Für meine Eltern
Asymmetric Bioreduction of 
$N$- and $O$-Substituted Alkenes 
Using Enoate Reductases

Diplomarbeit

Zur Erlangung des akademischen Grades

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vorgelegt von Christoph Winkler

Diese Arbeit wurde im Zeitraum August 2009 bis März 2010 am Institut für Chemie an der Karl-Franzens-Universität Graz unter der Betreuung von Prof. Dr. Kurt Faber durchgeführt.
“I have a friend who's an artist and he's sometimes taken a view which I don't agree with very well. He'll hold up a flower and say, "Look how beautiful it is." and I'll agree, I think. And he says, "You see, I as an artist can see how beautiful this is, but you as a scientist, oh, take this all apart and it becomes a dull thing." And I think he's kind of nutty.

First of all, the beauty that he sees is available to other people and to me, too, I believe, although I might not be quite as refined aesthetically as he is. But I can appreciate the beauty of a flower.

At the same time, I see much more about the flower that he sees. I could imagine the cells in there, the complicated actions inside which also have a beauty. I mean, it's not just beauty at this dimension of one centimeter: there is also beauty at a smaller dimension, the inner structure...also the processes.

The fact that the colors in the flower are evolved in order to attract insects to pollinate it is interesting -- it means that insects can see the color.

It adds a question -- does this aesthetic sense also exist in the lower forms that are...why is it aesthetic, all kinds of interesting questions which a science knowledge only adds to the excitement and mystery and the awe of a flower.

It only adds. I don't understand how it subtracts.”

Richard P. Feynman during a BBC Horicon interview 1981
Acknowledgements

During my studies I have received lots of aid and backup from many good people. This is where I want to thank them.

First of all I want to thank my parents Karl and Lisbet who supported me all my live. Without your example and your standby I would not have been able to reach this goal. I like to remember how during school you Mum made me study English very hard, even though you don’t speak the language yourself. And now I am writing a whole thesis in this language which used to be so hard for me. Also I want to thank you, Dad, for opening my mind for studying and education and for passing me your broad interest, not only in natural sciences but in everything.

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Special thanks go to my grandparents here in Graz, Franz and Elisabeth, my uncle and aunt, Manfred and Margret and my cousins Alex and Anna. During my first year in Graz I lived with you and I like to remember how caring you were.

I owe big thanks to all my great friends. Especially for the great environment I was allowed to live in at the KHG and the KHJ Graz. All of you really made Graz my second home. I want to thank my long time roommate Luki, for being so different from me and for all the good talks we had. And for your inspiring music, of course. And I want to thank my chemistry-friends and colleagues during the studies. For all the learning, the notes we shared, the beer after studying and all the XP we collected together. It is impossible to enumerate you all, every one of you is very special to me and I owe thanks to all of you!

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Abstract

During the last few years the biocatalytic asymmetric reduction of C=C bonds, performed by enoate reductases from the ‘old yellow enzyme’ (OYE) family, grew to be a field of high interest. In the present work the activity of enoate reductases towards new substrate classes beyond the usual types of simple enals, enones, α,β-unsaturated carboxylic acids & -esters, imides, lactones, nitroalkenes, etc., and also their application for asymmetric synthesis were investigated.

α,β-Unsaturated cyclic α-alkoxy ketones were tested as a new substrate class. The stereochemical outcome of the produced α-alkoxy functionalized ketones could be switched by variation of the α-alkoxy-substituent or the ring-size of the substrate. Whereas α-alkoxy substituted ketones were readily accepted as substrates, β-alkoxy substituted substrates were not converted at all. Also α,β-dehydro amino acid derivatives were tested as novel substrates. Most interestingly, for the asparagines precursors, the stereochemical outcome of the bioreduction could be switched by substrate engineering, i.e. via variation of the size of the N-acyl protective group, using OYE3.

As an application of enoate reductases, the fragrants Lilial™ and Helional™ were obtained by asymmetric bioreduction of α-methylcinnamaldehyde derivatives. Depending on the applied enzyme and the reaction conditions, both stereoisomers could be produced.

Furthermore, different O-protected derivatives of methyl 2-hydroxymethyl acrylate were employed to enoate reductases to furnish the corresponding O-protected species of the industrially relevant product [(R)-'Roche Ester'].

\[ \text{Enoate Reductase} \quad \text{[EC 1.3.1.31]} \]

\[ \text{NAD(P)H (recycling)} \]
Zusammenfassung

Während der letzten Jahre wurde die biokatalytische asymmetrische Reduktion von C=C Bindungen mit Hilfe von Enoat Reduktasen aus der 'Old Yellow Enzyme' (OYE) Familie zu einem stark beforschten Thema. In dieser Arbeit wurde die Aktivität dieser Enzyme für neue Substrate außerhalb des klassischen Substratspektrums (Enale, Enone, α,β-ungesättigte Carbonsäuren & -ester, Imide, Lactone, Nitroalkene, etc.), und auch ihre Anwendung für die asymmetrische Synthese untersucht.

α,β-Ungesättigte cyclische α-Alkoxyketone wurden als neue Substratklasse untersucht. Abhängig vom α-Alkoxy Substituenten oder der Ringgröße des Substrates konnten beide Enantiomere des Produkts, einem chiralen α-Alkoxy funktionalisierten Keton, hergestellt werden. Während α-Alkoxy substituierte Ketone gut als Substrate akzeptiert wurden, wurden β-Alkoxy substituierte Substrate nicht umgesetzt.

Auch α,β-Dehydroamino säuren wurden als neue Substrate getestet. Interesseranterweise konnte mit OYE3 am Asparagine Precursor durch Substrate Engineering über die Größe der N-Acyl Schutzgruppe bestimmt werden, welches Enantiomer gebildet wird.


## Content

1. **Introduction** ................................................................................................................................. 3

   1.1 **Asymmetric Reduction of C=C Bonds** .......................................................................... 3

   1.2 **Enzymes of the old yellow enzyme family** ................................................................. 4

      1.2.1 **Physiological role** ........................................................................................................ 6

      1.2.2 **Structure** ...................................................................................................................... 6

      1.2.3 **Mechanism** .................................................................................................................. 8

2. **Theoretical Background** ........................................................................................................ 11

   2.1 **Usage of whole cells or crude cell extracts** .............................................................. 12

   2.2 **Usage of isolated enzymes** ......................................................................................... 14

      2.2.1 **Coupled-substrate approach** .................................................................................. 14

      2.2.2 **Coupled-enzyme approach** .................................................................................... 15

      2.2.3 **Photoenzymatic cofactor regeneration** .................................................................... 17

   2.3 **Substrate types** ............................................................................................................. 18

      2.3.1 **α,β-Unsaturated aldehydes** .................................................................................... 18

      2.3.2 **α,β-Unsaturated ketones** ......................................................................................... 21

      2.3.3 **α,β-Unsaturated carboxylic acids and derivatives** .................................................. 25

      2.3.4 **α,β-Unsaturated nitro-alkenes** ................................................................................ 32

      2.3.5 **Non-enoate-reductase activity** .................................................................................. 35

3. **Literature** ................................................................................................................................... 38
4 Results and Publications .................................................................................................................43

4.1 Paper I: O-Protected Acyloins (first-authorship) .................................................................43

4.2 Paper II: α-methylcinnamaldehyde (co-authorship) .............................................................62

4.3 Paper III: Roche Ester (co-authorship) .................................................................................81

4.4 Paper IV: α,β-Dehydroamino Acid Derivatives (co-authorship) ...........................................97

5 Curriculum Vitae .........................................................................................................................113
1 Introduction

1.1 Asymmetric Reduction of C=C Bonds

The asymmetric reduction of C=C bonds generates up to two chiral centers, and is therefore an important technique in asymmetric synthesis. Several methods for asymmetric hydrogenation have been developed in the last decades. A whole arsenal of transition metal catalysts, performing cis-hydrogenations of C=C bonds with excellent chemo- and stereo-selectivity has been developed.\[1\] On the down side, transition-metal catalysts can be rather expensive and depend on heavy metals, which can be harmful to the environment. In the last years asymmetric hydrogenations of C=C bonds in a trans-fashion, using “man-made” organocatalysts have also been shown.\[2\]

Nature also evolved a way to asymmetrically hydrogenate C=C bonds. Enzymes with such an activity are called enoate reductases (EC 1.3.1.31) and are members of the old yellow enzyme family.\[3\] These enzymes are widely distributed in microorganisms - particularly in bacteria and fungi - and in plants. Enoate reductases are able to asymmetrically reduce activated C=C bonds in a trans-fashion, at the expense of one NAD(P)H equivalent (Figure 1). A wide range of electron-withdrawing groups have been shown to act as activating groups. Substrates bearing C=C bonds next to carbonyl-moieties (α,β-unsaturated aldehydes, ketones, carboxylic acids, esters, anhydrides, lactones, imides), nitro-groups and nitriles were successfully reduced chemo- and stereo-selectively using enoate reductases.\[4\]

![Figure 1: Asymmetric bioreduction of activated alkenes in a trans-fashion, performed by enoate reductases.](image)

In the next chapters an overview of the reactions these enzymes are able to catalyze and of the wide range of substrates they accept is given.
1.2 Enzymes of the old yellow enzyme family

In 1932 Warburg & Christian isolated the first yellow enzyme (old yellow enzyme, OYE) from brewer’s bottom yeast. This enzyme was the first enzyme shown to contain flavin as a cofactor which causes its yellow color. Five years later, when in 1938 a second, “new” yellow enzyme was isolated from yeast by Haas, Warburg’s enzyme was termed “old yellow enzyme”.

In the years after Warburg’s discovery several homologues of his enzyme were found and the family started to grow. Homologues have been found in fungi, bacteria, plants and in the nematode *Caenorhabditis elegans* (Table 1).

Recently N. S. Scruton et al. reported the isolation and characterization of a thermophilic old yellow enzyme member from *Thermoanaerobacter pseudethanolicus* E39, termed TOYE. This enzyme is able to perform asymmetric bioreductions the of $\alpha,\beta$ unsaturated C=C bond of cyclic ketones, maleimides, aldehydes and nitro-compounds, and is stable at higher temperatures.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Organism</th>
</tr>
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<tbody>
<tr>
<td>old yellow enzyme (OYE)</td>
<td><em>Gluconobacter suboxydans</em>[^8]</td>
</tr>
<tr>
<td>old yellow enzyme (OYE)</td>
<td><em>Saccharomyces carlsbergensis</em>[^9]</td>
</tr>
<tr>
<td>old yellow enzyme 2 and 3 (OYE2, OYE3)</td>
<td><em>Saccharomyces cerevisiae</em>[^10]</td>
</tr>
<tr>
<td>old yellow enzyme (OYE)</td>
<td><em>Candida macedoniensis</em>[^11]</td>
</tr>
<tr>
<td>trans-2-enoyl-coenzyme A reductase</td>
<td><em>Escherichia coli K12</em>[^12]</td>
</tr>
<tr>
<td>YqjM</td>
<td><em>Bacillus subtilis</em>[^13]</td>
</tr>
<tr>
<td>NCR</td>
<td><em>Zymomonas mobilis</em>[^14]</td>
</tr>
<tr>
<td>SYE1-4</td>
<td><em>Shewanella oneidensis</em>[^15]</td>
</tr>
<tr>
<td>Yers-ER</td>
<td><em>Yersinia bercovieri</em>[^16]</td>
</tr>
<tr>
<td>KYE</td>
<td><em>Kluyveromyces lactis</em>[^10]</td>
</tr>
<tr>
<td>xenobiotic reductase A (XenA)</td>
<td><em>Pseudomonas putida II-B, 86</em>[^17]</td>
</tr>
<tr>
<td>xenobiotic reductase B (XenB)</td>
<td><em>Pseudomonas fluorescensens</em>[^17]</td>
</tr>
</tbody>
</table>

[^1]: Table 1: Characterized homologues of OYE.
1.2.1 Physiological role

Despite numerous investigations the true physiological role of old yellow enzymes remains a mystery.

Since the enzymes have reductive potential, they are believed to play a role in antioxidation and detoxification processes. It is possible that they are involved in oxidative stress response and programmed cell death response.[30]

OPR3 from *Arabidopsis thaliana* was shown to be a part of the jasmonate biosynthesis. It converts (9S,13S)-12-oxophytodienoic acid to the corresponding reduced derivate, which is a precursor for jasmonic acid. Jasmonates are plant hormones which help regulate plant growth and development (Figure 2).[30a, 31]

![Figure 2: Bioreduction of (9S,13S)-12-oxophytodienoic acid by OPR3, a part of jasmonate biosynthesis.](image)

Recently, EasA, an old yellow enzyme homolog was shown to play a role in ergot alkaloid biosyntheses. It was demonstrated to reduce the aldehyde activated C=C bond of chanoclavine-I-aldehyde yielding dihydrochanoclavine aldehyde.[32]

1.2.2 Structure

Old yellow enzyme from brewer’s yeast (OYE1, *Saccharomyces carlsbergensis*) consists of two ~45 kDa subunits. The subunits of the dimer are folded as eight-stranded α/β barrels and each subunit has one non-covalently bound FMN in its active site (Figure 3).[30a, 33]
Some members of the old yellow enzyme family do not form dimers. There are examples known that form monomers and YqjM from Bacillus subtilis even forms a tetramer.\cite{13} Very recently TOYE from Thermoanaerobacter pseudethanolicus E39, a new family member was characterized and the tetrameric quaternary structure was shown to be the minimal oligomere. It was also shown to build octameric and dodecameric quaternary structures.\cite{7}

In an overlay of crystal structures of C\textsubscript{a} backbones of OYE1 (Saccharomyces carlsbergensis), OPR1 (12-oxophytodienoate reductase) and PETNr (pentaerythritol tetranitrate reductase from Enterobacter cloaceae st. PB2) was shown that the FMN cofactor is bound in a very similar manner for all three enzymes (Figure 4). Side chains or residues contacting the cofactor are conserved across the family. The residues lying immediately above the plane of the flavin are conserved as well, either playing a role in catalysis, or in forming the hydrophobic substrate binding site.\cite{30a}
1.2.3 Mechanism

The well studied catalytic mechanism of OYE1 (*Saccharomyces carlsbergensis*) shows a bi-bi ping-pong mechanism.\(^{[34]}\) Generally it is divided up into a reductive half-reaction and an oxidative half-reaction.

During the reductive half-reaction, flavin is reduced by the transfer of the \textit{pro-(R)}-hydride of NADPH to its N5-atom. An \(\alpha,\beta\)-unsaturated carbonyl-compound can be bond via a hydrogen bond to a histidine (His191) and an asparagine (Asn194).\(^{[34b]}\) If the substrate is coordinated like this, the activated \(\beta\)-carbon is aligned right above N5 of the flavin.
In the oxidative half-reaction (Figure 5) the N5 hydride of flavin is transferred to the $\beta$-carbon of the substrate, whereas a tyrosine (Tyr196) adds a proton (which is ultimately derived from the solvent) to the $\alpha$-carbon in *trans*-fashion from the opposite side which represents a Michael-type addition reaction.$^{[34a]}$ In Figure 6 the whole catalytic mechanism is depicted.

---

*Figure 5: Oxidative-half reaction of the catalytic mechanism of old yellow enzymes.*

*Figure 6: Reductive and oxidative half reaction of the catalytic mechanism of old yellow enzymes.*
A rare example of a *cis*-selective hydrogenation is known to be performed by an enzyme from *Nicotiana tabacum* (tobacco), but it seems to be an exception.\[35\]

As a consequence of this mechanism non-activated substrates bearing a C=C bond are completely unreactive.\[36\]
2 Theoretical Background

During the last years enoate reductases from the old yellow enzyme family grew to be a field of high interest and many groups have been working on their characterisation. Especially the investigation of their substrate spectrum and their application to asymmetric synthesis was topic of many publications.

In the present work we investigated the activity of enoate reductases towards new substrate classes beyond the usual types of simple enals, enones, $\alpha,\beta$-unsaturated carboxylic acids & -esters, imides, lactones, nitroalkenes, etc., and also their application for asymmetric synthesis. $\alpha,\beta$-unsaturated $\alpha$-alkoxy ketones and $\alpha,\beta$-dehydro amino acid derivatives were tested as new substrate classes. The industrially relevant product (R)-3-Hydroxy-2-methylpropanoate ('Roche Ester') was produced via asymmetric bioreduction by enoate reductases and the fragrants Lilial™ and Helional™ were obtained by bioreduction of $\alpha$-methylcinnamaldehyde - derivatives.

In the following an overview of the current knowledge about the substrate spectrum of enoate reductases and their practical application is given.
2.1 Usage of whole cells or crude cell extracts

The majority of bioreductions of activated C=C bonds have been performed using whole cells as catalyst. Whole cells can be applied as growing (fermenting) cells, or as resting (non-fermenting) cells.

With whole cells some problems can be avoided and the handling is easier. First of all, an external cofactor recycling is not necessary. And secondly, the troublesome isolation and purification of the enzyme can be avoided.\[^{[37]}\]

On the other hand, bioreductions performed with whole cells can have some disadvantages as well. The reaction work up can be difficult, although the main problem that arises is that due to other activities in the whole cell, the biotransformations are not necessarily chemo-selective.\[^{[37]}\] In the case of enoate reductases some often observed side activities are the bioreduction of carbonyl moieties by *prim-* and *sec-*alcohol dehydrogenases,\[^{[3, 38]}\] hydrolysis of ester moieties by hydrolases,\[^{[39]}\] C-C lyase activities leading to the loss of a C\(_2\) fragment or the oxidation of the aldehyde by an aldehyde dehydrogenase (Figure 7).\[^{[40]}\]

\[ \text{R}_1^1 \text{C} = \text{R}_2^2 \text{R}_3^3 \xrightarrow{\text{prim-alcohol dehydrogenase}} \text{NAD}(\text{P})\text{H} \quad \text{R}_1^1 \text{C} = \text{R}_2^2 \text{R}_3^3 \quad \xrightarrow{\text{sec-alcohol dehydrogenase}} \quad \text{NAD}(\text{P})\text{H} \]

\[ \text{R}_1^1 \text{C} = \text{R}_2^2 \text{R}_3^3 \quad \text{R}_1^1 \text{C} = \text{R}_2^2 \text{R}_3^3 \xrightarrow{\text{ester hydrolase} + \text{H}_2\text{O} - \text{ROH}} \]

\[ \text{R}_1^1 \text{C} = \text{R}_2^2 \text{R}_3^3 \quad \text{R}_1^1 \text{C} = \text{R}_2^2 \text{R}_3^3 \quad \text{aldehyde dehydrogenase} \quad \text{NAD}^+, \text{H}_2\text{O} \]

\[ \text{citral} \quad \text{citral lyase} \quad + \text{H}_2\text{O}, -\text{CH}_3\text{CHO} \]

\[ \quad \text{sulcatone} \]

*Figure 7: Whole cell side activities: prim- and sec-alcohol dehydrogenase-, ester-hydrolase-, aldehyde dehydrogenase-, and lyase-activities.\[^{[3, 38-40]}\]*
The most common problem is the stereo-selectivity of competing alcohol dehydrogenase- and enoate reductase-activities. Which product is obtained depends on the relative reaction rates of the enoate reductases and the alcohol dehydrogenases. In case of aldehyde-activated substrates, over-reduction of the carbonyl-moiety is common. Not only the substrate can be reduced to the allylic alcohol, but also the desired product can be over-reduced to its corresponding saturated alcohol. Because the relative reaction rate of sec-alcohol dehydrogenases is lower than the relative reaction rate of prim-alcohol dehydrogenases, the desired product will be formed in case of ketone-activated substrates (Figure 8).\[4\]

\[4\] Figure 8: Competing enoate reductase and alcohol dehydrogenase activities.\[4\]
2.2 Usage of isolated enzymes

Although the usage of isolated enzymes for bioreductions goes in hand with many advantages, such as increased chemoselectivity, they have one main disadvantage: the expensive and relatively unstable NAD(P)H is required as cofactor. In a very recent publication the possibility of replacing it with cheaper molecules via disproportionation was investigated (2.3.5.1 on page 35). Nevertheless cofactor recycling techniques are necessary.

A very easy approach would be the reduction of the cofactor by nonenzymatic reducing agents, like Na$_2$S$_2$O$_4$ (sodium dithionite), but turnover numbers using this method are very low (<100) and the reducing agent might deactivate the enzyme. Turnover numbers of efficient cofactor recycling systems should be $>10^5$ cycles or higher. Because their turnover numbers are too limited as well (<1000), electrochemical and photochemical methods are also not used very often.

In the following are the two dominant approaches that are used today, and a thired, recently developed photochemical method described.

In up to now unpublished work, Glieders group showed an oxidation reaction catalysed by an old yellow enzyme family member from *Geobacillus kaustophilus*. This enzyme was shown to be able to introduce hydroxyl groups and a double bond into testosterone. Although it never was shown, this opens the possibility of a coupled-substrate cofactor regeneration with enoate reductases.

2.2.1 Coupled-substrate approach

In the ‘coupled-substrate approach’ two substrates are applied in the reaction, one acting as oxidizing substrate (acceptor) and one acting as reducing auxiliary substrate (donor), both reactions are catalyzed by the same enzyme but in opposite directions (Figure 9).
Since the two substrates are competing for the active site of the enzyme, the activity of the enzyme is limited. In order to shift the equilibrium, the auxiliary substrate has to be applied in excess. Due to the high concentration of the auxiliary substrate, substrate-inhibition might occur.

2.2.2 Coupled-enzyme approach

In this approach, a second independent enzyme regenerates the cofactor. Most of the disadvantages of the coupled substrate approach do not arise. Two enzymes act parallel, one reducing the substrate, using NAD(P)H, the other oxidizing the auxiliary substrate, using NAD(P)⁺ (Figure 10).
Several systems using this approach have been applied to the bioreduction with enoate reductases.

