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The Organic–Synthetic Potential of Recombinant Ene Reductases: Substrate-Scope Evaluation and Process Optimization

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In this study an evaluation of the synthetic potential of a broad range of recombinant ene reductases was performed. In detail, a library of 23 ene reductases was used to screen the C=C reduction of 21 activated alkenes from different compound classes as substrates. The chosen set of substrates comprises nitroalkenes with an aryl substituent at the β-position and a methyl substituent at the α- or β-position, α,β-unsaturated carboxylic acids and their esters with and without substituents at the β-position, a range of cyclic α,β-unsaturated ketones with different ring sizes and substitution patterns and one α,β-unsaturated boronic acid. After we obtained insight into the substrate scope, several biotransformations were prioritised and further investigated in a screening of 41 reaction parameters (which included chaotropic and kosmotropic salts, polyols, buffer solutions, amino acids and organic solvents) towards their impact on the activity and enantioselectivity of the applied ene reductases. Under the optimised conditions, selected reduction processes were performed on an increased lab scale (up to 30 mL) with up to 10% substrate concentration, which led in general to both high conversion and (if chiral products were formed) enantioselectivity.

Introduction

During recent decades, ene reductases have become an efficient tool in organic synthesis for the enantioselective reduction of activated C=C bonds.[1–16] In the beginning of the studies on ene reductases the major focus was the identification of new ene reductases,[1,2] and numerous enzymes have been successfully identified, characterised and overexpressed recombinantly. Although many of those ene reductases have been applied in organic synthesis already, insight into their substrate scope mostly remains not thoroughly explored because of the use of only a narrow range of so-called model substrates.[1,2] Recently, however, an increased tendency in organic synthesis to apply ene reductases in target-driven asymmetric synthesis has been seen.[1,3] In such multi-step syntheses, reductions catalysed by ene reductase represent key steps within new retrosynthetic approaches to complex chiral molecules, for example, pharmaceuticals. Representative examples are new approaches towards β^2-amino acids[3a] (for which alternative chemocatalytic routes are still rare) as well as nitroalkane intermediates[3b] for the drugs tamsulosin and selegiline. Furthermore, ene reductases are also applied to an increasing extent in industry as underlined by the recent development of such a process technology at BASF[4] and recent process development work by Pfizer researchers on a route towards pregabalin.[5,6] The current accessibility of a range of enzymes in recombinant form and an expected broad synthetic utility (as alkenes activated by various electron-withdrawing groups as substituents represent potential substrates) are some of the advantages of ene reductases, which also make them attractive for applications in route screening in pharmaceutical research. However, it would be helpful to have a "synthetic-scope roadmap" of known recombinant ene reductases at hand to gain an insight into the synthetic properties of such enzymes. Such comprehensive screenings are rare in the literature with some notable exceptions from the Faber,[7] Bommarius,[8] and Bornscheuer and Mihovilovic groups.[9] Herein we report our results on the evaluation of 23 ene reductases available in recombinant form for the reduction of 21 substrates from different types of compound classes. In addition, we also report the process optimisation of selected biotransformations with prioritised ene reductases that result from the screening study.

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Results and Discussion

Enzyme screening

As one of the exciting features of ene reductases is their tolerance for a range of electron-withdrawing substituents at the C=C bond, we decided to test a variety of types of activated alkenes (21 substrates in total; Figure 1). In detail, we chose several nitroalkenes (**1**) that bear an aryl substituent at the β-position and a methyl substituent at the α- or β-position (as the resulting nitroalkane products can be converted by established methods into the corresponding amines, which also represent a class of products of pharmaceutical interest), α,β-unsaturated carboxylic acids and their esters with and without substituents at the β-position (**2**–**5** and **7**; which lead, e.g., to profene-type structures after C=C reduction in the case of **4** and **5**), a range of cyclic α,β-unsaturated ketones (**8**–**12**) with different ring sizes and substitution patterns and one boronic acid (**6**). An overview of the selected substrates **1**–**12** is given in Figure 1.

