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# Asymmetric bioreduction of activated alkenes to industrially relevant optically active compounds

Christoph K. Winkler[b], Gábor Tasnádi[a,b], Dorina Clay[b], Mélanie Hall[b], Kurt Faber[b,*]

[a] ACIB Co. c/o, Heinrichstrasse 28, 8010 Graz, Austria
[b] Department of Chemistry, Organic & Bioorganic Chemistry, University of Graz, Heinrichstrasse 28, 8010 Graz, Austria

## ARTICLE INFO

## ABSTRACT

Ene-reductases from the 'Old Yellow Enzyme' family of flavoproteins catalyze the asymmetric reduction of various α,β-unsaturated compounds at the expense of a nicotinamide cofactor. They have been applied to the synthesis of valuable enantiopure products, including chiral building blocks with broad industrial applications, terpenoids, amino acid derivatives and fragrances. The combination of these highly stereoselective biocatalysts with a cofactor recycling system has allowed the development of cost-effective methods for the generation of optically active molecules, which is strengthened by the availability of stereo-complementary enzyme homologues.

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## 1. Introduction

The increasing demand for small enantiopure molecules as chiral building blocks for the synthesis of biologically active compounds (most notably active pharmaceutical ingredients – API) has contributed to the development of highly specific synthetic strategies. The reduction of alkenes, for instance, is a powerful tool in modern asymmetric synthesis and various approaches are now available on industrial scale. Transition-metal based homogeneous catalysis has reached high standards (Knowles, 2002; Noyori, 2002), and related fields of catalysis are now becoming competitive in this area. Metal-free organocatalysis uses general acid-type catalysts to perform stereoselective transfer hydrogenation, but suffers from low atom economy due to the requirement for molar amounts of the 'Hantzsch ester' used as reductant (List and Yang, 2006; Yang et al., 2005). Nature, on the other hand, provides an attractive sustainable and cost-effective alternative. The biocatalytic analog relies on ene-reductases to perform the reduction of activated C=C bonds. These enzymes belong to the 'Old Yellow Enzyme' family of nicotinamide-dependent flavoproteins and have been intensely investigated over the past five years in view of their applicability in preparative-scale biotransformations (Hall et al., 2010; Stuermer et al., 2007; Toogood et al., 2010). They catalyze the highly stereoselective reduction of a broad variety of α,β-unsaturated compounds, affording excellent yields and enantiomeric excess, while working under mild conditions of pH and temperature. A whole set of homologous enzymes has been developed and several industrially relevant molecules could be obtained in nonracemic form. This review focuses on this new enzyme platform, presenting pertinent examples while stressing on general rules that should help chemists incorporate ene-reductases in the design of asymmetric synthetic routes.

## 2. System

### 2.1. Reaction mechanism

The mechanism of the ene-reductase-catalyzed reduction of α,β-unsaturated compounds has been studied in great detail (Kohli and Massey, 1998). The reaction was shown to proceed via the stereoselective transfer of a hydride (derived from the reduced flavin-cofactor) onto Cβ, while a Tyr-residue adds a proton (ultimately derived from the solvent) onto Cα from the opposite side (Fig. 1). The overall addition of [2H] onto a C=C bond resembles a Michael-type addition of a complex hydride and results with exclusive relative *trans*-stereospecificity.[1] Reduction of the

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[1] Rare cases for *cis*-addition were observed with plant cell cultures and flavin-independent reductases: Shimoda, K., Ito, D.I., Izumi, S., Hirata, T., 1996. Novel
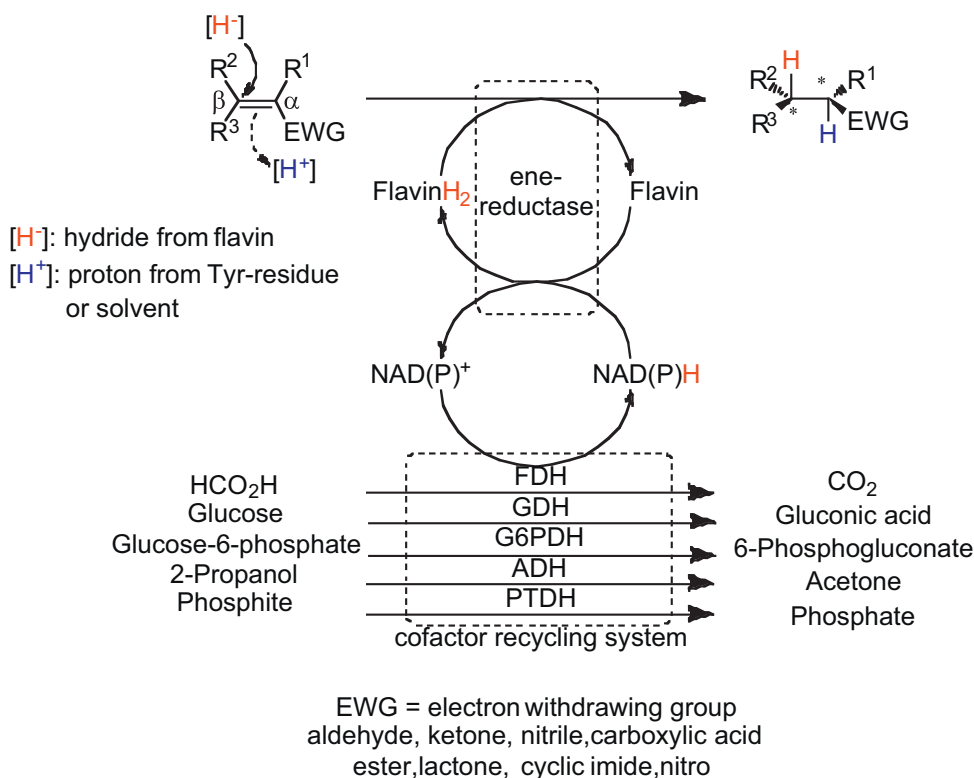
[H⁻]: hydride from flavin

