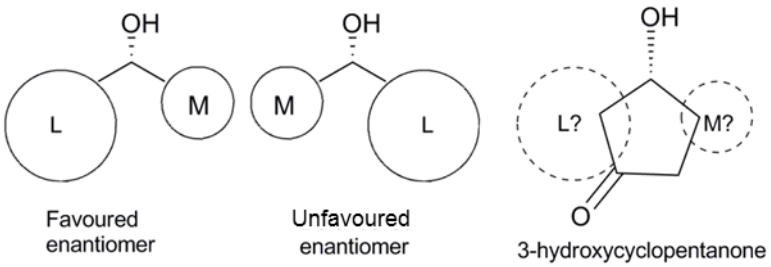


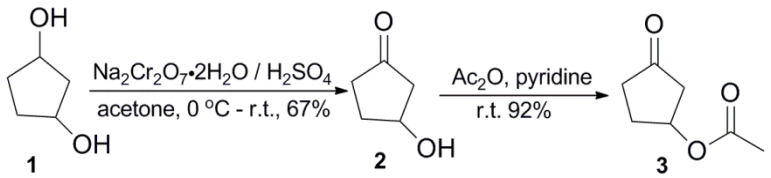
FIGURE 1. Kazlauskas' rule for kinetic resolution of secondary alcohols.

of a range of commercially available lipases and a subsequent characterization of absolute configuration and enantiomeric excess were performed.

It is interesting to note that a large number of substituted cyclopentanol derivatives have been subjected to kinetic resolutions. A general trend observed is that derivatives with large substituents are readily resolved,[21-33] while simple compounds similar to our target molecule tend to give low *E* value.[34-38] Our study is a very good starting point for the enantioselective preparation of secondary alcohols,[39] especially for optically active 4-hydroxy-5-alkylcyclopent-2-en-1-one derivatives[40] and tetrahydrofuran-3-yl acetate.[41]

4.2 RESULTS AND DISCUSSION

Racemic starting material 3-hydroxycyclopentanone **2** was readily prepared from commercial cyclopentane-1,3-diol **1** with freshly prepared Jones reagent (1.6 M in acetone) by a known method (Scheme 1).[42] The *rac*-3-hydroxycyclopentanone **2** was converted to *rac*-3-oxocyclopentyl acetate **3** *via* acylation in order to be used as a reference compound for chiral GC analysis.



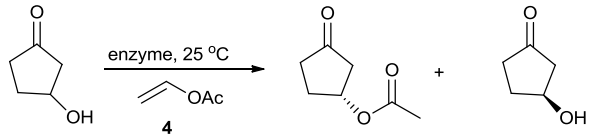
SCHEME 2. Preparation of *rac*-3-hydroxycyclopentanone for kinetic resolution and *rac*-3-oxocyclpentyl acetate for chiral GC analysis.

Kinetic resolution via hydrolysis of the ester is unsuitable since the resulting alcohol is highly hydrophilic and therefore difficult to extract from water. Thus, the *rac*-3-hydroxycyclopentanone **2** obtained was treated with lipases using vinyl acetate **4** as a solvent and acylating agent, whose green properties was recently evaluated.[19,43] Several different lipases (CAL-A, CAL-B, CRL, PCL immobilized on diatomite as Amano PS-DI and as free enzyme, *Pseudomonas stutzeri*, *Alcaligenes* sp., PFL, PPL,) were investigated for the kinetic resolution.

We initially performed the screening of the enantioselectivity using the same activity (450 U enzyme for 0.5 mmol substrate) at 25 °C of all lipases according to the reported guidelines (Table 1).[44] The screening revealed that lipases from *Alicaligenes* sp., *Pseudomonas stutzeri*, CRL, PPL, CAL-B furnished acetate 5 in low *ee* < 10% (entry 1-5); due to these low enantioselectivity values (*E*), they were not further investigated.

Next, we reduced the amount of enzyme from 450 U to 112 U for the same amount of substrate (0.5 mmol). The results showed an increase in the *ee* of (*R*)-3-oxocyclopentyl acetate **5** with Amano PS-DI (63% *ee*), PCL (60% *ee*), CAL-A (48% *ee*), PFL (30% *ee*) (entry 6-9). Among the lipases screened, we found that Amano lipase PS-DI (*P. cepacia* immobilized on diatomite, 2.97 U/mg) gave the enantiomerically enriched (*R*)-3-oxocyclopentyl acetate **5** in 57% yield and 63% *ee* (entry 6). The outcome of enzyme-catalyzed reactions revealed PCL as the most enantioselective enzyme. Immobilized as Amano lipase PS-DI it gave the highest *E*-value of 11, the best result among the nine commercially available lipases tested. A large scale reaction was performed after further optimization with Amano lipase PS-DI. At a conversion of 44% the enantiomerically enriched (*R*)-3-oxocyclopentyl acetate **5** and (*S*)-3-hydroxycyclopentanone **6** were obtained in 21% yield with 82% *ee* and in 47% yield with 66% *ee*, respectively, after isolation and purification by column chromatography. This corresponds to an $E = 20$. This is in line with the earlier observation that only cyclopentanol derivatives with large or many substituents give high *E* value in kinetic resolution.[21-33] Those with small and few substituents, such as cyclopetan-4-ene-1,3-diyl diacetate (80% yield, 20.5 % *ee*),[34] cyclohex-2-en-1-yl acetate,[35] 4-hydroxycyclopent-2-en-1-one (*E*=11),[36] 4-hydroxy-2-methyl-2-cyclopentenone (60% yield, 60% *ee*),[37] 3-bromocyclopent-2-enol[38] give less enantiopure products. An

TABLE 1. Enzyme screening for the kinetic resolution of 3-hydroxycyclopentanone



2 **5** **6**

Entry	Enzyme	Reaction Time (Hrs)	Unit U[a]	Acetate *ee*[b] %	Alcohol *ee*[d] %	Conversion [c] %	E[e]	Enantio preference
1	*Alicaligenes sp.*	4	450	<1	64	99	nd	*S*
2	*Pseudomonas stutzeri*	4	450	<1	17	98	nd	*R*
3	CRL	4	450	9	12	57	1	*R*
4	PPL	4	450	10	6	36	1	*R*
5	CAL-B	4	450	<1	45	99	nd	*R*
6	Amano PS-DI	4	112	63	83	57	11	*R*
7	PCL	4	112	60	83	58	10	*R*
8	CAL-A	4	112	48	44	48	5	*R*
9	PFL	4	112	30	33	52	2	*R*

[a] Enzyme activities were determined according to the reported procedure.[45]

[b] *ee* (%) of acetate **5** were determined by GC analysis using chiradex GTA column (see analytical methods in experimental).

[c] conversion (%) of alcohol **2** were determined by GC analysis using a CP WAX 52 CB column with decane as an internal standard (see analytical methods in experimental).

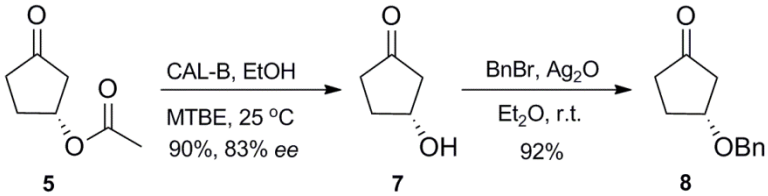
[d] *ee* (%) of alcohol **6** were calculated by using equation conversion of substrate $c = ee_s/(ee_s+ee_p)$.

[e] Enantioselectivities of the reaction (*E*) were determined from the equation $E = \ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$.

nd: not determined.

exception is the meso compounds,[46-52] however here the stereo differentiation is based on a different principle.

In general, Kazlauskas' rule predicts which enantiomer reacts faster in the acylation of racemic secondary alcohols (Figure 1). However, interestingly, for the substrate **2** as also shown in Figure 1, it is difficult to define the larger and medium size groups. Therefore Kazlauskas' rule might not apply. Thus, it is important and necessary to determine the absolute stereochemistry independently. In this case, the absolute configuration was assigned by comparison of the sign of the specific rotation with the benzyl derivative **8** that is reported in the literature.[15] The acetate group of **5** was deprotected using the essentially non-selective CAL-B (entry 5, Table 1) and ethanol/MTBE at 25 °C (Scheme 2). After 30 h, the enzyme was filtered off and the enantiomerically enriched (*R*)-3-hydroxycylopentanone **7** was obtained in



SCHEME 2. Derivatization of (*R*)-3-hydroxycyclopentanone for determination of the absolute configuration.

90% isolated yield. In order to determine the absolute stereochemistry of the product, compound **7** was converted by *O*-benzylation (BnBr, Ag_2O, Et_2O)[25] into (*R*)-**8**, $[\alpha]_D^{20}$ −41.3 (*c* 0.57, CH_2Cl_2) (Scheme 2). The known (*R*)-3-(benzyloxy)cyclopentanone (*R*)-**8** has $[\alpha]_D^{20}$ −43.0 (*c* 1.0, CH_2Cl_2).[15] The enantiomeric excess of compound **8** was determined to be ≥88% by chiral HPLC analysis using a chiralcel AD-H column (iso-PrOH/heptane = 7/93, flow rate = 1.0 mL/min, t_R (*R*) = 6.86 min and t_R (*S*) =7.32 min). After deprotection, the reobtained (*R*)-3-hydroxycyclopentanone **7** still had the same *ee*. Reprotecting as benzyl ether again yielded (*R*)-**8** with an *ee* of 83%, ruling out any loss of stereo information due to derivatisation.[53-55] This implies that the enzyme distinguishes the CH_2CO section of the cyclopentane ring as bulkier than the CH_2CH_2 part of this ring (Figure 1) according to Kazlauskas' rule.

4.3 CONCLUSTION

In summary, we have shown that enantiomerically enriched (*R*)-3-hydroxycyclopentanone **7** can be prepared with 82% *ee* and in acceptable chemical yield by kinetic resolution using commercially available lipase Amano PS-DI from *P. cepacia* immobilized on diatomite, accomplishing the first synthesis of optically active (*R*)-3-hydroxycyclopentanone **7**. This is a significant improvement on the biohydroxylation that only gave 40% *ee* in modest yields after deprotection.[15] The absolute stereochemistry was determined by derivatization into (*R*)-3-(benzyloxy)cyclopentanone, whose sign of the specific rotation matched that reported. After the kinetic resolution the unconverted (*S*)-enantiomer can equally be isolated.

4.4 EXPERIMEANTAL

4.4.1 General information

Cyclopentane-1,3-diol, benzyl bromide, silver oxide, sodium dichromate, sulfuric acid, diethyl ether, acetone, ethanol, MTBE, and vinyl acetate were purchased from Sigma-Aldrich and Acros Organics. Various lipases namely, *Candida antarctica* B (CAL-B, Novozyme 435, Novo Nordisk A/S LC200015), *Candida antarctica* A (CAL-A, Chirazyme L5, Roche Diagnostics), *Candida rugosa* (CRL, Sigma L1754), *Pseudomonas fluorescens* (PFL, Fluka 62312), *Pseudomonas stutzeri* (Meito Sangyo Co. Ltd, Lot No.TH8901), *Alcaligenes* sp. (Meito Sangyo Co. Ltd, Lot No.B3102), *Pseudomonas cepacia* (PCL, Sigma 62309), Amano lipase PS-DI (*P. cepacia* immobilized on diatomite, Aldrich 534870), and *Porcine pancreatic* lipase (PPL, Sigma L3126) were purchased or obtained as a gift. Enzyme activity was determined according to the reported procedure [21]. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Advance 400 (400 MHz and 100 MHz, respectively) instrument and were internally referenced to residual solvent signals. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration, coupling constant (Hz) and assignment. Data for ^{13}C NMR are reported in terms of chemical shift. Optical rotations were obtained with a Perkin-Elmer 241 polarimeter (sodium D line at 20 °C). Column chromatography was carried out with siliga gel (0.060-0.200 mm, pore diameter ca. 6 nm) and with mixtures of petroleum ether (PE) and ethyl acetate (EtOAc) as solvent. Thin-layer chromatography (TLC) was performed on 0.20 mm silica gel 60-F plates. Organic solutions were concentrated under reduced pressure on a Büchi rotary evaporator.

4.4.2 Analytical methods

GC analysis of the conversion (%) of 3-hydroxycylcopentanone **2** were followed with a Shimadzu type GC 2014 equipped with a CP WAX 52 CB column (50 m x 0.53 mm x 2 μm) using N_2 as the carrier gas. The following conditions were used for the nonchiral separation: injector 250 °C, detector (FID) 270 °C, FID hydrogen 30 oxygen 300, column flow 20 mL/min, maximum temp: 255 °C, temperature program: start 165 °C, hold time 11 min, rate 30 °C /min to 250 °C hold time 1 min. The quantification of 3-oxocyclopentyl acetate and 3-hydroxycyclopentanone was done by using

calibration curves with decane as an internal standard (7 standard samples marked 1, 2, 3, 4, 5, 6, 7; each sample contained 0.125 mmol/mL decane and 1 mmol/mL, 0.5 mmol/mL, 0.25 mmol/mL, 0.125 mmol/mL, 0.0625 mmol/mL, 0.03125 mmol/mL, 0.015625 mmol/mL 3-hydroxycyclopentanone in ethyl acetate, respectively). The retention times of 3-oxocyclopentyl acetate and 3-hydroxycyclopentanone were 6.63 and 8.78 min, respectively.