We used a library of 23 ene reductases that consist of literature-known recombinant ene reductases (OPR1,[10] OYE 2.6,[11] XenB,[12] NCR,[13] DBVPG,[14] OYE 3,[6] GOx-ER;[15] these enzymes were obtained by overexpression in *E. coli*; an overview of these enzymes and the applied plasmid systems is given in Table 1, entries 1–7 and 9) and 16 commercial ene reductases purchased from Codexis (Ene-101 to Ene-116;[16] Table 1, entry 8) as enzymes for the substrate screening. The enzymes were used as cell-free crude extracts (for overexpression of selected ene reductases, see Supporting Information) as this offers economic advantages over the use of highly purified enzymes as in the latter case additional enzyme purification step(s) are required, which make such a "biocatalyst formulation" less economically attractive. This is especially true if crude extracts of enzymes with high overexpression data are used. In addition, we also investigated the activity of the crude extract from native *E. coli* cells for each substrate because of potential "background reactions" of ene reductases from *E. coli* (as such an undesired side reaction has been reported[3b]).

Table 1. Screening enzymes, *E. coli* was used as host organism.

Entry	Enzyme[a]	Strain	Expression system
1	OPR1	*Lycopersicon esculentum*	pET28 or pQR1[b]
2	OYE 2.6	*Pichia stipitis*	pET28 or pQR1[b]
3	OYE3	*Saccharomyces cerevisiae*	pET28 or pQR1[b]
4	NCR	*Zymomonas mobilis*	pET28 or pQR1[b]
5	DBVPG	*Kazachstania lodderae*	pET28 or pQR1[b]
6	XenB	*Pseudomonas putida*	pET28 or pQR1[b]
7	GOx-ER	*Gluconobacter oxydans*	pET21a[c]
8	Ene-101 to Ene-116	–	–[d]
9	BL21(DE3)	*E. coli*	–[b]

[a] all enzymes were overexpressed in *E. coli* and used as cell-free crude extracts; [b] preparation performed by F. Hoffmann-La Roche Ltd., Biocatalysis research group; [c] preparation performed by the Hummel group; [d] purchased from Codexis.

In such a case, the use of highly purified enzymes would be preferred compared to the use of crude extracts to avoid undesired side reactions.

The screening was performed on a 0.5 mL scale using NADPH as a cofactor, glucose as co-substrate and a glucose dehydrogenase (GDH) to recycle the cofactor in situ. The reduction of each substrate was studied with all of the enzymes listed in Table 1.

The result of the initial screening of the 21 alkene substrates **1**–**12** with the 23 ene reductases is shown in Figure 2. The data given in the coloured boxes are related to the conversions with respect to the formation of the desired product, and the colours indicate the degree of conversion (which range from green for high conversion to red for no or very low conversion). A qualitative graphical indication if suitable enzymes were found for the corresponding compound classes is given in Figure 1 (blue: reasonable to high activity; red: no or low activity).

To start with the reduction of nitroalkenes, most of the enzymes accepted the investigated substrates **1a**–**d**. In particular, α-substituted nitroalkenes **1a**–**c** were suitable substrates for most of the ene reductases, whereas a stronger dependence on the type of enzyme was observed if we used α-unsubstituted and β-disubstituted nitroalkene **1d** as a substrate. Next, the enantioselectivity exemplified for nitroalkene **1a** was investigated, which led to the identification of the two promising enzymes NCR and Ene-111 that showed both high activity and enantioselectivity. Notably, the use of NCR and Ene-111 gave the opposite enantiomers of the corresponding nitroalkane (71% *ee* for the *R* enantiomer if NCR was used, and 84% *ee* for the *S* enantiomer if Ene-111 was used), which is of synthetic interest as both absolute configurations can be formed selectively by this reduction method catalysed by ene reductase.

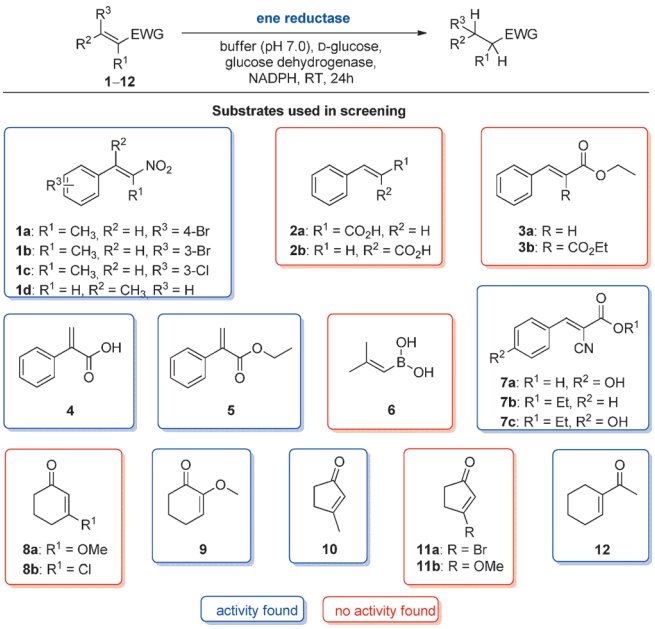


Figure 1. Overview of the substrates used in the ene reductase screening.