### 2.2.2.1 Formate dehydrogenase system

One of the most widely used methods is the formate dehydrogenase (FDH) system. Formate dehydrogenase catalyses the oxidation of formate to CO₂. The obvious advantage of this method is the easy removal of the coproduct from the equilibrium (Figure 11).[37, 42]

### 2.2.2.2 Oxidation of glucose or glucose-6-phosphate

In this method, glucose is oxidized by glucose dehydrogenase (GDH) forming gluconolactone at the expense of one NAD(P)⁺ equivalent. Similarly, glucose-6-phosphate can be oxidized by glycose-6-phosphate dehydrogenase (G6PDH). In both cases the furnished gluconolactone hydrolyses spontaneously and is thus removed from the equilibrium (Figure 11).[37, 42]

![Figure 11: Enzymatic regeneration of NAD(P)H][37, 42a]

FDH, GDH and G6PDH depend on essential metal ions. It has been observed that α,β-unsaturated dicarboxylic acids build complexes with these ions and thereby deactivate the recycling enzymes. For reductions of such substrates, a divalent metal ion e.g. Ca²⁺, Mg²⁺ or Zn²⁺, has to be added to the reaction in an equimolar concentration with respect to the substrate.[43]

There are several other NAD(P)H regeneration systems using the coupled enzyme approach, like the oxidation of phosphate with phosphate dehydrogenase or the oxidation of alcohols using alcohol dehydrogenases, which will not be further discussed.[37, 42]
2.2.3 Photoenzymatic cofactor regeneration

The photoenzymatic cofactor regeneration system was shown with ketoisophorone as model substrate. In this system the flavin-cofactor is regenerated directly by a cosubstrate (EDTA, ethylenediaminetetraacetic acid) which acts as donor, and a photocatalyst, which is oxidized by light (Figure 12). As photocatalysts different flavin-derivatives have been tested. During the light-induced reaction, the photocatalyst is reduced at the expense of EDTA. The reduced photocatalyst then regenerates the FMN in the active site of the enzyme.\cite{42b, 44}

**Figure 12: Photoenzymatic cofactor recycling.**

Because in this approach the flavin in the enzyme is regenerated directly, no redox-side-reactions can occur by activities of non-flavin enzymes.
2.3 Substrate types

Enoate reductases from the old yellow enzyme family are able to convert a huge variety of substrates, such as $\alpha,\beta$-unsaturated aldehydes, ketones, carboxylic acids, esters, anhydrides, lactones, imides, nitriles and nitro compounds. In Figure 13 a general bioreduction is displayed. The activating group is labeled as “X”, and in the following the carbon atom next to the activating group will be referred as “$\alpha$”-carbon, the second carbon atom next to the activating group as “$\beta$”-carbon.

![Figure 13: Asymmetric bioreduction of activated C=C bonds using enoate reductases.](image)

Additional electron withdrawing substituents usually enhance the reaction rate whereas substrates without sufficient activation are not converted.

2.3.1 $\alpha,\beta$-Unsaturated aldehydes

Very common for whole cell bioreductions are problems with insufficient chemoselectivity. An often observed side activity of reductions of the C=C bond of $\alpha,\beta$-unsaturated aldehydes with whole cells is the over-reduction of the C=O moiety with prim-alcohol dehydrogenases, furnishing the prim-alcohol. Other side-activities are C-C lyase activities, leading to the loss of a C2 fragment, or the oxidation of the aldehyde by an aldehyde dehydrogenase (Figure 7). In general the tolerance of substituents at the C=C bond for open-chain substrates is high (Figure 14).
As long as only one of the substituents is large, substrates with bulky R₁ or R₂ are accepted. Asymmetric bioreductions of substrates with (substituted) aryl and heteroaryl β-substituents have been shown. For R³ being electron-withdrawing groups, the reaction rate is enhanced.

A study on cinnamaldehydes with tri- and tetra-substituted double bonds reveals similar results. Tri-substituted substrates with small substituents are accepted; chloride-substituents enhance the reaction rate. Among the tetrasubstituted substrates the (E)-isomer was converted with higher e.e. than the (Z)-isomer.

Enals bearing the C=C bond in a cyclic system are accepted as substrates as well.

Because of the reversible alcohol dehydrogenase activity in whole cells, allylic alcohols are also accepted. In a first step, the alcohol is oxidized to the aldehyde by a prim-alcohol dehydrogenase.
Then the C=C bond is reduced by an enoate reductase, and in a third step the freshly built carbonyl is reduced back to the alcohol again (Figure 15).\textsuperscript{[49]}

The asymmetric reductions of α,β-unsaturated aldehydes using cloned\textsuperscript{[14]} or isolated\textsuperscript{[4, 7, 16, 50]} enoate reductases were investigated with great interest in the last few years. On the example of the bioreduction of citral, the potential of isolated enoate reductases to reduce α,β-unsaturated aldehydes in a chemo-, regio-, and stereoselective fashion is displayed in Figure 16.

Depending on the (E/Z)-configuration of the substrate, both enantiomers could be obtained by using either the (Z)-isomer of citral, furnishing the (S)-enantiomer of citronellal, or by using the (E)-isomer of citral, furnishing the (R)-enantiomer.\textsuperscript{[14]} A variation in enantioselectivity depending on the enzyme\textsuperscript{[14, 50a]} or depending on the recycling system was reported.\textsuperscript{[50a]} This variation may be explained by in situ (nonspecific) enzymatic (E/Z)-isomerisation of citral. The rate of (E/Z)-isomerisation and the rate of enzymatic reduction define the enantioselectivity of the product (Figure 17).

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure15.png}
\caption{Competing (E/Z)-isomerisation and C=C bond reduction.\textsuperscript{[50a]}}
\end{figure}
For \( \alpha, \beta \)-unsaturated aldehydes bearing \( \alpha \)-substituents, the activity is increased,\(^{[50c]} \) but the enantioselectivity goes down,\(^{[50b]} \) possibly due to spontaneous racemisation of the saturated aldehyde.\(^{[51]} \)

Volatile aldehydes are important key compounds in fragrance and flavor industry. Recently, the bioreduction of \( \alpha \)-methylcinnamaldehyde derivatives with enoate reductases to obtain the fragrance-aldehydes Lillial\(^{TM}\) and Helional\(^{TM}\) was reported.\(^{[52]} \) Although enoate reductases do not readily reduce bulky aldehydes like \( \alpha \)-methylcinnamaldehyde derivatives,\(^{[50c]} \) good results were obtained by using \( t \)-butyl-methyl ether as cosolvent.

### 2.3.2 \( \alpha, \beta \)-Unsaturated ketones

For the whole cell bioreductions of \( \alpha, \beta \)-unsaturated ketones the problem of over-reduction to the corresponding \( sec \)-alcohol, catalyzed by \( sec \)-alcohol dehydrogenases exists as well as for aldehydes.\(^{[38]} \) Since the ketone reduction by \( sec \)-alcohol dehydrogenases is slower than the aldehyde reduction by \( prim \)-alcohol dehydrogenases, the ratio of the competing reaction rates of C=C bond and C=O bond reduction shifts in favor of the C=C bond reduction and over-reduction is not as common as for aldehydes. It was shown, that the relative reduction rates of C=C vs. C=O depends on the reaction conditions, meaning pH and reaction time.\(^{[53]} \)

As long as \( R^4 \) is not too big, the substrate tolerance for open-chain \( \alpha, \beta \)-unsaturated ketones can be compared to the substrate tolerance for \( \alpha, \beta \)-unsaturated aldehydes (Figure 18).\(^{[3]} \)

![Figure 18: Asymmetric bioreduction of enones with whole-cells.\(^{[1]} \)](image)

Like aldehydes, enones are readily converted with bulkier \( R^1 \)- or \( R^2 \)-groups, as long as only one substituent is big.\(^{[53]} \) For aromatic \( R^1 \) or \( R^2 \), heteroaromatic substituents, or benzylic groups, substituted in \( m \)-position increase conversion and enantioselectivity.\(^{[54]} \)
Cyclic α,β-unsaturated ketones are good substrates as well. Usually five- or six-membered ring-derivatives with endo- or exo-cyclic C=C bonds are tested as substrates. According to Swiderska et al.,\[55]\ conversion drops significantly with increasing size of the alkyl substituent R¹ or R² (Figure 19).

\[\begin{align*}
\text{R}^1: \text{Me (high conversion)} & > \text{Et (low conversion)} \\
\text{R}^2: \text{Me (high conversion)} & > \text{Et > n-Pr > i-Pr > n-Bu (no conversion)}
\end{align*}\]

\textit{Figure 19: Asymmetric bioreduction of cyclohexenones by baker’s yeast, dropping conversion with increasing size of alkyl substituent.}\[55]\n
Substrates with substituents on positions other than α or β are usually readily converted.\[56]\ Cycloenones with \textit{exo}-cyclic C=C bonds are usually converted as well, although the conversion goes down with increasing size of the β-substituent (Figure 20).\[56]\n
\[\begin{align*}
\text{R}^1: \text{Me} \\
\text{R}^2: \text{Me, (Et)} \\
\text{R}^2: \text{substituents on other positions in the ring: not too big} \\
n = 1 - 2
\end{align*}\]

\textit{Figure 20: Asymmetric bioreduction of cyclohexenones bearing endo- and exo-cyclic bonds by whole-cells.}\[56\]
A prominent example for an industrial application of this bioreduction technique is the production of (R)-levitone. Due to careful reaction control during the bioreduction of ketoisophorone (reaction depicted in Figure 23), the product could be obtained with only minimal over-reduction in a yield of 80%. The production was performed at a 13 kg scale and the product is used for 3-hydroxycarotenoid production.

In the last years many asymmetric reductions of α,β-unsaturated ketones have been performed using isolated enoate reductases. Open-chain ketones as well as cyclic ketones are accepted as substrates.

Open-chain ketones with aromatic β-substituents do not seem to be good substrates although the conversion and enantioselectivity improve drastically with a substituent in m-position. The electronic effect of the substituent seems to be more important than its steric effect. Methyl vinyl ketone was accepted as substrate. Furthermore, the reduction of a C=C triple bond, conjugated to a ketone was reported (Figure 21).

Many of the recently isolated enoate reductases were characterized using cyclic α,β-unsaturated ketones as substrates (Figure 22). In general, the conversion for substrates with alkyl-substituents in the α-position is better than for substrates with alkyl-substituents in β-position.
When the ring-size of the α-methyl-substituted substrate is increased from five to six atoms, the stereopreference is switched from (S) to (R). This is not the case for the β-methyl-substituted substrate. This switch may be explained by assuming a flipped orientation of the substrates in the active site.\textsuperscript{50c, 60}

![Figure 22: Asymmetric bioreductions of cyclic α,β-unsaturated ketones by isolated enoate reductases;\textsuperscript{44, 7, 30} OYE1-3 = old yellow enzyme 1-3 from Saccharomyces carlsbergensis; YqIM from Bacillus subtilis; TOYE from Thermoanaerobacter pseudethanolicus E39; NCR from Zymomonas mobilis; OPR1 = 12-oxophytodienoate reductase 1 from Lycopersicon Esculentum; OPR3 = 12-oxophytodienoate reductase 3 from Lycopersicon Esculentum; PETNr = pentaerythritol tetranitrate reductase from Enterobacter cloacae PB2; N-ethylmaleimide reductases (NemA) from Escherichia coli; Eestrogen binding protein (EBP1) from Candida albicans.]

Recently Padhi et al.\textsuperscript{60} investigated the effect of site-saturation mutagenesis of tryptophan 116 of \textit{Saccharomyces pastorianus} OYE on acceptance of bulky β-substituted cyclohexanones. Unexpectedly, with some Trp 116 mutants the substrate changed its orientation in the active site and they obtained opposite stereoselectivity. This effect may be similar to the different stereochemical behavior of α-methylated cyclohexenones and cyclopentenones.

![Figure 23: Asymmetric bioreductions of cyclic α,β-unsaturated ketones (carvone to dihydrocarvone; ketalosporone to (R)-levitone) with additional substituents by isolated enoate reductases;\textsuperscript{44, 7, 30} OYE1-3 = old yellow enzyme 1-3 from Saccharomyces carlsbergensis; YqIM from Bacillus subtilis; TOYE from Thermoanaerobacter pseudethanolicus E39; NCR from Zymomonas mobilis; OPR1 = 12-oxophytodienoate reductase 1 from Lycopersicon Esculentum; OPR3 = 12-oxophytodienoate reductase 3 from Lycopersicon Esculentum; PETNr = pentaerythritol tetranitrate reductase from Enterobacter cloacae PB2; N-ethylmaleimide reductases (NemA) from Escherichia coli; morphinone reductase (MorR) from Pseudomonas putida M10; Eestrogen binding protein (EBP1) from Candida albicans.]
(R)-Levitone, the product of the asymmetric bioreduction of ketoisophorone is due to its industrial application one of most popular reactions performed by enoate reductases. A huge number of isolated enoate reductases were tested and yield (R)-levitone in high conversion and enantioselectivity.\cite{4, 7, 50} On the example of ketoisophorone and carvone it is displayed that substrates with alkyl substituents on positions other than α or β usually are tolerated and their stereoconfiguration has no impact on the stereoconfiguration of the reduced product\cite{50c} (Figure 23; an additional example can be found in Figure 2).

### 2.3.3 α,β-Unsaturated carboxylic acids and derivatives

One can conclude from the name ‘enoate reductases’, that α,β-unsaturated carboxylic acids are readily transformed by these enzymes. In fact a large variety of substrates with carboxyl-activated C=C bonds are tolerated. The activating groups range from simple carboxylic acids and carboxylic acid esters to lactones, imides and cyclic carboxylic acid anhydrides.\cite{4}

### 2.3.3.1 α,β-Unsaturated carboxylic acids and esters

A broad substrate spectrum of α,β-unsaturated carboxylic acids was investigated with whole cells and isolated enzymes from of anaerobes like Clostridium and Proteus sp. by the group of H. Simon (Figure 24).\cite{61}

![Figure 24: Asymmetric bioreduction of α,β-unsaturated carboxylic acids by Clostridium or Proteus sp.\cite{61}](image)

R¹ and R² could be even the same carbon atom, the α,β-C=C bond of allenic carboxylic acids could be reduced. Only the activated α,β-C=C bond was reduced chemo- and stereo-selectively to
the \((R)\)-enantiomer. Both enantiomers of the substrate gave opposite \((E/Z)\)-stereoisomeric products (Figure 25).\[^3\]

![Figure 25: Asymmetric bioreduction of allenic carboxylic acids.\[^\text{11}\]\]

Most recently, an example of a bioreduction of \(\alpha,\beta\)-unsaturated carboxylic acids bearing a \(\beta\)-nitrile substituent using anaerobic bacteria was reported\[^62\]\ during the course of an investigation towards a short route to chiral \(\beta\)-aryl-\(\gamma\)-amino acids (Figure 26).

![Figure 26: Bioreduction with whole cells of \(\beta\)-nitrile substituted \(\alpha,\beta\)-unsaturated carboxylic acids, synthetic route to chiral \(\beta\)-aryl-\(\gamma\)-amino acids.\[^\text{62}\]\]

The main problem using anaerobes for bioreduction is that even traces of oxygen disturb the system. This problem does not arise by using whole cells of baker’s yeast which was shown by the bioreduction of \(\alpha\)-chloroacrylic acid esters. Due to a hydrolase activity in baker’s yeast, the ester was hydrolysed in the first step, and the resulting \(\alpha\)-chloroacrylic acid was reduced in the second step (Figure 27). The absolute configuration of the product depended on the \((E/Z)\)-configuration of the substrate and the \(\beta\)-haloalkyl substituents seemed to act as additional activator, enhancing the reaction rate.\[^39\]
Figure 27: Asymmetric bioreduction of α-chloracrylic acids.\textsuperscript{109}\textsuperscript{109}

Ester hydrolysis is the main side reaction in whole cell bioreductions of α,β-unsaturated carboxylic acid esters. This problem can be avoided by performing the bioreduction with isolated enzymes (Figure 28).\textsuperscript{4, 43}

Figure 28: Asymmetric bioreduction of α,β-unsaturated carboxylic acids and esters by isolated enoate reductases.\textsuperscript{43, 50d} YqjM from Bacillus subtilis; OPR1 = 12-oxophytodienoate reductase 1 from Lycopersicon Esculentum; OPR3 = 12-oxophytodienoate reductase 3 from Lycopersicon Esculentum; PETNε = pentaerythritol tetranitrate reductase from Enterobacter cloacae PB2; N-ethylmaleimide reductases (NemA) from Escherichia coli; morphinone reductase (MorR) from Pseudomonas putida M10; Eestrogen binding protein (EBP1) from Candida albicans.

In two studies different isolated enoate reductases were tested with diesters as substrates.\textsuperscript{43, 50d} Substrate 2b was not accepted by any of the enzymes. OPR3 from Lycopersicon esculentum (tomato) accepted only 1a and 1c as substrates, 2c was accepted, yielding only very low conversion. Overall, the diesters were better substrates than the corresponding diacids and the exo-methylene species proved to be difficult substrates. For YqjM from Bacillus subtilis depending on the applied (E/Z)-isomer of the applied diesters, opposite stereopreference was achieved. Whereas 1a furnished (R)-3, 1b gave (S)-3.
As example for a bioreduction of \(\alpha,\beta\)-unsaturated carboxylic acid esters with an electron-withdrawing \(\beta\)-substituent other than a \(\beta\)-ester moiety, the bioreduction of \(\beta\)-nitroacylates is depicted in Figure 29.\(^{[63]}\)

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{R}^1 \\
\text{CO}_2\text{Et} & \\
\text{NO}_2 & \quad \text{R}^2 \\
\text{CO}_2\text{Et} & \\
\to & \\
\text{O}_2\text{N} & \quad \text{R}^1 \\
\text{CO}_2\text{Et} & \\
\text{NO}_2 & \quad \text{R}^2 \\
\text{CO}_2\text{Et} & \\
\end{align*}
\]

\(\text{R}^1\): Me (high conversion, low e.e.), Et (low conversion, low e.e.)
\(\text{R}^2\): Me (low conversion and e.e.) < Et < \(\gamma\)-Pr < \(\delta\)-Pr < Ph (high conversion and e.e.)

Figure 29: Asymmetric bioreduction of \(\beta\)-nitroacylates by an isolated OYE from Saccharomyces carlsbergensis.\(^{[63]}\)

\(\alpha\)-Substituted \(\beta\)-nitroacylates seem to be better substrates than the \(\beta\)-substituted \(\beta\)-nitroacylates, although for a good e.e. sterically demanding \(\alpha\)-substituents were necessary.

A detailed investigation of the substrate spectrum of PETNr (pentaerythritol tetranitrate reductase from Enterobacter cloacae PB2) showed, that it seems to have no activity towards \(\alpha,\beta\)-unsaturated carboxylic acids and esters.\(^{[50c]}\)

### 2.3.3.2 \(\alpha,\beta\)-Unsaturated lactones

Not much is known about the asymmetric bioreduction of \(\alpha,\beta\)-unsaturated lactones. \(\beta\)-Substituted \(\alpha,\beta\)-unsaturated \(\gamma\)-lactones were converted by baker’s yeast yielding good e.e.s. Depending on the polarity of the group R, the conversion and optical purity rose. Excellent yield and stereoselectivity were obtained with the thioether, low yield and stereoselectivity with the more polar sulfone by reduction with baker’s yeast.\(^{[4, 64]}\) Shimoda \textit{et al.}\(^{[65]}\) showed the asymmetric bioreduction of different \(\alpha,\beta\)-unsaturated \(\gamma\)-lactones with an isolated enoate reductase form Marchantia polymorpha (i.e. liverwort). Whereas the \(\alpha\)-substituted substrates were readily converted, the \(\beta\)-methyl substituted substrate showed no conversion at all, which has also been observed with \(\beta\)-alkyl substituted cyclic \(\alpha,\beta\)-unsaturated ketones (2.3.2, page 21). Even less is known about \(\delta\)-lactones. They are accepted as substrates, but do not seem to be converted with high yield and stereoselectivity. However, the reason for the low conversion may be the steric...
 demand of the substrate, the low e.e. may be due to the distance to the chiral center in δ–position of the substrate, displayed in Figure 30.\textsuperscript{[66]}

\[
\begin{align*}
\text{R} = \text{SPh} > \text{SOPh} > \text{SO}_2\text{Ph}; & \quad \text{OAc, OCH}_2\text{Ph} \\
\end{align*}
\]

\[
\begin{align*}
\text{good conversion and enantioselectivity: } R^1 = \text{H, OH, Me}; & \quad R^2 = \text{H}; \\
\text{no conversion: } R^1 = \text{H}; & \quad R^2 = \text{Me}; \\
\end{align*}
\]

\textit{Figure 30: Asymmetric bioreduction of α,β-unsaturated lactones.\textsuperscript{[4, 64-66]}}

Isolated xenobiotic reductase A (XenA) from \textit{Pseudomonas putida} was shown to reduce 8-hydroxy coumarin and coumarin (Figure 31).\textsuperscript{[67]}

\[
\begin{align*}
\text{R} = \text{H, OH} \\
\end{align*}
\]

\textit{Figure 31: Asymmetric bioreduction of 8-hydroxy coumarin and coumarin with isolated XenA from \textit{Pseudomonas putida}.\textsuperscript{[67]}}
2.3.3.3 \( \alpha, \beta \)-Unsaturated carboxylic acid anhydrides

\( \alpha, \beta \)-Unsaturated carboxylic acid anhydrides have been reduced by isolated enoate reductases.\(^{16, 65}\)

![Figure 32: Asymmetric bioreduction of \( \alpha, \beta \)-unsaturated carboxylic acid anhydrides by isolated enoate reductases.\(^{16, 65}\) ](image)

2.3.3.4 \( \alpha, \beta \)-Unsaturated imides

A range of \( \alpha, \beta \)-unsaturated maleimides have been tested as substrates for both, whole cell bioreductions and bioreductions with isolated enoate reductases, respectively. According to a study with whole cells of \textit{Marchantia polymorpha},\(^{68}\) the size of the \( N \)-alkyl group does not affect the conversion. However, substrates with \( p \)-methoxy substituted aromatic \( R^3 \) give, due to electronic reasons less conversion. An \( \alpha \)-methyl group has no effect on conversion and enantioselectivity, substrates bearing both, an \( \alpha \)- and a \( \beta \)-methyl group are converted much slower. Also substrates with exo-cyclic \( C=C \) bonds were tested with \textit{Marchantia polymorpha}, and showed no conversion whereas a thiazolidine-2,4-dione bearing an exo-cyclic \( C=C \) bond was readily reduced by \textit{Rhodotorula} or \textit{Rhodosporidium} spp.\(^{69}\)
Figure 33: Asymmetric whole-cell bioreduction of substituted maleimides.\cite{68-70}

A range of isolated enoate reductases have been tested with substituted maleimids as well, and proved a great tolerance of the \( \text{N} \)-group. All substrates were accepted in high conversion and stereoselectivity.\cite{4, 7, 16, 50a, 50b, 50d}

Figure 34: Asymmetric bioreduction of substituted maleimids with isolated enoate reductases;\cite{4, 7, 16, 50a, 50b, 50d} OYE1-3 = old yellow enzyme 1-3 from Saccharomyces carlsbergensis; YqjM from Bacillus subtilis; TOYE from thermoanaerobacter pseudethanolicus E39; NCR from Zymomonas mobilis; XenA = xenobiotic reductase A from Pseudomonas putida II-B; OPR1 = 12-oxophytodienoate reductase 1 from Lycopersicon Esculentum; OPR3 = 12-oxophytodienoate reductase 3 from Lycopersicon Esculentum; PETNr = pentaerythritol tetranitrate reductase from Enterobacter cloacae PB2; Yers-ER from Yersinia bercovieri; N-ethylmaleimide reductases (NemA) from Escherichia coli; morphinone reductase (MorR) from Pseudomonas putida M10; Eestrogen binding protein (EBP1) from Candida albicans.
2.3.3.5 \( \alpha,\beta \)-Unsaturated nitriles

The nitrile-group seems to act also as activating group for the bioreduction, catalyzed by enoate reductases. In Figure 35 several substrates are shown, that have been converted by enoate reductases in high conversions and enantioselectivities.\(^{[71]}\)

Isolated PETNr (pentaerythritol tetranitrate reductase from \textit{Enterobacter cloacae PB2}) however, is reported not to accept nitrile-activated substrates.\(^{[50c]}\)

2.3.4 \( \alpha,\beta \)-Unsaturated nitro-alkenes

Substrates bearing a C=C bond activated with a nitro group are accepted by enoate reductases as well. The reduction occurs in a stepwise fashion. In the first step, a nitronate intermediate is formed by hydride transfer from the flavin to the \( \beta \)-carbon of the substrate. Then the nitronate is protonated by a Tyr in the active site (Figure 36).\(^{[30a, 72]}\)
A huge variety of substrates were reduced with whole cells of different microorganisms: anaerobes (Clostridium and Eubacterium spp.), aerobic (red) yeasts (Candida, Torulopsis, Rhodotorula) and bacteria (Klebsiella, Bacillus subtilis, Rhodococcus, Nocardia, Escherichia coli) (Figure 37).\textsuperscript{[4, 70, 73]}

During the reaction two chiral centers are produced. The β-center is stable, the chiral center on the α-carbon undergoes spontaneous racemisation and enantiomeric mixtures are produced.\textsuperscript{[4]} The substrate spectrum is very broad, allowing alkyl, and aryl substituents on the C=C bond ranging from small to medium size. During a recent study on the reduction of β,β- and α,β-disubstituted nitroalkenes with extracts of Clostridium sporogenes some trends were uncovered: \emph{p}-substituted aromatic \emph{R}^3 generally increased reaction time. The conversion and reaction rates of halogen \emph{p}-
substituents followed the trend: F > Cl > Br. The reaction time was much shorter, and the conversion much higher for methoxy substituents in p- than in o-position.