The enantiomeric excess (*ee*) of 3-oxocyclopentyl acetate **5** was determined with a Shimadzu type GC 2012 equipped with a chiradex GTA column (50 m x 0.25 mm x 0.12 μm) using Helium as the carrier gas. The following conditions were used for the chiral separation: injector 200 °C, detector 220 °C, split 60, FID hydrogen 30 oxygen 300, column flow: 0.49 mL/min, maximum temp: 175 °C, temperature program: start 150 °C, hold time 10 min, rate 25 °C /min to 170 °C hold time 1.20 min. The retention times of (*R*) and (*S*)-3-oxocyclopentyl acetate were 4.70 and 4.89 min, respectively.

4.4.3 General procedures

*4.4.3.1. Racemic 3-hydroxycyclopentanone (**2**)*

4

In a round-bottom flask (100 mL) fitted with a mechanical stirrer, cyclopentane-1,3-diol **1** (3.52 g, 34.44 mmol) was dissolved in 40 mL of acetone. The solution was cooled in an ice bath, and a solution of $Na_2Cr_2O_7 \bullet 2H_2O$ (3.49 g, 11.72 mmol), concentrated H_2SO_4 (2 mL), and H_2O (14 mL) was added over 25 min. The green-blue solution was allowed to warm to room temperature over 15 min. The reaction mixture was filtered through Celite and the solid was washed by acetone, then the solvent was evaporated. The residue was purified by flash column chromatography (eluent: PE/EtOAc 1:2) to give **2** (2.36 g, 67%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.98–2.08 (m, 1H), 2.08–2.32 (m, 3H), 2.32–2.54 (m, 2H), 2.78 (s, 1H), 4.38–4.84 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 31.38, 35.40, 47.91, 69.72, 217.80.

*4.4.3.2. Racemic 3-oxocyclopentyl acetate (**3**)*

Acetic anhydride (2.0 mL, 60.00 mmol) was added to a solution of 3-hydroxycyclopentanone **2** (700 mg, 6.99 mmol) in pyridine (0.5 mL). The reaction mixture was stirred at room temperature for 12 h. Then the solvent was evaporated, and the residue was purified by chromatography on silica gel

(eluent: EtOAc/PE 2:1) to give compound **3** (914 mg, 92%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.99 (s, 3H), 2.28–2.42 (m, 5H), 2.54 (dd, *J* = 19.0, 6.0 Hz, 1H), 5.46 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 20.70, 28.73, 34.30, 44.12, 71.74, 169.85, 215.98.

4.4.3.3. Kinetic resolution of 3-hydroxycyclopentanone using different lipases

The screening of lipases for kinetic resolution was performed at 25 °C in 2 mL of freshly distilled vinyl acetate **4** containing 0.5 mmol of *rac*-3-hydroxycyclopentanone **2** and 450 (or 112.5) U lipases activity. After 4 h, 100 μL sample was taken from the reaction mixture and added 100 μL of internal standard solution (0.25 mmol/mL decane in ethyl acetate). Then the mixture was centrifuged to separate the lipase. All of the obtained samples were analyzed by GC to determine the conversion of substrate **2** and the *ee* of 3-oxocyclopentyl acetate **5**.

4.4.3.4. Kinetic resolution of 3-hydroxycyclopentanone using Amano lipase PS-DI

To a round-bottom flask (100 mL) was added *rac*-3-hydroxycyclopentanone **2** (700 mg, 7.00 mmol), freshly distilled vinyl acetate **4** (26 mL) and Amano lipase PS-DI (1568 U, 412 mg). The reaction mixture was stirred at 25 °C for 4 h. Then the lipase was filtered off and the solvent was evaporated. The residue was purified by flash chromatography on silica gel (eluent: EtOAc/PE 2:1) to give the enantiomerically enriched (*S*)-3-hydroxycyclopentanone **6** and (*R*)-3-oxocyclopentyl acetate **5** in 47% and 21% yield, respectively.

*4.4.3.5. Ethanolysis of (R)-3-oxocyclopentyl acetate (**5**) using Candida antarctica lipase*

To a round-bottom flask was added (*R*)-3-oxocyclopentyl acetate **5** (160 mg, 1.13 mmol), ethanol (1 mL), MTBE (1 mL) and CAL-B (330 U, 83 mg). The reaction mixture was stirred at 25 °C for 30 h. Then the enzyme was filtered off and the solvent was evaporated. The residue was purified by flash chromatography on silica gel (eluent: EtOAc/PE 2:1) to yield (*R*)-3-hydroxycyclopentanone **7** (101 mg, 90 %) as a colorless oil.

*4.4.3.6. (R)-3-(benzyloxy)cyclopentanone (**8**)*

To a solution of (*R*)-3-hydroxycyclopentanone **7** (60 mg, 0.6 mmol) in 20 mL of diethyl ether were added benzyl bromide (0.15 mL, 1.52 mmol) and silver oxide (400 mg, 1.73 mmol). After stirring in the dark for two days at room temperature, the mixture was filtered through Celite and concentrated in vacuo. Flash chromatography (eluent: EtOAc/PE 1:6) afforded compound **8** (105 mg, 92%, 88% *ee*) as a colorless oil. $[\alpha]_D^{20}$ −41.3 (*c* 0.57, CH_2Cl_2) {lit.[15] $[\alpha]_D^{20}$ −43.0 (*c* 1.0, CH_2Cl_2)}; {The enantiomeric ratio was determined by HPLC analysis using a chiralcel AD-H column (iso-PrOH/heptane = 7/93, flow rate = 1.0 ml/min, t_R (*R*) = 6.86 min and t_R (*S*) =7.32 min)}; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.71-1.84 (m, 1H), 2.01–2.23 (m, 3H), 2.25-2.56 (m, 2H), 4.11-4.36 (m, 1H), 4.55 (s, 2H), 7.28-7.42 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 28.21, 35.14, 44.63, 65.60, 71.01, 126.74, 127.21, 128.22, 137.55, 217.68.

4.4.3.7. Reproduction of (R)-3-hydroxycyclopentanone by catalytic transfer hydrogenolysis

To 10% Pd/C (500 mg) was added a solution of compound **8** (380 mg, 2 mmol) in anhydrous EtOH (16 mL) and formic acid (2 mL). The mixture was stirred for 5 h at room temperature under a nitrogen atmosphere. The reaction mixture was filtered and concentrated under reduced pressure. The oily residue was purified by flash column chromatography on silica gel eluting with EtOAC-PE (2:1) to give compound **7** (164 mg, 1.64 mmol).

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5

RHODOCOCCUS RHODOCHROUS AS NOVEL SOURCE FOR ENE-REDUCTASE ACTIVITY

In this chapter, a series of substrates, including activated ketones, aldehydes, amines and nitro-compounds were screened for ene-reductase activity using whole cells of *Rhodococcus rhodochrous* ATCC 17895. This showed that *R. rhodochrous* is a very promising catalyst for the reduction of C = C bonds and harbours ene-reductases. Indeed, three candidates were observed from gene annotation of the genome of *R. rhodochrous* ATCC 17895 and were classified as ene-reductases by amino acid sequence alignment. Thus, the putative ene-reductase genes from *R. rhodochrous* ATCC 17895 were heterologously overexpressed in *Escherichia coli* and the encoded proteins were purified and characterized for their biocatalytic and biochemical properties.

This chapter will be the basis for a paper

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Manuscript in preparation.

5.1 INTRODUCTION

The asymmetric reduction of conjugated C = C bonds using ene reductases (ERs, EC 1.3.1.31) is receiving great interest in preparative organic chemistry,[1-6] because of its huge potential for applications in the pharmaceutical, fine chemical and advanced material industries. The name "ene reductase" derives from the flavin cofactor of its first representative, the old yellow enzyme isolated from *Saccharomyces carlsbergensis* in 1932.[6] Over the past decades, the number of isolated and characterized ene reductases has grown significantly and their use in recombinant form (either as isolated enzymes or recombinant whole cells) is now generally favored over wild-type whole cells. Many homologous enzymes of this class of flavoproteins have been found in yeasts, bacteria and plants.[8-16]

Overall, ene reductases have been discovered from *Arabidopsis thaliana* (OPR1, OPR2, OPR3),[17] *Lycopersicon esculentum* (OPR1, OPR2, OPR3),[18] *Saccharomyces pastorianus* (OYE1),[19] *Synechococcus elongates* (SynER), *Agrobacterium tumefaciens* (NerA),[20] *Pseudomonas fluorescens* (XenB),[21] *Zymomonas mobilis* (NCR),[22] *Gluconobacter oxydans* (EnR, GOX),[23] *Pseudomonas putida* (XenA, MR),[24] *Escherichia coli* (NemA),[25] *Enterobacter cloacae* (PETNR),[26] *Candida albicans* (EBP1),[27] *Pichia stipites* (OYE2.6),[28] *Kluyeromyces lactis* (KYE1),[13] *Kluyveromyces marxianus* (OYE),[29] *Saccharomyces cerevisiae* (OYE2, OYE3),[30] *Shewanella oneidensis* (SYE1, SYE3, SYE4),[31] *Lactobacillus casei* (LacER),[32] *Thermus scotoductus* (CrS),[33] *Geobacillus kaustophilus* (OYE),[34] *Bacillus subtills* (Yqjm),[35] *Agrobacterium radiobacter* (NER),[36] *Yersinia bercovieri* (Yer-ER),[23] *Thermoanaerobacter pseudethanolicus* (OYE).[37]

Herein we describe three new ene reductases isolated from *Rhodococcus rhochochrous* for the first time. The initially unexpected stereoselective reduction of conjugated C = C bonds was discovered during the studies on the enantioselective Michael addition of water using whole-cells of *Rhodococcus* strains.[38] The cells reduced α,β-unsaturated cyclic ketones into the corresponding ketones as initially undesired side reaction for the addition of water to C = C bonds.

A series of substrates, including activated ketones, aldehydes, amines and nitro-compounds were screened for ene-reductase activity using whole cells of *Rhodococcus rhodochrous*. This showed that *R. rhodochrous* is a very

promising catalyst for the reduction of C = C bonds and harbours ene-reductases. Generally, gene annotation offers an excellent opportunity for the discovering novel enzymes with the development of genome sequencing technology. Indeed, three candidates were annotated as OYE family NADH flavin oxidoreductases from gene annotation[39] of the genome of *R. rhodochrous* and were classified as ene-reductases by amino acid sequence alignment.

Thus, the putative ene-reductase genes from *R. rhodochrous* were heterologously overexpressed in *Escherichia coli* and the encoded proteins were purified and characterized for their biocatalytic and biochemical properties.

5.2 RESULTS AND DISCUSSION

5.2.1 Substrate specificity for ene-reductase activity of *R. rhodochrous* ATCC 17895

Since the ene reductases from *R. rhodochrous* were never reported in the literature previously, we were interested in the substrate scope and limitation. Therefore, we selected a series of structurally diverse α,β-unsaturated compounds[40] bearing a ketone, aldehyde, cyclic imide and nitro group as electron-withdrawing group using whole cells of *R. rhodochrous*. Different substrates require different reaction times to reach the best conversion, therefore all the reactions were carried out at pH 7.0 and at 30 °C according to pH and temperature optimun (data not shown) and the results are summarized in Table 1. The reduction product of 2-cyclohexenone was isolated and characterized by ^{1}H and ^{13}C NMR, comfirming that the reduction occured at the C = C bonds.

The results showed that *R. rhodochrous* was promisingly active for the reduction towards aliphatic and cyclic α,β-unsaturated carbonyl aldehydes and ketones. Among all of the tested substrates, 2-methylcyclopentenone showed the highest activity. For cyclic α,β-unsaturated ketones, substituted groups on the ring greatly affect the enzyme activity. For example, *R. rhodochrous* showed high activity on 2-methylcyclopentenone while no activity was detected for 3-methylcyclopentenone, the same is true for 2-methylcyclohexenone (accepted) and 3-methylcyclohexenone (not accepted).