substrate	OPR1 (pET28)	OPR1 (pQR1)	OYE 2.6 (pET28)	OYE 2.6 (pQR1)	XenB (pET28)	XenB (pQR1)	NCR (pET28)	NCR (pQR1)	DBVPG (pET28)	DBVPG (pQR1)	OYE3 (pET28)	OYE3 (pQR1)	Ene-101	Ene-102	Ene-103	Ene-104	Ene-105	Ene-106	Ene-107	Ene-108	Ene-109	Ene-110	Ene-111	Ene-112	Ene-113	Ene-114	Ene-115	Ene-116	GOx-ER	BL21(DE3)
1a	96	94	14	35	96	97	97	96	97	11	6	97	73	86	35	94	59	67	68	42	57	64	91	39	86	51	84	43	90	12
1b	100	95	17	22	93	49	91	94	100	8	6	93	81	72	35	85	64	69	70	65	60	70	81	61	94	55	52	53	94	21
1c	97	98	7	22	98	43	99	98	99	5	3	98	80	68	35	89	68	75	72	70	51	62	77	62	96	52	58	60	96	13
1d	4	41	0	1	6	1	99	100	17	1	0	16	12	95	95	37	95	99	97	22	83	70	100	100	10	95	3	4	99	1
2a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2b	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
3a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3b	4	0	1	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	15	6	0	0	0	0	3	7	0	13	4	0	0	0	0	0	0	0	0	10	3	2	0	0	0	0	0	0	52	0
5	10	2	0	0	0	0	0	0	0	11	3	0	0	0	0	0	0	0	0	11	3	2	0	0	0	9	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7a	0	0	0	0	0	0	11	26	0	0	0	0	0	4	0	0	6	0	0	0	0	0	3	0	0	0	0	0	19	0
7b	37	60	3	3	36	6	3	5	14	2	2	13	17	5	15	11	12	5	84	70	48	41	32	17	19	89	5	4	40	2
7c	3	23	6	3	14	4	4	8	4	0	0	2	28	10	7	4	6	3	84	25	29	34	21	11	10	88	46	20	33	8
8a	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8b	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	2	14	27	2	5	1	13	36	2	1	0	1	1	26	12	2	85	20	84	11	84	89	54	88	8	41	2	1	10	0
10	0	1	1	0	0	0	4	11	1	1	1	1	1	9	9	1	49	7	2	2	17	15	33	32	2	1	1	2	11	0
11b	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	4	61	33	4	62	6	96	100	7	4	1	4	57	98	98	10	96	99	99	32	96	98	99	81	17	86	4	2	100	1

Figure 2. Results of the ene reductase screening. The numbers indicate product-related conversion (%; determined by GC or HPLC, for details, see the Supporting Information; defined as the ratio of the amount of product to the sum of the amounts of (remaining) substrate, (formed) product and all side products).

In contrast to the successful reduction of nitroalkenes with a range of enzymes, *cis*- and *trans*-cinnamic acid (**2a**,**b**) as well as *trans*-ester **3a** gave no or a negligible conversion in all cases (0–1% conversion). The more activated cinnamate derivative **3b** led to very low conversions of 4% at best. At first glance, it appears that a carboxylic acid function does not sufficiently activate the C=C bond for reduction. However, in contrast for the α-phenyl-substituted carboxylic acid and ester analogues **4** and **5**, the ene reductase screening yielded promising candidates for each substrate albeit with a low conversion of approximately 10–15% in most cases. Two enzymes, OPR-1 and Ene-108, accept acid **4** as well as ester **5**. This result is noteworthy as an acid (in deprotonated form) and ester moiety are very different types of functional groups with different requirements for binding in the active site of an enzyme. The highest conversion was found if GOx-ER was used with 54% conversion for the free acid **4**, although ester **5** was not tolerated as a substrate in this case. Interestingly, the enantioselectivities were high. For example, the reduction of **5** was performed with >99% *ee* using Ene-114, and GOx-ER, which is the only enzyme that showed activity towards **4**, also gave an excellent >99% *ee*.