The bioreduction of nitroalkenes has also been investigated with isolated enoate reductases. Their enantioselectivity seems to differ widely. Depending on the applied enzyme, opposite enantiomers are produced (Figure 38).\textsuperscript{[4, 50]} To rationalize these results, docking-studies on the substrate binding in OPR1 and OPR3 have been performed.\textsuperscript{[50b]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure38.png}
\caption{Asymmetric bioreduction of nitroalkenes with isolated enzymes;\textsuperscript{[4, 50]} OYE1-3 = old yellow enzyme 1-3 from Saccharomyces carlsbergensis; YqjM from Bacillus subtilis; NCR from Zymomonas mobilis; OPR1 = 12-oxophytodienoate reductase 1 from Lycopersicon Esculentum; OPR3 = 12-oxophytodienoate reductase 3 from Lycopersicon Esculentum; PETNr = pentaerythritol tetraniitrate reductase from Enterobacter cloacae PB2; Yers-ER from Yersinia bercovieri; N-ethylmeleimide reductases (NemA) from Escherichia coli; morphinone reductase (MorR) from Pseudomonas putida M10; Eestrogen binding protein (EBP1) from Candida albicans.}
\end{figure}

In a recent publication\textsuperscript{[74]} a huge number of nitroalkenes have been tested with PETNr (pentaerythritol tetraniitrate reductase from Enterobacter cloacae PB2) (Figure 39).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure39.png}
\caption{Asymmetric bioreduction of nitroalkenes with isolated PETNr from Enterobacter cloacae PB2.\textsuperscript{[74]}}
\end{figure}

Because of the similarity between nitroreductases and enoate reductases, in a very recent paper, a nitro reductase from Salmonella typhimurium was tested for its enoate reductase activity, aiming to find an enzyme with a broader acceptance of nitro-activated substrates. Substrates, activated by a nitro group are readily accepted, \(\alpha,\beta\)-unsaturated ketones and aldehydes showed only little conversion. In general the stereo-preference was low.\textsuperscript{[75]}

\[34\]
2.3.5 Non-enoate-reductase activity

The members of the yellow enzyme family have also shown activities that are not related to reductions of an electron-withdrawing activated C=C bond.

2.3.5.1 Disproportionation

In a very recent publication the disproportionation of 2-cyclohexenone furnishing cyclohexanone and phenol, catalyzed by enoate reductases was investigated in detail.\cite{76} This phenomenon has been known for old yellow enzymes (Figure 40).\cite{10, 77}

![Figure 40: Disproportionation of 2-cyclohexenone.\cite{76}](image1)

It was tried to find a suitable pair of compounds, one being reduced and acting as substrate, one being oxidized and acting as cosubstrate, to finally get a NAD(P)H independent system. Best results were obtained with YqjM from *Bacillus subtilis* and cyclohexane-1,4-dione as cosubstrate, yielding hydroquinone as coproduct (Figure 41).

![Figure 41: NAD(P)H independent bioreduction of C=C bonds via disproportionation.\cite{76}](image2)
2.3.5.2 Bioreduction of activated C-C triple bonds

Mueller et al. reported the bioreduction of carbonyl-activated C-C triple bonds by cloned enoate reductases OYE1 (*Saccharomyces carlsbergensis*), OYE2 and 3 (*Saccharomyces cerevisiae*) and NCR (*Zymomonas mobilis*) (Figure 21).[59]

2.3.5.3 Nitroreductase, denitrification and reductive denitration activities

Because of the production and use of huge amounts of 2,4,6-trinitrotoluene (TNT) for military use, TNT contamination has grown to be a problem. Enol reductases have proven to be useful in the biodegradation of this compound.[78]

Enzymes from the old yellow enzyme family are known to have type I nitroreductase activity, which means they catalyze the reduction of nitro species to the amine. To accomplish this, the nitro species is first reduced to the nitroso-species, then the hydroxylamine is formed which finally reacts to the amine.[79] A second activity, useful for the TNT biodegradation is the denitration. During the denitration pathway the hydrides are delivered directly onto the electron-deficient aromatic system, reducing it to the non-aromatic Meisenheimer complexes, which are stabilized by elimination of nitrite. The nitroreduction is catalyzed by all members of the old yellow enzyme family, the denitration is catalyzed by only few (Figure 42).[80]

![Figure 42: TNT degradation by enol reductases via A: the denitration pathway, B: the type I nitroreductase pathway.][80]
Another important biodegradation mechanism, catalyzed by the old yellow enzyme family is the reductive denitration. During this activity, nitroesters are cleaved, possibly by formation of a nitroso ester intermediate, yielding the corresponding alcohol and nitrite (Figure 43).\cite{23, 81}

\[
\begin{align*}
\text{R}^1\text{O}^+\text{N}^+\text{O}_2^- \text{ O}_2^- + \text{OYE} \xrightarrow{\text{NAD(P)H}} \\
\text{spontaneous} \rightarrow \text{R}^1\text{OH} + \text{NO}_2^-
\end{align*}
\]

\textit{Figure 43: General pathway of reductive denitration of nitroesters.}\cite{23, 81}

\subsection{2.3.5.4 Reduction of aliphatic nitro-compounds (biocatalytic Nef-Reaction)}

In a study of G. Stephens \textit{et al.} the reduction of 2-nitro-1-phenylpropene by an anaerobe whole cell biocatalyst was investigated and it was shown, that not only the C=C bond was reduced, but also the nitro group was reduced, furnishing the corresponding amine.\cite{82}

Very recently some members of the old yellow enzyme family were shown to catalyze the reduction of aliphatic nitro-compounds to the corresponding carbonyl-species. In the proposed mechanism the aliphatic nitro-species is firstly reduced by the enzyme, furnishing the nitroso species. Tertiary nitroso-compounds are known to dimerize, secondary nitroso species quickly tautomerize to the more stable oximes. In the next step, the second hydride is delivered via the flavin cofactor, reducing the oxime to the corresponding imine, which rapidly hydrolyzes to the carbonyl compound. This reaction represents a biocatalytic equivalent to the Nef-reaction (Figure 44).\cite{80c}

\[
\begin{align*}
\text{R}^1\text{H} & \xrightarrow{\text{NADH} - \text{H}_2\text{O}} \text{R}^1\text{H}_2\text{N}^+\text{O}_2^- \\
\text{spontaneous tautomerisation} & \rightarrow \text{R}^1\text{H}_2\text{N}^+\text{O}_2^- \\
\text{Flavin Reductase} & \xrightarrow{\text{NADH} - \text{H}_2\text{O}} \text{R}^1\text{NH} \\
\text{Flavin Reductase} & \xrightarrow{\text{NADH} - \text{H}_2\text{O}} \text{R}^1\text{H}_2\text{N}^+\text{O}_2^- \\
\text{spontaneous hydrolysis} & \rightarrow \text{R}^1\text{H}_2\text{N}^+\text{O}_2^- \\
\end{align*}
\]

\textit{Figure 44: Reduction of aliphatic nitro-compounds (biocatalytic Nef-Reaction).}\cite{80c}
3 Literature


[52] C. M. Stueckler, Nicole J.; Winkler, Christoph k.; Glueck Silvia M.; Gruber, Karl; Steinkellner, Georg; Faber, Kurt, *Dalton Transactions* **2010**.


4 Results and Publications

4.1 Paper I: O-Protected Acyloins (first-authorship)

Asymmetric Synthesis of O-Protected Acyloins Using Enoate Reductases: Stereochemical Control via Protective-Group Modification


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Abstract

O-Protected cyclic acyloins were obtained in nonracemic form via asymmetric bioreduction of α,β-unsaturated alkoxy ketones using eleven different enoate reductases from the ‘Old Yellow Enzyme’ family. The stereochemical outcome of the biotransformation could be switched by variation of the O-protective group or by the ring-size of the substrate, which allows to access both stereoisomers in up to >97% e.e. Whereas α-alkoxy enones were readily accepted as substrates, β-analogs were not converted. Overall, α-alkoxyenones represent a novel type of substrate for flavin-dependent ene-reductases.

Introduction

Due to their bifunctionality containing a nucleophilic and an electrophilic group, nonracemic acyloins are important building blocks in organic synthesis. They can be converted to alcohols, diols, epoxides, amines, hydroxylamines and haloketones, usually with a high degree of chirality transfer. Consequently, they were frequently employed as building blocks for the asymmetric synthesis of bioactive compounds.[1,2,3] For the synthesis of nonracemic acyloins, classic approaches include the acyloin- and the benzoin-condensation, both of which have been performed in an asymmetric fashion.[4,5] The most common strategy to obtain nonracemic acyloins is based on the oxidation of enolates by chiral N-sulfonyloxaziridines derived from camphor.[6,7,8]

For the production of nonracemic acyloins, a broad range of enzymes belonging to different classes, including oxidoreductases (peroxidases, carbonyl reductases and oxidases), hydrolases (lipases) and lyases (hydroxynitrile lyases and transketolases)[3] were successfully used, including most prominently the thiamine-dependent C-C lyases.[9]

During our investigation of the exploration of the substrate spectrum of flavin-dependent enoate reductases from the old yellow enzyme family [EC 1.3.1.X], [10,11] we envisaged that O-functionalised α,β-unsaturated ketones might be suitable substrates for these enzymes, which would furnish O-protected acyloins as the reduction product. Although the asymmetric hydrogenation of α,β-unsaturated α-alkoxycarboxylic acids and esters using rhodium-catalysts bearing chiral phosphine ligands[12] was reported, to the best of our knowledge, α-alkoxy functionalized enones were not investigated as substrates for asymmetric (bio)reductions.[13]
Results and Discussion

In contrast to electronically activated enol ethers derived from open-chain $\beta$-dicarbonyl compounds, which proved to undergo spontaneous hydrolysis in aqueous buffer under standard conditions, cyclic analogues were sufficiently stable under the reaction conditions. Thus, alkoxy functionalized cyclohexen-2-ones (1a-4a) and cyclopenten-2-ones (5a, 6a) together with the corresponding $\beta$-alkoxy analogues 7a and 8a were synthesized from the corresponding 1,2- and 1,3-diones. In order to explore the influence of the size of the $\alpha/\beta$-substituent on the stereoselectivity, the $O$-protective group was varied from small (methyl) via medium ($n$-propyl/allyl) to large (benzyl). In addition, the allyl$^{[14]}$ and benzyl-moieties$^{[15]}$ were chosen for their easy removal without endangering the newly generated stereocenter (Scheme 1). The results from the enzymatic reduction are summarised in Table 1.

![Scheme 1](image)

**Scheme 1.** Asymmetric bioreduction of $\alpha$- and $\beta$-alkoxy substituted enones 1a-8a.
Table 2. Conversion, absolute configuration and enantiomeric excess of products 1b-6b.

| Substrate | Product | Cofactor[a] | OYE1 | OYE2 | OYE3 | OPR1 | OPR3 | YqiM | NCR | XenA | XenB | NerA | EBP1 |
|-----------|---------|-------------|------|------|------|------|------|------|------|------|------|------|------|------|
|           |         |             | c    | ee   | c    | ee   | c    | ee   | c    | ee   | c    | ee   | c    | ee   |
| (R)- or (S)-1b | NADH  | 37 | 92 | 26 | 90 | 25 | 84 | <5 | 9 | 59 | 31 | 24 | 46 | 77 |
| (S)-2b   | NADH  | <3 | nd | <3 | nd | <3 | nd | 91 | 11 | 64 | 26 | 64 | 94 | 97 |
|          | NADH  | <3 | nd | <3 | nd | <3 | nd | 99 | 15 | 38 | 31 | 62 | 82 | 96 |
| (S)-3b   | NADH  | <3 | nd | <3 | nd | <3 | nd | 98 | 83 | 12 | 98 | 12 | 83 | 18 |
| (S)-4b   | NADH  | 13 | 87 | 12 | 87 | S | 6 | 67 | 98 | 70 | 56 | 64 | 91 | 99 |
|          | GDH    | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| (S)-5b   | NADH  | 10 | 58 | 58 | n.d | 25 | 66 | nc | nd | <3 | nd | 55 | 84 | 10 |
|          | FDH    | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 9  | 88 |
| (S)-6b   | NADH  | <3 | nd | <3 | nd | <3 | nd | 98 | 95 | 5  | >99 | 3  | 92 | 74 |
|          | FDH    | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

[a] Reaction conditions: [b] Reaction conditions.
[a] Standard condition: substrate (10 mM), enzyme (75-125 µg/mL), Tris-HCl-buffer (0.8 mL, 50 mM, pH 7.5), NADH (15 mM); GDH = NAD\(^+\) (100 µM) / glucose dehydrogenase or formate dehydrogenase (10U) / glucose or formate, resp., (20 mM); [b] TBME was added as a cosolvent (v:v 20%) to solubilise the substrate; abbreviation of enzymes: old yellow isoenzyme OYE1 from *Saccharomyces carlsbergensis*, OYE2 and OYE3 from *Saccharomyces cerevisiae*, 12-oxophytodienoic acid reductase isoenzymes OPR1 and OPR3 from *Lycopersicon esculentum*, old yellow enzyme homolog YqiM from *Bacillus subtilis*, nicotinamide-dependent cyclohexenone reductase NCR from *Zymomonas mobilis*, xenobiotic reductase XenA and XenB from *Pseudomonas putida* and *P. fluorescens*, resp., glycerol trinitrate reductase NerA from *Agrobacterium radiobacter*, estrogen-binding protein EBP1 from *Candida albicans*; c = conversion; ee = enantiomeric excess; nc = no conversion; nd = not determined.

The identity of products was confirmed by comparison with racemic reference material obtained via catalytic hydrogenation and the absolute configuration of products was elucidated via co-injection with authentic nonracemic samples synthesised from (1S,2S)-cyclohexane-1,2-diol and (1S,2S)- or (1R,2R)-cyclopentane-1,2-diol via mono-alkylation of the diol, followed by Jones-oxidation of the unprotected hydroxy moiety to the corresponding O-protected acyloin as described in the experimental section. Overall, the influence of the substrate-size on the stereochemical outcome of the bioreduction followed a very consistent pattern (Table 1): The cyclohexenone bearing the shortest protective group (1a) was accepted by all enolate reductases, except for NerA. However, the reactivities ranged from low (OPR3) to good (XenA). Interestingly, both enantiomeric products were obtained depending on the enzyme: Whereas most enzymes furnished (R)-1b in up to 92% e.e. (OYE1), XenA gave the mirror-image product (S)-1b in 47% e.e. The best data sets obtained using OYE1 and OYE2 were repeated in presence of cofactor-recycling [glucose dehydrogenase (GDH)/glucose/NAD\(^+\)], which yielded satisfactory results.

Substrates bearing a medium-sized allyl- (2a) or n-propyl-protective group (3a) gave almost identical results: While EBP1 was unreactive, the majority of enzymes showed low to modest reactivities, only OPR1, NCR, XenA and XenB were sufficiently active. No stereochemical switch was observed with XenA and all enzymes furnished (S)-2b and (S)-3b; excellent selectivities were obtained using XenB (96% and 98% e.e., respectively).
The cyclohexenone derivative bearing the largest benzyl group (4a) could be reduced by all enzymes with varying efficiencies, the best conversions were obtained with OPR1, YqiM, NCR and XenA. Again, only (S)-4b was formed in up to 96% e.e. using XenB. The preparative applicability of this method was exemplified by repeating the best data sets in presence of the GDH/glucose-cofactor recycling system.

The cyclopentenone derivative bearing the short O-methyl-protective group (5a) could not be reduced with sufficient rates by most enzymes, only NCR and XenB were reasonably active, showing conversions of 55 and 94%, respectively, with e.e.s of 76 and 90%. In contrast to the cyclohexenone analog 1a, no stereoselectivity-switch depending on the enzyme was observed with 5a and all enzymes produced (S)-5b in up to 98% e.e. (XenB). This trend is in line with the results using structurally closely related α-methyl substituted cyclohexenone and cyclopentenone derivatives using OYE1-3, NCR,[10c] OPR1, OPR3, YqiM[10a,b] and PETN-reductase,[10c,16] which predominantly formed (R)-2-methylcyclohexanone but (S)-2-methylcyclopent-2-anone. This stereochemical switch was recently explained by a ‘flipped’ orientation of the substrate in the active site.[16,17] When the steric demand of the α-alkoxy substituent was increased from methyl (5a) to benzyl (6a), the stereoselectivity of all active enzymes increased, best overall results were obtained with OPR1 and NCR. Again, cofactor recycling proved the preparative feasibility of the method.

\[
\begin{align*}
(R)-1b & \quad [H^+] \\
[O-R] & \quad [H^-]
\end{align*}
\]

\[
\begin{align*}
R = \text{Me, Allyl, } n-\text{Propyl, Benzyl} \\
[S]-\text{2b-4b} \quad [H^+] \\
[O-R] & \quad [H^-]
\end{align*}
\]

**Scheme 2.** Substrate-based stereocontrol for α-alkoxy-cyclohex-2-enone derivatives due to ‘flipped’ orientation of the substrate within the active site, forced by a gradual increase of the steric demand of the O-protective group R.

With β-alkoxy-functionalized cyclohexenone (7a) and cyclopentenone (8a) derivatives, no conversion could be achieved. This might be explained by a +M-effect[18] exerted by the O-atom, which enhances the electron-density at Cβ, thereby quenching its δ+-charge, which is required to allow the nucleophilic attack of the hydride delivered from N5 of the flavin cofactor. Again, an analogous effect was observed for β-methyl
substituted cyclic alkenones, which were reduced at considerably lower rates than the corresponding α-substituted substrate analogs.\textsuperscript{[10a-c,10e,16]}

Careful monitoring of the bioreductions occasionally revealed the formation of phenolic by-products \textbf{1c}, \textbf{2c} and \textbf{4c}. In most cases they were only observed in trace amounts, with the exception of OYE1, OPR1, and EBP1, which formed \textbf{1b}, \textbf{2b} and \textbf{4b} together with \textbf{1c}, \textbf{2c} and \textbf{4c} from \textbf{1a}, \textbf{2a} and \textbf{4a}, respectively, significant amounts (OYE1: 29\% \textbf{1c}; OPR1: 22\% \textbf{1c}; OYE1: 2\textbf{c} 20 \%; EBP1: \textbf{4c} 30 \%). The latter represents a disproportionation of enones, which was reported as a side-activity of OYEs,\textsuperscript{[19]} and which was recently exploited as a nicotinamide-independent method for the asymmetric bioreduction of activated alkenes using β-methylcyclohexenone or cyclohexane-1,4-dione as a hydrogen-donor.

![Scheme 3. Disproportionation of 2-methoxycyclohex-2-enone derivatives catalysed by OYE1, OPR1 and EBP1.](image)

\textbf{Conclusion}

We could demonstrate that conjugated α-alkoxycycloenones are a novel type of substrate for flavin-dependent enoate reductases, which upon bioreduction yield \textit{O}-protected acyloins in up to >99\% \textit{e.e.} The stereochemistry could be efficiently controlled by variation of the size of the \textit{O}-protective group, which — for cyclohexenone derivatives — furnished \textit{(R)}-configured acyloins with short-chain (methyl) groups and mirror-image \textit{(S)}-products with long-chain (\textit{n}-propyl, allyl or benzyl) analogues in very high \textit{e.e.s} (97-98\%). No switch was detected with cyclopentenone derivatives, which invariably produced \textit{(S)}-acyloins in up to >99\% \textit{e.e.}

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Experimental

General:
TLC plates were run on silica gel Merck 60 (F_{254}) and compounds were visualized by spraying with Mo-reagent [(NH\(_4\))\(_6\)Mo\(_7\)O\(_{24}\).4H\(_2\)O (100 g/L), Ce(SO\(_4\)).4H\(_2\)O (4 g/L) in H\(_2\)SO\(_4\) (10%)]) or by UV (254 nm). Silica gel 60 from Merck was used for flash chromatography. GC–MS analyses were performed on an Agilent 7890A GC system equipped with an Agilent 5975C mass-selective detector (electron impact, 70 eV) using a (5%-phenyl)-methylpolysiloxane phase column (Agilent HP-5ms, 30 m x 0.25 mm, 0.25 µm film). Helium was used as carrier gas (column flow: 2 mL/min). GC–FID analyses were carried out on a Varian 3800 by using H\(_2\) as a carrier gas (14.5 psi). NMR were done on a Bruker Avance III 300 MHz NMR spectrometer. Chemical shifts are reported relative to TMS (δ = 0.00) and coupling constants (J) are given in Hz. Cyclohexane-1,2-dione, hexamethyl disilazane (HMDS), 2-chlorocyclopentane-1-ol, cyclohexane-1,3-dione, cyclopentane-1,3-dione, pyrocatechol, 2-(benzyloxy)phenol, 2-methoxyphenol, (1\(S\),2\(S\))-cyclohexane-1,2-diol, (1\(S\),2\(S\))-cyclopentane-1,2-diol and (1\(R\),2\(R\))-cyclopentane-1,2-diol were purchased from Aldrich.