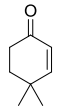
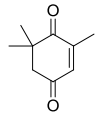
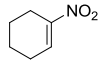
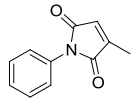
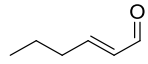
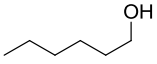
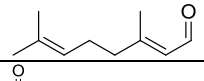
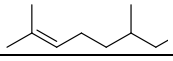
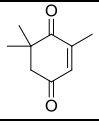
These results indicate that substituents in β-postition are a challenge for the ene reductase activity of *R. rhodochrous*, which can also be found from the examples of 4,4-dimethylcyclohexenone (accepted), ketoisophorone (accepted) and isophorone (not accepted). However, the enzyme could not reduce (*R*)-(-)-carvone or (*S*)-(+)-carvone, which have substituents in α-position, probably due to their more bulky stuctures. Here it should be mentioned that since the whole-cell (wild-type) approach is used, the additional carbonyl reduction also occurs in almost all the substrates due to the presence of alcohol dehydrogenase. Especially for *trans*-2-hexenal and citral, within 1h, all the substrates were reduced to the corresponding aldehydes and then further reduced to the alcohols. While, interestingly, the whole-cells reduction was still performed with high regio- and chemo-selectivity, the second double bond of citral stayed untouched. Nevertheless, intact (wild-type) cells offer the simplest and readily available methodology for biocatalytic alkene reductions because the reducing equivalents (usually supplied by NAD(P)H can be regenerated inexpensivly by cellular metabolism and because the actual enzyme(s) responsible were unknown.

With regard to the stereoselectivity of ene-reductases, different enzymes from different organisms generally reveal (*R*)-selectivitiy to ketoisophorone.[8-17,19-34] To date, only two putative styrene monooxygenase reductases from *Thermus thermophilus* (subsequently named *Tt*ENR) and from *Pyrococcus horikoshii* (subseqently named *Ph*ENR) were reported to reduce ketoisophorone with *S*-selectivity, and the corresponding product (*S*)-levodione was obtained with 8% conversion and 14% *ee*, 28% conversion and 87% *ee*, respectively.[41] Despite different protein sizes, folding types, and low sequence identities, both of them showed very similar arrangements of active site functional groups. The active site constellations in *Ph*ER and *Tt*ER show approximate mirror symmetry to the OYEs structures. Interestingly, our studies using wild-type whole-cells of *R. rhodochrous* ATCC 17895 yielding 14% (*S*)-levodione with 80% *ee,* demonstrated that *R. rhodochrous* ATCC 17895 habours an interesting, unusual and new (*S*)-selective ene reductase.

5.2.2 Classification of the ene-reductases

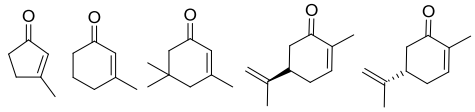
Substrate screening using whole-cells of *R. rhodochrous* showed that *R. rhodochrous* is a very promising catalyst for the reduction of C = C bonds

TABLE 1. Substrate screening for the reduction activity of whole-cells of *R. rhodochrous* ATCC 17895.

entry	Substrate	Reaction time (h)	Conversion of substrate (%)	Yield of product (%)	Ee (%)
1		8	96	86	-
2		2	98	74	-
3		2	98	58	-
4		1	99	99	4(*R*)
5		22	13	5	91 (*S*)
6		1	40	28	-
7		1	17	14	81 (*S*)
8		1	87	57	-
9		24	77	29	n.d.
10[a]		1	99		-
11[a]		1	99		-
control[b]		1	0	0	n.d.

[a]All the desired products were overreduced into alcohols; [b]Without the addition of whole-cells; n.d.: not determined.

Unconverted substrates:



(Table 1). In order to identify whether this strain harbours ene-reductases, bioinformatics studies were performed. We have sequenced and annotated the genome of *R. rhodochrous* ATCC 17895 (Chapter 3). Indeed, three candidates were annotated as "OYE family NADH flavin oxidoreductase" in strain *R. rhodochrous* ATCC 17895 during draft genome studies.[40] The predicted amino acid sequences were designated as RhrER 301, RhrER 2718 and RhrER 5439 and a multiple sequence alignment with some known ene reductases is performed using the Clustal W alignment tool.[42] The sequence identities of RhrER 301 with OYE 1 (*Saccharomyces pastorianus*), OYE 2 (*Saccharomyces cerevisiae*), OYE 3 (*Saccharomyces cerevisiae*), KYE1 (*Kluyveromyces lactis*), Yqjm (*Bacillus subtilis*), GOX (*Gluconobacter oxydans*), LacER (*Lactobacillus casei*) are 19.7%, 19.7%, 19.6%, 21.0%, 22.1%, 25.3%, 23.9% respectively. The sequence identities of RhrER 2718 with OYE 1 (*Saccharomyces pastorianus*), OYE 2 (*Saccharomyces cerevisiae*), OYE 3 (*Saccharomyces cerevisiae*), KYE1 (*Kluyveromyces lactis*), Yqjm (*Bacillus subtilis*), GOX (*Gluconobacter oxydans*), LacER (*Lactobacillus casei*) are 24.8%, 21.9%, 24.0%, 21.9%, 45.2%, 27.3%, 25.2%, respectively. The sequence identities of RhrER 5439 with OYE 1 (*Saccharomyces pastorianus*), OYE 2 (*Saccharomyces cerevisiae*), OYE 3 (*Saccharomyces cerevisiae*), KYE1 (*Kluyveromyces lactis*), Yqjm (*Bacillus subtilis*), GOX (*Gluconobacter oxydans*), LacER (*Lactobacillus casei*) are 21.4%, 17.9%, 20.8%, 20.0%, 23.2%, 21.6%, 27.5%, respectively. Notably, as shown in Figure 1, the residues of substrate binding, FMN binding and the most important catalytic site (it can be Histidine – Histidine – Tyrosine or Histidine - Asparagine – Tyrosine[41]) are highly conserved. Furthermore, phylogenetic analysis was also performed for the three putative ERs from *Rhodococcus rhodochrous* and 28 OYEs that have been reported to reduce activated alkenes in Figure 2, clearly indicating the position of the three new ERs.

5.2.3 Cloning, protein expression and purification

Standard cloning techniques were used to create three different recombinant plasmids and all of them were heterologously expressed in *E. coli* cells with N-terminal His6-tag. All of them contain the ene reductase genes. Due to the fact that all the three recombinant proteins show the same selectivity to ketoisophorone (*R*-selectivivity) and only RhrER2718 was predominantly present in the soluble fraction (Figure 3; RhrER 301 and RhrER 5439 were not

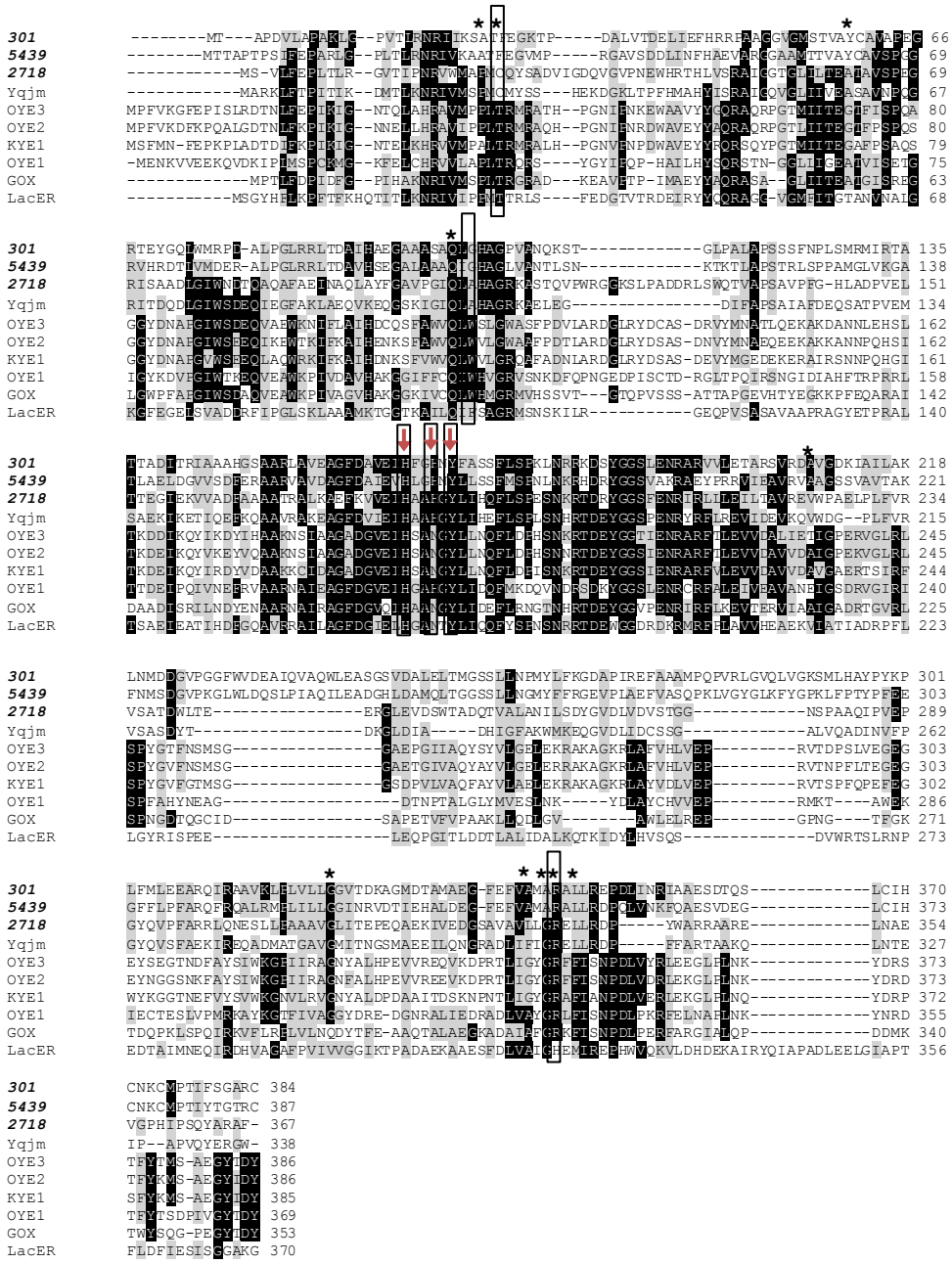


FIGURE 1. Amino acid sequence alignment of the three ene reductases from *R. rhodochrous* with several reported OYEs. The sequence of OYE 1 (*Saccharomyces pastorianus*), OYE 2 (*Saccharomyces cerevisiae*), OYE 3 (*Saccharomyces cerevisiae*), KYE1 (*Kluyveromyces lactis*), Yqjm (*Bacillus subtilis*), GOX (*Gluconobacter oxydans*), LacER (*Lactobacillus casei*) are compared with the protein sequence of the ene

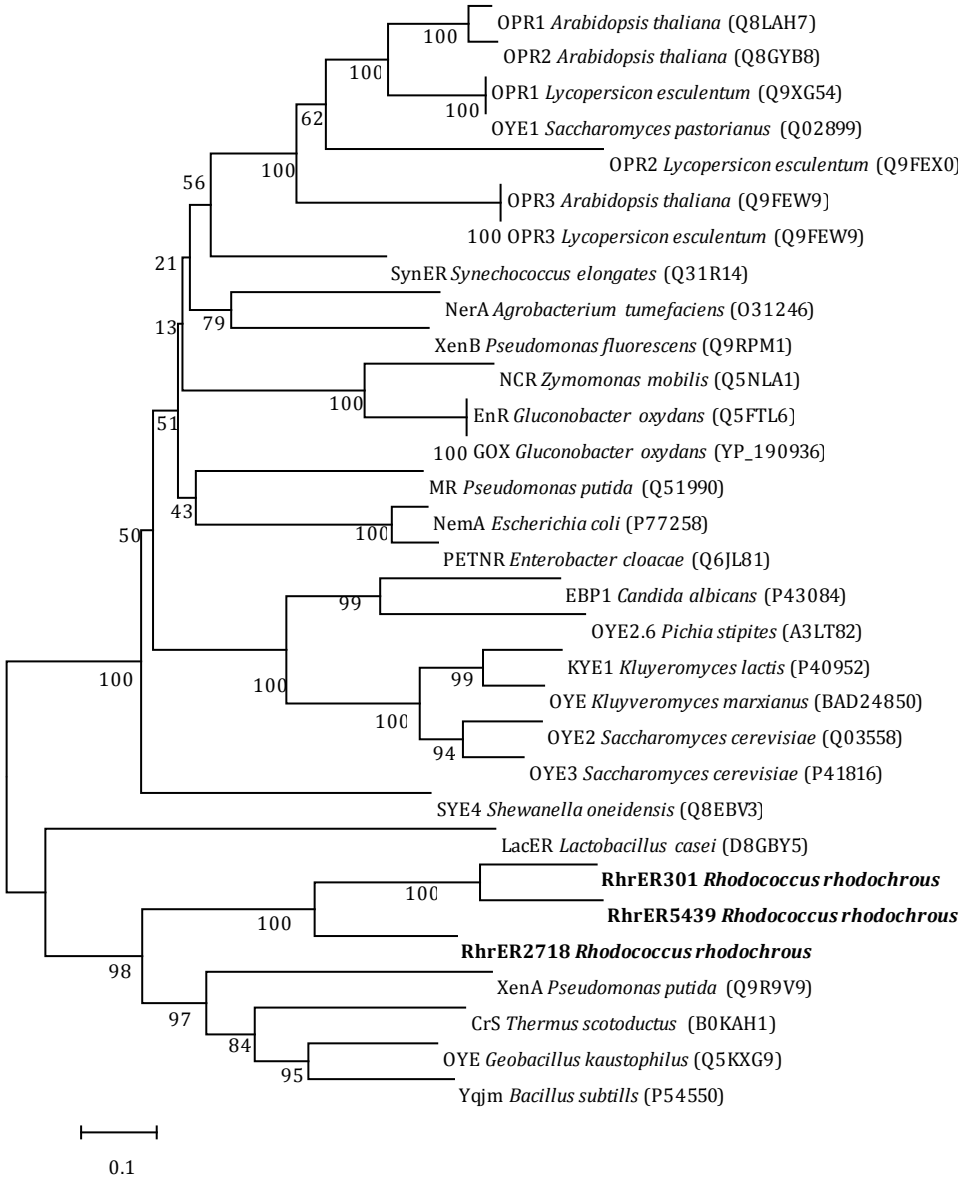


FIGURE 2. Phylogenetic relationship of RrhERs (Bold) to the other OYEs with known function. Amino acid sequences were used to generate the distance tree by Clustal W with default settings and a distance neighbor-joining tree was then constructed by using Mega (version 5.1). Bootstrap values expressed as percentages of 1,000 replications are shown at the nodes. The names of the proteins are indicated with the Genbank accession nos. given in parentheses.

reductases from *R. rhodochrous*. The alignment was created using the Clustal W alignment tool. Shaded boxes in black indicate conserved amino acid, grey ones highlight similar amino acids. We considered the following amino acids as similar: (A, S, T), (D, E), (F, Y, W), (I, L, M, V), (N, Q) and (R, K). The amino acid residues involved in substrate binding (black frames), FMN binding (*), and the catalytic sites (↓) are indicated.