For the α,β-unsaturated boronic acid **6**, however, no enzyme was suitable, whereas for (substituted and non-substituted) α-cyano cinnamic acid and esters **7a–c** a range of enzymes catalysed the desired C=C bond reduction with conversions of up to 89%. Although only moderate activities were found towards **7a**, in general, higher activities were observed if the alkene bears an ester instead of an acid moiety (**7b** and **7c**). For example, for **7b** as a model substrate, the highest activities were obtained with Ene-107 and Ene-114.

In the screening of different types of ketones (**8**–**12**) a strong dependence of the activity of the enzymes on their substitution pattern was observed. Although a halogen or methoxy as a substituent in the β-position of both the cyclic five- and six-membered ketones (**8** and **11**) led to no or negligible activity (0–1% conversion) with all of the applied enzymes, β-methyl-substituted cyclopentanone **10** was tolerated by some of them to lead to conversions of up to 49%. In addition, the use of cyclic enone **9**, which bears a methoxy substituent in the α-position, revealed numerous enzymes as suitable catalysts to lead to the reduced product with up to 89% conversion. Thus, it appears that cyclic ketones that are not substituted in the β-position are favoured substrates. As an example of a non-cyclic enone, compound **12** was studied and high conversions were achieved with a range of enzymes.

Reaction parameter screening

Next, we prioritised the most promising hits from the enzyme screening and for each of these biotransformations (which are based on the use of substrates **1a**, **4**, **7b** and **12**) we studied the influence of 41 reaction parameters on reactivity and enantioselectivity. The biotransformations were performed on a 1 mL scale using a glucose/GDH system to recycle NADPH in situ. As reaction parameters different buffer solutions (arranged in order of pH), chao- and kosmotropic salts (arranged according to their position in the Hofmeister series; see Supporting Information), polyols, amino acids and organic solvents (arranged according to their log*P* value; see Supporting Information) were investigated. Additives often have a significant impact on biotransformations, for example, as demonstrated by the influence of salt and solvent additives on enzyme activity studied by Dordick et al.,[17] Klibanov,[18] Halling et al.[19] and Bommarius et al.[20] For the example, in the latter case the influence of salt additives along the Hofmeister series as well as water-immiscible organic solvents on the enzyme activity of glucose dehydrogenase mutants was studied intensively.[20]

As a first reaction, we focused on the reduction of nitroalkene **1a** with the enzyme NCR (Figure 3). If NCR was tested with **1a** as the substrate, the initial "standard experiment" that was performed in piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer gave 35% conversion and 31% *ee* with preference for the *R* enantiomer. Among the salt additives, none of the applied salts led to an increase of the conversion. Although KCl and LiSCN caused the most significant decrease in activity, the kosmotropic sulfates were able to increase the enantioselectivity up to 45% *ee* (K_2SO_4). With regard to the tested water-miscible solvents, there was no significant correlation between log*P* and conversion. In biphasic systems, solvents with highest log*P* values gave the best results (cyclohexane: log*P* = 3.35, 26% conversion; heptane: log*P* = 4.40, 26% conversion). In general, however, organic solvents and salt additives did not result in a significant improvement (and sometimes caused a loss of activity and/or selectivity). We found that polyethylene glycol (PEG) had a positive influence on the reactivity and led to 42% conversion at expense of the enantioselectivity, which decreased to 15% *ee*. With regard to the enantioselectivity, a positive influence of 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) buffer was noted (20% conversion, 52% *ee*). Notably, a 20-fold increase of the amount of enzyme not only led to a higher conversion but also an increased enantioselectivity of up to 72% *ee* (Figure 3). The combination of beneficial parameters, namely, Tris buffer and a high enzyme loading, yielded an improved enantiomeric excess of 80% *ee* (Supporting Information).