[H⁺]: proton from Tyr-residue or solvent

EWG = electron withdrawing group
aldehyde, ketone, nitrile, carboxylic acid
ester, lactone, cyclic imide, nitro

**Fig. 1.** Asymmetric bioreduction of activated alkenes using ene-reductases.

oxidized flavin cofactor at the expense of NAD(P)H closes the catalytic cycle (Fig. 1). Ene-reductases often show relaxed specificities for NADH or NADPH as cofactor, which allows to choose the recycling system on a case-to-case basis. The enzymes have been shown to tolerate organic co-solvents very well, especially water immiscible ones, in up to 50%, v:v (Stueckler et al., 2010a; Yanto et al., 2011).

### 2.2. Substrates

Only C=C-bonds that are electronically activated by a conjugated electron-withdrawing group (EWG) are reduced, non-activated (isolated) alkenes are unreactive. The following functional groups may serve as 'activators':

(i) α,β-Unsaturated carboxaldehydes (enals) are good substrates and yield the expected saturated aldehydes as products when pure ene-reductases are used (Stueckler et al., 2010a). However, in whole-cell biotransformations (using e.g. baker's yeast), carbonyl reduction is a dominant side reaction forming saturated *prim*-alcohols via over-reduction of the product or allylic alcohols by depleting the substrate (Hall et al., 2006; Mueller et al., 2006).

(ii) α,β-Enones are usually well accepted, competing carbonyl reduction is less dominant as with enals.

(iii) Conjugated nitroalkenes are highly activated and are thus readily reduced. Whereas chiral centers in the β-position of the

nitroalkanes thus formed are stable, α-analogs are somewhat labile due to the acidity of the α-H.

(iv) Depending on their degree of activation, α,β-unsaturated carboxylic acids or esters behave as 'borderline'-substrates: whereas simple α,β-unsaturated *mono*-carboxylic acids or -esters are not easily reduced by ene-reductases, they are good substrates for 'enoate-reductases' from anaerobic organisms, which possess an additional (oxygen-sensitive) ferredoxin Fe₄S₄-cofactor (Ferraboschi et al., 1987; Tischer et al., 1979). However, mono-acids or -esters can be activated by an additional electron-withdrawing group, such as a second acid- or ester-group, a halogen or a nitrile (Brenna et al., 2011c; Kitazume and Ishikawa, 1984). Consequently, *di*-carboxylic acids and -esters are well accepted. Cyclic imides, bearing two activating carbonyl groups next to the C=C bond are good substrates in general.

(v) α,β-Unsaturated nitriles are only slightly activated and also count as 'borderline'-substrates, although complex nitrile-containing molecules have been successfully reduced (Kosjek et al., 2008).

### 2.3. Enzymes

Enzymes from the 'Old Yellow Enzyme' family are widely distributed in microorganisms and in plants. Some of them occur in well-defined pathways, e.g. in the biosynthesis of jasmonic acid or the metabolism of morphine (Barna et al., 2002; Schaller et al., 2000), others are involved in the detoxification of xenobiotics (Williams et al., 2004), such as trinitrotoluene (TNT, Barna et al., 2001). Over recent years, a great variety of new homologues has been identified and their potential as biocatalysts was investigated. Table 1 gives a summary of ene-reductases used in isolated form in asymmetric bioreduction reactions.

reductase participation in the *syn*-addition of hydrogen to the C=C bond of enones in the cultured cells of *Nicotiana tabacum*. J. Chem. Soc., Perkin Trans. 1. 355–358; Bougioukou, D.J., Stewart, J.D., 2008. Opposite stereochemical courses for enzyme-mediated alkene reductions of an enantiomeric substrate pair. J. Am. Chem. Soc. 130, 7655–7658.