Source of Enzymes:
12-Oxophytodienoic acid reductase isoenzymes OPR1 and OPR3 from *Lycopersicon esculentum* and the OYE-homolog YqjM from *Bacillus subtilis* were overexpressed and purified as reported.\(^{[10a,21]}\) The cloning, purification and characterisation of old yellow iso-enzymes from yeast (OYE1 from *Saccharomyces carlsbergensis*, OYE2 and OYE3 from *Saccharomyces cerevisiae*) and nicotinamide-dependent cyclohexenone reductase (NCR) *Zymomonas mobilis* reductase were performed according to literature.\(^{[22]}\) Xenobiotic reductases XenA and XenB from *Pseudomonas putida* and *P. fluorescens*, resp., glycerol trinitrate reductase NerA from *Agrobacterium radiobacter* and estrogen binding protein EBP1 from *Candida albicans* were obtained as recently published.\(^{[10e,23]}\)

General procedure for the enzymatic bioreduction of 1a-8a:
An aliquot of enzyme (OYE1-3, OPR1, OPR3, YqjM, NCR, XenA, XenB, NerA, and EBP1, 60-100 µg, protein concentration in biotransformations 75-125 µg/mL) was added to a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (10 mM) and the cofactor NADH (15 mM). In case of 6a, the substrate was solubilised by addition of
tBuOMe (v:v 20%). The mixture was shaken at 30 °C and 120 rpm. After 24 h products were extracted with EtOAc (2 x 0.5 mL). The combined organic phases were dried over Na₂SO₄ and analyzed on achiral GC to determine the conversion and on chiral GC to determine the enantiomeric excess. For experiments in presence of cofactor recycling, NADH was replaced by the oxidized form of the cofactor (NAD⁺, 100 µM), the cosubstrate (glucose or formate, 20 mM) and the recycling enzyme (glucose dehydrogenase or formate dehydrogenase, 10 U), respectively.

**Determination of absolute configuration of products 1b-6b.**

![Scheme 4](image)

Scheme 4. Asymmetric synthesis of reference material for (S)-2b, (S)-4b, (S)-5b and (R)-6b.

(1S,2S)-2-(Allyloxy)cyclohexanol and (1S,2S)-2-methoxycyclopentanol: The corresponding (1S,2S)-dial (0.86 mmol) was dissolved in THF (5 mL) and NaH (40 mg, 1 mmol, 60% in mineral oil) was added. After 10 min of stirring, allyl- or methyl-bromide (0.5 mmol) was added and the mixture was stirred for 1 h at room temperature. Then more NaH was added (40 mg; 1 mmol; 60% in mineral oil) and the mixture was stirred at room temperature for 16 h. Then HCl (5mL, 1 M) was added and the mixture was extracted with ethyl acetate (3 x 10 mL). The organic phase was dried with Na₂SO₄ and the solvent was removed *in vacuo* to yield a mixture containing 15% of the desired product mono-alkylated dial, which was used without further purification for the next step. (1S,2S)-2-(Benzyloxy)cyclohexanol and (1R,2R)-2-(benzyloxy)cyclopentanol: The corresponding (1S,2S)- and (1R,2R)-dial (0.86 mmol) and benzyl bromide (152.9 mg, 0.9 mmol) were dissolved in CH₂Cl₂ (5 mL) and silver(I)oxide (1.35 mmol, 310 mg) was added. The mixture was stirred at room temperature for 24 h and then HCl (2mL, 1 M) was added and the mixture was extracted with ethyl acetate (2 x 10 mL). The organic phase was dried with
Na$_2$SO$_4$ and the solvent was removed in vacuo to yield a mixture containing 80% of the of monobenzylated diol, which was used without further purification for the next step.$^{[24]}$

General procedure for the oxidation of mono-ethers of cyclopentane- and cyclohexane-1,2-diols to the corresponding O-protected acyloins (S)-2b, (S)-4b, (S)-5b and (R)-6b:

(S)-2-(Allyloxy)cyclohexanone [(S)-2b], (S)-2-(benzyloxy)cyclohexanone [(S)-4b], (S)-2-methoxycyclopentanone [(S)-5b], and (R)-2-benzyloxyxycyclopentanone [(R)-6b] were obtained by Jones-oxidation of the corresponding mono-ether derivatives of 1,2-diols as prepared above: Jones reagent CrO$_3$ (7g, 70 mmol) was dissolved in water (50 mL) and H$_2$SO$_4$ (6.1 mL, conc.) was added dropwise under cooling. The mono-ether derivative was dissolved in CH$_2$Cl$_2$ (2 mL) and Jones reagent (2 mL) was added dropwise over 30 min with stirring. Excess oxidant was quenched by addition of 2-butanol (2 mL). Water (10 mL) was added and products were extracted with CH$_2$Cl$_2$ (2 x 10 mL). The organic phase was filtered through cellite, dried with Na$_2$SO$_4$ and the solvent was removed in vacuo to afford (S)-2-(allyloxy)cyclohexanone [(S)-2b], (S)-2-(benzyloxy)cyclohexanone [(S)-4b], (S)-2-methoxycyclopentanone [(S)-5b] and (R)-2-benzyloxyxycyclopentanone [(R)-6b].

(S)-2-Propoxycyclohexanone [(S)-3b]: (S)-2-(Allyloxy)cyclohexanone [(S)-2b] was hydrogenated according to method B, yielding (S)-2-propoxycyclohexanone [(S)-3b]. The absolute configuration of 1b was determined by co-injection of enantioenriched reference material obtained by reduction of 1a with EBP1 [20 mg, e.e. 78% (R)]. $\left[\delta\right]_D^{20}$ (CH$_2$Cl$_2$) = +29.5, for (R)-1a.$^{[25]}$
References and Notes


53


Electronic Supporting Information

Synthesis of substrates

2-Methoxycyclohex-2-enone (1a): Cyclohexane-1,2-dione (0.41 g, 3.7 mmol) was dissolved in methanol (5 mL) and H₂SO₄ (conc., 0.5 mL) was added. The solution was heated under reflux for 5 h. General workup (method A): The reaction mixture was cooled to room temperature, neutralized with NaOH (1 M), diluted with ethyl acetate (5 mL) and washed with saturated aqueous NaHCO₃ solution (5-10 mL). The organic phase was dried with Na₂SO₄ and the solvent was removed in vacuo. The crude product was purified by flash chromatography on silica gel (ethyl acetate/petroleum ether 1:4) to obtain 1a (0.25 g, 54%, 2.0 mmol) as a yellow oil.

1H-NMR (CDCl₃, 300 MHz): δ [ppm] = 1.95-2.05 (2H, m, J = 6.9), 2.42-2.47 (2H, q, J = 4.8), 2.51-2.55 (2H, t, J = 6.9), 3.61 (3H, s); 5.85-5.88 (1H, t, J = 4.5); 13C-NMR (CDCl₃, 75 MHz): δ [ppm] = 23.0, 24.4, 38.8, 54.7, 116.3, 151.5, 194.4.[1]

2-(Allyloxy)cyclohex-2-enone (2a): To a solution of cyclohexane-1,2-dione (0.65 g, 5.8 mmol) and allyl alcohol (5 mL) in freshly distilled THF (10 mL), H₂SO₄ (conc., 0.2 mL) was added. The solution was heated under reflux for 24 h and worked up using method A. The crude product was purified by flash chromatography on silica gel (ethyl acetate/petroleum ether 1:5) to obtain 2a (0.27 g, 31%, 1.8 mmol) as a yellow oil. 1H-NMR (CDCl₃, 300 MHz): δ [ppm] = 1.93-2.53 (6H, m), 4.30-4.31 (2H, d, J = 4.2), 5.21-5.30 (1H, m), 5.34-5.36 (1H, t, J = 1.2 ), 5.89-5.92 (1H, t, J = 1.5), 5.94-6.05 (1H, m); 13C-NMR (CDCl₃, 75 MHz): δ [ppm] = 22.9, 24.5, 38.8, 68.7, 118.0, 132.9, 150.3, 194.4.[1]

2-n-Propoxycyclohex-2-enone (3a): To a mixture of cyclohexane-1,2-dione (0.12 g, 0.9 mmol) and 1-propanol (5 mL), H₂SO₄ (conc., 0.2 mL) was added. After 4 h of reflux, the TLC showed complete conversion of the educt and a second portion of cyclohexane-1,2-dione (0.51 g, 4.5 mmol) and 1-propanol (7 mL) were added. This solution was refluxed for 4 more hours and afterwards worked up using method A. The crude product was purified by flash chromatography on silica gel (ethyl acetate/petroleum ether 1:4) to afford 3a (0.27 g, 32%, 1.75 mmol) as a yellow oil. 1H-NMR (CDCl₃, 300 MHz): δ [ppm] = 0.96-1.01 (3H, t, J = 7.5), 1.73-1.85 (2H, m), 1.93-1.99 (2H, m), 2.41-2.49 (2H, q, J = 6, J = 10.8), 3.62-3.67 (2H, t, J = 6.9), 5.85-5.88 (1H, t, J = 4.5); 13C-NMR (CDCl₃, 75 MHz): δ [ppm] = 10.4, 22.0, 22.9, 24.5, 38.9, 69.6, 117.3, 150.9, 194.5.[1]

Benzyloxytrimethylsilane: To a mixture of benzyl alcohol (88 mmol, 10 mL, 9.6 g) and acetonitrile (50 mL), hexamethyl disilazane (44 mmol, 9.34 mL, 7.1 g) was added. The
mixture was stirred at room temperature for 1 h and then heated to reflux for 18 h. The reaction mixture was cooled to room temperature, neutralized with NaOH (1 M), diluted with ethylacetate (100 mL) and washed with saturated aqueous NaHCO₃ solution (100 mL). The organic phase was dried with Na₂SO₄ and the solvent was removed in vacuo, to afford benzyloxytrimethylsilane (15.3 g, 95 %) which was used without further purification for the next step.[¹]

2-(Benzyloxy)cyclohex-2-enone (4a): To a mixture of cyclohexane-1,2-dione (1 g, 8.8 mmol) and benzyloxytrimethylsilane (17.6 mmol, 2 eq.) in CH₂Cl₂ (10 mL), trifluoromethanesulfonic acid (0.01 eq., 13.2 mg) was added. After 18 h of reflux the reaction mixture was cooled to room temperature and worked up using method A. The crude product was purified by flash chromatography on silica gel (ethyl acetate/petroleum ether 1:5) to afford 4a (1.07 g, 60 %, 5.3 mmol) as a yellow oil. ¹H-NMR (CDCl₃, 300 MHz): δ [ppm] = 1.94-2.02 (2H, m), 2.37-2.43 (2H, q, J = 5.5, J = 5.4), 2.51-2.56 (2H, t, J = 6.7), 4.87 (2H, s), 5.92-5.95 (1H, t, J = 4.5), 7.28-7.39 (5H, m); ¹³C-NMR (CDCl₃, 75 MHz): δ [ppm] = 22.9, 24.5, 38.9, 119.3, 127.7, 128.6, 136.5, 150.4, 194.4.[¹]

Cyclopentane-1,2-dione: A solution of 2-chlorocyclopentane-1-ol (11.9 g, 0.10 mol) in water (120 mL) was heated under stirring to 100 °C. Then FeCl₃·6H₂O (50 g, 0.19 mol) dissolved in water (25 mL) was added within 10 minutes. After cooling to 40 °C, ammonium sulfate (36 g, 0.27 mol) was added. The black-brown mixture was washed with ethoxyethane, the organic phase was dried with Na₂SO₄ and the solvent was removed in vacuo to afford cyclopentane-1,2-dione (7.86 g, 80%) which was used without further purification for the next step.[²]

2-Methoxycyclopent-2-enone (5a): Cyclopentane-1,2-dione (0.80 g, 8.2 mmol) was dissolved in methanol (5 mL) and H₂SO₄ (conc., 0.5 mL) was added. The solution was heated to reflux for 18 h and then worked up using method A. The crude product was purified by flash chromatography on silica gel (ethyl acetate/petroleum ether 1:2) to afford 5a (0.42 g, 46 %, 3.8 mmol) as a yellow oil. ¹H-NMR (CDCl₃, 300 MHz): δ [ppm] = 2.44 (2H, t, J = 4.8), 2.53 (2H, q, J = 3.0, J = 8.1), 3.74 (3H, s), 6.40 (1H, t, J = 3.0); ¹³C-NMR (CDCl₃, 75 MHz): δ [ppm] = 21.8, 33.2, 57.1, 126.9, 157.6, 202.4.[¹]

2-(Benzyloxy)cyclopent-2-enone (6a): To a mixture of cyclopentane-1,2-dione (980 mg, 10 mmol) and benzyloxytrimethylsilane (20 mmol, 2 eq.) in CH₂Cl₂ (10 mL), trifluoromethanesulfonic acid (0.01 eq., 30 mg) was added. After 18 h of reflux the reaction mixture was cooled to room temperature and worked up using method A. The crude product
was purified by flash chromatography on silica gel (ethyl acetate/petroleum ether 1:9) to afford 6a (1.44 g, 77%, 7.7 mmol) as a yellow oil. \(^1\)H-NMR (CDCl\(_3\), 300 MHz): \(\delta [ppm] = 2.44 (2H, t, J = 3.3), 2.51 (2H, q, J = 3, J = 4.8), 4.99 (2H, s), 6.41 (1H, t, J = 3), 7.27-7.40 (5H, m); \(^{13}\)C-NMR (CDCl\(_3\), 75 MHz): \(\delta [ppm] = 22.0, 33.1, 71.7, 127.5, 128.2, 128.6, 128.7, 135.8, 156.3, 202.5.\)\(^{[1]}\)

3-Methoxycyclohex-2-enone (7a): Cyclohexane-1,3-dione (0.88 g, 7.8 mmol) was dissolved in methanol (15 mL) and \(\text{H}_2\text{SO}_4\) (conc., 0.2 mL) was added. The solution was heated to reflux for 18 h and then worked up using method A to afford 7a (0.90 g, 90%, 7.1 mmol) as a yellow oil. The product needed no further purification. \(^1\)H-NMR (CDCl\(_3\), 300 MHz): \(\delta [ppm] = 1.93-2.01 (2H, m), 2.31-2.36 (2H, t, J = 6.6), 2.38-2.42 (2H, t, J = 6.3), 3.68 (3H, s), 5.36 (1H, s); \(^{13}\)C-NMR (CDCl\(_3\), 75 MHz): \(\delta [ppm] = 21.2, 28.8, 36.7, 55.5, 102.3, 178.6, 199.6.\)\(^{[3]}\)

3-Methoxycyclopent-2-enone (8a): Cyclopentane-1,3-dione (1.0 g, 10.2 mmol) was dissolved in methanol (5 mL) and \(\text{H}_2\text{SO}_4\) (conc., 5 mL) was added. The solution was heated to reflux for 18 h and then worked up using method A to afford 8a (0.76 g, 67%, 6.8 mmol) as a yellow oil. The product needed no further purification. \(^1\)H-NMR (CDCl\(_3\), 300 MHz): \(\delta [ppm] = 2.48 (2H, t, J = 4.8), 2.59 (2H, t, J = 8.4), 3.84 (3H, s), 5.32 (1H, s); \(^{13}\)C-NMR (CDCl\(_3\), 75 MHz): \(\delta [ppm] = 28.2, 33.1, 55.2, 103.3, 189.9, 205.4.\)\(^{[4]}\)

**Synthesis of reference material**

**General procedure for the catalytic hydrogenation of 1a-6a to obtain rac-1b-6b (method B):**

Alkenones (1a-6a, 1 mmol) were dissolved in THF (5 mL) and hydrogenated under \(\text{H}_2\) with Pd/C (10%, 5 mg) at atmospheric pressure and room temperature by stirring the mixture overnight at room temperature. The mixture was filtered through Celite and concentrated to yield rac-1b-6b as reference materials with >99% conversion.

**rac-2-Methoxycyclohexanone (1b):** \(^1\)H-NMR (CDCl\(_3\), 300 MHz): \(\delta [ppm] = 1.64-1.78 (3H, m), 1.92-1.99 (2H, m), 2.21-2.36 (2H, m), 2.40-2.56 (1H, m), 3.43 (3H, s), 3.67-3.86 (1H, m); \(^{13}\)C-NMR (CDCl\(_3\), 75 MHz): \(\delta [ppm] = 23.1, 27.7, 34.2, 40.6, 57.7, 84.3, 210.1.\)\(^{[5]}\)

**rac-2-(Allyloxy)cyclohexanone (2b):** \(^1\)H-NMR (CDCl\(_3\), 300 MHz): \(\delta [ppm] = 1.25-1.30 (4H, m), 1.66-1.74 (2H, m), 1.94-2.43 (2H, m), 3.98-4.08 (1H, m), 4.36 (2H, d, J = 7.2), 5.18-5.36
(2H, m), 5.87-5.98 (1H, m); $^{13}$C-NMR (CDCl$_3$, 75 MHz): $\delta$ [ppm] = 23.0, 27.7, 34.2, 40.4, 71.8, 82.9, 125.5, 135.8, 210.6.

**rac-2-Propoxycyclohexanone (3b):** $^1$H-NMR (CDCl$_3$, 300 MHz): $\delta$ [ppm] = 0.91-0.96 (3H, t, J = 7.5), 1.24-1.29 (2H, m), 1.60-1.75 (5H, m), 2.05-2.32 (3H, m), 3.53-3.81 (2H, m), 3.76-3.81 (1H, m); $^{13}$C-NMR (CDCl$_3$, 75 MHz): $\delta$ [ppm] = 10.4, 22.9, 23.0, 27.7, 34.5, 40.4, 71.8, 82.8, 210.7.

**rac-2-(Benzyl oxy)cyclohexanone (4b):** $^1$H-NMR (CDCl$_3$, 300 MHz): $\delta$ [ppm] = 1.62-1.99 (5H, m), 2.19-2.33 (2H, m), 2.53-2.59 (1H, m), 4.72 (2H, s), 7.28-7.37 (5H, m); $^{13}$C-NMR (CDCl$_3$, 75 MHz): $\delta$ [ppm] = 23.1, 27.7, 34.6, 40.7, 65.4, 81.7, 127.0, 127.8, 128.4, 137.9, 210.3.$^6$

**rac-2-Methoxycyclopentanone (5b):** $^1$H-NMR (CDCl$_3$, 300 MHz): $\delta$ [ppm] = 1.72 (2H, m), 2.03 (2H, m), 2.22 (2H, m), 3.52 (3H, s), 3.68 (1H, t, J = 9.0); $^{13}$C-NMR (CDCl$_3$, 75 MHz): $\delta$ [ppm] = 17.2, 29.1, 35.3, 58.1, 82.7.

**rac-2-(Benzyloxy)cyclopentanone (6b):** $^1$H-NMR (CDCl$_3$, 300 MHz): $\delta$ [ppm] = 1.72-1.92 (2H, m), 2.0-2.1 (1H, m), 2.21-2.32 (3H, m), 3.82 (1H, t, J = 9), 4.68-4.87 (2H, dd, J = 12, J = 33), 7.28-7.39 (5H, m); $^{13}$C-NMR (CDCl$_3$, 75 MHz): $\delta$ [ppm] = 17.4, 29.6, 35.5, 72.0, 80.0, 127.8, 127.9, 128.4, 137.7, 216.4.

2-(Allyloxy)phenol (2c): Pyrocatechol (1 g, 9 mmol) and allyl bromide (840 mg, 7 mmol) were dissolved in CH$_2$Cl$_2$ (20 mL) and NaH (300 mg, 60% in mineral oil) was added with stirring. After 5 h of stirring at room temperature more NaH (150 mg) was added. The mixture was stirred for another 18 h and then neutralized with HCl (1 M), diluted with ethyl acetate (20 mL) and washed with brine (20 mL). The organic phase was dried with Na$_2$SO$_4$ and the solvent was removed in vacuo. The crude product was purified by flash chromatography on silica gel (ethyl acetate/petroleum ether 1:5) to obtain 2c (102 mg, 10%). $^1$H-NMR (CDCl$_3$, 300 MHz): $\delta$ [ppm] = 4.62-4.64 (2H, d, J = 5.1), 5.33-4.36 (1H, d, J = 10.4), 5.41-5.47 (1H, d, J = 17.4), 5.79 (1H, s, OH), 6.0-6.12 (1H, m), 6.81-7.00 (4H, m); $^{13}$C-NMR (CDCl$_3$, 75 MHz): $\delta$ [ppm] = 69.8, 112.3, 114.8, 118.3, 120.1, 121.8, 132.9, 145.6, 145.9.$^7$

2-Propoxyphenol (3c): 2-(Allyloxy)phenol (2c) was hydrogenated according to method B, yielding 2-propoxyphenol (3c). $^1$H-NMR (CDCl$_3$, 300 MHz): $\delta$ [ppm] = 0.95-1.00 (3H, t, J =
7.5), 1.75-1.88 (2H, m), 3.60-3.65 (2H, m), 5.70 (1H, s, OH), 6.87-7.26 (4H, m); $^{13}$C-NMR (CDCl$_3$, 75 MHz): $\delta$ [ppm] = 10.4, 22.3, 69.7, 112.4, 114.3, 119.1, 121.3, 146.1, 145.9.$^{[7]}$

Analytical procedures

Table 1. Achiral GC-analysis for determination of conversion.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Column$^a$</th>
<th>Conditions/Program$^b$</th>
<th>$t_R$ [min]</th>
<th>1a-8a</th>
<th>1b-6b</th>
<th>1c-4c</th>
</tr>
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<tbody>
<tr>
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<td>A</td>
<td>a</td>
<td>6.19</td>
<td>4.22</td>
<td>4.98</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>b</td>
<td>7.10</td>
<td>5.50</td>
<td>6.20</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>c</td>
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<td>3.73</td>
<td>4.33</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C$^c$</td>
<td>f$^c$</td>
<td>12.69</td>
<td>11.24</td>
<td>11.86</td>
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</tr>
<tr>
<td>5</td>
<td>A</td>
<td>e</td>
<td>8.76</td>
<td>3.72</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>C$^c$</td>
<td>f$^c$</td>
<td>9.71</td>
<td>10.18</td>
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</tr>
<tr>
<td>7</td>
<td>A</td>
<td>d</td>
<td>15.17</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>A</td>
<td>d</td>
<td>13.10</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Column: A = J&W Scientific DB-1701, 14% cyanopropyl-phenyl phase capillary column, 30 m x 0.25 mm, 0.25 $\mu$m film; B = Varian CP-1301, 6% cyanopropyl-phenyl phase capillary column, 30 m x 0.25 mm, 0.25 $\mu$m film; C = J&W HP-5 MS 5% phenylmethylpolysiloxane capillary column, 30 m x 0.25 mm, 0.25 $\mu$m film.