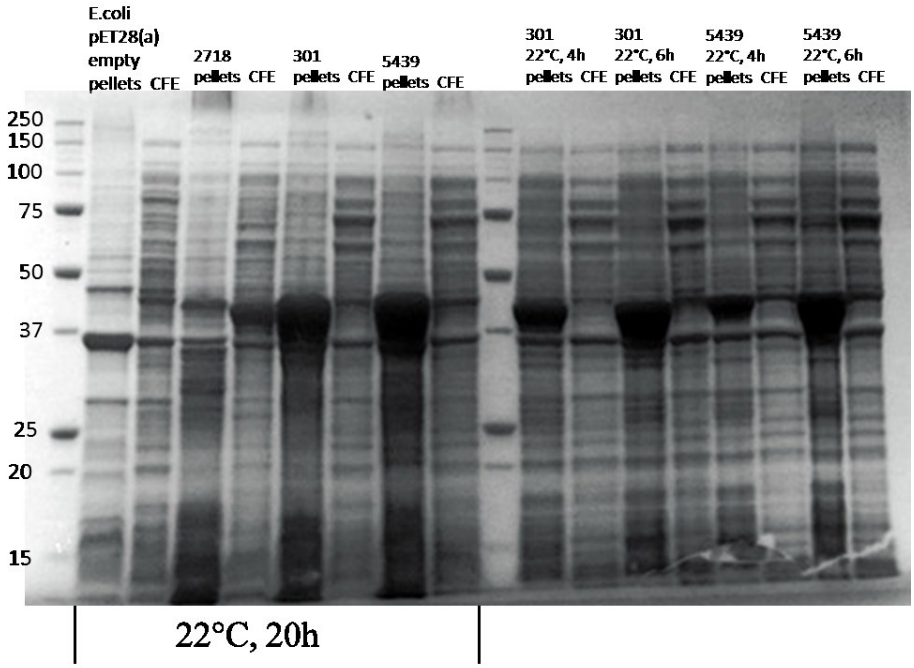


FIGURE 3. SDS-PAGE gel analysis revealed the optimal cultivation conditions. Lane 1 and 10, molecular weight marker (BenchMark Protein Ladder Invitrogen); lane 2, pellets of *E. coli* pET28a (+) empty; lane 3, crude cell-free extract of *E. coli* pET28a (+) empty; lane 4, pellets of RhrER2718 obtained after 20 h at 22 °C; lane 5, crude cell-free extract of RhrER2718 obtained after 20 h at 22 °C; lane 6, pellets of RhrER301 obtained after 20 h at 22 °C; lane 7, crude cell-free extract of RhrER301 obtained after 20 h at 22 °C; lane 8, pellets of RhrER5439 obtained after 20 h at 22 °C; lane 9, crude cell-free extract of RhrER5439 obtained after 20 h at 22 °C; lane 11, pellets of RhrER301 obtained after 4 h at 22 °; lane 12, crude cell-free extract of RhrER301 obtained after 4 h at 22 °C; lane 13, pellets of RhrER301 obtained after 6 h at 22 °C; lane 14, crude cell-free extract of RhrER301 obtained after 6 h at 22 °C; lane 15, pellets of RhrER5439 obtained after 4 h at 22 °C; lane 16, crude cell-free extract of RhrER5439 obtained after 4 h at 22 °C; lane 17, pellets of RhrER5439 obtained after 6 h at 22 °C; lane 18, crude cell-free extract of RhrER2718 obtained after 6 h at 22 °C;

soluble in all different culture conditions: 4 h at 22 °C, 6 h at 22 °C, 20 h at 22 °), RhrER2718 was purified by nickel affinity chromatography and used for all further investigations

The resulting specific activity of the ene reductase RhrER2718 on 2-methylcyclopentenone was 0.92 U/mg after the purification. This corresponds to a threefold improvement in specific activity compared to the crude extract. SDS-PAGE analysis revealed that a single band with an apparent molecular size of 40 kDa (Figure 4, lane 1), corresponds to the enzyme, confirming the theoretically calculated molecular mass (39.84 kDa) derived from the amino acid sequence. The native molecular weight was determined to be 75 kDa by Native polyacrylamide gel electrophoresis (PAGE), which suggests a dimeric structure for RhrER 2718, the same as *Ts*ER from *Thermus scotoductus*[33], OYE1 from *Saccharomyces pastorianus*[28] and OYE2.6 from *Pichia stipitis*.[28] In summary, a purification of the recombinant ene reductase to homogeneity (>95%) was achieved by a single purification step (Figure 4). Studies concerning cofactor dependency of the ene reductase RhrER2718 revealed that the cofactor NADH is highly preferred, almost no activity was detected when NADPH was tested as cofactor with 2-methylcyclopentenone as substrate. As such, RhrER2718 is a NADH-dependent ene reductase.

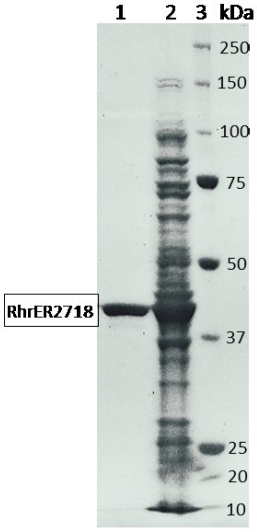


FIGURE 4. SDS-PAGE gel analysis of the purified ene reductase RhrER2718 from *R. rhodochrous*. Lane 3, molecular weight marker (BenchMark Protein Ladder Invitrogen); lane 1, purified ene reductase, lane 2, crude extract.

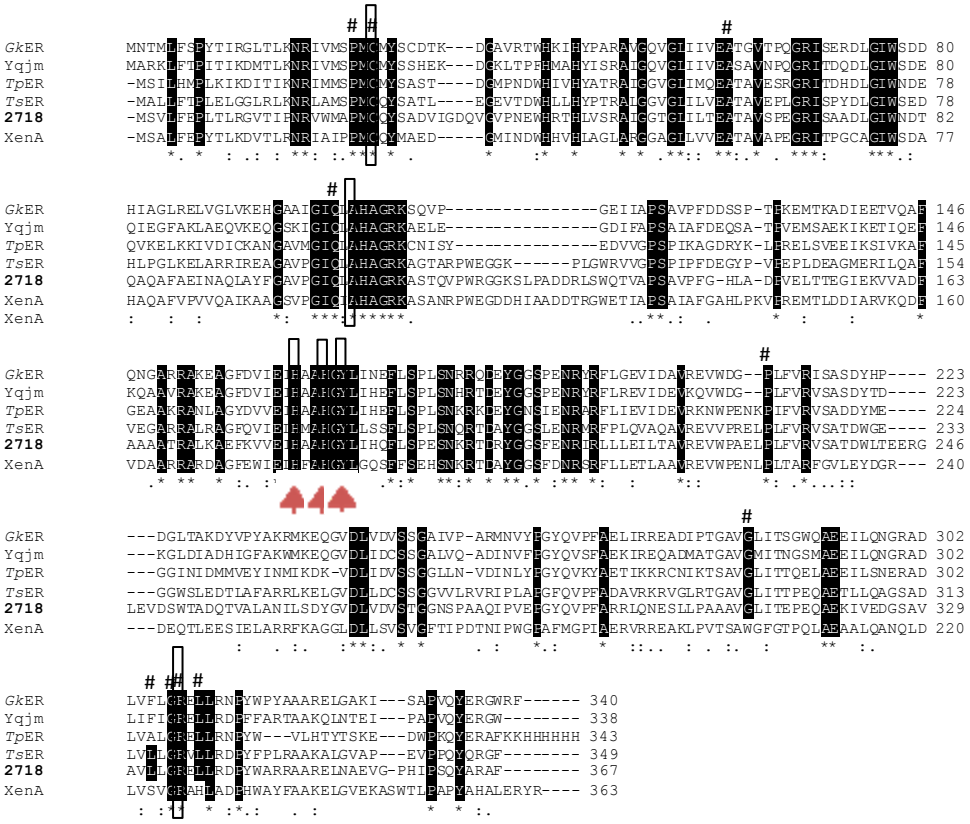


FIGURE 5. Amino acid sequence alignment of RhrER2718 from *R. rhodochrous* with the GenBank BLAST similar known ene reductases: TsER (3HF3), GkER (3GR7), XenA (3N14), TpER (3KRU) and Yqjm (1Z41A). Symbol indicate identical residues (*), conserved substitutions (:), and semiconserved substitutions (.). The amino acid residues involved in substrate binding (black frames), FMN binding (#), and the catalytic sites ▲ are indicated.

In addition, the coenzyme requirement of the ene reductase was investigated. BLAST analysis of the amino acid sequence of RhrER2718 against the GenBank showed the highest identity (51%) to be with an OYE homoloque (*Ts*ER) from *Thermus scotoductus*. High identity with the OYE (*Gk*ER) from *Geobacillus Kaustophilus* (49%), the thermostable OYE (*Tp*ER) from *Thermoanaerobacter Pseudethanolicus* (46%), the OYE homologue (YqjM) from *Bacillus subtilis* (45%), xenobiotic reductase (XenA) from *Pseudomonas putida* 86 (45%), were also found. Therefore multiple alignments of the gene product with TsER (3HF3), GkER (3GR7), XenA (3N14), TpER (3KRU) and

Yqjm (1Z41A) are shown in Figure 5. We can clearly see that identical catalytic tyrosine regions, the residues for substrate binding and FMN binding are present. These results suggest that FMN is the coenzyme of RhrER2718. This is confirmed by the observation of FMN in the HPLC analysis of the denatured enzyme solution. Since only FMN was detected we could demonstrate that the ene reductase requires FMN.

5.2.4 Effect of pH and temperature, thermostability, and kinetic parameters

The influence of temperature and pH on the enzyme activity and stability were investigated by monitoring the change in enzyme activity towards 2-methylcyclopentenone (Figure 6). The temperature profile of the purified RhrER 2718 was studied over a range of 20-60 °C (Figure 6A). The enzyme exhibited an optimal activity at 40 °C, while it steeply decreased over 50 °C, with only 10% relative activity at 60 °C. The activity-pH profile of the enzyme gave a broad peak over the range of pH 5.0-9.0 (Figure 6B). However, the enzyme showed no detectable activity at pH 10.0 or higher. The optimal activity was observed at pH 7.0 in potassium phosphate buffer. Studies concerning thermostability were performed at different temperatures from 20 to 70 °C (Figure 6C). The enzyme solutions were kept for 1h at each temperature before the sample were withdrawn to measure the residual activity. The result shows that the purified RhrER 2718 was relatively stable under 50 °C, but lost its activity almost completely after incubation at 60 °C for 1h. It is worthy to mention that this enzyme is stable at 30 °C for 24 h without activity loss (data not shown).. For 2-methylcyclopentenone, the Michaelis–Menten Plot (Figure 6D) allowed calculation of the affinity constant K_m to be 0.88 mM and the maximal specific activity (V_{max}) to be 0.93 μmol·min^{-1}·mg^{-1}, respectively.