**Table 1**
Ene-reductases from the 'Old Yellow Enzyme' family.

| Enzyme | Organism |
| --- | --- |
| Fungi | |
| Old yellow enzyme 1 (OYE1) | *Saccharomyces pastorianus* (formerly *S. carlsbergensis*) (Saito et al., 1991) |
| Old yellow enzyme 2 and 3 (OYE2 and 3) | *Saccharomyces cerevisiae* (Karplus et al., 1995) |
| Old yellow enzyme (OYE) | *Candida macedoniensis* AKU4588 (Kataoka et al., 2002, 2004) |
| Estrogen binding protein (EBP1) | *Candida albicans* (Buckman and Miller, 1998) |
| *Kluyveromyces lactis* yellow enzyme 1 (KYE1) | *Kluyveromyces lactis* (Chaparro-Riggers et al., 2007) |
| Old yellow enzyme 2.6 (OYE 2.6) | *Pichia stipitis* CBS 6054 (Padhi et al., 2009) |
| Bacteria | |
| YqjM | *Bacillus subtilis* (Fitzpatrick et al., 2003) |
| NAD(P)H-dependent 2-cyclohexen-1-one reductase (NCR) | *Zymomonas mobilis* (Mueller et al., 2007) |
| Xenobiotic reductase A (XenA) | *Pseudomonas putida* II-B (Blehert et al., 1999) |
| Xenobiotic reductase B (XenB) | *Pseudomonas fluorescens* I-C (Blehert et al., 1999) |
| Pentaerythritol tetranitrate reductase (PETNr) | *Enterobacter cloacae* PB2 (French et al., 1996) |
| TOYE | *Thermoanaerobacter pseudoethanolicus* E 39 (Adalbjornsson et al., 2010) |
| SYE1-4 | *Shewanella oneidensis* (Brige et al., 2006) |
| *Gk*OYE | *Geobacillus kaustophilus* DSM 7263 (Schittmayer et al., 2010) |
| Chromate reductase (CrS) | *Thermus scotoductus* SA-01 (Opperman et al., 2008, 2010) |
| Morphinone reductase (MR) | *Pseudomonas putida* M10 (French and Bruce, 1994) |
| YersER | *Yersinia bercovieri* (Chaparro-Riggers et al., 2007) |
| *Gluconobacter oxidans* ene-reductase | *Gluconobacter oxidans* DSM 2343 (Richter et al., 2011) |
| *N*-ethylmaleimide reductase (NemR) | *Escherichia coli* (Miura et al., 1997) |
| Glycerol trinitrate reductase (NerA) | *Agrobacterium radiobacter* (Snape et al., 1997) |
| Plants | |
| 12-Oxophytodienoate reductase 1–3 (OPR1-3) | *Arabidopsis thaliana* (Biesgen and Weiler, 1999; Costa et al., 2000; Schaller and Weiler, 1997) |
| 12-Oxophytodienoate reductase 1–3 (*Le*OPR1-3) | *Solanum lycopersicum* (formerly *Lycopersicon esculentum*) (Strassner et al., 1999, 2002) |
| Commercially available | |
| ERED101-114 | Source not available (Kosjek et al., 2008) |

## 2.4. Cofactor regeneration

A major limitation to the broad application of nicotinamide-dependent enzymes for synthetic purpose lies in the prohibitive cost of these natural cofactors, especially the reduced form (~500 €/g NADH and 1400 €/g NADPH, from chemical suppliers). Fortunately, advances in cofactor regeneration techniques now allow the use of catalytic amounts of NAD(P)H and various systems have been exploited with ene-reductases for in situ recycling (Fig. 1) (Faber, 2011; Hall and Bommarius, 2011), while often serving as driving force to overcome thermodynamic equilibrium limitations (Park et al., 2011).

A common strategy for the regeneration of NADH is the formate dehydrogenase (FDH)-catalyzed oxidation of formate to $CO_2$. FDH has been successfully employed with ene-reductases, although substrate and product depletions were observed with an enal (citronellal), resulting from carbonyl reduction caused by *prim*-ADH impurities in the commercial FDH preparation; likewise, racemisation of α-substituted cycloalkanones was observed (Hall et al., 2008a). Recently, alcohol dehydrogenase ADH-'A' was successfully combined with several ene-reductases, using only 2 equivalents of 2-propanol as H-donor, thereby producing acetone. As above, aldehydes are not suitable substrates due to over-reduction of the CH═O moiety (Tauber et al., 2011). Glucose-6-phosphate dehydrogenase (G6PDH) and glucose dehydrogenase (GDH) are commonly used as cofactor recycling systems for ene-reductases (Hall et al., 2007, 2008b). While G6PDH only accepts $NADP^+$, GDH can be employed with both nicotinamide cofactors.

Occasionally, the nature of the substrate had a dramatic influence on the efficiency of the recycling system. For instance, FDH, GDH and G6PDH were inactive in presence of a *cis*-configurated α,β-unsaturated dicarboxylic acid (citraconic acid), which acts as strong chelator for divalent metal ions. The addition of metals (e.g. $Ca^{2+}$, $Mg^{2+}$ or $Zn^{2+}$) to the reaction medium proved necessary to overcome deactivation of the recycling enzymes and to make this substrate amenable to bioreduction (Stueckler et al., 2007).

While FDH produces $CO_2$ and ADH forms highly volatile acetone, GDH and G6PDH furnish gluconolactone/gluconic acid and 6-phosphogluconate respectively, both unstable compounds that hydrolyse spontaneously. This renders all four systems practically irreversible and thereby shifts the equilibrium towards reduction. Phosphite dehydrogenase (PTDH) (Vrtis et al., 2002; Woodyer et al., 2003) has also been applied to the reduction of α,β-unsaturated nitriles, which provides in situ pH-control as phosphate is being produced throughout the reaction (Kosjek et al., 2008).

While all these systems represent coupled-enzyme approaches requiring two proteins, the first example of a coupled-substrate single-enzyme approach applied to ene-reductases was recently published, where advantage was taken of the disproportionation of enones catalyzed by a single ene-reductase. With the enone substrate being reduced to the corresponding saturated ketone, a sacrifial enone co-substrate served as artificial H-donor and was oxidized, thereby rendering the reduced flavin for a subsequent catalytic cycle. 2-Enones and 1,4-diones were particularly good co-substrates as their oxidized forms spontaneously tautomerized to phenol and hydroquinone derivatives, respectively, thus driving the reaction to the desired product side (Stueckler et al., 2010b).