$^b$Conditions: a = 14.5 psi H$_2$ at 80 °C hold 2 min, 20 °C/min to 160 °C, 30 °C/min to 220 °C, hold 2 min; b = 14.5 psi H$_2$ at 80 °C hold 2 min, 5 °C/min to 200 °C; c = 14.5 psi H$_2$ at 110 °C hold 2 min, 15 °C/min to 200 °C hold 2 min; d = 14.5 psi H$_2$ at 80 °C hold 2 min, 5 °C/min to 230 °C hold 5 min; e = 14.5 psi H$_2$ at 80 °C hold 5 min, 5 °C/min to 180 °C, hold 5 min; f = column flow 0.55 ml/min He at 100 °C hold 0.5 min, 10 °C/min to 300 °C;

$^c$measured by GC-MS;
Table 2. Chiral GC-analysis for determination of the enantiomeric excess.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Column&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Conditions/ Program&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1a-6a&lt;sup&gt;c&lt;/sup&gt;</th>
<th>(S)-1b-6b</th>
<th>(R)-1b-6b</th>
<th>1c-4c</th>
</tr>
</thead>
<tbody>
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<td>1</td>
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<td>a</td>
<td>14.25</td>
<td>12.00</td>
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<td>B</td>
<td>b</td>
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</tr>
<tr>
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<td>B</td>
<td>b</td>
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<td>24.75</td>
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</tr>
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<td>B</td>
<td>c</td>
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</tr>
<tr>
<td>5</td>
<td>A</td>
<td>d</td>
<td>20.69</td>
<td>13.77</td>
<td>14.29</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>A</td>
<td>e</td>
<td>59.25</td>
<td>50.31</td>
<td>49.72</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup>Column: A = Hydrodex-β-TBDAc (Macherey-Nagel), β-cyclodextrin capillary column, 25 m x 0.25 mm; B = Chiraldex B-TA (Supelco), β-cyclodextrin trifluoroacetyl capillary column, 40 m x 0.25 mm, 0.12 µm.

<sup>b</sup>Conditions: a = 14.5 psi H₂ at 80 °C hold 1 min, 2.5 ºC/min to 100 ºC, 15 ºC/min to 180 ºC, hold 5 min; b = 14.5 psi H₂ at 40 ºC hold 2 min, 4 ºC/min to 120 ºC, hold 1 min, 15 ºC/min to 180 ºC, hold 5 min; c = 14.5 psi H₂ at 130 ºC, hold 40 min, 2 ºC/min to 180, hold 10 min; d = 14.5 psi H₂ at 50 °C, hold 5 min, 10 ºC/min to 80 °C, hold 5 min, 20 ºC/min to 180 °C, hold 10 min; e = 14.5 psi H₂ at 100 °C, hold 20 min, 10 ºC/min to 130 °C, hold 30 min, 15 ºC/min to 175 °C, hold 20 min.
References


4.2 Paper II: α-methylcinnamaldehyde (co-authorship)

Bioreduction of α-Methylcinnamaldehyde Derivatives:

Chemo-Enzymatic Asymmetric Synthesis of Lilial™ and Helional™

(Dalton Transactions 2010)

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Summary

Nonracemic aryl-substituted α-methyldihydrocinnamaldehyde derivatives employed as olfactory principles in perfumes (Lilial™, Helional™) were obtained via enzymatic reduction of the corresponding cinnamaldehyde precursors using cloned and overexpressed ene-reductases. Whereas (R)-enantiomers were obtained using the old-yellow-enzyme (OYE) homolog YqiM from Bacillus subtilis and 12-oxophytodienoic acid reductase isoenzyme OPR1 from tomato (e.e. max 53%), isoenzyme OPR3, nicotinamide 2-cyclohexene-1-one reductase NCR from Zymomonas mobilis and yeast OYE isoenzymes 1-3 furnished (S)-aldehydes in up to 97% e.e. under optimised reaction conditions in the presence of t-butyl methyl ether as co-solvent. The stereochemical outcome of the reduction of α-methylcinnamaldehyde using NCR and OYE1-3 [previously reported to be (R)] was unambiguously corrected to be (S).

Introduction

Due to their volatility and their olfactory properties, aldehydes constitute important active ingredients in fragrance and flavour applications. Since the enantiomers of α- and β-substituted aldehydes often considerably differ in odour, their application in nonracemic form is required. Whereas β-substituted aldehydes are chirally stable, α-substituted analogues are prone to racemisation, which requires sophisticated methods for their preparation. Among them, the desymmetrisation of conjugated enals via asymmetric hydrogenation is the method of choice. Whereas numerous protocols using chirally modified homogeneous (transition-metal) containing catalysts have been reported, metal-independent organocatalysts for the reduction of enals at the expense of a nicotinamide-mimic ('Hantzsch-ester') as hydride source were developed more recently. To date, chirally surface-modified heterogeneous catalysts are not competitive. As an alternative to the variety of chemo-catalytic methods, bioreduction has been envisaged by using various types of redox enzymes. In order to circumvent tedious protein purification and external cofactor-recycling, whole microbial cells - most prominent baker's yeast - were employed for the reduction of enals. Due to the presence of competing ene- and carbonyl-reductases, the chemo- and stereoselective bioreduction of enals was impossible, because undesired carbonyl reduction always overruled the desired C=C-bond reduction, thereby causing substrate- and product-depletion via formation of the corresponding allylic and/or saturated alcohols.
It was only recently, that oxygen-stable ene-reductases from the Old Yellow Enzyme family became available in sufficient amounts, which allowed the chemo- and stereoselective bioreduction of activated C=C-bonds in enones and enals by leaving C=O-moieties untouched.\textsuperscript{[9,10]} Encouraged by our recent results,\textsuperscript{[11]} we investigated the application of these enzymes for the preparation of nonracemic \(\alpha\)-methyl dihydrocinnamaldehyde derivatives used in perfumery applications.\textsuperscript{[10]}

**Results and Discussion**

The reduction product of \(p\)-tert-butylcinnamaldehyde (1b) is the olfactory principle of the lily-of-the-valley\textsuperscript{[12c,9]} and is marketed under the trade name Lilial\textsuperscript{TM} or Lysmeral\textsuperscript{TM}, whereas the \(m,p\)-methylenedioxy aldehyde 2b is the active ingredient of various perfumes and is marketed as Helional\textsuperscript{TM} or Tropional\textsuperscript{TM}.\textsuperscript{[1,13]}

**Scheme 1.** Asymmetric bioreduction of \(\alpha\)-methylcinnamaldehyde derivatives 1a - 3a.

The bioreduction of 1a under standard conditions in neat aqueous buffer pH 7.5 proved to be disappointingly slow using a variety of ene-reductases (data not shown). However, when the solubility of the lipophilic substrate was enhanced by addition of a small amount of diisopropyl ether (5\%, v:v), reaction rates picked up markedly (Table 1, entries 1-7). Among all enzymes, YqiM and isoenzyme OPR1 gave \((R)\)-1b, albeit in low enantiomeric excess (e.e.\textsubscript{max} 21\%). In contrast, OPR3, NCR and OYE1-3 furnished \((S)\)-1b with slightly enhanced stereoselectivities, but they still were insufficient for synthetic purposes (e.e.\textsubscript{max} 64\%). Since the co-solvent seemed to have a strong influence on the reaction rate, we anticipated that it might also have an impact on the stereoselectivity of the ene-reductases. An increased amount of diisopropyl ether (20\%, v:v) caused a drop in reaction rates, without altering the stereoselectivities significantly, similar effects (reduced rates and slightly diminished stereoselectivities) were observed when \(i\)-Pr\textsubscript{2}O was replaced with ethyl acetate or \(n\)-hexane (20\%, v:v, data not shown). A switch to the water-miscible co-solvent ethanol (20\%, v:v) enhanced the rates (e.e.\textsubscript{max} 80\%) for OPR1, NCR and OYE1-3 going in hand with a decrease of stereoselectivities (e.e.\textsubscript{max} 51\%, entries 8-12). YqiM and OPR3 were only marginally active.
(data not shown). Finally, a switch to t-butyl methyl ether proved to be an ideal solution: Excellent stereoselectivities were obtained with OYE1s 1-3 going in hand with a significant drop in reaction rates (e.e. \( \text{max} > 95\% \), entries 13-19).

In order to tune the system, 1a was reduced using OYE3 at increasing proportions of t-butyl methyl ether. As may be deduced from figure 1, a clear inverse correlation between reaction rate and stereoselectivity (plotted as conversion and e.e. versus TBME concentration) was observed at increasing amounts of co-solvent. Overall, a fraction of 20% (v:v) of t-BuOMe seemed to be a good compromise between a decrease of activity and an increase of stereoselectivity. Consequently, all further studies were performed at this co-solvent ratio.

**Figure 1.** Dependence of reaction rate and stereoselectivity on proportion of organic co-solvent (t-BuOMe, v:v) in the reduction of 1a using OYE3.

![Figure 1](image_url)

Under optimised conditions, 2a was accepted by all ene-reductases (entries 20-26). In line with previous observations, YqjM and OPR1 showed a weak preference to furnish (R)-2b. Excellent stereoselectivities (e.e. \( \text{max} 97\% \)) and rates (up to full conversion) were obtained with NCR and OYE1s 1-3 for (S)-2b. Interestingly, the structurally and mechanistically closely related OYE-homologs N-ethylmaleimide-(NEM)-reductase, morphinone reductase and pentaerythritol tetranitrate-(PETN)-reductase showed insufficient stereoselectivities (e.e.s 0-14%) with good activities (conversion up to 79%) with 1a-3a or vice versa: Only NEM-
Reducase furnished (S)-1b and (S)-2b with e.e. max 57% (conversion 18%) and e.e. max 18% (conversion 64%), respectively.\textsuperscript{[14]}

**Table 1.** Conversion and enantiomeric excess of bioreduction products 1b-3b.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Enzyme\textsuperscript{a}</th>
<th>Conditions\textsuperscript{b}</th>
<th>Product</th>
<th>Conv. [%]</th>
<th>E.e. [%]</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1a</td>
<td>YqjM</td>
<td>buffer / i-Pr\textsubscript{2}O (95:5)</td>
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<td>13 (R)</td>
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<tr>
<td>2</td>
<td>1a</td>
<td>OPR1</td>
<td>buffer / i-Pr\textsubscript{2}O (95:5)</td>
<td>37</td>
<td>21 (R)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1a</td>
<td>OPR3</td>
<td>buffer / i-Pr\textsubscript{2}O (95:5)</td>
<td>4</td>
<td>22 (S)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1a</td>
<td>NCR</td>
<td>buffer / i-Pr\textsubscript{2}O (95:5)</td>
<td>66</td>
<td>59 (S)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1a</td>
<td>OYE1</td>
<td>buffer / i-Pr\textsubscript{2}O (95:5)</td>
<td>48</td>
<td>52 (S)</td>
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<td>1a</td>
<td>OYE3</td>
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<td>64 (S)</td>
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<td>1a</td>
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<td>buffer / EtOH (80:20)</td>
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<td>NCR</td>
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<td>26</td>
<td>&gt;95 (S)</td>
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<td>1a</td>
<td>OYE2</td>
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<td>26</td>
<td>&gt;95 (S)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>1a</td>
<td>OYE3</td>
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<td>&gt;95 (S)</td>
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<td>10</td>
<td>13 (R)</td>
<td></td>
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<tr>
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<td>2a</td>
<td>OPR1</td>
<td>buffer / t-BuOMe (80:20)</td>
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<td>6 (R)</td>
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<tr>
<td>22</td>
<td>2a</td>
<td>OPR3</td>
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<td>buffer / t-BuOMe (80:20)</td>
<td>72</td>
<td>96 (S)</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>3a</td>
<td>YqjM</td>
<td>buffer / t-BuOMe (80:20)</td>
<td>26</td>
<td>33 (R)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>3a</td>
<td>OPR1</td>
<td>buffer / t-BuOMe (80:20)</td>
<td>&gt;99</td>
<td>53 (R)</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>3a</td>
<td>OPR3</td>
<td>buffer / t-BuOMe (80:20)</td>
<td>22</td>
<td>28 (S)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>3a</td>
<td>NCR</td>
<td>buffer / t-BuOMe (80:20)</td>
<td>&gt;99</td>
<td>76 (S)</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>3a</td>
<td>OYE1</td>
<td>buffer / t-BuOMe (80:20)</td>
<td>&gt;99</td>
<td>94 (S)</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>3a</td>
<td>OYE2</td>
<td>buffer / t-BuOMe (80:20)</td>
<td>&gt;99</td>
<td>96 (S)</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>3a</td>
<td>OYE3</td>
<td>buffer / t-BuOMe (80:20)</td>
<td>84</td>
<td>90 (S)</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) YqjM = Old Yellow Enzyme homolog from Bacillus subtilis;\(^{[15]} \) OPR1 and OPR3 = 12-oxophytodienoic acid reductase isoenzymes from Lycopersicon esculentum (tomato);\(^{[16]} \) NCR = nicotinamide 2-cyclohexen-1-one reductase from Zymomonas mobilis;\(^{[10]} \) OYE = Old Yellow Enzymes from Saccharomyces carlsbergensis (OYE1) and from S. cerevisiae (OYE2, OYE3);\(^{[17]} \)

\( ^b \) Tris-HCl buffer 50 mM, pH 7.5.
The absolute configuration of products 1b and 2b was deduced by comparison of optical rotation values of 1b and 2b obtained using OYE2 with literature data (for details see experimental part), which proved to be (S) for both substrates. However, the pronounced stereochemical preference of NCR and OYEs 1-3 to yield (S)-1b and (S)-2b is in conflict with the (R)-preference of these enzymes on the close homolog α-methyldihydrocinnamaldehyde (3a), as reported by B. Rosche et al.\textsuperscript{10} According to this report, the bioreduction of 3a furnished (R)-3b in 50% and ca. 75% e.e. using NCR and OYEs 1-3, resp. using slightly different reaction conditions (recombinant whole cells of \textit{E. coli} expressing NCR, NADPH, MES-buffer pH 6.8, 50 mM, 1-PrOH 10% v:v). In order to clarify this discrepancy, we re-investigated substrate 3a using all ene-reductases (entries 27-33). Again, the absolute configuration of 3b obtained by using OYE2 was deduced by comparison of optical rotation values with literature data and proved to be (S). This result was double-checked by chemical reduction of the aldehyde (S)-3b obtained via bioreduction using NaBH\textsubscript{4} to yield 2-methyl-3-phenyl-1-propanol (3c), which was proven to be (S)-configured on the basis of its optical rotation. Overall, the stereochemical outcome of the reduction of 3a nicely matched our previous results, as may be expected since substrates 1a-3a represent a structurally homologous series: while YqiM and OPR1 furnished (R)-3b with modest stereoselectivities (e.e.\textsubscript{max} 53 % using OPR1), OPR3, NCR and OYE1-3 gave predominantly (S)-3b in up to 96 % e.e. In view of these results, the stereochemical assignment of 3b - reported to be (R)\textsuperscript{10} - have to be corrected to be (S).

In order to test whether the stereochemical preference of OYE1 and NCR to yield (S)-3b from 3a could be predicted via molecular modelling, substrate complexes were modelled using docking simulations and molecular mechanics optimization (see Experimental Section). In the case of OYE1, a single binding mode of 3a was obtained (Figure 2) which clearly indicates that the (S)-configured product will be formed by trans-hydrogenation (hydride transfer from the flavin onto Cβ of 3a and protonation of Cα by Tyr-196). Initial attempts of docking 3a into the active site of NCR (assuming a rigid enzyme structure) failed to produce a productive binding mode, because the active site region was partially blocked by side chains of neighbouring residues. Thus, the modelled structure of the OYE1 complex (Figure 2, left) was used to build a model of the corresponding complex with NCR. Only small movements of the interfering residues (Trp-64, His-126 and Tyr-341), however, were enough to yield exactly the same optimized binding mode as in the case of OYE1, again predicting the formation of (S)-3b (Figure 2 right). Especially in the case of His-126, the crystallographic B-factors indicate significant flexibility of this part of the structure.
Figure 2. Modelled binding modes of 3a in the active sites of OYE1 from Saccharomyces cerevisiae (left) and NCR from Zymomonas mobilis (right). The active site pockets are shown in a semi-transparent surface representation. Amino acids are shown in green, the FMN cofactor in yellow and the bound substrate in pink. Hydrogen bonding interactions of 3a with Asn-194 and His-191 in OYE1 and with Asn-173 and His-170 in NCR respectively are indicated as dashed lines. Close contacts (3.4-3.8 Å) between Cα of 3a and Tyr-196 (in OYE1) or Tyr-175 (in NCR) as well as between Cβ of 3a and N5 of the corresponding FMN cofactor are also shown as dashed lines. The conformations of Trp-64, His-126 and Tyr-341 as observed in the NCR structure before molecular mechanics optimization are shown as orange thin lines. The figures were prepared using the programme PyMOL (http://www.pymol.org/).

Conclusions

A convenient chemo-enzymatic synthesis for the fragrance aldehydes Lilial™ (1b) and Helional™ (2b) was developed via asymmetric bioreduction of α-methylldihydrocinnamaldehyde derivatives 1a and 2a catalysed by cloned and overexpressed ene-reductases. Whereas (R)-1b and (R)-2b were formed in modest e.e.s using YqiM and OPR1, NCR and OYEs 1-3 yielded (S)-antipodes in up to 97% e.e., when the reactions were run in an aqueous-organic biphasic system containing t-butyl methyl ether (20%, v:v). The stereochemical outcome of the reduction of α-methylcinnamaldehyde 3a using NCR and OYE1-3 - previously reported to be (R)\textsuperscript{[10]} - was unambiguously corrected to be (S). Our biocatalytic method compares favourably with asymmetric hydrogenation protocols based on iridium-phosphanodihydrooxazole catalysts (e.e.s up to 94%),\textsuperscript{[15]} asymmetric alkylation using
SAMP/RAMP-hydrazone (e.e.s up to 90%)\textsuperscript{[1]} and counteranion-directed organocatalytic transfer hydrogenation (e.e.s up to 98%).\textsuperscript{[17]}

**Acknowledgements:** The authors would like to express their cordial thanks for financial support by BASF SE and to R. Stürmer, B. Hauer, K. Ditrich for stimulating discussions, and W. Hoeffken (Ludwigshafen) for providing the coordinates of NCR. N. C. Bruce and H. Housden (York) are thanked for the generous donation of PETN-, NEM- and morphinone reductase.

**Electronic Supporting Information**

General experimental conditions, source of substrates, synthesis of reference compounds, spectroscopic data of substrates and products, and analytical procedures for the determination of conversion and enantiomeric excess of products are given in the ESI, which is available via the Internet.

**Experimental**

**Source of enzymes**

The open reading frame of *Lycopersicon esculentum* OPR1 was cloned into pET-21a and overexpressed as a C-terminal hexahistidine tagged protein in *E. coli* BL21 cells. The overexpressed recombinant protein was purified on a Ni-NTA affinity column (Invitrogen) according to the manufacturer’s protocol. *Lycopersicon esculentum* OPR3 and YqjM from *Bacillus subtilis* were overexpressed and purified as reported recently.\textsuperscript{[18,19]} The cloning, purification and characterisation of old yellow iso-enzymes from yeast (OYE1 from *Saccharomyces carlsbergensis*, OYE2 and OYE3 from *Saccharomyces cerevisiae*) and *Zymomonas mobilis* reductase (NCR) were performed according to literature.\textsuperscript{[10,20]} NEM-reductase (from *E. coli*), PETN-reductase (from *Enterobacter cloacae*) and morphinone-reductase (from *Pseudomonas putida* M10) were provided by N. C. Bruce (Department of Biology, University of York, York, UK).\textsuperscript{[21]}
General procedure for the enzymatic bioreduction under standard conditions
An aliquot of enzyme (OPR1, OPR3, YqjM, OYE1-3, NCR, NEM-reductase, morphinone reductase, PETN-reductase, protein concentration in biotransformations: 75 -125 µg/mL) was added to a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (10 mM) and the cofactor NADH (10 mM). The mixture was shaken at 30 °C and 120 rpm. After 24 h the products were extracted with EtOAc (2 x 0.5 mL). The combined organic phases were dried over Na₂SO₄ and analysed on achiral GC to determine the conversion and on chiral GC or HPLC, respectively, to determine the enantiomeric excess.

General procedure for cofactor recycling
An aliquot of enzyme (see above) was added to a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (10 mM) the oxidized form of the cofactor (NAD⁺, 100 µM), the cosubstrate (glucose, 20 mM) and the recycling enzyme (glucose dehydrogenase, 10 U). The mixture was shaken at 30 °C and 120 rpm for 24 h and worked up as described above.

General procedure for the enzymatic bioreduction using organic cosolvents
An organic co-solvent (EtOH, i-Pr₂O, tert-BuOMe, ethyl acetate and n-hexane) was employed in a ratio of 20% (v:v). The substrate (10 mM) was dissolved in the organic solvent (200 µL, for the cosolvent concentration study in 50 - 250 µL of t-BuOMe) and added to a Tris-HCl buffer solution (0.75 - 0.95 mL, 50 mM, pH 7.5) containing either the cofactor NADH (10 mM) or the cofactor recycling system (see above) followed by the addition of an aliquot of enzyme (see above). The mixture was shaken at 30 °C and 120 rpm for 24 h and worked up as described above.

Determination of the absolute configuration
The absolute configuration of the products 1b-3b was determined by comparison of its optical rotation value ([α]D20) with literature data. Enantioenriched material for 1b-3b was obtained by repeating the bioreduction of 1a-3a 40 times in parallel experiments in presence of cofactor recycling. The combined aqueous phases were extracted, dried (Na₂SO₄) and evaporated. Compound 1b was purified by flash chromatography (petroleum ether / ethyl acetate 20:1), 2b and 3b were directly used for optical rotation measurements.
The absolute configuration of \(3b\) was independently double-checked by comparison of the optical rotation value of \(3c\) (2-methyl-3-phenylpropan-1-ol), which was obtained by chemical reduction of \(3b\) (derived via bioreduction of \(3a\) using OYE2) using NaBH\(_4\).

### Table 2. Optical rotation values of products.

<table>
<thead>
<tr>
<th>Compound</th>
<th>([\alpha]_D^{20})</th>
<th>Conditions(^a)</th>
<th>E.e. [%]</th>
<th>Config.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1b)</td>
<td>+4.5</td>
<td>c = 1.8, CHCl(_3)</td>
<td>83</td>
<td>(S)</td>
<td>this study</td>
</tr>
<tr>
<td>(1b)</td>
<td>-5.2</td>
<td>c = 1, CHCl(_3)</td>
<td>95</td>
<td>(R)</td>
<td>ref.(^{[22]})</td>
</tr>
<tr>
<td>(2b)</td>
<td>-2.9</td>
<td>c = 2.2, CHCl(_3)</td>
<td>95</td>
<td>(S)</td>
<td>this study</td>
</tr>
<tr>
<td>(2b)</td>
<td>-2.8</td>
<td>c = 1.07, CHCl(_3)</td>
<td>≥90</td>
<td>(S)</td>
<td>ref.(^{[13]})</td>
</tr>
<tr>
<td>(3b)</td>
<td>-4.1</td>
<td>c = 0.5, MeOH</td>
<td>96</td>
<td>(S)</td>
<td>this study</td>
</tr>
<tr>
<td>(3b)</td>
<td>+7.0</td>
<td>c = 0.7, MeOH</td>
<td>76</td>
<td>(R)</td>
<td>ref.(^{[23]})</td>
</tr>
<tr>
<td>(3b)</td>
<td>-4.42</td>
<td>c = 4, MeOH</td>
<td>94</td>
<td>(S)</td>
<td>ref.(^{[24]})</td>
</tr>
<tr>
<td>(3c)</td>
<td>-11.3</td>
<td>c = 1.0, CHCl(_3)</td>
<td>96</td>
<td>(S)</td>
<td>this study</td>
</tr>
<tr>
<td>(3c)</td>
<td>-14.0</td>
<td>c = 0.25, CHCl(_3)</td>
<td>87</td>
<td>(S)</td>
<td>ref.(^{[25]})</td>
</tr>
</tbody>
</table>

\(^a\) Concentration [g/100 mL]; \(^b\) obtained by using OYE2; \(^c\) 2-methyl-3-phenyl 1-propanol.