5.2.5 Biotransformation with purified RhrER2718

In order to ensure comparability, we screened the same substrates which were accepted by whole cells of *R. rhodochrous* ATCC 17895 as described in Table 1, to assess the substrate scope and the stereospecificity of RhrER 2718

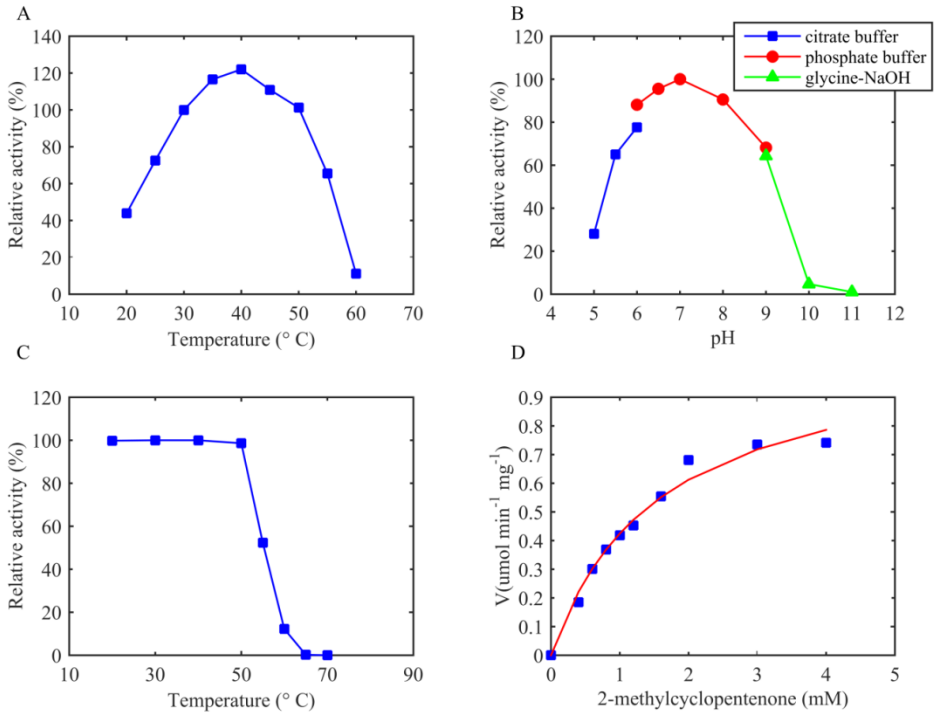


Figure 6. Temperature optima (A), pH optima (B), thermostability (C) and Michaelis-Menten kinetics (D) of the purified RhrER 2718. The activity was measured using the standard UV assay towards 2-methylcyclopentenone. (A): All the reaction mixtures were kept at given temperatures for 5 min before NADH and enzyme solution were added to initiate the reaction; (B): The activity was assayed in the following 50 mM buffers: (i) sodium citrate (pH 5.0–6.0) (■), (ii) potassium phosphate (pH 6.0–9.0) (•), and (iii) glycine-NaOH (pH 9.0–11.0) (▲) after incubate reaction mixture at 30 oC for 5 min before NADH and enzyme solution were added to initiate the reaction; (C): The enzyme solutions were kept for 1h at each temperature before the samples were withdrawn to measure the residual activity in the soluble protein content; (D): From these plots, V_{max} and K_m were measured as 0.931 µmol.min-1.mg-1 and 0.88 mM, respectively.

(Table 2). As expected, RhrER 2718 also catalysed the reduction of cyclic and linear alkenes having carbonyl and nitro group, following the same general trend observed in *Rhodococcus* as discussed above. The biotransformation was performed aerobically on 1.0 mL scale with 10 mM of substrate and 12.5

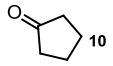
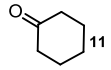
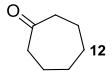
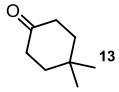
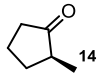
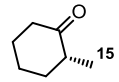
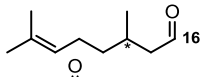
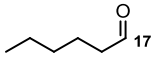
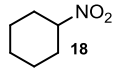
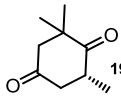
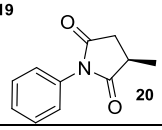
mM of NADH, in potassium phosphate buffer (pH 7.0, 50 mM) at 30 °C for 4h. Among all the tested substrates, the best activity was obtained in the reduction of 2-methylcyclopentenone (entry 5), yielding 99% (*S*)-2-methylcyclopentanone with 96% *ee* within 4h with 200 µg/mL of enzyme. Here it should be mentioned that the racemisation of the corresponding reduction product (*S*)-2-methylcyclopentanone (**14**) in aqueous media was observed, with loss enantiopurity over time (data not shown). A similar time-dependent loss of enantiopurity for this compound was also seen with other ene-reductases,[46,47] although it was not investigated in detail. Only one previously reported publication[48] carefully evaluated all the conditions influencing the loss enantiopurity of the products. And it concluded that the reduction products with a stereogenic centre at α-carbon (like compound **14**, **15**, **19**) racemised quickly in aqueous media over time. It also further demonstrated that the racemisation is primarily a non-enzymatic, chemical process, depending on the nature of the product. For this reason, the enzyme concentration for 2-methylcyclopentenone was used as 200 µg/mL since the enzymatic reaction completed within 4 h under this condition, while 400 µg/mL of enzyme concentration was used for the rest of substrates in order to obtain the complete conversion within 4h.

In a second study, the reduction products of 2-methylcyclopentenone was isolated from a scale-up reaction (25 mL, with 80% isolated yield and 86 % *ee*) and characterized by GC-MS, confirming that the reduction occurred at C=C bond. The absolute configuration of **14** was done by measurement of the optical rotation of the purified product and comparison with literature data.[52] The determination of the absolute configuration of levodione **19** was identified by chiral GC analysis with the isolated and characterized (*S)*-levodione which was prepared by *R. rhodochrous* ATCC 17895 as described above.

5.2.6 Purification of the ene reductase directly from *R. rhodochrous* ATCC 17895

The isolation of (*S*)-selective enzyme(s) harbours ene-reductase activity from *R. rhodochrous* ATCC 17895 is currently underway in our laboratory.

Table 3. Substrate scope for the reductions of α,β-unsaturated carbonyl compounds (**1-9**) by RhrER 2718[a]

Entry	Substrate	Product	Yield (%)[c]	Ee (%)[c]
1	**1a**		90	n.a.[d]
2	**1b**		96	n.a.[d]
3	**1c**		93	n.a.[d]
4	**1d**		85	n.a.[d]
5[b]	**2a**		99	96 (*S*)
6	**2b**		57	99 (*R*)
7	**4**		52	n.d.[e]
8	**5**		83	n.a.[d]
9	**6**		97	n.a.[d]
10	**8**		99	93 (*R*)
11	**9**		92	n.d.[e]

[a]Conditions: [substrate]=10 mM, [NADH]=12.5 mM, [RhrER 2718]=400 μg/mL, T = 30 °C, reaction time= 4 h;
[b][RhrER 2718] = 200 μg/mL
[c]determined by GC analysis;
[d]n.a.= not applicable;
[e]n.d. = not determined;

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5.3 CONCLUSTION

In summary, ene reductase activity was discovered from *Rhodococcus* strains for the first time. The enzyme-catalysed asymmetric C = C reduction of a series activated alkenes as substrates comprising nitroalkenes, carboxylic acids and esters, using whole-cells of several *Rhodococcus* strains were investigated. Examining the annotated genome of *R. rhodochrous* ATCC 17895 showed three candidates (RhrER301, RhrER2718 and RhrER5439) which were further classified as ene reductases by amino acid sequence alignment and phylogenetic relationship with several reported ene-reductases. All the three

genes were successfully cloned and expressed in *E. coli* BL 21 (DE3), although only RhrER2718 is predominantly present in the soluble fractions. In order to solubilize RhrER301 and RhrER5439, different culture conditions were investigated but no improvement was achieved to date. Therefore only the encoded protein RhrER2718 was purified and characterized.

The stereoselectivity of a couple of α,β-unsaturated ketones using wild-type whole-cells of *R. rhodochrous* ATCC 17895 was measured by chiral GC analysis. Interestingly, ketoisophorone was reduced into the corresponding saturated carbonyl compound levodione with *S*-selectivity (81% *ee* and 14% yield). It is worthy of noting that all of the known ene reductases only gave *R*-selectivity to ketoisophorone. Only two enzymes *Ph*ENR and *Tt*ENR, which have been reported to have promiscuous ene-reductase activity, yielded *S*-levodione in 8% conversion with 14% *ee* and 28% conversion with 87% *ee*, respectively. Looking for the stereoselectivity of the three encoded proteins, RhrER 301, RhrER 2718 and RhrER 5439 only displayed *R*-selectivity towards ketoisophorone, emphasizing the *S*-selective ene reductase is still missing within the database.

Furthermore, bioinformatic studies based on the sequences of *Ph*ENR and *Tt*NER did not give any hint on the *S*-selective ene reducatse from *R. rhodochrous* ATCC 17895. Further work is currently under way to isolate *S*-selective enzyme(s) from *R. rhodochrous* ATCC 17895 by HPLC in our laboratory.

The physiological reductant of ene-reductases is assumed to be NADPH. A majority of characterized ene reducatases prefer NADPH over NADH or are not specific in this respect. The purified RhrER 2718 was identified as a NADH dependent ene reductase, thus, belonging to the few family members of ene-reductases with a preference for NADH to date.

5.4 EXPERIMEANTAL

5.4.1 Material and method

Vector pET-28a(+) was purchased from Novagen (Merk, Germany). Reduced form (NADH and NADPH) of nicotinamide adenine dinucleotide were from Sigma-Aldrich. All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) and were used without further purification unless otherwise

specified. The culture media components were obtained from BD (Becton, Dickinson and Company, Germany).

^{1}H and ^{13}C NMR spectra were recorded with Bruker Advance 400 or Varian 300 (400 MHz and 100 MHz, respectively) instrument and were internally referenced to residual solvent signals. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration, coupling constant (Hz) and assignment. Data for ^{13}C NMR are reported in terms of chemical shift. Optical rotations were obtained at 20 °C with a Perkin-Elmer 241 polarimeter (sodium D line). Column chromatography was carried out with silica gel (0.060-0.200 mm, pore diameter ca. 6 nm) and with mixtures of petroleum ether (PE) and ethyl acetate (EtOAc) as solvents. Thin-layer chromatography (TLC) was performed on 0.20 mm silica gel 60-F plates. Organic solutions were concentrated under reduced pressure with a rotary evaporator.

Conversion of substrates and yield of products were quantified by GC using calibration lines with dodecane as an internal standard (specifications and temperature programs given in Table 3) and the optical purity of the products were determined using chiral GC (specifications and temperature programs given in Table 3).

5.4.2 Biotransformation

5.4.2.1 General biotransformation procedure for substrate screening of Rhodococcus strains

Whole-cells of *Rhodococcus* strains were obtained as described previously.[15] Reactions were carried out in 1.5 mL screw-capped glass vials to prevent evaporation of substrate/product. 50 mg lyophilized cells were resuspended in 1 mL of potassium phosphate buffer (pH 7.0, 50 mM) and the substrate was added to a final concentration of 10 mM. The mixture was incubated at 30 °C in a heated table top shaker for the given hours. Control experiments were performed in the same reaction system in the absence of cells. For work-up, the cells were discarded after centrifugation at 13000 rpm for 2 mins and the aqueous reaction mixtures (0.8 mL) were saturated with NaCl followed by extraction with ethyl acetate (containing internal standard)

TABLE 3. GC retention times for the determination of conversion and *ee*

Column CP Sil 5CB (50 m x 0.53 mm x 1.0 µm) A Column Mega-Dex Det Beta (25 m x 0.25 mm x 0.25 µm) B Column CP-Chirasil-Dex CB (25 m x 0.32 mm x 0.25 µm) C			
Column	**Program**	**Compound**	**Retention time (min)**
A	50/3/3/65/1/30/150/2/50/250/1/50/2 70/1/50/330/1	2-cyclopenten-1-one cyclopentanone	4.6 3.9
A	50/3/3/65/1/30/150/2/50/250/1/50/2 70/1/50/330/1	2-cyclohexen-1-one cyclohexanone	7.6 6.4
A	50/3/3/65/1/30/150/2/50/250/1/50/2 70/1/50/330/1	2-cyclohepten-1-one cycloheptanone	10.7 10.2
A	50/3/3/65/1/30/150/2/50/250/1/50/2 70/1/50/330/1	2-methylcyclopentenone 2-methylcyclopentanone	8.8 5.1
A	50/3/3/60/3/3/70/1/30/150/2/50/270 /1/50/330/1	2-methylcyclohexenone 2-methylcyclohexanone	11.0 8.5
A	60/1/10/65/10/30/135/2/35/330/2	4,4-dimethylcyclohexenone 4,4-dimethylcyclohexanone	9.6 9.0
A	50/3/3/65/1/30/150/2/50/250/1/50/2 70/1/50/330/1	1-nitro-1-cyclohexene nitrocyclohexane	12.0 11.2
A	50/3/3/65/1/30/150/2/50/250/1/50/2 70/1/50/330/1	trans-2-hexenal hexanal	5.4 4.2
A	50/1/30/90/20/50/250/1/50/330/1	citral citronellal citronellol geraniol	15.8/18.3 10.1 15.5 17.5
A	50/1/30/150/2/30/180/5/30/250/1/3 0/270/1/30/330/1	2-methyl-*N*-phenylmaleimide 2-methyl-*N*-phenylsuccinimide	8.5 9.0
B	75/6/10/110/2/30/230/2	(*S*)-2-methylcyclopentanone (*R*)-2-methylcyclopentanone 2-methylcyclopentenone	9.3 9.6 11.6
B	75/2/10/110/7/30/230/2	(*S*)-2-methylcyclohexanone (*R*)-2-methylcyclohexanone 2-methylcyclohexenone	10.0 10.2 12.8
C	120/3/10/150/4/15/225/1	ketoisophorone (*R*)-levodione (*S*)-levodione	7.1 7.7 7.9
Program: initial temperature (°C) / hold time (min) / slope (°C/min) / temperature (°C) / hold time (min) / slope (°C/min) / temperature (°C) / hold time (min) / slope (°C/min) / temperature (°C) / hold time / slope (°C/min) / temperature (°C) / hold time			

(2 × 0.4 mL). Combined organic layers were dried over Na_2SO_4 and measured on GC (for method see Table 3).