Nonconventional regeneration methods are also being developed (Hollmann et al., 2010). A light-driven system was designed with YqjM, where irradiation with white light in the presence of external free flavin and EDTA allowed the full conversion of 4-ketoisophorone to levodione. The system, however, was plagued by the non-stereoselective background reaction catalyzed by free flavin, leading to reduced product enantiopurity (Taglieber et al., 2008).

## 3. Applications

### 3.1. Synthesis of amino acid derivatives

Natural and non-natural amino acids are valuable building blocks and key intermediates for a number of pharmaceuticals, heterocycles or modified peptides (Goodman et al., 2007; Hughes and Moody, 2007; Trabocchi et al., 2005). Ene-reductases have been successfully used for the synthesis of various α- and β-amino acid analogs. In a process developed by Swiderska and Stewart
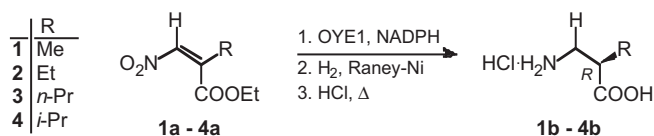
**Fig. 2.** Route to β²-amino acids.



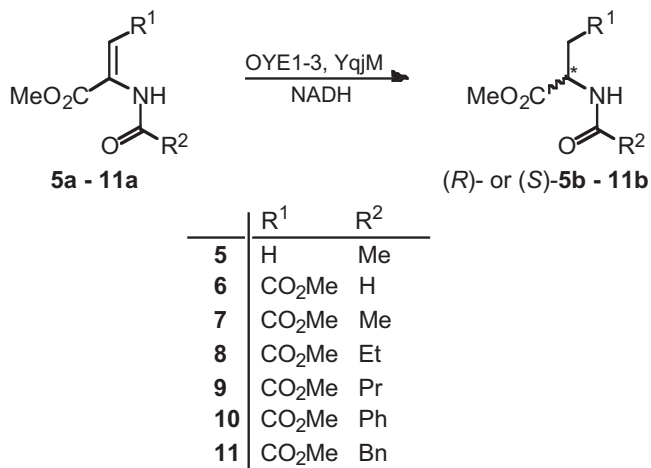| | R¹ | R² |
|---|---|---|
| **5** | H | Me |
| **6** | CO₂Me | H |
| **7** | CO₂Me | Me |
| **8** | CO₂Me | Et |
| **9** | CO₂Me | Pr |
| **10** | CO₂Me | Ph |
| **11** | CO₂Me | Bn |

**Fig. 3.** Reduction of α,β-dehydroamino acid derivatives by ene-reductases.

(2006), β-nitroacrylates **1a–4a** were stereoselectively reduced by OYE1 to the corresponding β-nitro carboxylic acid esters as the key step in the asymmetric synthesis of optically active β²-amino acids (Fig. 2). Since the bioreduction of the C=C bond proceeded chemoselectively, the nitro group was subsequently reduced with Raney-Ni. Ethyl α-alkyl-β-nitroacrylates were reduced with high stereoselectivity (ee ≥ 87%) and β²-amino acids were isolated as their hydrochloride salts (**1b–4b**) in good overall yield (57–73%). β-Alkyl-β-nitroacrylates (β³-amino acid precursors) on the other hand were reduced with low stereoselectivities, most likely due to the α-protonation occurring after product release from the active site.

Recently, α,β-dehydroamino acid derivatives have been identified as novel substrates for members of the OYE family (Fig. 3) (Stueckler et al., 2011). While an α-amino acid precursor having an additional methyl group at Cβ (methyl 3-methyl-2-acetamidoacrylate) and an α-alanine precursor (methyl 3-acetamidoacrylate) were unreactive, N-acyl derivative of alanine (**5a**) and aspartic acid ester (**8a**) were reduced by YqjM to the corresponding (S)-enantiomers (**5b**, 41% conv., 97% ee; **8b**, up to quantitative yield and 99% ee). A switch of stereopreference in the reduction of aspartic acid derivatives **6a–11a** could be induced with OYE3 via substrate engineering by variation of the size of the N-acyl protective group. While **6a**, **7a** and **10a** were reduced to the (S)-amino acid derivatives (23% to >99% ee), the (R)-enantiomers were obtained from **8a**, **9a** and **11a** (61% up to 92% ee). ²H-labelling experiments in D₂O revealed that the opposite stereochemical

outcome by OYE3 was due to a flipped "bottom/top" orientation of the substrate, resulting in an exchange of the activating ester as docking group in the active site. This switch of the activating group opens new perspectives for the asymmetric synthesis of β-amino acids.