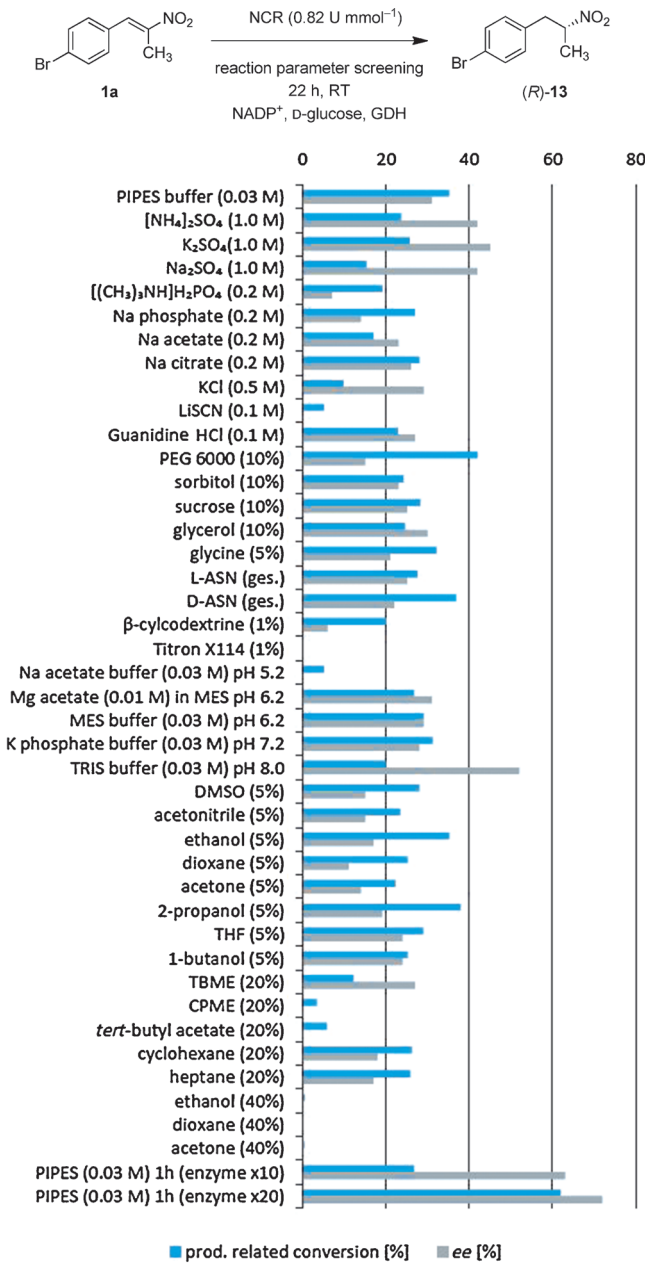


Figure 3. Reaction parameter screening with substrate **1a** and ene reductase NCR (enzyme×10 (20) means the use of a 10 (20)-fold amount of enzyme compared to the other experiments, see also Experimental Section).

This enantioselectivity of 80% is an excellent value as it is known[3b] that there is an *E. coli* background reactivity caused by the native *E. coli* ene reductase NemA in the case of nitroalkene reduction. In this study we gained an insight into the impact of this side reaction with **1b** as a substrate (Supporting Information) and found that the crude cells lysate produced the opposite enantiomer preferably compared to that if the ene reductase NCR was used. However, the conversions achieved with the *E. coli* crude cell extract were very low so the background reaction does not have a significant negative impact on the reduction if the ene reductase NCR is used (Figure 3). According to the initial screening (Figure 2), the *E. coli* background reactivity is negligible for most substrates except the nitroalkenes and **7c**. Furthermore, sodium dodecyl sulfate (SDS) gels of most applied enzyme formulations show good overexpression (Supporting Information), which is generally associated with a low impact of the *E. coli* background reaction. However, an effect that has to be considered is the racemisation of the produced nitroalkane (*R*)-**13** (which was addressed in the later process optimisation).

The reduction of nitroalkene **1a** was also studied with the ene reductase Ene-111 as the opposite enantiomer of the resulting product **13** is produced with this enzyme. Thus, with enzymes NCR and Ene-111 enantio-complementary biocatalysts are available that give selective access to both enantiomeric forms of nitroalkanes of type **13**. In the presence of Ene-111, the substrate **1a** was reduced to (*S*)-**13** with 21% conversion and 18% *ee* under the initial standard reaction conditions (Figure 4). Notably, there was a positive influence of glycerol and glycine as additives on the enantioselectivity. For example, the use of 5% of glycine gave a significantly improved enantioselectivity of 34% *ee* at a similar conversion of 19%. A dramatic negative impact on both conversion and enantioselectivity, however, was observed with a range of salts. The best conversions with salt additives were achieved with phosphates (20 and 19% conversion, respectively), but all other salts decreased enzyme activity significantly. In addition, the use of any type of (water-miscible or -immiscible) organic solvent led to a strong decrease of enantioselectivity and often also to a very low conversion. For example, acetonitrile gave 28% conversion and 0% *ee*, whereas 2-propanol led to 26% conversion and 3% *ee*. We found no correlation between log*P* and conver-