5.4.2.2 Molecular cloning of the ene-reductases genes

Chromosomal DNA of strain *R. rhodochrous* ATCC 17895 was isolated, sequenced and annotated as described previously.[45] The sequences of the three putative ene-reductases (RhrER 301, RhrER 2718 and RhrER 5439; 301, 2718, 5439 means the number of annotated enzymes) were taken from the gene annotation of the genome of *R. rhodochrous* ATCC 17895. The three putative ene reductase genes were coden optimized for *E. coli*, synthesized, and cloned in expression vector pET28a(+) between the NdeI and HindIII restriction sites by BaseClear (http://www.baseclear.com/).

5.4.2.3 Heterologous expression and purification of the ene-reductase

The recombinant plasmid was subsequently transformed into *Escherichia coli* BL21 (DE3) cells, which were cultivated overnight at 37 °C in 15 mL LB medium containing 50 µg/mL kanamycin. For expression in conical flasks, a final concentration of 0.05 (OD_{600}) was used to inoculate different amounts of LB medium containing 50 µg/mL kanamycin at 37 °C. When OD_{600} reached 0.5-0.6, the production of the recombinant ene reductase was induced by addition of isopropyl thio-β-D-galactoside (IPTG) to a final concentration of 0.1 mM. For the determination of the optimal conditions cultures were grown after induction at 22 and 30 °C, and assayed after a period of 4, 6 and 20 h. *E. coli* pET28a(+) empty was cultivated and induced with the same system as control experiments. Cells were harvested by centrifugation (10000 rpm, 20 min, 4 °C) and washed two times with potassium phosphate buffer (pH 7.0, 50 mM). Harvested cells were resuspended in potassium phosphate buffer (pH 7.0, 50 mM) again and disrupted by sonication on ice (5 min (25% power output), 5 min cooling, five cycles in total). After a centrifugation step (13000 rpm, 30 min, 4 °C), the cleared crude extract was used for protein purification on Shimadzu using a HisTrap affinity column (5 mL, GE Healthcare). The column was equilibrated with potassium phosphate buffer (pH 7.0, 50 mM). After loading, the sample and elution of non-bound proteins with equilibration buffer a linear gradient of imidazole (0-500 mM, 60 min) in potassium

phosphate buffer was performed. The ene reductases elutes at 130 mM imidazole. After a desalting step (PD-10 desalting column) with potassium phosphate buffer (pH 7.0, 50 mM), the enzyme was stored at -20 °C prior to use.

5.4.2.4 Protein analysis

Protein concentration was determined using the Bradford assay with BSA as a standard. SDS-PAGE was carried out on 4-12% (or 12% or 10%) Bis-Tris gels running in MOPS buffer, SeeBlue Plus2 Protein standard (both Invitrogen, Darmstadt, Germany) was used for molecular weight.

5.4.2.5 Activity assay

The ene reductase activity was assayed by monitoring the oxidation of NADH through the decrease in UV at 340 nm using a molar absorption coefficient of 6.22 $mM^{-1}cm^{-1}$. 2-methylcyclopentenone was used as standard substrate. One unit of activity was defined as the amount of enzyme catalysing the oxidation of 1 µmol NADH per minute under standard conditions (pH 7.0, 30 °C). The standard assay mixture (1 mL) was composed of 960 µL substrate solution [10 mM in potassium phosphate buffer (pH 7.0, 50 mM)], 20 µL NADH (12.5 mM in distilled water) and 20 µL enzyme solution. Reactions were started by addition of the enzyme solution and measured for 1 min. The activity of the crude extract was determined under the control of *E. coli* pET28(a) empty with the same conditions.

5.4.2.6 Determination of pH and temperature optima, stability, kinetic parameters

The optimum pH was determined by standard activity assay at different pH in the range of 5.0-11.0, with sodium citrate buffer (50 mM) for a pH range from 5.0-6.0, potassium phosphate buffer (50 mM) for a pH range from 6.0-9.0, glycine-NaOH buffer (50 mM) for pH 9.0-11.0. For the determination of the temperature optima, standard activity assy was performed at different temperatures in the range of 20-60 °C. The reaction mixtures were kept at each temperature for 5 min before NADH and enzyme solution were added to

initiate the reaction. And activity measure at standard condition (pH 7.0, 30 °C) was taken as 100%.

In order to determine the thermostability, the enzyme was incubated at different temperatures (20-70 °C) for 1h, and the residual activity was measured using 2-methylcyclopentenone as the substrate at 30 °C by standard activity assay. The activity of the enzyme without incubation at the given temperature was defined as 100%.

The kinetic parameters were performed by the standard activity assay with 2-methylcyclopentenone as substrate in duplicate at various concentrations (0.1-5 mM) and a constant NADH concentration (0.25 mM). The K_m and K_{cat} values were calculated from non-linear regression of Michaelis-Menten plots.

5.4.2.7 Biotransformation of activated alkenes

When possible, stock solutions (100 mM) of the substrates were prepared in potassium phosphate buffer (pH 7.0, 50 mM). *N*-Phenyl-2-methylmaleimide, that is not soluble in water, was dissolved in DMF (1% final DMF concentration). The final concentration of substrate in reaction system was 10 mM.

The biotransformations were carried out in 1.5 mL screw-capped glass vials to prevent evaporation of substrate/product. Reactions were preformed aerobically in a mixture (1.0 mL) containing potassium phosphate buffer (50 Mm, pH 7.0), 10 mM of substrate, 12.5 mM of NADH, 200 µg/mL (or 400 µg/mL) of purified RhrER 2718. The reaction mixture was shaken for 4h at 30 °C with 1000 rpm in a thermoshaker and then extracted with 2 x 0.5 mL of ethyl acetate (containing internal standard) by shaking for 2 mins. The combined organic layer was dried over on Na_2SO_4 and measured with GC for conversion, yield and *ee* (for method see Table 3).

5.4.2.8 Determination of the flavin species

The pure enzyme sample was incubated for 10 min at 99 °C to release the cofactor, denatured protein was removed by centrifugation at 14000 rpm for 30 min. The supernatant was spun through a Microcon YM3 (Millipore, MWCO, 3000 Da) centrifugal concentrator device (14000 rpm, 30 min) to

remove residual protein. The resulting sample was analyzed by the high performance liquid chromatography (HPLC) to identify the flavin cofactor. For the separation and quantification of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), a reverse phase C18 HPLC column connected to a Shimadzu LC10Ai HPLC system was used. Ammonium acetate (50 mM, pH 6.0) and 70% acetonitrile in ammonium acetate (50 mM, pH 6.0) were used as mobile phase.[22] The retention times of FAD and FMN are 6.53 and 9.12 min, respectively.

5.4.2.10 Preparative synthesis of (S)-2-methylcyclopentanone

For isolation and characterization of the reduction product, the reaction was carried out on preparative scale. 250 mg of the lyophilized cells of *E. coli* expressing RhrER 2718 were resuspended in 50 mL of potassium phosphate buffer (50 mM, pH 7.0), and substrate 2-methylcyclopentenone (**2a**; 98 mg, 1 mmol) and NADH (1 mmol) was added. Reaction was incubated at 30 °C and shaken at 180 rpm for 4 h. Then the cells were removed by centrifugation and the supernatant was saturated with NaCl and then extracted with ethyl acetate. The extract was then concentrated under reduced pressure and purified by flash column chromatography on silica gel (eluent: PE/EtOAc 1:1) to yield (*S*)-2-methylcyclopentanone (80 mg, 0.80 mmol, 80% yield, 86% *ee*) as a colorless oil; $[\alpha]_D^{20}$ = + 102.5° (*c* 0.44, methanol) {lit.[49] [$[\alpha]_D^{20}$ = + 109.5° (*c* 1.06, methanol) for *S*-isomer }; GC-MS and GC analysis were identical with the data of the commercial sample.

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6

CONCLUSIONS AND FUTURE RESEARCH

This chapter will summarize the key finds of the research presented in this thesis, followed by further research in future.

This thesis describes a direct, enanioselective Michael addition of water in water to conjugated C = C bonds, using whole-cells of *Rhodococcus* strains. This study aims to enhance our understanding of the chemistry of water addition to C = C bonds, which is almost unexploited. Since most of the hydratases are involved in the primary and secondary metabolism, it might also well be expected that more hydratases can be found from natural sources or obtained via protein engineering. One of the most important factors of the chemistry of hydration reaction is the stereoselectivity. When the research described in this thesis started, we realized that limited or no literature was available on the stereoselectivity determination of the Michael addition products (β-hydroxycarbonyl compounds) and hence the preparation of enantiomerically enriched β-hydroxycarbonyl compounds by kinetic resolution was established. In order to identify the new hydratase, genome sequence and annotation of *Rhodococcus* strain was performed. Looking for the annotated hydratase only showed known hydratases with their narrow substrate specificity, emphasizing the hydratase of this study has not been described before. Moreover, we unexpectedly found three putative ene-reductase from the annotated genome. With the results presented in the previous chapters, the main objectives as mentioned in Chapter 1 have been met and the main conclusions and future research are summarized in this chapter.

6.1 CONCLUSION

1. Since the chemical hydration reactions display an almost exclusive preference for the *anti*-addition, Chapter 1 provides unifying ideas for stereochemistry of the enzymatic water addition to carbon–carbon double bonds. Based on understanding the enzymatic reaction mechanism new chemical catalysts or artificial hydratases can be developed (Chapter 1).

2. A direct Michael addition of water in water for the preparation of chiral β-hydroxy carbonyl compounds was established, employing whole cells from several *Rhodococcus* strains harboring a Michael hydratase (Chapter 2).

3. Our studies suggests that the Michael hydratase is not soluble but membrane-bound or cell wall-associated. This natural immobilization of the Michael hydratase allows the high reusability of the whole cells (Chapter 2).

4. None of the substrates investigated in Chapter 2 are known to be part of primary metabolic pathways, it might well be expected that more hydratases can be found from natural sources or obtained *via* protein engineering (Chapter 1 and 2).

5. Direct Michael addition of water to C = C bonds opens up an entirely new approach to the synthesis of chiral 3-hydroxy carbonyl compounds (Chapter 2).

6. The features of *Rhodococcus rhodochrous* ATCC 17895, together with the complete genome sequence and annotation is described (Chapter 3).

7. A straightforward approach to enantiomerically enriched (*R*) and (*S*)-3-hydroxycyclopentanone is described by kinetic resolution. The isolated alcohols are used for stereoselectivity determination of Michael addition products (Chapter 2 and 4).

8. Ene-reductase activitity is discovered from *Rhodococcus* strains for the first time (Chapter 2 and 5).

9. Three candidates were isolated from *R. rhodococcus* ATCC 17895 and classified as ene-reductases by amino acid alignment with known OYEs. They were successfully cloned and expressed in *E. coli* BL 21 (DE3) (Chapter 5).

10. All the three ene-reductases are identified as a NADH dependent ene reductase, belonging to the few family members of Old Yellow Enzymes with a preference for NADH (Chapter 5).[1-3]

6.2 FUTURE RESEARCH

6.2.1. Michael hydratase isolation from *Rhodococcus* strains

As mentioned in Chapter 2, the desired hydratase activity was only found in the cell pellets, rather than in the crude cell-free extract after whole-cells of *R. rhodochrous* ATCC 17895 were broken. Furthermore, no significant difference was found between the initial rate of whole-cells and pelleted cell debris. These results indicate that the Michael hydrase is not a soluble protein but bound to either the membrane or cell wall. It is important to further note whether the Michael hydratase is membrane-bound or cell wall-associated. Differential centrifugation as shown in Figure 1 can be used to separate crude cell-free extract, membrane and cell wall. The desired hydratase activity was only found in cell wall, rather than in membrane or crude cell-free extract, indicating the Michael hydratase is cell wall-associated. Apparently, isolation, solubilisation and purification of cell wall-associated protein is a more challenging task, especially since *R. rhodochrous* ATCC 17895 is a Gram-positive bacteria.