### 3.2. Terpenoids

Terpenoids are one of the largest classes of natural products offering a great variety of biologically active compounds and chiral intermediates. Enantiomers of dihydrocarvone (**12b**) are minor components of essential oils produced by plants and have been used as chiral starting compounds in the synthesis of natural products (e.g. striatenic acid, pechueloic acid) (Aubin et al., 2006; Blay et al., 2007; Harrowven et al., 2005), antimalarial drugs (Dong et al., 2010) and valuable chiral synthons (de Rouville et al., 2009; Krawczyk et al., 2007). In the course of exploring the substrate specificity of PETN reductase (Fryszkowska et al., 2009), (5R)- and (5S)-carvone (**12a**) were quantitatively reduced into the diastereomeric products (**12b**) with the same absolute (R)-configuration on the newly generated centre at C2 in 95% and 88% diastereomeric excess, respectively (Fig. 4).

Both enantiomers of citronellal (**13b**), a key intermediate in menthol synthesis, have been prepared with excellent ee values (> 95%) starting from (E/Z)-citral (**13b**) using various OYEs (Fig. 5) (Bougioukou et al., 2010; Fryszkowska et al., 2009; Hall et al., 2007, 2008a,b; Mueller et al., 2010). While (S)-citronellal [(S)-**13b**] could be produced quantitatively, (R)-citronellal [(R)-**13b**] was obtained with 69% conversion. It was observed that the (E/Z)-configuration of citral played a crucial role in the stereoselectivity of OYEs 1–3 (Mueller et al., 2007). Whereas whole cells generally led to over-reduction of the product to the corresponding saturated alcohol (Hall et al., 2006; Mueller et al., 2006), isolated OYE-enzymes furnished the aldehyde **13b** as single product.

### 3.3. Fragrance compounds

α-Methyl dihydrocinnamaldehyde derivatives (**14b** and **15b**) are of commercial importance (Brenna et al., 2003), with **14b** being the olfactory principle of the lily-of-the-valley odor (Enders and Dyker, 1990), marketed under the trade name Lilial™ or Lysmeral™, while **15b**, marketed as Helional™ or Tropional™, is the active ingredient of various perfumes (Enders and Backes, 2004). A convenient enzymatic strategy for the synthesis of **14b** and **15b** was developed (Stueckler et al., 2010a) via bioreduction of α-methyl cinnamaldehydes (**14a** and **15a**) with OYEs. The (S)-antipodes were produced with OYE1-3 in an aqueous-organic biphasic system (containing 20% t-BuOMe) in >95% ee and quantitative yield (Fig. 6).

### 3.4. Chiral building blocks

(6R)-Levodione (**16b**), obtained by asymmetric bioreduction of 4-ketoisophorone (**16a**), represents an important industrial intermediate for carotenoide synthesis (e.g. zeaxanthin, cryptoxanthin,



**Fig. 4.** Total asymmetric synthesis of striatenic and pechueloic acid via ene-reductase catalyzed reduction of carvone **12a** producing the key intermediate **12b**.
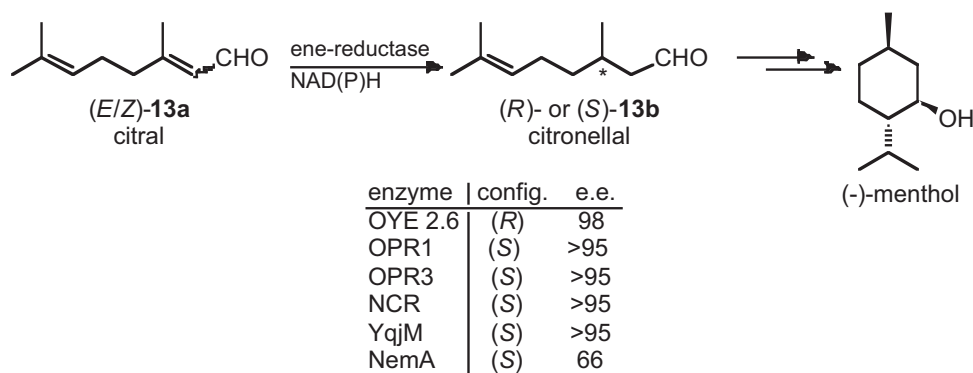
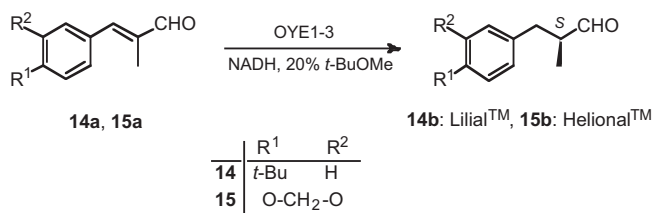**Fig. 5.** Bioreduction of citral (**13a**) to citronellal (**13b**).



**Fig. 6.** Fragrance production with ene-reductases.

xanthoxin) (Demole and Enggist, 1974). So far, all OYE family members have yielded strictly the (R)-enantiomer (up to >99% ee) (Toogood et al., 2010). A one-pot two-step enzymatic cascade was developed leading to (4R,6R)-actinol (**16c**). The first ene-reduction was catalyzed by OYE2, expressed in *E. coli* and used as cell extract, to furnish (6R)-levodione (**16b**) as intermediate. The latter was subsequently reduced at the carbonyl group to actinol (**16c**) with levodione reductase from *Corynebacterium aquaticum* M-13, also expressed in *E. coli*. Glucose dehydrogenase was used for the regeneration of NADH, which allowed the quantitative formation of (4R,6R)-actinol (**16c**) in 94% ee (Fig. 7) (Wada et al., 2003).