### Docking and minimization

A molecular model of \(3a\) was docked into the structures of OYE1 from *Saccharomyces cerevisiae* and of NCR from *Zymomonas mobilis* using AutoDock 4.0\(^{[26]}\) as implemented in YASARA Structure.\(^{[27,28]}\) Protein coordinates for OYE1\(^{[29]}\) were taken from the PDB (entry code: 1OYB), while the structure of NCR was kindly provided by Wolfgang Hoeffken (BASF SE, unpublished results). The flavin cofactor was modelled as reduced FMNH\(^-\) in both structures, protonation and tautomerisation states of His residues were chosen according to hydrogen bonding networks. Asp, Glu, Arg, and Lys residues were treated as charged. A
model of the substrate was built and optimized within YASARA, and AM1-BCC partial charges\textsuperscript{[30]} were applied. The position and orientation of the ligand as well as one torsion angle were allowed to vary while the protein was kept rigid. The search was restricted by a 15 Å\textsuperscript{3} box around the N5 atom of the cofactor. Twenty independent simulations were performed employing a genetic algorithm (population size 150, number of generations 20000). The lowest energy structures of each independent run were clustered with an r.m.s-tolerance of 2.0 Å. The docking mode with the overall lowest energy was subjected to an additional molecular mechanics optimization in YASARA. Rigid docking failed to yield meaningful results in the case of NCR. Thus, the final structure of the complex of 3a and OYE1 was used to build a model of the corresponding complex with NCR.
References and Notes


[14] PETN-reductase gave (R)-3b with e.e.\textsubscript{max} 14%.


Electronic Supporting Information

Experimental

General

TLC plates were run on silica gel Merck 60 (F_{254}) and compounds were visualized by spraying with Mo-reagent [((NH₄)₆Mo₇O₂₄·4H₂O (100 g/L), Ce(SO₄)₂·4H₂O (4 g/L) in H₂SO₄ (10%)] or by UV (254 nm). Conversion and enantiomeric excess were determined via GC or HPLC analysis, respectively. GC analysis was carried out on a Varian 3800 gas chromatograph equipped with a FID detector using H₂ as carrier gas (14.5 psi), using an achiral stationary phase [for the determination of conversion (Varian CP-1301, 6 % cyanopropyl-phenyl phase capillary column, 30 m, 0.25 mm, 0.25 μm), column A] or a chiral stationary phase [for the determination of the enantiomeric excess (Hydrodex-β-6TBDM, modified β-cyclodextrin capillary column, 25 m x 0.25 mm), column B]. Temperature of the injector and detector were 180 and 250 °C, respectively, using a split ratio of 20:1. Chiral HPLC analyses were carried out on a Shimadzu system equipped with a Chiralcel OD-H column (column C, 0.46 x 25 cm) or a Chiralcel OJ column (column D, 0.46 x 25 cm) for the determination of the enantiomeric excess. NMR spectra were measured in CDCl₃ using a Bruker AMX spectrometer at 360 (¹H) and 90 (¹³C) MHz. Chemical shifts are reported relative to TMS (δ 0.00) and coupling constants (J) are given in Hz. Optical rotation values ([α]D) were measured on a Perkin-Elmer polarimeter 341 at 589 nm (Na-line) in a 1 dm cuvette and are given in units of [(deg x mL)/(g x dm)].

(E)-3-(4-tert-Butylphenyl)-2-methylpropenal (1a): ¹H (360 MHz, CDCl₃) δ = 1.37 (s, 9H), 2.11 (s, 3H), 7.27 (s, 1H) 7.48-7.53 (m, 4H), 9.59 (s, CHO); ¹³C (90 MHz, CDCl₃) δ = 10.96, 31.15, 34.89, 125.72, 130.07, 149.96, 195.70.

(E)-3-(1,3-Benzodioxole-5-yl)-2-methylpropenal (2a) and (E)-α-methylcinnamaldehyde (3a) were provided by BASF (Ludwigshafen), NADH and NAD⁺ were purchased from Biocatalytix/Codexis, glucose was obtained from Fluka and glucose dehydrogenase from Jülich Chiral Solutions.

(E)-3-(4-tert-Butylphenyl)-2-methylpropenal (1a): ¹H (360 MHz, CDCl₃) δ = 1.37 (s, 9H), 2.11 (s, 3H), 7.27 (s, 1H) 7.48-7.53 (m, 4H), 9.59 (s, CHO); ¹³C (90 MHz, CDCl₃) δ = 10.96, 31.15, 34.89, 125.72, 130.07, 149.96, 195.70.

(E)-3-(1,3-Benzodioxole-5-yl)-2-methylpropenal (2a): ¹H (360 MHz, CDCl₃) δ = 2.07 (s, 3H), 6.05 (s, 2H), 6.9-7.08 (m, 3H) 7.16 (s, 1H), 9.53 (s, 1H); ¹³C (90 MHz, CDCl₃) δ = 10.95, 101.62, 108.65, 109.62, 125.81, 129.44, 136.58, 148.09, 148.88, 149.75, 195.40.
**General procedure for the synthesis of reference materials via catalytic hydrogenation**

*(E)-Alkene (1a-3a, 0.5 mmol)* was dissolved in THF (10 mL) and hydrogenated under H$_2$ at atmospheric pressure and at room temperature employing Pd/C (10%, 5 mg) as catalyst. After the mixture was stirred overnight at room temperature, the reaction mixture was filtered through Celite and concentrated to yield racemic reference materials (*rac*-1b-3b) at 99% conversion. Thus were obtained:

*rac*-3-(4-tert-Butylphenyl)-2-methylpropanal (*Lysmeral*™, *Lilial*™, *rac*-1b): $^1$H (360 MHz, CDCl$_3$) $\delta$ = 1.11-1.12 (d, 3H, $J$ = 6.8Hz), 1.33 (s, 9H), 2.59-2.63 (m, 2H), 3.05-3.09 (m, 1H), 7.11-7.13 (d, 2H, $J$ = 8.2), 7.32-7.35 (d, 2H, $J$ = 8.2), 9.74-9.75 (d, CHO, $J$ = 1.4); $^{13}$C (90 MHz, CDCl$_3$) $\delta$ = 13.30, 31.37, 34.39, 36.16, 48.02, 125.41, 128.67, 204.62.

*rac*-3-(1,3-Benzodioxole-5-yl)-2-methylpropanal (*Tropional*™, *Helional*™, *rac*-2b): $^1$H (360 MHz, CDCl$_3$) $\delta$ = 1.09-1.11 (d, 3H, $J$ = 6.8), 2.52-2.66 (m, 2H), 2.99-3.04 (m, 1H), 5.95 (s, 2H), 6.62-6.76 (m, 3H) 9.72 (s, CHO); $^{13}$C (90 MHz, CDCl$_3$) $\delta$ = 13.18, 36.40, 48.22, 100.91, 108.25, 109.30, 121.94, 132.50, 146.11, 147.73, 204.41.

*rac*-2-Methyl-3-phenylpropanal (*rac*-3b): $^1$H (360 MHz, CDCl$_3$) $\delta$ = 1.10-1,12 (d, 3H, $J$ = 6.8Hz), 2.59-2.73 (m, 2H), 3.09-3.14 (m, 1H), 7.18-7.34 (m, 5H), 9.74-9.75 (d, CHO, $J$ = 1.3Hz); $^{13}$C (90 MHz, CDCl$_3$) $\delta$ = 13.21, 36.65, 48.04, 126.42, 128.53, 129.02, 138.83, 204.39.

Reference material for *rac*-2-methyl-3-phenylpropan-1-ol (*rac*-3c)$^{[1]}$ was obtained by NaBH$_4$-reduction (1.5 equiv.) of *rac*-3b in MeOH at r.t. for 2h: $^1$H (360 MHz, CDCl$_3$) $\delta$ = 0.95 (d, 3H, $J$ = 6.7), 1.96-1.98 (m, 1H), 2.45 (dd, 1H, $J$ = 6 and 13), 2.79 (dd, 1H, $J$ = 5 and 13), 3.46-3.58 (m, 2H), 7.20-7.30 (m, 5H, ArH); $^{13}$C (90 MHz, CDCl$_3$) $\delta$ = 16.48, 37.76, 39.72, 67.52, 125.90, 128.28, 129.17, 140.66.
**Determination of conversion and enantiomeric excess.**

The conversion and enantiomeric excess were determined via GC or HPLC analysis, respectively.

**Table 3.** Determination of conversion via achiral GC-analyses.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Column</th>
<th>Conditions</th>
<th>$t_R$ [min]</th>
<th>(1a-3a)</th>
<th>(1b-3b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>E</td>
<td>8.23</td>
<td>5.45</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>E</td>
<td>8.99</td>
<td>5.78</td>
<td></td>
</tr>
<tr>
<td>3(\textsuperscript{[2]})</td>
<td>A</td>
<td>E</td>
<td>3.80</td>
<td>3.10</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Column: A = Varian CP-1301, 6 % cyanopropyl-phenyl phase capillary column;

\(b\) conditions: E = 14.5 psi \(H_2\) at 180 °C, hold for 11 min.

**Table 4.** Determination of enantiomeric excess via chiral GC- and HPLC-analyses.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Column</th>
<th>Conditions</th>
<th>(t_R) [min]</th>
<th>((R))</th>
<th>((S))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>C</td>
<td>G</td>
<td>29.45</td>
<td>29.73</td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>D</td>
<td>H</td>
<td>12.5</td>
<td>13.5</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>B</td>
<td>F</td>
<td>11.8</td>
<td>12.7</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Column: B = Chiralcel OJ column (HPLC); C = Hydrodex-\(\beta\)-6TBDM, modified \(\beta\)-cyclodextrin capillary column (GC); D = Chiralcel OD-H column (HPLC);

\(b\) conditions: F = \(n\)-heptane/\(i\)-propanol 99:1 (isocratic) at 18 °C, flow 1 mL/min, \(\varepsilon\) = 190 nm, 205 nm, 215 nm; G = 14.5 psi \(H_2\) at 130 °C, hold for 0 min, heat rate 1 °C/min to 165 °C, heat rate 20 °C/min to 180 °C, hold for 7 min; H = \(n\)-heptane/\(i\)-propanol 98:2 (isocratic) at 18 °C, 0-15 min: flow 1 mL/min, 15-20 min: flow 1.5 mL/min, \(\varepsilon\) = 205 nm, 235 nm, 285 nm.
References and Notes

Asymmetric Synthesis of
(R)-3-Hydroxy-2-methylpropanoate (‘Roche Ester’) and Derivatives via Biocatalytic C=C-Bond Reduction

(Advanced Synthesis and Catalysis 2010; submitted)

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Department of Chemistry, Organic and Bioorganic Chemistry, University of Graz, Heinrichstrasse 28, 8010-Graz, Austria;

R = H, allyl, benzyl
c >99%, e.e. up to >99%

*Corresponding author: phone +43-316-380-5332; fax: +43-316-380-9840; <Kurt.Faber@Uni-Graz.at>
Abstract

Enoate reductases from the ‘Old Yellow Enzyme’ family were employed for the asymmetric bioreduction of methyl 2-hydroxymethylacrylate and its O-allyl, O-benzyl- and O-TBDMS-derivatives to furnish (R)-configured methyl 3-hydroxy-2-methylpropionate products in up to >99% e.e. Variation of the O-protective group had little influence on the stereoselectivity, but a major impact on the reaction rate.

Introduction

The asymmetric reduction of C=C bonds creates (up to) two chiral carbon centres and is thus one of the most widely employed strategies for the production of chiral materials. The biocatalytic variant, which is applicable to activated alkenes bearing an electron-withdrawing substituent is catalysed by enoate reductases [EC 1.3.1.X],[1,2] which are members of the ‘Old Yellow Enzyme’ (OYE) family.[3] Over the past few years, increasing attention has been devoted to these flavo-proteins[4] in view of their substrate scope,[5] encompassing α,β-unsaturated carbonyl compounds (such as enals and enones), as well as carboxylic acids and derivatives thereof (such as esters, cyclic imides, nitriles, lactones) and nitroalkenes. As a rule of thumb, the degree of activation of the C=C-bond exerted by the electron-withdrawing effect of the activating substituent goes in hand with the substrate acceptance, which ensures generally fast reaction rates for enals, enones and nitroalkenes, whereas (di)carboxylic acids and esters are transformed more slowly.

To illustrate the importance of this enzyme class for asymmetric synthesis, we aimed at their applicability for an industrially relevant product, i.e. (R)-3-hydroxy-2-methylpropanoate, which is commonly denoted as the ‘Roche-ester’. The latter is a popular chiral building block for the synthesis of vitamins (e.g. α-tocopherol[6]), fragrance components (e.g. muscone[7]), and antibiotics (e.g. calcimycin,[8] palinurin,[9] rapamycin,[10] 13-deoxytedanolide,[11] dictyostatin[12]) and natural products (e.g. spiculoic acid A,[13]). Classical methods for its preparation include the diastereoselective addition of nonracemic alcohols as chiral auxiliaries,[14] the transformation of a chiral homoallylic acetate[15] or involve aldol condensation[16] and — most prominent — the transition-metal catalysed asymmetric hydrogenation of acrylate esters using Rh[17] (e.e. up to 99%) or Ru[18] (e.e. up to 94%). For the biocatalytic synthesis of the ‘Roche ester’ only few examples are reported: the stereoselective oxidation of 2-methyl-1,3-propanediol by Gluconobacter and Acetobacter
spp.\textsuperscript{[19]} (e.e. up to 97\%), the asymmetric reduction of ethyl 4,4-dimethoxy-3-methylcrotonate using baker's yeast\textsuperscript{[20]} and the stereoseletive (formal) β-hydroxylation of isobutyric acid using 	extit{Pseudomonas putida} (ATCC 21244).\textsuperscript{[21]} All of these biotransformations were performed using whole (fermenting) microbial cells with several enzymes being involved. Only recently, it was shown that a non-flavin NADH-dependent Δ\textsuperscript{4,5}-steroid-5β-reductase from 	extit{Arabidopsis thaliana} was able to reduce ethyl 2-hydroxymethylacrylate, however, the stereochemistry of the product was not examined in view of its absolute configuration and enantiomeric composition.\textsuperscript{[22]}

Herein we report on the synthesis of the 'Roche-ester' via biocatalytic reduction of methyl 2-hydroxymethylacrylate using eleven OYE's. In order to test the influence of the overall substrate structure regarding its size and polarity on the relative rate and/or stereoselectivity, various protective groups on the hydroxy moiety were investigated.

\textbf{Results and Discussion}

Old yellow enzymes OYE1-3 from \textit{Saccharomyces} sp.,\textsuperscript{[23]} 12-oxophytodienoic acid reductase isoenzymes OPR1 and OPR3 from \textit{Lycopersicon esculentum},\textsuperscript{[24]} YqiM from \textit{Bacillus subtilis},\textsuperscript{[25]} nicotinamide-dependent cyclohexenone reductase (NCR) from \textit{Zymomonas mobilis},\textsuperscript{[26]} xenobiotic reductases A And B (XenA, XenB) from \textit{Pseudomonas putida},\textsuperscript{[27]} glycerol trinitrate reductase (NerA) from \textit{Agrobacterium radiobacter}\textsuperscript{[28]} and estrogen-binding protein (EBP1) from \textit{Candida albicans}\textsuperscript{[29]} were tested for their ability to reduce four methyl 2-hydroxymethacrylate derivatives (Scheme 1, Table 1).

![Scheme 1. Asymmetric bioreduction of methyl 2-hydroxymethylacrylate derivatives 1a-4a.](image-url)
Table 1. Conversion and enantiomeric excess of bioreduction products 1b–4b.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Enzyme</th>
<th>Cofactor(^{[a]})</th>
<th>1b</th>
<th>2b</th>
<th>3b</th>
<th>4b</th>
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<tr>
<td></td>
<td></td>
<td>c [%]</td>
<td>e.e. [%]</td>
<td>c [%]</td>
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<td>OYE1</td>
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<td>14</td>
<td>&gt;99 (R)</td>
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<tr>
<td>2</td>
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<td>&gt;99 (R)</td>
<td>5</td>
<td>&gt;99 (R)</td>
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<td>&gt;99 (R)</td>
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<td>&gt;99 (R)</td>
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<td>NADH</td>
<td>14</td>
<td>98 (R)</td>
<td>26</td>
<td>&gt;99 (R)</td>
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<td>YqjM</td>
<td>NAD(^+[b])</td>
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<td>94 (R)</td>
<td>69</td>
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<td>8</td>
<td>NCR</td>
<td>NADH</td>
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<td>n.d.</td>
<td>34</td>
<td>&gt;99 (R)</td>
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<td>NAD(^+[b])</td>
<td>n.d.</td>
<td>n.d.</td>
<td>22</td>
<td>&gt;99 (R)</td>
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<td>n.d.</td>
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<td>&gt;99 (R)</td>
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<tr>
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<td>&gt;99 (R)</td>
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<tr>
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<td>NADH</td>
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<td>63 (R)</td>
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<tr>
<td>15</td>
<td></td>
<td>NAD(^+[b])</td>
<td>28</td>
<td>60 (R)</td>
<td>97</td>
<td>&gt;99 (R)</td>
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</table>

\(^{[a]}\) Standard conditions: substrate 1a-4a (10 mM), NADH (15 mM), Tris-HCl-buffer 50 mM, pH 7.5, 30°C, 24h; \(^{[b]}\) NAD\(^+\) (100 µM)/glucose dehydrogenase (10U)/glucose (20mM); c = conversion; n.d. = not determined.
The unprotected Roche ester precursor methyl 2-hydroxymethylacrylate (1a) was reduced to (R)-methyl 3-hydroxy-2-methylpropionate (1b) by four of the eleven enoate reductases tested (entries 1-7) with perfect stereoselectivity (e.e. up to >99%), only XenA showed limited stereoselectivities (e.e. max 63%). Despite these encouraging selectivities, insufficient reaction rates led to incomplete conversion of this substrate (c_max 37%). Since the prim-allylic alcohol group of 1a is considerably hydrated in aqueous solution, the corresponding allyl-, benzyl and TBDMS-ethers we tested as more lipophilic substrate analogues.

Overall, the masking of the hydroxy-group of the initial substrate by easily removable allyl- and benzyl-ether groups enhanced the substrate acceptance drastically. The O-allyl-analog 2a and O-benzyl ether 3a were converted by OyEs1-3 and YqiM with approximately three-fold enhanced rates (c_max 90%, entry 5). In the case of NCR, NerA and OPR1 this substrate-modification even turned the inactive substrate 1a into a suitable target molecule for these enzymes (c_max 89%, entry 12), and the conversion of 2b using XenA was almost quantitative (entry 15). In addition to improved reaction rates, the incomplete stereoselectivities of YqiM and XenA were corrected from e.e.s 94% and 60% to >99%, respectively. However, the stereopreference remained constant for all modified substrates by yielding (R)-2b and (R)-3b.

Since the stereopreference of enoate reductases sometimes may be controlled by the substrate shape, such as (E/Z)-configuration of the C=C-bond,[4a,5c,5e] we attempted to obtain the (S)-‘Roche-ester’ by increasing the steric bulkiness of the hydroxy protective group even further in order to enforce a flipped substrate-orientation within the active site of the enzyme. Unfortunately, this attempt for selectivity-control failed, as the bulky tert-butyldimethylsilyloxy derivative 4a invariably furnished (R)-4b in >99% e.e., going in hand with a negative effect on the reaction rates: The activities dropped significantly (for YqiM, NCR and OPR1) or were completely erased (for OYE1-3 and NerA), only XenA was able to reduce 4a quantitatively with absolute selectivity for the (R)-enantiomer (entry 15). None of the substrates could be reduced with sufficient rates using OPR3, XenB and EBP1 (c <2%).

Conclusions

In conclusion, an efficient method for the preparation of (R)-methyl 3-hydroxy-2-methylpropionate [(R)-Roche ester’] and related O-protected derivatives was developed via bioreduction of the corresponding acrylate precursors was developed using enoate reductases from the ‘Old Yellow Enzyme’ family. In order to ensure full conversion, the allylic hydroxy-
moiety had to be protected with an allyl- or benzyl-ether group to render a more lipophilic substrate analog.

**Experimental Section**

**Source of enzymes**
OPR1 and OPR3 from *Lycopersicon esculentum* and YqjM from *Bacillus subtilis* were overexpressed and purified as reported recently.[5a,24,25] The cloning, purification and characterisation of OYE3 from *Bacillus subtilis,* OYE2 and OYE3 from *Saccharomyces carlsbergensis,* OYE2 and OYE3 from *Saccharomyces cerevisiae* and nicotinamide-dependent cyclohexenone reductase (NCR) from *Zymomonas mobilis* was performed according to literature.[26,30] Xenobiotic reductases A (XenA) and B (XenB) from *Pseudomonas putida,* glycerol trinitrate reductase NerA from *Agrobacterium radiobacter* and estrogen-binding protein (EBP1) from *Candida albicans* were obtained as recently published.[31]

**General procedure for the bioreduction of 1a-4a:**
An aliquot of enzyme (OYE1-3, OPR1, OPR3, YqjM, NCR, XenA, XenB, Ner, and EBP1, protein concentration 75-125 µg/mL) was added to a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (10 mM) and the cofactor NADH (15 mM). The mixture was shaken at 30 °C and 120 rpm. After 24 h the products were extracted with EtOAc (2 x 0.5 mL). The combined organic phases were dried over Na₂SO₄ and analysed on achiral GC to determine the conversion and on chiral GC or HPLC, respectively, to determine the enantiomeric excess. For cofactor recycling, the oxidized form of the cofactor (NAD⁺, 100 µM), the cosubstrate (glucose 20 mM) and the recycling enzyme (glucose dehydrogenase, 10 U) were used.