Gram-positive bacteria have a thick cell wall composed of primarily of peptidoglycan, a large macromolecule of acetamido sugars and amino acids.[4] Most Gram-positive cell walls are resistant to dissolution with lysozyme.[5] A convenient and efficient mutanolysin extraction method for the solubilization of cell wall- associated proteins from *S. pyogenes* has been reported by Walker and co-workers.[6] If this mutanolysin extraction method allows the

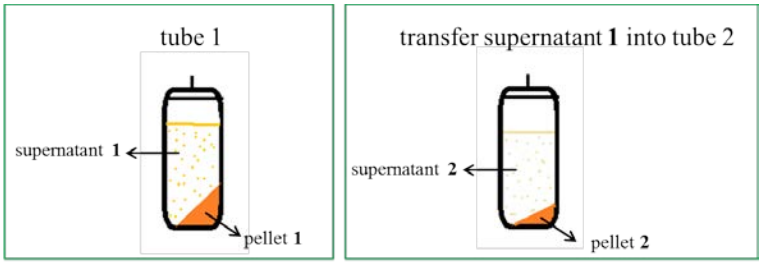

FIGURE 1. Differential centrifugation used to separate crude cell-free extract, membrane and cell wall:
1) Tube 1 contains cell suspension after disruption with a French Press;
2) Supernatant 1 (containing crude cell-free extract and membrane) and pellet 1 (containing unbroken cells and cell wall) were separated by centrifugation tube 1 at 4 °C with 4000 rpm for 10 min;
3) Transfer supernatant 1 (containing crude cell-free extract and membrane) into a clean tube 2;
4) Supernatant 2 (containing crude cell-free extract) and pellet 2 (containing membrane) were separated by centrifuge tube 2 at 4 °C with 20000 rpm for 60 min.

solubilisation of the Michael hydratase from the cell wall of *R. rhodochrous* ATCC 17895, a one-step (or more) standard HPLC purification might be performed. The fractions with the desired hydratase activity can be applied to a NATIVE-PAGE gel. All the protein bands will be excised from the gel and measured by nano-LC-ESI-QTOF tandem mass spectrometry. Peptide sequence will be used to look for similar annotated enzyme from the whole genome sequence of *R. rhodochrous* ATCC 17895 in order to identify the Michael hydratase.

6.2.2. Preparation of other chiral 3-hydroxy ketones by kinetic resolution using lipases

A straightforward approach to enantiomerically enriched (*R*)- and (*S*)-3-hydroxycyclopentanone has been described in chapter 4. The key step involves a kinetic resolution of racemic 3-hydroxycyclopentanone using commercial *Pseudomonas cepacia* lipase immobilized on diatomite (Amano lipase PS-DI). This methodology allows to perform the kinetic resolution by screening different lipases with the other β-hydroxy ketones structurally closely related to 3-hydroxycyclopentanone, for instance, 4-hydroxy-4-

phenylbutan-2-one and 4-hydroxypentan-2-one, especially those that are not commercially available or very expensive.

6.2.3. *S*-selective ene reductase isolation from *R. rhodochrous* ATCC 17895

Looking for annotated ene reductases only gave *R*-selective ene reductases from the genome of *R. rhodochrous* ATCC 17895, emphasizing the *S*-selective ene-reductase has not been described in the database. Actually only two ene reductases from *Thermus thermophiles* (subsequently named *Tt*ENR) and *Pyrococcus horikoshii* (subsequently named *Ph*ENR) have been reported very recently with this *S*-selectivity. Therefore, on one hand, further bioinformatics studies should be performed between known *S*-selective ene-reductases and whole genome of *R. rhodochrous* ATCC 17895 in order to have the gene information of the missing *S*-selective ene reductase. On the other hand, crude cell-free extract of wild-type *R. rhodochrous* ATCC 17895 might be applied to a one-step (or more) HPLC purification, afterwards, the fraction with *S*-selective ene reductase activity can be loaded to a NATIVE-PAGE gel. All the protein bands will be excised from the gel and measured by nano-LC-ESI-QTOF tandem mass spectrometry. Peptide sequence will be used to look for similar annotated enzymes from the whole genome sequence of *R. rhdochrous* ATCC 17895 in order to identify the *S*-selective ene-reductase.

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SUMMARY

β-Hydroxy carbonyl compounds are an important class of compounds often found as a common structural motif in natural products. Although the molecules themselves look rather simple, their synthesis can be challenging. Water addition to conjugated C = C bonds opens up a straightforward route for the preparation of β-hydroxy carbonyl compounds. Moreover, water addition to C = C bonds benefits a lot from its simplicity and excellent atom economy. However, the enantioselective addition of water to α,β-unsaturated carbonyl (Michael) acceptors still represents a chemically very challenging reaction, due to the poor nucleophilicity of water and its small size, which make regio- and stereoinduction difficult. Equally, the often unfavorable equilibrium of water-addition reactions remains to be solved. In contrast, enzymes such as fumarase, malease, citraconase, aconitase, and enoyl-CoA hydratase have been successfully used on industrial scale, and their excellent (enantio-) selectivities are highly valued. Unfortunately, most hydratases are part of the primary metabolism where perfect substrate specificity is required. This very high substrate selectivity severely limits their practical applicability in organic synthesis. Thus, a straightforward approach with broad applicability still had not been described. The aim of the research presented in this thesis was to take up this challenge and dedicated to the search for a Michael hydratase with a more relaxed substrate specificity for the preparation of important β-hydroxy carbonyl compounds.

The stereospecificity of enzyme-catalysed reactions has been a fruitful source of information about the mechanisms of enzyme catalysis and vice versa; the application of stereospecifically labelled substrates allows for studying the course of the reaction. It offers a very promising opportunity to comprehensively understand the precise mechanistic and kinetic details of even the most complex enzymatic reactions. Thus **Chapter 1** provides unifying ideas for stereochemistry of the enzymatic water addition to C = C bonds. This enhances our understanding of the chemistry of water addition to C = C bonds, and further allows us to find more hydratases from natural sources or obtained *via* protein engineering.

In **Chapter 2**, a direct, enantioselective Michael addition of water in water to prepare important β-hydroxy carbonyl compounds using whole cells of *Rhodococcus* strains is described. Good yields and excellent enantioselectivities were achieved with this method. This opens up an entirely new approach for the preparation of important β-hydroxy carbonyl compounds. Deuterium labelling studies demonstrate that a Michael hydratase catalyzes the water addition exclusively with *anti*-stereochemistry, which belongs to the family members of hydratases: oleate hydratase, fumarase, malease, aconitase and type II dehydroquinase with a preference for the *anti*-addition; whereas, type I dehydroquinase, enoyl-CoA hydratase and artificial hydratase exclusive prefer for the *syn*-addition, as discussed in Chapter 1. The biocatalytic reaction system was carefully optimized for gram-scale synthesis, resulting in good conversions and excellent enantioselectivities. Under the optimized conditions, whole cells could be reused for 4 cycles without significant loss of activity while maintaining up to 90% *ee*.

Since whole cells from *Rhodococcus* strains were used to catalyse the Michael addition of water in water to a series of α,β-unsaturated carbonyl compounds, and when the work presented in Chapter 2 started, no genomic information of *Rhodococcus* strains was publically available, we sequenced and annotated the strain *R. rhodochrous* ATCC 17895. This is described in **Chapter 3** together with features of the *R. rhodochrous* ATCC 17895. It is a Gram-positive aerobic bacterium with a rod-like morphology. The 6,869,887 bp long genome contains 6,609 protein-coding genes and 53 RNA genes. Our study suggests the Michael hydratase has not been described before.

In the work presented in Chapter 2, we found that most β-hydroxy ketones are not commercially available or commercially expensive as we mentioned in the first paragraph, which made the stereoselectivity determination of Michael addition products difficult. Indeed, many seemingly simple molecules have to be prepared *via* multi-step syntheses, in particular so if they are optically active. Therefore a straightforward approach to enantiomerically enriched (*R*)- and (*S*)-3-hydroxycyclopentanone was established by kinetic resolution in **Chapter 4**. This methodology allows us to prepare more β-hydroxy carbonyl compounds structurally closely related to 3-hydroxycyclopentanone.

The isolated chiral alcohols were used to determine the stereochemistry of the Michael addition of water in Chapter 2, saving us a lot of laboratory work.

Moreover, unexpected stereoselective reduction of conjugated C = C bonds was discovered during studies on the enantioselective Michael addition of water. As mentioned in Chapter 2, the whole cells of *R. rhodochrous* ATCC 17895 reduced α,β-unsaturated cyclic ketones into the corresponding ketones as initially undesired side reaction for the addition of water to C = C bonds. Therefore, ene-reductase activity was also investigated in **Chapter 5**. A series of substrates, including activated ketones, aldehydes, amines and nitro-compounds were screened for ene-reductase activity using whole cells of *R. rhodochrous* ATCC 17895. This showed that *R. rhodochrous* is a very promising catalyst for the reduction of C = C bonds and harbours ene-reductases. Indeed, looking for the annotated ene reductase from the genome of *R. rhodochrous* ATCC 17895 as described in Chapter 3, three candidates were observed and were classified as ene-reductases by amino acid sequence alignment with the known Old Yellow Enzymes (OYEs). Thus, the putative ene-reductase genes from *R. rhodochrous* ATCC 17895 were heterologously overexpressed in *Escherichia coli* and one of the encoded proteins was purified and characterized for their biocatalytic and biochemical properties.

Based on these accomplishments it can be concluded that we have discovered a new Michael hydratase and three new ene reductases from *Rhodococcus* strains. Genome sequence and annotation of strain *R. rhodochrous* ATCC 17895 has been done, offering an excellent opportunity for the discovering novel enzymes, for instance, the Michael hydratase and *S*-selective ene reductase. The important chiral β-hydroxy carbonyl compounds can be prepared by kinetic resolution of racemic alcohols using lipases or the direct enantioselective Michael addition of water using whole cells of *Rhodococcus* strains. The isolated products from kinetic resolution were readily used for the stereochemistry determination of Michael addition of water in water, completes the story of water addition to C = C bonds.

SAMENVATTING

β-Hydroxycarbonylverbindingen zijn een belangrijke klasse van stoffen die vaak gevonden worden als een algemeen structureel motief in natuurlijke producten. Alhoewel de moleculen er nogal simpel uitzien, kan hun synthese behoorlijk complex zijn. Water additie aan geconjugeerde C = C bindingen resulteert in een directe route voor de productie van β-hydroxycarbonylverbindingen. Bovendien is water additie aan C = C bindingen simpel en heeft een excellente atoomefficiëntie. Helaas is de enantioselectieve additie van water aan niet-verzadigde α,β-carbonyl (Michael) acceptoren nog steeds een erg uitdagende reactie omdat water een slechte nucleofiel is en een klein molecuul, wat regio- en stereoinductie moeilijk maakt. Tegelijkertijd moet ook het vaak ongunstige evenwicht van water-additie reacties opgelost worden. Enzymen, zoals fumarase, malease, citraconase, aconitase en enoyl-CoA hydratase, worden daarentegen succesvol gebuikt op industriële schaal en hun excellente (enantio)selectiviteit wordt erg gewaardeerd. Helaas zijn de meeste hydratases onderdeel van het primaire metabolisme waar perfecte substraat specificiteit essentieel is. Deze nauwe substraatspecificiteit beperkt hun praktische gebruik in organische synthese behoorlijk. Dus een rechtstreekse benadering met brede toepassingen is nog niet beschreven. Het doel van het onderzoek dat in dit proefschrift beschreven wordt, was om deze uitdaging aan te gaan en is gewijd aan de zoektocht naar een Michael hydratase met een bredere substraatspecificiteit voor de productie van belangrijke β-hydroxycarbonylverbindingen.

De stereospecificiteit van enzym gekatalyseerde reacties is een vruchtbare bron van informatie wat betreft de mechanismes van enzymkatalyse en omgekeerd; het toepassen van stereospecifiek gelabelde substraten maakt het mogelijk om het verloop van de reactie te bestuderen. Dit biedt een grote kans om de complete en precieze mechanistische en kinetische details van zelfs de meest complexe enzymatische reacties te begrijpen. Daarom worden in **Hoofdstuk 1** ideeën over de stereochemie van de enzymatische water additie aan C = C bindingen bij elkaar gebracht. Dit verbetert ons begrip van de achterliggende chemie van water additie aan C = C bindingen en helpt ons om meer hydratases te vinden uit natuurlijke bronnen of verkregen *via* eiwit veranderingen (protein engineering).