Due to its broad acceptance as a substrate by a large number of OYE homologues, 4-ketoisophorone (**16a**) emerged as a standard test-substance for the characterization of ene-reductases [(OPR1 and OPR3 (Hall et al., 2007, 2008a), YqjM (Hall et al., 2008a), OYE1-3 and NCR (Hall et al., 2008b), PETNr (Fryszkowska et al., 2009; Mueller et al., 2010), NemR, MR and EBP1 (Mueller et al., 2010), *Gluconobacter oxidans* ER (Richter et al., 2011), XenA (Chaparro-Riggers et al., 2007; Yanto et al., 2010), TOYE (Adalbjornsson et al., 2010), CrS (Opperman et al., 2010), YersER and KYE1 (Chaparro-Riggers et al., 2007), OYE from *Candida macedoniensis* (Kataoka et al., 2004)], the screening for novel ene-reductase activity in organisms (Goretti et al., 2011; Raimondi et al., 2010) and the development of novel cofactor regeneration systems (Taglieber et al., 2008, 2010; Tauber et al., 2011).

Chiral acyloins (**17b**–**21b**) are important building blocks in asymmetric synthesis (Adam et al., 1999; Demir et al., 2007; Patel, 2006). They can be converted into nonracemic diols,

epoxides, aminoalcohols, hydroxylamines, and haloketones (Fig. 8). In addition to the classical asymmetric synthesis involving *N*-sulfonyloxaziridines (Davis and Chen, 1992; Davis et al., 1986; Hughes et al., 2005), and several biocatalytic systems (Adam et al., 1999; Demir et al., 2007; Patel, 2006), an additional biocatalytic alternative was recently provided through the asymmetric reduction of α,β-unsaturated alkoxy ketones (Winkler et al., 2010). Stereocomplementary routes to *O*-protected acyloins were developed via substrate engineering through variation of the size of the *O*-protecting group. Both enantiomers of α-alkoxy enones could be obtained in up to >99% ee, while β-analogs were not converted. The *O*-protected acyloins thus obtained can be used in further synthetic steps; particularly allyl- or benzyl-moieties can be easily removed under mild conditions.

Enantiopure lactones are valuable synthetic precursors. For instance, γ-butyrolactone (**22c**) has been utilized as building block in the synthesis of natural products such as milbemycin β₃, jasplakinolide and amphidinolides (Fig. 9) (Korpak and Pietruszka, 2011). Two of its four possible stereoisomers were recently obtained via an enzymatic two-step one-pot cascade. In the first step, OYE1 was employed for the generation of the first stereocenter, where reduction of the two (E/Z)-isomers of starting material **22a** was stereoconvergent and yielded the (R)-enantiomer **22b**. In the second step, various alcohol dehydrogenases (ADH) were used for carbonyl reduction leading to the γ-hydroxy ester, followed by spontaneous lactonization to **22c** (Korpak and Pietruszka, 2011). The carbonyl reduction proceeded with enzyme-based stereocontrol, where proper choice of the catalyst allowed both (2R,4S)-**22c** (with Prelog-type ADH-T from *Thermoanaerobacter* species) and (2R,4R)-**22c** (with anti-Prelog-type ADH-LK from *Lactobacillus kefir*) in good yields (up to 80%) and perfect stereoselectivity (>99% ee).

(R)-3-Hydroxy-2-methylpropanoate (**23b**), commonly denoted as 'Roche-Ester', is a popular chiral building block for the synthesis of vitamins (vitamin E), fragrance compounds (muscone), antibiotics (rapamycin) and natural products (Stueckler et al., 2010c). Prominent routes for its preparation include enzymatic oxidation of prochiral diols (Molinari et al., 2003) or the transition metal-catalyzed asymmetric hydrogenation of acrylate esters using Rh- (Holz et al., 2008; Qiu et al., 2009; Wassenaar
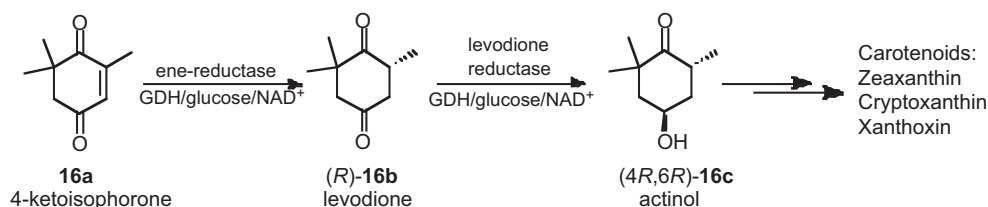