**Determination of absolute configuration:**
The absolute configuration of 1b, 2b and 4b was determined by co-injection with reference material of known absolute configuration. The absolute configuration of 3b was determined by comparison with a published chiral HPLC analysis (Chiralcel OD-H 0.46 x 25 cm, 98:2 heptane:2-propanol mobile phase at 1.5 mL/min flow. The (R)-enantiomer eluted at 3.88 min, the (S)-enantiomer eluted at 4.17 min.).[32]
Electronic Supporting Information:

General information on commercially obtained compounds and materials, the synthesis of substrates 1a-4a, the synthesis of reference material for rac-1b, rac-3b and rac-4b, and analytical methods for the determination of conversion and enantiomeric excess are described in the electronic supporting information.
References and Notes


Electronic Supporting Information

Experimental Section

General:
TLC plates were run on silica gel Merck 60 (F254) and compounds were visualized by spraying with Mo-reagent \([(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot4\text{H}_2\text{O} (100 \ g/L), \text{Ce(SO}_4)_2\cdot4\text{H}_2\text{O} (4 \ g/L) \text{ in } \text{H}_2\text{SO}_4 (10\%)]\) or by UV (254 nm). Silica gel 60 from Merck was used for flash chromatography. GC analyses were carried out with a Varian 3800 equipped with FID using \(\text{H}_2\) as a carrier gas (14.5 psi). Chiral HPLC analyses were carried out on a Shimadzu system equipped with a Chiralcel OJ column (column D, 0.46 x 25 cm) or a Chiralcel OD-H column (column C, 0.46 x 25 cm) for the determination of the enantiomeric excess. NMR spectra were measured on a Bruker Avance III 300 MHz NMR spectrometer. \((R)-\text{nd}(S)\)-Methyl 3-hydroxy-2-methylpropionate \([(R)-1b, (S)-1b]\) were purchased from Aldrich.

Synthesis of substrates and reference materials

**Methyl 2-hydroxymethylacrylate (1a):**\(^{[1b]}\) Methyl acrylate (34.8 mmol, 3.14 mL), formaldehyde solution (37\%, 2.17 mL) and DABCO (0.65 equiv.) were added to 100 mL of a 1,4-dioxane/H\(_2\)O (1:1) mixture. The reaction was stirred at room temperature for 24h. The mixture was neutralized with HCl and extracted with EtOAc (2 x 100 mL). The combined organic phases were washed with brine, dried over Na\(_2\)SO\(_4\) and evaporated. The product was purified via silica gel chromatography (petroleum ether/EtOAc 2:1) yielding 11% 1a (452 mg, 3.9 mmol) as colourless oil. \(^1\text{H}-\text{NMR (CDCl}_3, 300 \text{ MHz)}: \delta = 2.36 (\text{OH, br s}); 3.80 (3\text{H, s}); 4.34 (2\text{H, s}); 5.85 (1\text{H, s}); 6.27 (1\text{H, s}). \(^{13}\text{C}-\text{NMR (CDCl}_3, 75 \text{ MHz)}: \delta = 51.9, 62.5, 125.9, 139.3, 166.8.**

**Methyl 2-(allyloxymethyl)acrylate (2a):** (stabilized with approx. 1\% phenothiazine):\(^{[2]}\) To astirred solution of allyl alcohol (17.22 mmol, 1.0 g) in methylene chloride (17.2 mL) at room temperature, triethylamine (17.22 mmol, 1.7 g) and methyl-2-(bromomethyl)acrylate (16.4 mmol, 2.9 g) were added dropwise in an exothermic reaction while the temperature was maintained between 24–34 °C. After 6 days of stirring under reflux (further methylene chloride was added), the reaction mixture was washed with 10 mL of dist. H\(_2\)O. The organic and aqueous phase were separated, the organic layer was washed with further 10 mL of 1M HCl, dried over MgSO\(_4\), and the solvent was removed under reduced pressure. Substrat 2a
(0.65 g, 4.2 mmol) was obtained as a colourless liquid in 24% yield. No further purification was necessary (>95%). To avoid polymerization, 1% of phenothiazine was added as a stabilizer. $^1$H-NMR (CDCl$_3$, 500 MHz): $\delta$ = 3.77 (3H, s); 4.05 (2H, d, $J$ = 5.4 Hz, allyl-CH$_2$), 4.20 (2H, br s, CH$_2$); 5.26 (2H, dd, $J_1$ = 33.3, $J_2$ = 10.5 Hz, allyl-=CH$_2$); 5.90 (1H, br s, =CH); 5.88-5.99 (1H, m, allyl-=C-H), 6.31 (1H, br s, =CH). $^{13}$C-NMR (CDCl$_3$, 125 MHz): $\delta$ = 51.8, 68.3, 71.7, 117.1, 125.8, 134.6, 137.3, 166.3.

**Methyl 2-(benzyloxymethyl)acrylate (3a)** (stabilized with approx. 1% phenothiazine). To a stirred solution of 1a (43 mmol, 5 g) in 1,2-dimethoxyethane (86 mL) at room temperature were added benzyl alcohol (215 mmol, 23.3 g) and PdCl$_2$ (4.3 mmol, 0.76 g). The reaction mixture was stirred at 50 °C for 24 h. Then solids were filtered and the residue was purified by distillation (bp: 52–62 °C at 7.1 mbar). Substrate 3a (4.6 g, 22.3 mmol) was obtained as a colourless liquid in 52% yield and 80% chemical purity. To avoid polymerization, 1% of phenothiazine was added as a stabilizer. $^1$H-NMR (CDCl$_3$ and DMSO, 500 MHz): $\delta$ = 3.71 (3H, s, OCH$_3$); 4.20 (2H, s, PhCH$_2$); 4.54 (2H, s, OCH$_2$); 5.90 (1H, s, =CH); 6.28 (1H, s, =CH); 7.22-7.40 (5H, m, Ph). $^{13}$C-NMR (CDCl$_3$, 125 MHz): $\delta$ = 50.6, 67.2, 71.4, 124.7 126.5, 127.2, 136.1, 137.0, 164.9.

**Methyl 2-((tert-butyldimethylsilyloxymethyl)acrylate (4a).** To a stirred solution of 1a (43 mmol, 5 g) in toluene (110 mL) at room temperature were added triethylamine (52 mmol, 5.2 g) and DMAP (4.3 mmol, 0.53 g), followed by dropwise addition of a solution of tert-butyldimethylsilyl chloride (47 mmol, 14.3 g) in toluene (50% w/w) at 0 °C. The reaction mixture was allowed to reach room temperature and was stirred for 20h at ambient temperature. After addition of 40 mL MTBE, solids were filtered off and the solvent was removed under reduced pressure. The resulting crude product was taken up in 50 mL MTBE and extracted with 30 mL sat. NH$_4$Cl solution. The aqueous phase was washed two times with 50 mL MTBE. The combined organic layers were washed with 50 mL sat. NaHCO$_3$ solution and 50 mL sat. NaCl solution, dried over MgSO$_4$, filtered, and concentrated under reduced pressure. Product 4a (5.7 g, 24.7 mmol) was obtained as a slightly yellow, cloudy solution in 57% yield and was used without further purification (chemical purity determined by NMR >95%). $^1$H-NMR (CDCl$_3$, 500 MHz): $\delta$ = 0.08 (6H, s, SiMe$_2$); 0.91 (9H, s, tBuSi); 3.75 (3H, s, OCH$_3$); 4.36 (2H, s, OCH$_2$); 5.91 (1H, s, =CH); 6.25 (1H, s, =CH). $^{13}$C-NMR (CDCl$_3$, 75 MHz): $\delta$ = -5.5, 18.3, 25.9, 51.7, 61.5, 123.9, 139.6, 166.4.
Synthesis of reference material for rac-1b, rac-3b and rac-4b (method A): Compounds 1a-4a were hydrogenated over Pd/C (10%, 5mg) in EtOAc (10 mL) in a two-necked round bottom flask equipped with a septum. H₂ was provided via a balloon and the mixture was stirred under H₂ atmosphere overnight at room temperature. The mixture was filtered through Celite and evaporated to dryness. Thus were obtained:

rac-Methyl 3-hydroxy-2-methylpropionate (rac-1b): Methyl 2-hydroxymethylacrylate (1a) (103 mg, 0.89 mmol) was reduced according to method A yielding 97% rac-1b (102 mg, 0.86 mmol) as colourless oil. ¹H-NMR (CDCl₃, 300 MHz): δ = 1.18 (3H, d, J = 7.2 Hz), 2.46 (OH, br s), 2.65-2.71 (1H, m), 3.72 (3H, s). ¹³C-NMR (CDCl₃, 75 MHz): δ = 13.4, 41.7, 51.8, 64.5, 176.0.

rac-Methyl 3-allyloxy-2-methylpropionate (rac-2b) was obtained by mixing (R)- and (S)-enantiomers (see below).

rac-Methyl 3-benzyloxy-2-methylpropionate (rac-3b): Methyl 2-(benzyloxymethyl)acrylate (3a) (107 mg, 0.47 mmol), was reduced according to method A (reaction time 20 min) yielding 98% rac-3b (106 mg, 0.46 mmol) as colourless oil. ¹H-NMR (CDCl₃, 300 MHz): δ = 1.20 (3H, d, J = 7.2 Hz), 2.81 (1H, qt, J = 6.7Hz), 3.60 (2H, dd, J₁ = 40.2, J₂ = 6.0 Hz), 3.72 (3H, s), 4.54 (2H, s), 7.28-7.39 (5H, m). ¹³C-NMR (CDCl₃, 75 MHz): δ = 14.0, 40.2, 51.7, 51.7, 72.0, 73.1, 127.6, 128.4, 138.2, 175.3.

rac-Methyl 3-(tert-butyldimethylsilyloxy)methylpropionate (rac-4b): Methyl 2-(tert-butyldimethylsilyloxy)methylacrylate (4a) (99 mg, 0.43 mmol) was reduced according to method A yielding 98% rac-4b (97 mg, 0.42 mmol) as colourless oil. ¹H-NMR (CDCl₃, 300 MHz): δ = 0.05 (6H, s), 0.88 (9H, s), 1.15 (3H, d, J = 7.2 Hz), 2.66 (1H, qt, J = 6.9 Hz), 3.64-3.79 (2H, m), 3.69 (3H, s). ¹³C-NMR (CDCl₃, 75 MHz): δ = 5.5, 13.5, 18.2, 25.4, 42.5, 51.5, 65.2, 175.5.

(R)- and (S)-methyl 3-(allyloxy)methylpropionate [(R)-2b, (S)-2b]: In a round bottom flask wrapped with aluminium foil and under argon, (S)-methyl 3-hydroxy-2-methylpropionate [(S)-1b], 100 µL, 0.9 mmol) was added to a suspension of MgSO₄ (107 mg, 0.9 mmol) and Ag₂O (270 mg, 1.2 mmol) in petroleum ether (4 mL). The mixture system was stirred for 1 h at room temperature, then cooled with an ice bath and allyl bromide (117 µL, 1.35 mmol) was
added slowly. After 30 min a second portion of Ag₂O was added (270 mg, 1.2 mmol). The mixture was stirred at room temperature overnight, filtered through Celite, dried over Na₂SO₄, concentrated in vacuo and purified via silica gel chromatography (petroleum ether/EtOAc, 90:10) yielding 38% (S)-2b (54 mg, 0.34 mmol) as colourless oil.⁵ ¹H-NMR (CDCl₃, 300 MHz): δ = 1.19 (3H, d, J = 7.2 Hz), 2.77 (1H, qt, J = 6.7 Hz), 3.56 (2H, dd, J₁ = 40.8, J₂ = 6.0 Hz), 3.71 (3H, s), 3.99 (2H, d, J = 5.7 Hz), 5.20-5.31 (2H, m), 5.83-5.96 (1H, m). ¹³C-NMR (CDCl₃, 75 MHz): δ = 14.0, 40.2, 51.7, 71.9, 72.1, 117.0, 134.6, 175.4. The same procedure was used to prepare (R)-methyl 3-(allyloxymethyl)propionate [(R)-2b] from (R)-methyl 3-hydroxy-2-methylpropionate [(R)-1b] yielding 49% of (R)-2b (70 mg, 0.44 mmol) as colourless oil.

(S)-Methyl 3-(tert-butyldimethylsilyloxy)methyl)propionate [(S)-4b]: A mixture of (S)-methyl 3-hydroxy-2-methylpropionate (100 µL, 0.9 mmol), tert-butyldimethylsilyl chloride (96 mg, 1.3 mmol) and imidazole (170 mg, 2.5 mmol) in anhydrous DMF (0.5 mL) was stirred at room temperature under argon overnight. The reaction was quenched with hexane (30 mL), washed with brine (2x 10 mL), H₂O (20 mL), the organic phase was dried (Na₂SO₄), evaporated and the residue was purified by silica gel chromatography (petroleum ether/EtOAc, 90:10) yielding 52% [(S)-4b] (110 mg, 0.47 mmol) as colourless crystals.⁶

Analytical methods

Determination of conversion

Conversion of methyl 2-hydroxymethylacrylate (1a), methyl 2-(allyloxymethyl)acrylate (2a), methyl 2-(benzylxoymethyl)acrylate (3a) and methyl 2-(tert-butyldimethylsilyloxy)methyl)acrylate (4a) were analysed by GC using a 14% cyanopropyl-phenyl phase capillary column (J&W Scientific DB-1701, 30 m x 0.25 mm, 0.25 µm film), detector temperature 250 °C, split ratio 20:1. Temperature program: 40 °C, hold for 2 min, heat rate 20 °C/min to 180 °C, hold for 11 min. Retention times: 1b: 5.55 min; 1a: 6.08 min; 2b: 6.28 min; 2a: 6.66 min; 3b: 10.41 min; 3a: 11.11 min; 4b: 7.32 min 4a: 7.77 min.

Determination of enantiomeric excess

The enantiomeric excess of methyl 3-hydroxy-2-methylpropionate (1b) was determined by GC-FID using a β-cyclodextrin capillary column (CP-Chirasil-DEX CB, 25m x 0.32 mm, 0.25 µm film), detector temperature 200 °C, split ratio 20:1. Temperature program: 60 °C, hold for 1 min, heat rate 10 °C/min to 100 °C, hold for 10 min, then heat 15 °C/min to 160 °C.
Retention times: (R)-1b: 7.70 min; (S)-1b: 8.03 min; 1a: 8.50 min. The enantiomeric excess of methyl 3-allyloxy-2-methylpropionate (2b) and methyl 3-(tert-butyl dimethylsilyloxy)-2-methylpropionate (4b) was determined by GC-FID using a β-cyclodextrin capillary column (Hydrodex-β-6TBDM, 25 m x 0.25 mm), detector temperature 200 °C, split ratio 20:1. Temperature program for 2b: 40 °C hold for 10 min, heat rate 5 °C/min to 75 °C, hold for 3 min, heat rate 15 °C/min to 180 °C. Retention times: (S)-2b: 20.08 min; (R)-2b: 20.71 min; 2a: 22.57 min. Temperature program for 4b: 100 °C, hold for 2 min, heat rate 20 °C/min to 180 °C, hold for 6 min. Retention times: (S)-4b: 7.52 min; (R)-4b: 7.76 min; 4a: 10.94 min. The enantiomeric excess of methyl 3-benzyloxy-2-methylpropionate (3b) was determined by HPLC-analysis on a Chiracel-OJ column (25 x 0.46 cm) using n-heptane/i-PrOH 98:2 (isocratic, flow rate of 0.25 mL/min, 18 °C). Retention times: (S)-3b: 70.87 min; 3a: 74.04 min; (R)-3b: 94.39 min.
References and Notes


Stereo-Controlled Asymmetric Bioreduction of α,β-Dehydroamino Acid Derivatives

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Abstract

α,β-Dehydroamino acid derivatives were tested as a novel substrate class for the bioreduction using enoate reductases from the ‘Old Yellow Enzyme’ family. (S)-Alanine was formed in 97% e.e. and (R) or (S)-Asparagin could be obtained. By substrate engineering, i.e. via variation of the size of the N-acyl protective group, the stereochemical outcome of the bioreduction using OYE3 could be switched and furnished the corresponding D- or L-amino acid derivatives, resp.

Introduction

The asymmetric reduction of C=C bonds creates (up to) two chiral carbon centres and is thus one of the most widely employed strategies for the production of chiral materials. The biocatalytic variant, which is applicable to activated alkenes bearing an electron-withdrawing substituent is catalysed by enoate reductases [EC 1.3.1.X],[1,2] which are members of the ‘Old Yellow Enzyme’ (OYE) family.[3] Over the past few years, increasing attention has been devoted to these flavo-proteins[4] in view of their substrate scope,[5] encompassing α,β-unsaturated carbonyl compounds (such as enals and enones), as well as carboxylic acids and derivatives thereof (such as esters, cyclic imides, nitriles, lactones) and nitroalkenes.

We focused to apply this method on the production of optical pure amino acids by reduction of α,β-dehydroamino acid derivatives. While efficient metal catalysis[6] and artificial metalloenzymes[7] provide tools for the enantioselective hydrogenation of N-protected dehydroamino acids a direct biocatalytic route has not been reported till now. A two step synthesis via reduction of β-nitroacrylates by Saccharomyces carlsbergensis old yellow enzyme and a sequential chemical conversion to β²-amino acids has been investigated recently.[8]

Here we report the scopes and limitations of the reduction of α,β-dehydroamino acid derivatives as a new substrate class using enoate reductases OYE1-3 from Saccharomyces sp.,[9] OPR1 and OPR3 from Lycopersicon esculentum,[10] YqjM from Bacillus subtilis[11] and NCR Reductase from Zymomonas mobilis.[12] Furthermore the stereochemical control via variation of the N-protective group could be shown for the first time.
Results and Discussion:

Working in an aqueous buffer system the use of zwitter ions showed various drawbacks (e.g. extraction and analytical problems). Therefore the methyl ester derivatives of the free dehydroamino acids and an acetyl protective group for the amino moiety were used. The most simple dehydroamino acid derivatives methyl 2-acetamidoacrylate (1a) was selected as the first candidate and showed poor to moderate activity with good selectivity with five of the seven enoate reductases (OYE1-3, YqjM, OPR1). YqjM from Bacillus subtilis gave best conversion (41%) with excellent optical purity (e.e. 97%). All active enzymes yield the (S)-enantiomer of the alanine derivative (e.e. 85 to 97%).

Scheme 1. Asymmetric bioreduction of substrate 1a-3a

Substrate 2a bearing an extra methyl group on the Cβ is completely inactive towards reduction. The electron donor and acceptor effects on the C=C double bond do play a crucial role in the bio-catalytic reduction using enoate reductases.

To our surprise even the β-dehydroamino acid derivative (3a) showed no conversion with the tested enzymes. Thus we are limited to highly activated α-dehydroamino acid derivatives.
Scheme 2. Asymmetric bioreduction of substrate 4a-9a

Table 1. Conversion and enantiomeric excess of bioreduction products 1b–9b

<table>
<thead>
<tr>
<th>Product</th>
<th>OYE1</th>
<th>OYE2</th>
<th>OYE3</th>
<th>YqjM</th>
<th>OPR1</th>
<th>OPR3</th>
<th>NCR</th>
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<td></td>
<td>c.</td>
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<tr>
<td>1b</td>
<td>30</td>
<td>(S) 95</td>
<td>6</td>
<td>(S) 85</td>
<td>15</td>
<td>(S) 95</td>
<td>41</td>
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<tr>
<td>2b</td>
<td>no conversion</td>
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<td></td>
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<tr>
<td>3b</td>
<td>no conversion</td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>4b</td>
<td>&gt;99 (S) 99</td>
<td>89</td>
<td>(S) &gt;99</td>
<td>&gt;99 (S) 88</td>
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</tr>
<tr>
<td>5b</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>n.c.</td>
<td>n.d.</td>
<td>36</td>
<td>(S) 23</td>
<td>26</td>
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<tr>
<td>6b</td>
<td>7</td>
<td>n.d.</td>
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<td>&gt;99</td>
<td>(R) 61</td>
<td>&gt;99</td>
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<td>7b</td>
<td>24</td>
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<td>29</td>
<td>(S) 82</td>
<td>98</td>
<td>(R) 68</td>
<td>95</td>
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<tr>
<td>8b</td>
<td>34 (S) &gt;99</td>
<td>35</td>
<td>(S) &gt;99</td>
<td>47</td>
<td>(S) &gt;99</td>
<td>12</td>
<td>(S) &gt;99</td>
</tr>
<tr>
<td>9b</td>
<td>&lt;5 n.d.</td>
<td>&lt;5</td>
<td>n.d.</td>
<td>41</td>
<td>(R) 92</td>
<td>&lt;5</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Standard conditions: substrate 1a-9a (10 mM), NADH (15 mM), Tris-HCl-buffer 50 mM, pH 7.5, 30°C, 24h; c = conversion; n.d. = not determined.

One of the highest activated systems is the asparagin precursor. Leaving the N-protective group the same and having the second ester group on beta position (5a) showed moderate conversion (19%-36%) with three of the enzymes. While YqjM and OPR1 showed perfect stereoselectivity [e.e. >99% (S)] OYE3 was unselective with a slight preference to the (S)-enantiomer.
In order to stress the steric demand of the substrate a variation of protective groups at the amino moiety was introduced (abb. 2). To our surprise, an “Old Yellow Enzyme” isoenzyme (OYE3) showed a remarkable stereochemical behaviour with respect to the size of the N-acyl protective group. By variation of the size of the N-acyl protective group, the stereochemical outcome using OYE3 could be switched: While small formyl- (4a) and acetyl- (5a) groups gave the (S)-product in up to 88% e.e., bulky N-propionyl- (6a), N-butanoyl- (7a) and N-phenylacetyl- (9a) moieties furnished the (R)-enantiomer in up to 92% e.e. Much to our surprise the N-benzoyl- (8a) moiety furnished the (S)-enantiomer despite its steric demand. Thus, stereocontrol in the asymmetric bioreduction of activated C=C bonds via substrate engineering was successfully proven for the first time. In addition to the variation of the size of the protective group, the stereochemical outcome could also be controlled by the choice of enzyme. Whereas OYE1-2, OPR1, OPR3, YqjM and NCR yielded the (S)-amino acid product in high conversions and excellent e.e. (YqjM gave the best conversion and e.e. in case of the N-propionyl- (6a) and N-butanoyl- (7a), OPR1 in case of the N-phenyl-acetyl (9a) moieties), OYE3 gave the (R)-enantiomer in up to 92% e.e.

Conclusion

Optical enriched alanine derivative and asparagine derivatives could be obtained by biocatalytic reduction of their α-dehydroamino acid precursors. β-Dehydroamino acid were not accepted as target molecules. A switch in selectivity of the asparagine product could be forced by variation of the size of the N-acyl protective group.