In **Hoofdstuk 2** wordt een directe enantioselectieve Michael additie van water in water beschreven om belangrijke β-hydroxycarbonylverbindingen te maken met hele cellen van *Rhodococcus* stammen. Een goede opbrengst en uitstekende enantioselectiviteit werden bereikt met deze methode. Dit opent een geheel nieuwe aanpak voor de productie van belangrijke β-hydroxycarbonylverbindingen. Experimenten waarbij producten met deuterium gemerkt worden, tonen aan dat sommige Michael hydratases de water additie exclusief met *anti*-stereochemie katalyseren, zoals oleate hydratase, fumarase, malease, aconitase en type II dehydroquinase, terwijl type I dehydroquinase, enoyl-CoA hydratase en artificieel hydratase een exclusieve voorkeur hebben voor de *syn*-additie, zoals in Hoofdstuk 1 is besproken. Het biokatalytische reactie systeem is zorgvuldig geoptimaliseerd voor een gram-schaal synthese wat resulteerde in goede omzettingen en excellente enantioselectiviteiten. Onder de geoptimaliseerde condities konden hele cellen vier maal hergebruikt worden zonder significante activiteit te verliezen, terwijl de enantioselectiviteit boven de 90% *ee* bleef.

Aangezien in Hoofdstuk 2 hele cellen van *Rhodoccoccus* stammen werden gebruikt om de additie van water in water te katalyseren, maar op dat moment nog geen genomische informatie van alle *Rhodococcus* stammen bekend was, hebben we de stam *R. rhodochrous* ATCC 17895 gesequenced en geannoteerd. Dit is beschreven in **Hoofdstuk 3** samen met de karakteristieken van *R. rhodochrous*. Het is een Gram-positieve aerobe bacterie met een staafvormige morfologie. Het 6.869.887 bp lange genoom bevat 6609 genen die voor eiwitten coderen end 53 RNA genen. Onze studie suggereert de Michael hydratase die nog niet eerder is beschreven.

Tijdens het werk dat beschreven staat in Hoofdstuk 2, kwamen we erachter dat de meest β-hydroxyketonen niet commercieel verkrijgbaar zijn of erg duur, zoals in de 1e paragraaf staat aangegeven, wat de opheldering van de stereoselectiviteit van de Michael additie producten erg moeilijk maakte. Veel ogenschijnlijk simpele moleculen moeten immers *via* synthese in meerdere stappen geproduceerd worden, vooral als ze optisch actief zijn. Daarom werd een eenduidige methode opgezet om enantiomeer verrijkt (*R*)- en (*S*)-3-hydroxycyclopentanon te produceren met behulp van kinetische resolutie in **Hoofdstuk 4**. Deze methode kan gebruikt worden om andere β-hydroxycarbonylverbindingen te maken die sterk verwant zijn aan 3-

hydroxycyclopentanon. De geïsoleerde chirale alcoholen werden gebruikt om de stereochemie te bepalen van de Michael additie van water in Hoofdstuk 2, wat ons veel labwerk bespaarde.

Tijdens de studies naar de enantioselectieve Michael additie van water werd er een onverwachte stereoselectieve reductie van geconjugeerde C = C dubbele bindingen ontdekt. Zoals in hoofdstuk 2 werd genoemd, reduceren hele cellen van *R. rhodochrous* ATCC 17895 niet-verzadigde cyclische α,β-ketonen naar de overeenkomstige ketonen als een ongewild bijproduct voor de water additie aan C = C dubbele bindingen. Daarom werd ene-reductase activiteit ook onderzocht in **Hoofdstuk 5**. Een serie substraten, waaronder geactiveerde ketonen, aldehydes, amines en nitro-verbindingen, werden gescreened op ene-reductase activiteit van hele cellen van *R. rhodochrous* ATCC 17895. Dit toonde aan dat *R. rhodochrous* een veelbelovende katalysator is voor de reductie van C = C dubbele bindingen en ene-reductases bevat. In het genoom van *R. rhodochrous* ATCC 17895 zoals beschreven in Hoofdstuk 3 waren inderdaad ene-reductases geannoteerd. Drie kandidaten werden gevonden en geclassificeerd als ene-reductases door een aminozuur sequentie vergelijking met de welbekende Old Yellow Enzymes (OYEs). Deze mogelijke ene-reductase genen van *R. rhodochrous* ATCC 17895 werden heteroloog tot overexpressie gebracht in *Escherichia coli* en de gevormde eiwitten werden gezuiverd en hun biokatalytische en biochemische eigenschappen werden gekarakteriseerd.

Gebaseerd op deze ontwikkelingen kan geconcludeerd worden dat we een nieuw Michael hydratase en drie nieuwe ene-reductases uit *Rhodococcus* stammen hebben ontdekt. Het genoom van *R. rhodochrous* ATCC 17895 is gesequenced en geannoteerd, wat een geweldige kans biedt voor het ontdekken van nieuwe enzymen, bijvoorbeeld het Michael hydratase en een *S*-selectieve ene-reductase. De belangrijke β-hydroxycarbonylverbindingen kunnen worden geproduceerd door kinetische resolutie van racemische alcoholen met behulp van lipases of door de directe enantioselectieve Michael additie van water met hele cellen van *Rhodococcus* stammen. De geïsoleerde producten van de kinetische resolutie werden direct gebruikt voor de bepaling van de stereochemie van de Michael additie van water in water, waarmee de cirkel van water additie aan C = C bindingen rond is.

ACKNOWLEDGEMENT

Finished, at least almost. At this point I would like to thank all the important people who have contributed to this thesis and my PhD life in one way or another.

First of all, I would like to express my deepest gratitude and respect to my promotor and supervisor Prof. dr. ir. Ulf Hanefeld. Ulf, thank you very much for support and inspiration and encouragement, for being positive and also strict, when it was necessary. Thanks for leaving your office door open all the time, making yourself always available when I needed advice, inspiration or critical comments. Now I looked back the past days, I found that every time I was able to discuss my "fresh" results with you at the first moment no matter it was a success or disaster. Thank you for coming around to lab to see how I was performing the experiments and give me the idea how to change or continue, which was a great help for my project indeed. Thank you for being an enthusiastic and helpful and critical supervisor. It is remarkable that having a work meeting every two weeks and writing a detailed report every two months really gave me the right direction and control to go for my project. I really appreciate that throughout those 4 years I never needed an appointment to reach you, your fast and detailed responses to my questions and reports and papers really enables me to finalize my thesis on time. I am very glad that I have finished my thesis under your guidance and persistent enthusiasm and I will always be grateful for your supports you have shown during my graduation!

Then my special appreciation goes to Prof. dr. ir. Isabel Arends, who was involved in the procedure to select me as a PhD in the Biocatalysis group. Thank you for giving me the chance to do my PhD in the group and I made it. I always remembered the first email you replied to me when I wrote to you asking for a position. You said "your background suits to work in our group", which encouraged me a lot to prepare for the skype interview and scholarship application. Thank you for being positive, supportive and friendly during the Go/ No Go meeting in my first year, which really encouraged me to take one step further. Thank you for treating all the foreigners in the group the way we feel like home!

I would like to thank all the committee members for the willingness to read, supply useful corrections and approve my thesis: Harald Gröger, Verena Resch, Gerard Roelfes, Willem van Berkel, Fred Hagen, Frank Hollmann, Isabel Arends!

I would also like to thank Dr. Linda Otten, who taught me a lot about molecular biology as my Master degree was in organic chemistry. Although I had no prior experience with microorganisms, you explained me everything, both practical and the theory behind it, in a concise way, so I can quickly grasp why I have to do things in a certain way. For me, you are my daily supervisor in MB-Lab. Without you, it is impossible for me to finish my work with microorganisms. Thank you for helping me to check, analyse and improve all the results. Your absolutely supportive, positive and critical attitude towards molecular biology of my research was a great help.

Heel hartelijk bedankt to Maarten Gorselin, who always helped me intensively with all the equipment, analytics, any small and big problems. Without your technical help at the beginning, I will never get my research started. Thank you very much for always being in positive mood and willing to help and I am for sure not the only one who appreciates everything you have done. No matter what technical problem I had, you always managed to solve it very quickly with saying " Don't worry, we will figure it out"!

I also would like to thank Remco van Oosten for all the help on GC measurement and chemical ordering. You helped me to order a new chiral GC column on GC 3 and set up a series of new methods for separation of many of the compounds presented in this thesis, which really saves me a lot of time on ee analysis. Thank you very much!

Special thanks go to our secretary-Mieke van der Kooij. Thank you for all your help in administrative matters and beyond. It is amazing how can you keep track on everything and be able to answer all the questions I asked. Although, we did not have many opportunities to talk, I always enjoyed our few short conversations in your office.

I would like to thank Laura Koekkoek-van der Weel, Marc Strampraad and Esengül Yildirim for all your technical help working in MB-Lab. Thank you for introducing me to the MB-Lab, guiding the very first experiments with microorganisms.

I extend my gratitude to Jin Jianfeng and Yan Ni, who explained me many basics in enzymatic catalysis at the very beginning. I am also very grateful to my officemates and labmates Fayene Ribeiro, Seyhan Tukel, Guzman Torrelo Villa, Kathrin Möller. We started our research in our group almost at the same time and we were the first people who started to use the big office with lots of windows. We have made a very big, positive change into the office together. We discovered the Netherlands together and adapted together to the culture. Thank you very much for all your support! I always consider you to be my very good friends during the whole PhD.

I would like to acknowledge all the co-authors of my articles. Special thanks to Verena Resch and Linda Otten for a pleasant and productive collaboration. We started the hydratase and genome sequence together at the very beginning and it is the most important to have a good and positive start, which made the follow-up research comprehensible and easier going. You made a great contribution to my thesis!

I would like to express my appreciation to my colleague Caroline and Guzman, who helped me a lot with advices, discussion and some important experimental methods when I was working on ene-reductases. Michelle van der Helm, my great student, thank you for helping me to start this project together and the beginning was always the most difficult part but we managed to have a very good and positive start. Without you three, it is impossible for me to have so many nice results within one year. I also would like to thank my colleague Jonathan and Kristina for help with NMR, especially D-NMR measurement and analysis. Thank you very much!

I would like to thank cordially all members and guests of the BOC group- Ulf, Guzman, Fayene, Seyhan, Kathrin, Verena, Lorina, Paula, Nicolas, Nelleke, Michelle, Katja, Adeline, Sanjib, Jin, Tobias, Enggar, Emanuele, Jereon, Wuyuan, Florian, Jonathan, Kristina, Sibel, Stephen, Yan Ni, Yi Cheng, Caroline, Elena, Alvaro, Jörg, Son, Selin, Serena, Ekaterina, Frank, Mieke, Mauro, Remco, Maarten, Linda, Laura, Marc, Esengül, Sinan, Kourosh, Corey, Simon, Peter-Leon, Isabel, Fred, Rosario, who were always ready to help, share experience and give advices. Many thanks to my Chinese friends in the Netherlands- Wuyuan, Yan Ni, Yi Cheng, Yixiao, Jingyi, Xuedong, Ran Shang, Peng Wei, Cuijie, Ping Liu, Liguo, Jing Chu, Jinglang, Lan Song, Yaqing, Wenbo, Wei Meng, Xuefei, Lu Wang, Hongkai, jianzhi, Xin Tian, Wei Shao, Lixia, Peng Yao, Min Su, Ying Li,

Linfeng, Yong Zhang for the great time in the relaxing social events and funny discussions during lunches in the CITG department.

I would like to express my appreciation to Huayang, who helped me to design the cover of my thesis. I am thankful to Linda for translating my Summary to Samenvatting, Propositions to Stellingen, and for all the linguistic advices.

I am great grateful for the finance support from China Scholarship Council (CSC) for my PhD time. And I also would like to thank Franca Post, who helped me so much applying for a visa, making appointments for ING, IND and so on at the very beginning. Thank you very much!

All the supports from my family and my family in-law are highly appreciated. I am indebted to them. Especially I would like to thank my parents and parents in-law for their unconditional love and unwavering support for my PhD study in the Netherlands (谢谢您们，我亲爱的爸爸妈妈，谢谢您们营造出来的轻松活泼的家庭气氛，让我们一家人之间相亲相爱，无话不说；谢谢您们热情开朗又开明的性格，总是在我难过的时候及时开导我，让我可以很快忘却烦恼，也让我拥有了热情开朗的性格；没有您们无私的关爱和义无反顾的支持，就没有我美好的今天和明天).

Finally, my greatest thanks goes to my friend, my soul mate, my beloved husband Huayang Cai who made my life in Delft easy and comfortable. I am very glad that we have met each other and been together since our bachelor studies in Sun Yat-Sen University (pure love). I was so afraid to do a PhD, and even afraid to live in a foreign country when I was still a Master student. You were the person who convinced me to come to the Netherlands and start my PhD, and it was your love, support and encouragement which helped me to also manage all the difficult phases of my PhD time. Thank you for being always positive, always giving me a smile and always making me see things better. I enjoyed my PhD time together with you in Delft and finally made it within 4 years. Thank you for all your love, support and understanding. I am looking forward to our new life in Guangzhou, China.

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Delft, 06 July, 2015

LIST OF PUBLICATIONS

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Education

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1. **Chen B.-S.**, Yang L.-H., Ye J.-L., Huang T., Ruan Y.-P., Fu J., Huang P.-Q. Diastereoselective synthesis and bioactivity of long-chain *anti*-2-amino-3-alkanols, *Eur. J. Med. Chem.*, **2011**, *46*, 5480-5486.
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