**Fig. 7.** Ene-reductase catalyzed reduction of 4-ketoisophorone (**16a**) to (R)-levodione (**16b**) and (4R,6R)-actinol (**16c**).
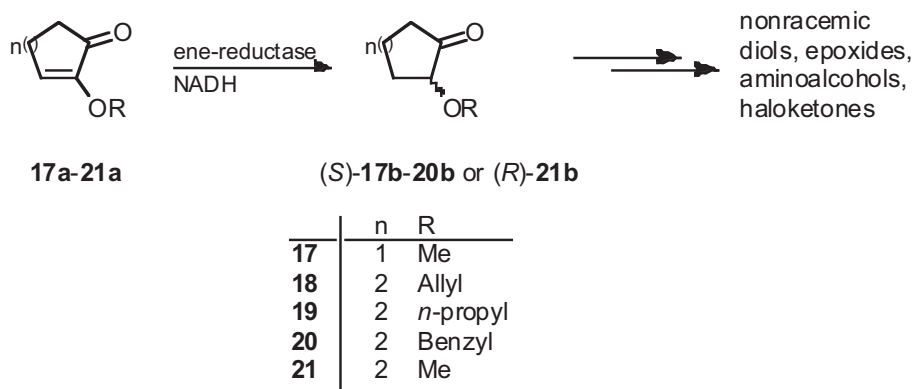
**Fig. 8.** Production of chiral acyloins via ene-reductases using OYE1-3, YqjM, NerA, OPR1, OPR3, XenA, XenB, EBP1 and NCR.
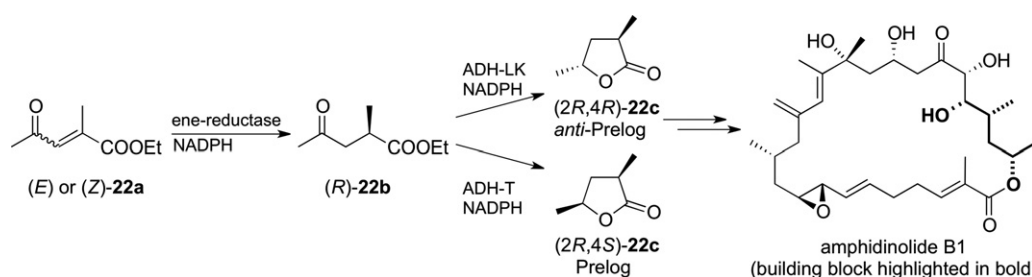


**Fig. 9.** A two-step one-pot cascade leading to γ-butyrolactones (**22c**).

et al., 2008) or Ru-catalysts (Pautigny et al., 2008). A biocatalytic equivalent was shown using ene-reductases. The reaction proceeded via strict (*R*)-stereoselective reduction of methyl 2-hydroxymethylacrylate derivatives (>99% *ee* in almost all cases; Fig. 10), with ene-reductases showing overall broad acceptance for this type of compounds (Stueckler et al., 2010c). Substrate engineering via hydroxyl-group protection (allyl-, benzyl- or TBDMS-ethers) enhanced the reaction rate significantly (up to >99% conversion) and hence allowed direct access to protected (*R*)-'Roche-Ester' (**23b**), a convenient intermediate for further synthesis.

Chiral α-halogenated carboxylic acids and esters are useful synthons since they can be transformed into a broad range of derivatives by stereospecific nucleophilic substitution reactions with nitrogen (Righi et al., 2006), oxygen (Hesek et al., 2009; Yang et al., 2001) and sulfur (Narendra et al., 2010; Seki et al., 2000) nucleophiles. Enantiopure α-haloesters in particular are valuable chiral synthons for the synthesis of several therapeutic agents used for the treatment of non-insulin dependent type 2 diabetes mellitus (T2DM) (Brenna et al., 2011c). Brenna and co-workers investigated the bioreduction of various methyl α-halo-β-substituted acrylates using isolated OYE1-3 and baker's yeast (Fig. 11) (Brenna et al., 2011b). OYE3 furnished the corresponding (*S*)-products in good to excellent stereoselectivity (*ee* ≥ 88%). The conversion strongly depended on the substitution pattern of the aromatic ring. In general, electron-donating groups on the ring lowered the reaction rate (**31a** and **33a**, conversion

up to 20%), while electron-withdrawing groups increased conversion levels (**28a–30a**, conversion 58–91%) in comparison with the non-substituted derivatives (**27a** and **32a**, conversion 37–38%). The latter can be explained by the varying degree of polarization of the C=C bond. Both chloro- and bromo-substituents at the α-position were accepted by the enzyme. (*S*)-α-Chlorocinnamates **28b** and **30b** were recovered from baker's yeast fermentation and subsequently transformed into non-natural D-phenylalanine derivatives (**28c** and **30c**), thus offering a new route to enantiomerically pure non-natural α-amino acid derivatives. A library of α,β-unsaturated α-halo esters bearing various alkyl chains was also tested (**34–37a**, Fig. 11) (Brenna et al., 2011c). Most interestingly, in contrast to the opposite stereopreference observed in baker's yeast-mediated reduction of (*E/Z*)-isomers of α,β-unsaturated α-chloroesters [(Utaka et al., 1989), also confirmed with isolated OYE1-3 acting on methyl 2-chloro-4-methylpent-2-enoate (Brenna et al., 2011a)], both (*E/Z*)-isomers of the α-bromo-analogs were converted to the (*S*)-product (*ee* up to 97%).