Experimental

General:
TLC plates were run on silica gel Merck 60 (F254) and compounds were visualized by spraying with Mo-reagent [(NH₄)₆Mo₇O₂₄·4H₂O (100 g/L), Ce(SO₄)₂·4H₂O (4 g/L) in H₂SO₄ (10%)] or by UV (254 nm). Silica gel 60 from Merck was used for flash chromatography. GC-MS analyses were performed on a HP 6890 Series GC system equipped with a 5973 mass selective detector and a 7683 Series injector using a (5%-phenyl)-methylpolysiloxane capillary column (HP-5MS, 30 m, 0.25 mm, 0.25 µm). GC-FID analyses were carried out on a Varian 3800 using H₂ as carrier gas (14.5 psi). NMR were done on a Bruker Avance III 300
MHz NMR spectrometer. Chemical shifts are reported relative to TMS ($\delta = 0.00$) and coupling constants ($J$) are given in Hz. Methyl 2-acetamidoacrylate (1a), methyl 2-acetamidobut-2-enoate (2a), (Z)-methyl 3-acetamido-2-methylacrylate (3a), (Z)-dimethyl 2-formamidofumarate (4a), (Z)-dimethyl 2-acetamidofumarate (5a), (Z)-dimethyl 2-propionamidofumarate (6a) and (Z)-dimethyl 2-benzamidofumarate (8a) were provided by BASF-Ludwigshafen. Ethoxy formyl chloride, N-aminopyridinium iodide, dimethyl fumarate, silicic acid, L-aspartic dimethylester hydrochloride, formyl chloride, acetyl chloride, propionyl chloride, butanoyl chloride, benzyl chloride and phenylacetyl chloride were purchased from Aldrich.

Source of enzymes:
12-Oxophytodienoic acid reductase isoenzymes OPR1 and OPR3 from *Lycopersicon esculentum* and the OYE-homolog YqiM from *Bacillus subtilis* were overexpressed and purified as reported.$^{[5a,10,11]}$ The cloning, purification and characterisation of old yellow isoenzymes from yeast (OYE1 from *Saccharomyces carlsbergensis*, OYE2 and OYE3 from *Saccharomyces cerevisiae*) and nicotinamide-dependent cyclohexenone reductase (NCR) *Zymomonas mobilis* reductase were performed according to literature.$^{[9]}$

General procedure for the enzymatic bioreduction of 1a-9a:
An aliquot of enzyme (OYE1-3, OPR1, OPR3, YqiM, and NCR, protein concentration in biotransformations 75-125 µg/mL) was added to a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (10 mM) and the cofactor NADH (15 mM). In case of 8a and 9a, the substrate was solubilised by addition of $t$-BuOMe (v:v 20%). The mixture was shaken at 30 °C and 120 rpm. After 24 h products were extracted with EtOAc (2 x 0.5 mL). The combined organic phases were dried over Na$_2$SO$_4$ and analysed on achiral GC to determine the conversion and on chiral GC to determine the enantiomeric excess. For experiments in presence of cofactor recycling, NADH was replaced by the oxidized form of the cofactor (NAD$^+$, 100 µM), the cosubstrate (formate, 20 mM) and the recycling enzyme (formate dehydrogenase, 10 U).
Synthesis:

*N*-Ethoxycarbonyliminopyridinium ylide:

![Scheme 3. synthesis of *N*-ethoxycarbonyliminopyridinium ylide](image)

Ethoxy formyl chloride (0.369 g, 3.4 mmol) was added dropwise to a solution of *N*-aminopyridinium iodide (0.511 g, 2.3 mmol) in KOH (10 mL, 10 %). The reaction mixture was stirred for 48 hours at room temperature. During the reaction the solution changed its colour from red to dark brown. The progress was controlled by TLC (CH$_2$Cl$_2$/MeOH 95:5; R$_f$ of *N*-ethoxycarbonyliminopyridinium ylide: 0.78). Water (30 mL) was added and the mixture was extracted two times with chloroform (30 mL), dried with Na$_2$SO$_4$ and the solvent was removed in vacuo yielding a dark blue powder, which was dissolved in methanol (50 mL) and stirred for 10 minutes with activated charcoal (0.2 g) for further purification. After filtration, methanol was removed in vacuo to yield brown crystals of *N*-ethoxycarbonyliminopyridinium ylide (0.34 mg, 87.6 %)

$^1$H-NMR (DMSO, 300 MHz): δ [ppm] = 1.16 (3H, t, J = 6.9), 3.96 (2H, q, J = 6.9), 7.80 (2H, t, J = 6.9), 8.05 (1H, t, J = 7.8), 8.75 (2H, d, J = 6.0); $^{13}$C-NMR (DMSO, 75 MHz): δ [ppm] = 15.5, 59.3, 127.1, 137.4, 143.5, 163.4.
Dimethyl 2-aminofumarate:

\[
\begin{align*}
\text{Dimethyl fumarate} & \quad \text{N-ethoxycarbonyliminopyridinium ylide} \\
\text{Silicic acid} & \quad \text{Xylene}
\end{align*}
\]

**Scheme 4: Synthesis dimethyl 2-aminofumarate**

Dimethyl fumarate (3.52 g, 24.4 mmol) was dissolved in xylene (160 mL) and \text{N-ethoxycarbonyliminopyridinium ylide} (4.09 g, 24.4 mmol) and silicic acid (14 g) were added. The mixture was heated to 130 °C for 48 h. The reaction progress was controlled by TLC (ethyl acetate/petroleum ether 1:5; \text{Rf: Xylene: 0.9; dimethyl fumarate: 0.5; dimethyl 2-aminofumarate: 0.4}). After the reaction cooled down the solvent was removed via dry-flash chromatography (1000 ml petroleum ether to wash the xylene off, then ethyl acetate/petroleum ether 1:5 until dimethyl 2-aminofumarate was eluted.) This yielded 1.6 g of a mixture of dimethyl 2-aminofumarate (38 %) and dimethyl 2-(ethoxycarbonylamino)fumarate.\[14\] The product was not further purified.

(Z)-Dimethyl 2-butyramidofumarate (7a):

In \text{CH}_2\text{Cl}_2 (15 mL) dimethyl 2-aminofumarate (38% mixture with dimethyl 2-(ethoxycarbonylamino)fumarate, 0.8 g, 1.9 mmol) was dissolved and pyridine (1.9 mmol, 152 µL) and butanoyl chloride (1.9 mmol, 1 eq.) was added at -20 °C. The mixture was stirred at this temperature for 2 h and then extracted with NaOH (0.3 M, 10 mL) and concentrated in vacuo. The product was purified by column chromatography (ethyl acetate/petroleum ether 1:5) which yielded 7a (88 mg, 20 %).

(Z)-Dimethyl 2-(phenylacetamido)fumarate (9a):

In \text{CH}_2\text{Cl}_2 (15 mL) dimethyl 2-aminofumarate (38% mixture with dimethyl 2-(ethoxycarbonylamino)fumarate, 0.8g, 1.9 mmol) was dissolved and pyridine (1.9 mmol, 152 µL) and phenylacetyl chloride (5.6 mmol, 3 eq.) were added. The mixture was stirred at room temperature for 48 h and then extracted with NaOH (0.3 M, 10 mL) and concentrated in
vacuo. The product was purified by column chromatography (ethyl acetate/petroleum ether 1:5) to yield 9a (250 mg, 47 %).

**General procedure for the hydrogenation (rac-1b,4b-9b)**

The alkene (1a, 4a-9a, 1 mmol) was dissolved in THF (5 mL) and hydrogenated under H₂ at atmospheric pressure at room temperature employing 10 % Pd/C (5 mg) as catalyst. The mixture was stirred overnight at room temperature. Afterwards the reaction mixture was filtered through Celite and concentrated to yield the rac-mixture of the reduced reference material (rac-1b, 4b-9b) in 99 % conversion.

**rac-N-Acetyl-alanine methyl ester (rac-1b):**

$^1$H NMR (CDCl₃) $\delta$ = 1.40 (3H, d, J = 7.1); 2.02 (3H, s); 3.75 (3H, s); 4.58-4.61 (1H, m); 6.15 (NH, br. s); $^{13}$C NMR (75 MHz, CDCl₃) $\delta$ = 18.3, 23.0, 48.0, 52.5, 170.1, 173.7.

**rac-Dimethyl 2-formamidosuccinate (rac-4b):**

$^1$H NMR (CDCl₃) $\delta$ = 2.88 (1H, dd, J₁ = 17.4, J₂ = 4.5), 3.09 (1H, dd, J₁ = 17.3, J₂ = 4.3), 3.69 (3H, s), 3.77 (3H, s), 4.90-4.96 (1H, m), 6.83 (NH, br. s), 8.21 (1H, s); $^{13}$C NMR (75 MHz, CDCl₃) $\delta$ = 36.0, 47.0, 52.1, 52.9, 160.8, 170.7, 171.4.

**rac-Dimethyl 2-acetamidosuccinate (rac-5b):**

$^1$H NMR (CDCl₃) $\delta$ = 2.05 (3H, s), 2.87 (1H, dd, J₁ = 17.3, J₂ = 4.4), 3.05 (1H, dd, J₁ = 17.3, J₂ = 4.3), 3.71 (3H, s), 3.78 (3H, s), 4.85-4.89 (1H, m), 6.53 (NH, br. s); $^{13}$C NMR (75 MHz, CDCl₃) $\delta$ = 23.1, 36.1, 48.4, 52.0, 52.8, 169.9, 171.2, 171.7.

**rac-Dimethyl 2-propionamidosuccinate (rac-6b):**

$^1$H NMR (CDCl₃) $\delta$ = 1.17 (3H, t, J = 7.8), 2.28 (2H, q, J = 7.8), 2.86 (1H, dd, J₁ = 17.1, J₂ = 4.5), 3.05 (1H, dd, J₁ = 17.4, J₂ = 4.5), 3.70 (3H, s), 3.77 (3H, s), 4.86-2.91 (1H, m), 6.50 (NH, br. s); $^{13}$C NMR (75 MHz, CDCl₃) $\delta$ = 9.5, 29.5, 36.1, 48.3, 52.0, 52.8, 171.3, 171.7, 173.6.

**rac-Dimethyl 2-butyramidosuccinate (rac-7b):**

$^1$H NMR (CDCl₃) $\delta$ = 0.96 (3H, t, J = 7.5), 1.65-1.72 (2H, m), 2.23 (2H, t, J = 7.5), 2.87 (1H, dd, J₁ = 17.1, J₂ = 4.5), 3.05 (1H, dd, J₁ = 17.1, J₂ = 4.5), 4.87-4.92 (2H, m), 6.50 (NH, br. s); $^{13}$C NMR (75 MHz, CDCl₃) $\delta$ = 13.7, 19.0, 36.1, 38.4, 48.3, 52.0, 52.8, 171.2, 171.7, 172.8.

**rac-Dimethyl 2-benzamidosuccinate (rac-8b):**

$^1$H NMR (CDCl₃) $\delta$ = 3.00 (1H, dd, J₁ = 17.3, J₂ = 4.5), 3.16 (1H, dd, J₁ = 17.3, J₂ = 4.2), 3.72 (3H, s), 3.81 (3H, s), 5.05-5.11 (1H, m), 7.25
(NH, br. s), 7.44-7.54 (3H, m), 7.83 (2H, d, J = 7.5); ^13^C NMR (75 MHz, CDCl$_3$) δ = 36.1, 48.9, 52.1, 52.9, 127.2, 128.6, 131.9, 133.6, 166.9, 171.3, 171.8.

rac-dimethyl 2-(phenylacetamido)succinate (rac-9b): ^1^H NMR (CDCl$_3$) δ = 2.83 (1H, dd, J$_1$ = 17.1, J$_2$ = 4.5), 3.00 (1H, dd, J$_1$ = 17.1, J$_2$ = 4.2), 3.62 (2H, s), 3.63 (3H, s), 3.74 (3H, s), 4.82-4.88 (1H, m), 6.48 (NH, br. s), 7.23-7.41 (5H, m); ^13^C NMR (75 MHz, CDCl$_3$) δ = 35.6, 43.5, 48.5, 52.0, 52.8, 127.4, 128.9, 129.3, 134.3, 170.8, 171.0, 171.3.

Synthesis of chiral reference material

(S)-Dimethyl 2-formamidosuccinate [(S)-4b]:

L-Aspartic dimethylester hydrochloride (943 mg, 5 mmol) and formic acid (460 mg, 40 mmol) were dissolved in toluene (50 mL). After 6 h of refluxing in a Dean-Stark apparatus, triethylamine (505 mg, 5 mmol) was added, the mixture was extracted two times with ethyl acetate (20 mL) and the combined organic layers were shaken once with a saturated solution of NaHCO$_3$ (20 mL). The solution was dried with Na$_2$SO$_4$ and concentrated in vacuo, which provided (S)-4b (503 mg, 53 %). The product was used without further purification.

(S)-Dimethyl 2-acetamidosuccinate [(S)-5b]; (S)-Dimethyl 2-propionamidosuccinate [(S)-6b]; (S)-Dimethyl 2-butyramidosuccinate [(S)-7b]; (S)-Dimethyl 2-benzamidosuccinate [(S)-8b]; (S)-dimethyl 2-(phenylacetamido)succinate [(S)-9b]:

L-aspartic dimethylester hydrochloride (200 mg, 1 mmol), acidchloride (3 mmol) and triethylamine (202 mg, 2 mmol) were dissolved in toluene CH$_2$Cl$_2$ (20 mL). After 24 hours of refluxing the mixture was extracted two times with ethyl acetate (10 mL) the combined organic layers once with a saturated solution of NaHCO$_3$ (10 mL). The solution was dried with Na$_2$SO$_4$ and concentrated in vacuo, which provided the (S)-amides [(S)-5b-9b]. The product was used without further purification for co-injection.

Analytical procedures:

_Determination of conversion:_ Conversion of methyl 2-acetamidoacrylate (1a) was determined by GC-FID using a 6% cyanopropyl-phenyl phase capillary column (Varian CP-1301, 30 m, 0.25 mm, 0.25 µm), detector temperature 240 °C, injector temperature 250 °C, split ratio 30:1. Temperature program: 120 °C hold 2 min, 10 °C/min to 160 °C, 30 °C/min to 200 °C, hold 2
Retention times: 1a 4.89 min, 1b 5.12 min. Conversion of (Z)-dimethyl 2-formamidofumarate (4a), (Z)-dimethyl 2-acetamidofumarate (5a), (Z)-dimethyl 2-propionamidofumarate (6a) and (Z)-Dimethyl 2-butyramidofumarate (7a) was determined by GC-FID using a 14% cyanpropyl-phenyl phase capillary column (J&W Scientific DB-1701, 30m, 0.25 mm, 0.25 µm), detector temperature 240 °C, injector temperature 250 °C, split ratio 30:1. Temperature program: 40 °C hold 2 min, 20 °C/min to 180 °C, hold 11 min. Retention times: 4a 10.77 min, 4b 11.18 min; 5a 9.96 min, 5b 10.47 min; 6a 10.67 min, 6b 11.18 min; 7a 11.64 min, 7b 12.32 min;

Conversion of (Z)-dimethyl 2-benzamidofumarate (8a) and (Z)-dimethyl 2-(phenylacetamido)fumarate (9a) was determined by GC-MS using a (5%-phenyl)-methylpolysiloxane capillary column (HP-5MS, 30 m, 0.25 mm, 0.25 µm film). Temperature program: 200°C hold 0.5 min, 5°C/min to 300°C, hold 2 min. Retention times: 8a 14.98 min, 8b 14.56; 9a 15.24 min, 9b 14.90 min.

**Determination of enantiomeric excess and absolute configuration:**
The enantiomeric excess of 1b and 4b-7b was determined by GC-FID using a modified β-cyclodextrin capillary column (ChiralDex B-TA, 40 m, 0.25 mm). Detector temperature 200 °C, injector temperature 180 °C, split ratio 20:1. Temperature program for 1b: 130 °C hold 3 min, 15 °C/min to 180 °C, hold 2 min. Retention times: (R)-1b 4.85 min, (S)-1b 5.00 min; Temperature program for 4b: 110 °C, hold 5 min, 1 °C/min to 140 °C, 5 °C/min to 180 °C, hold 2 min; Retention times: (S)-4b 35.67 min, (R)-4b 36.21 min; Temperature program for 5b and 6b: 110 °C, hold 5 min, 10 °C/min to 180 °C, 15 °C/min to 190 °C, hold 2 min. Retention times: (R)-5b 12.38 min, (S)-5b 12.44 min; (R)-6b 12.90 min, (S)-6b 12.96 min; Temperature program for 7b: 40 °C, hold 2 min, 10 °C/min to 140 °C, hold 17 min, 1 °C/min to 155 °C, 15 °C/min to 180 °C. Retention times: (R)-7b 33.65 min, (S)-7b 34.65 min; The enantiomeric excess of 8b was determined by HPLC-analysis on a Chiralcel OJ column (25 cm, 0.46 cm) using n-heptane/2-propanol 90:10 (isocratic, flow rate of 1ml/min at 18 °C). Retention times: (S)-8b 27.10 min, (R)-8b 30.40 min; The enantiomeric excess of 9b was determined by HPLC-analysis on a Chiralpak AD (25 cm, 0.46 cm) using n-heptane/2-propanol 90:10 (isocratic, flow rate of 1ml/min at 18 °C). Retention times: (S)-9b 35.62 min, (R)-9b 38.68 min;
The absolute configuration of 1b and 4b-9b was determined by co-injection with independently synthesised chiral reference material.

NMR:

All NMR experiments were acquired at 300 K on a Bruker Avance III 300 MHz NMR spectrometer using a broadband 5 mm probe with z-axis gradients, except the data for CHW-42 which were obtained on a Bruker Avance DRX 500 MHz spectrometer using a TXI triple-resonance probe with z-axis gradients at 303 K. For 1D $^1$H and $^{13}$C spectra 16 scans of 32 k or 1024 scans of 64 k data points were accumulated, respectively. Two-dimensional proton-carbon correlated HSQC (4 scans per increment) and HMBC (16 scans per increment) spectra consisting of 2048 x 256 ($F_2 \times F_1$) data points were used to complete the assignment of all proton and carbon resonances. For the 2D NOESY spectra data matrices of 2048 x 256 ($F_2 \times F_1$) points were accumulated with 8 scans per increment. The mixing time was 800 ms. For all spectra in all dimensions 60° phase-shifted squared sine-bell window function were employed. All spectra were processed and analyzed in MestReNova 6.0.

Results

Complete proton and carbon assignments were obtained using 1D $^1$H and $^{13}$C spectra as well as 2D $^1$H-$^{13}$C HSQC and HMBC experiments. NOESY spectra were used to differentiate between E and Z isomers. All compounds tested, showed clear NOEs between the amide proton and methyl group “II” as well as between the methine proton “a” and both methyl groups “I” and “II”. On the other hand very weak or no NOEs were found between the amide proton and methine proton “a”. All these NOEs or their absence are strong indications for Z-isomers.
\(^1\)H chemical shifts

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References and Notes


Christoph Winkler

Billrothgasse 46, A-8047 Graz
Mobile.: 0043/660/7677541, E-mail: winkler_christoph@hotmail.com

Personal Information:

Date of Birth: april, 8; 1985
Nationality: Austrian - Swiss
Marital Status: single

Education:

1990 - 1995 Elementary school in Altaussee, Austria
1995 - 1999 Junior high school in Bad Aussee, Austria
1999 - 2004 Technical high school for chemistry in Wels, Austria
2004 - current Student of chemistry at the Karl Franzens University of Graz and the TU Graz;

Honoured with scholarship awards in 2009, 2010;
Member of the scholarship program PRO SCIENCIA;

Qualifikation and Skills:

- English (fluent written and spoken)
- German (native)
- Spanish (basic)
- Completed a course on quality control
- Completed a first aid course
- Member of the auxiliary fire brigade; Skills in fire fighting and knowledge in fire safety
- Programming skills, in particular in web–programming
- Software skills: Microsoft Office, SciFinder, Beilstein, ChemOffice
Internships:

**August 2000**: Karl Franzens University of Graz, Department of Biochemistry
work as an intern;

work as a summer-substitute in the laboratory for quality control;

**July - August 2003**: Saline Austria GmbH, Altaussee (Austria)
work in the metal-workshop;

**October - December 2008**: Karl Franzens University of Graz, Department of Chemistry
teaching assistant for the laboratory course for organic synthesis;

**July 2008**: TU Graz, Department of Chemistry
summer student at the groups of Univ.-Prof. Dr.rer.nat. Rolf Breinbauer and Ao.Univ.-
Prof. Dr.phil Norbert Klempier;

**August 2008**: Karl Franzens University of Graz, Department of Chemistry
summer student at the groups of O.Univ.-Prof. Dr.phil. Kurt Faber and Ao.Univ.-Prof.
Dipl.-Ing. Dr.techn. Wolfgang Kroutil; (Publication list entries: Journals 1.; Lectures
1.; Posters 2.)

**October 2008 – January 2009**: TU Graz, Department of Chemistry
teaching assistant for the laboratory course for chemistry for Health Care
Engineering;

**October 2009 – January 2010**: Karl Franzens University of Graz, Department of
Chemistry
teaching assistant for the laboratory course for inorganic synthesis;

**September 2009 – March 2010**: Karl Franzens University of Graz, Department of
Chemistry
work on my diploma thesis in the field of enoate reductases of the ‘old yellow
enzyme’ family; (Publication list entries: Journals 2.-4.; Posters 1.,3.,4.)

**March – September 2010**: Karl Franzens University of Graz, Department of Chemistry
summer student at the group of O.Univ.-Prof. Dr.phil. Kurt Faber, work in the field of
enoate reductases of the ‘old yellow enzyme’ family;
Publication List

Journals:


4. “Stereo-Controlled Asymmetric Bioreduction of α,β-Dehydroamino Acid Derivatives”. C. Stueckler, C. K. Winkler, M. Hall, B. Hauer, M. Bonnekessel, K. Zangger and K. Faber, 2010. (manuscript to be submitted)

Lectures:


Forthcoming Lectures:

3. "Biocatalytic C=C-Bond Reduction ".
C. Stueckler, C. K. Winkler, S. M. Glueck, K. Faber; Biocatalysis Zing-Conference, Puerto Morelos, Mexico, December 10-13, 2010.

4. "Extending the Substrate Scope for the Biocatalytic C=C-Bond Reduction".

Posters:

1. "Stereo-Controlled Asymmetric Bioreduction of α,β-Dehydroaminoacid Derivatives".

2. "Asymmetric Bioreduction of Cinnamic Aldehyde Derivatives Using Ene-Reductases".
C. Stueckler, C. K. Winkler, N. J. Mueller, S. M. Glueck, B. Hauer, R. Stuermer, K. Faber;

5th International Congress on Biocatalysis, Hamburg, Germany, August 29 – September 2, 2010.

4. "Asymmetric Synthesis of (R)-3-Hydroxy-2-methylpropanoate ('Roche Ester') via Biocatalysed C=C Double Bond Reduction ".
C. Stueckler, C. K. Winkler, M. Bonnekessel, K. Faber
5th International Congress on Biocatalysis, Hamburg, Germany, August 29 – September 2, 2010.

5. "Nicotinamide-Independent Asymmetric Bioreduction of C=C Bonds via Disproportionation of Enones Catalyzed by Enoate Reductases".
C. Stueckler, C. K. Winkler, T. Reiter, N. Baudendistel, K. Faber
5th International Congress on Biocatalysis, Hamburg, Germany, August 29 – September 2, 2010.