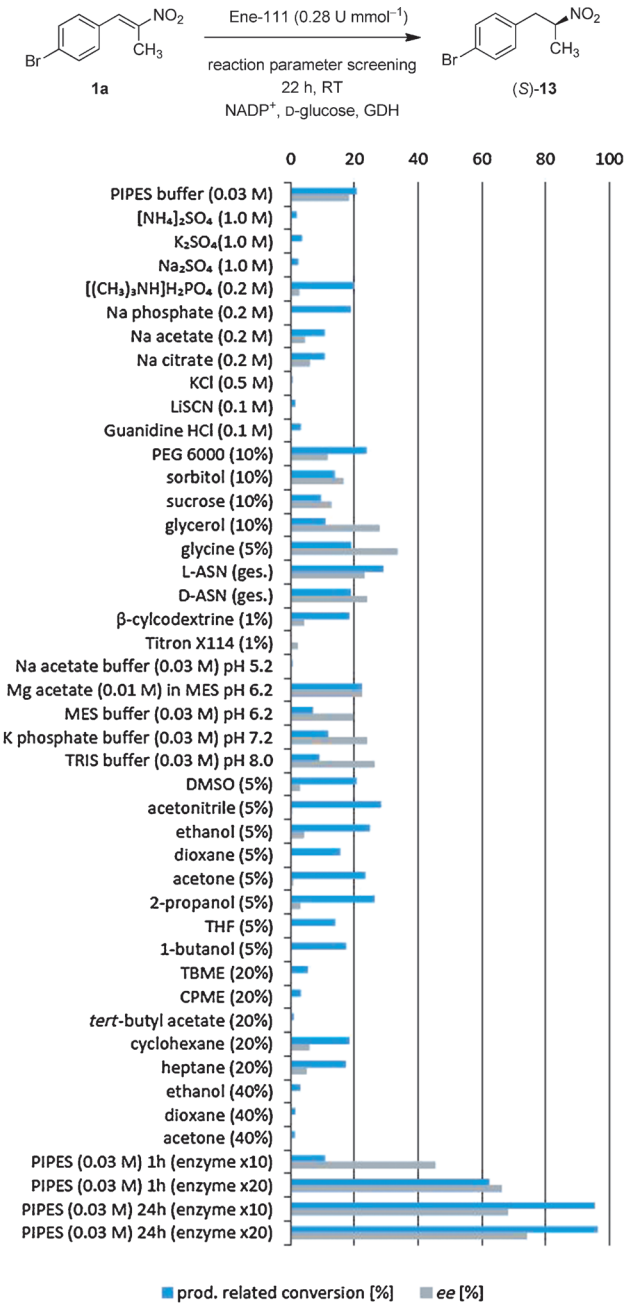


Figure 4. Reaction parameter screening results with substrate **1a** and Ene-111. Enzyme×10 (20) means, compared to the other experiments, a 10 (20)-fold amount of enzyme was added.

sion or enantioselectivity for water-miscible solvents. For water-immiscible solvents, the best conversions were achieved with cyclohexane (18%) and heptane (17%). As demonstrated before for the enzyme NCR, with Ene-111 both higher conversion and enantioselectivity (up to 74% *ee*) were obtained with an increased amount of enzyme, which indicates that the presence of an undesired side reaction (racemisation) might play a dominant role with a lower amount of enzyme. The combination of the beneficial parameters found in this study (Figure 4), namely, an increased amount of enzyme, glycine as an additive and 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer that contained Mg ions, further increased the activity and enantioselectivity (see Supporting Information and the next sub-chapter about process development).