Enantiopure nitriles are versatile chiral building blocks due to their chemical reactivity, allowing further transformation into numerous functional groups (e.g. carboxylic acids, amines or aldehydes). For instance, nitrile **42b** contains a spiropiperidine backbone and is relevant for pharmaceutical research (Fig. 12) (Jia et al., 2007; Limanto et al., 2008; Lu et al., 2007). In a study with commercially available ene-reductases, the C=C bond of a series of α,β-unsaturated nitriles were reduced in high yields and stereoselectivities (up to 99% *ee*, Fig. 12) (Kosjek et al., 2008). While all
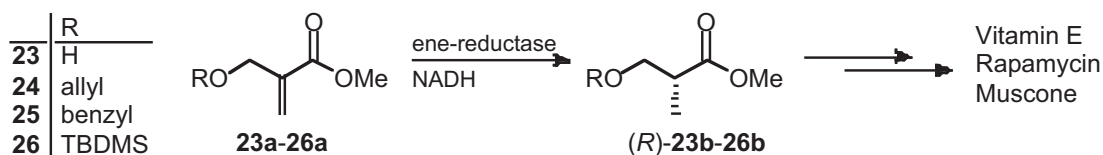


**Fig. 10.** Roche ester production via ene-reductases using OYE1-3, YqjM, NCR, NerA, OPR1, OPR3 and XenA.
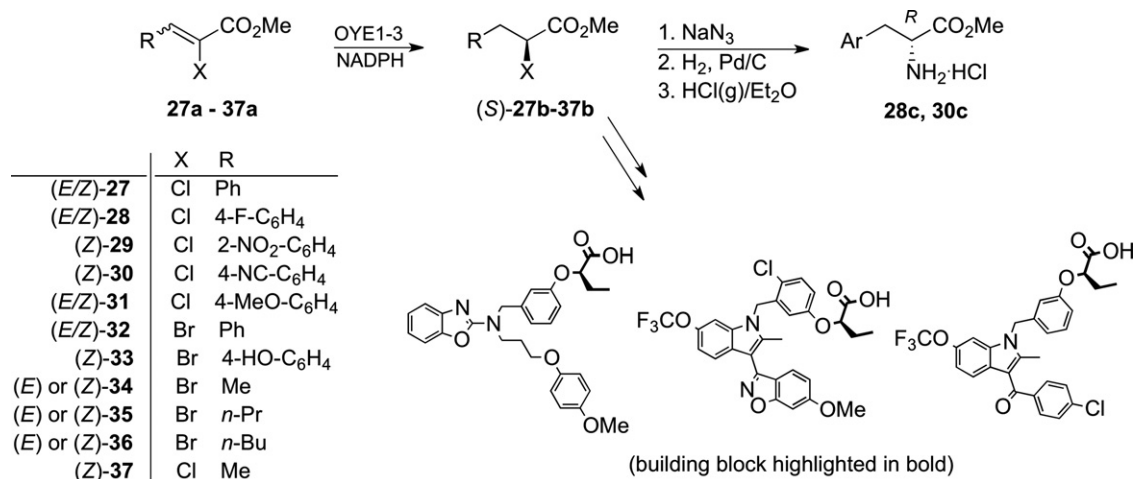
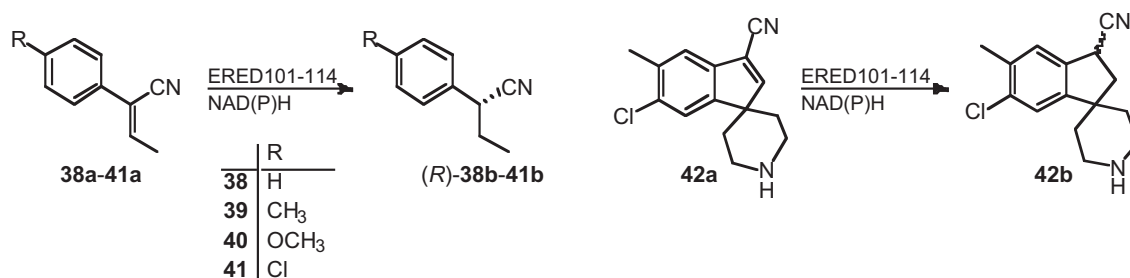**Fig. 11.** Ene-reductase-catalyzed reduction of α-halo-esters and further transformation into chiral products.



**Fig. 12.** Ene-reductase-catalyzed production of enantiopure nitriles.

enzymes showed (R)-selectivity for substrates **38a**–**41a**, the absolute configuration of **42b** was not assigned.

## 4. Concluding remarks

Ene-reductases from the 'Old Yellow Enzyme' family of flavoproteins have attracted increasing interest from synthetic chemists over the last years due to their exquisite chemo-, regio-, and stereoselectivities. Their use in the reduction of various α,β-unsaturated compounds has been successfully developed to allow the synthesis of enantiopure molecules with high synthetic value and industrial potential. The implementation of a nicotinamide cofactor regeneration renders the process cost-effective and highly competitive, while the availability of numerous OYE-homologues with stereocomplementary activities provides access to both enantiomeric forms of many synthons. Substrate engineering also revealed to be a powerful tool to control the stereoselectivity of the reaction.

With the advances of molecular biology tools, it can be expected that protein engineering applied to ene-reductases will further broaden their applicability. Structure-guided approaches combined with directed evolution have been recently used to enhance their catalytic properties, where few mutations were sufficient to reverse the stereopreference and/or increase reaction rates (Bougioukou et al., 2009, 2010; Hall and Bommarius, 2011). The remaining challenges to promote ene-reductases for large-scale applications are the improvement of enzyme stability under operational conditions – TTNs are still limited to $10^3$–$10^4$ (Yanto et al., 2010) – and the creation of successful 'designer bugs', where co-expression of ene-reductases and a suitable cofactor regeneration system will definitely establish these biocatalysts as robust and versatile synthetic tools for large-scale applications.

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