A further biotransformation prioritised from the screening is the reduction of α-phenyl acrylic acid (**4**) with GOx-ER as an ene reductase (Figure 5). The resulting product **14** belongs to the compound class of α-aryl propanoates, which are of broad pharmaceutical interest because of the importance of, for example, (*S*)-naproxene and other profen drugs. Thus, the reduction of α-aryl acrylates offers an attractive option for their synthesis, which is complementary to "classical" synthetic approaches. To start with the reduction of **4** using GOx-ER under

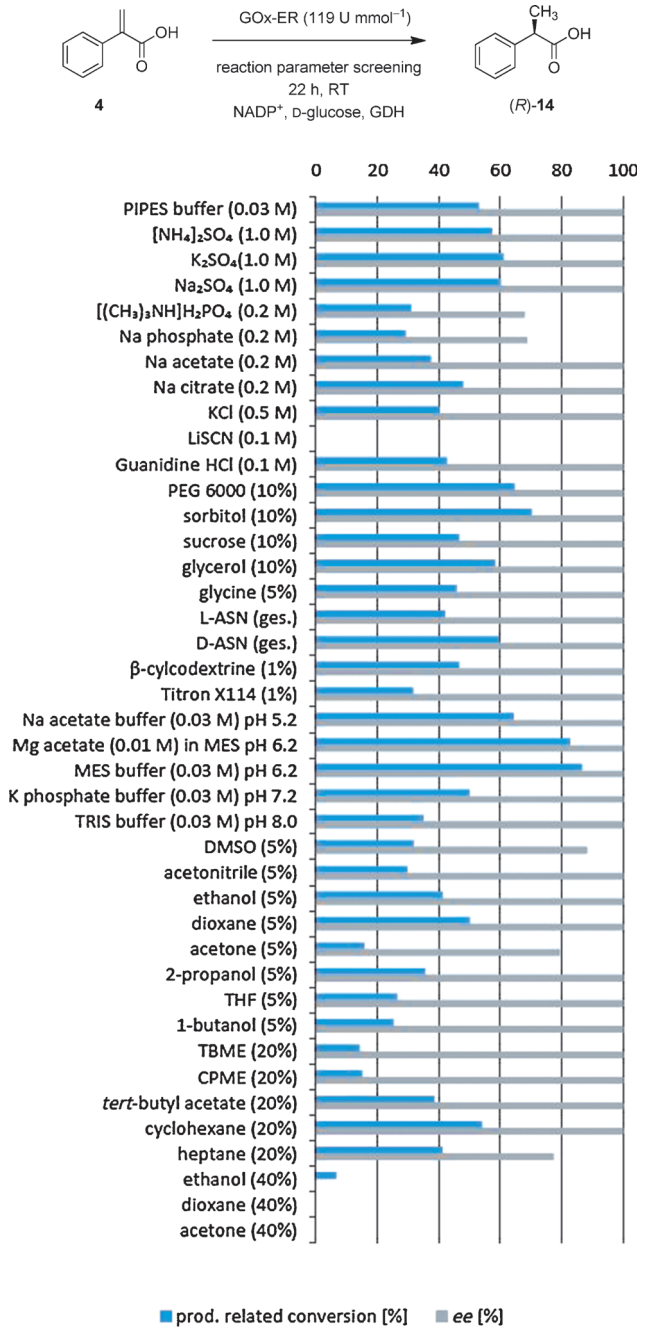


Figure 5. Reaction parameter screening results with substrate **4** and GOx-ER.

standard conditions, a conversion of 53% and an excellent enantioselectivity of >99% *ee* with preference for the *R* enantiomer[21] was obtained. Notably, independent of the reaction parameters changed in this screening, in nearly all cases the reduction of **4** proceeds with >99% *ee* (in contrast to previous results in nitroalkene reductions).

Among the salt additives, only the cosmotropic sulfates gave similar conversions to those achieved in case of the standard experiment. Chaotropic LiSCN led to complete enzyme deactivation. The enzyme activity and thus overall conversion was increased by additives such as PEG and sorbitol, which led to 65 and 70% conversion, respectively. Furthermore, the use of MES buffer (pH 6.2) was beneficial and led to 83 and 87% conversion in the presence and absence of magnesium acetate, respectively. In contrast, organic solvents in most cases led to a decrease of conversion as a result of enzyme deactivation, although a few solvents were compatible with the GOx-ER and led to similar or even slightly superior conversions (e.g., dioxane, log*P* = −0.27, 50% conversion; cyclohexane: 54% conversion). With a combination of MES buffer, PEG and cyclohexane, a further increase of conversion was achieved (Supporting Information).

Another prioritised reaction from the enzyme screening is the reduction of substrate **7b** with OPR1 as ene reductase (Figure 6).

Under the standard conditions, a conversion of 55% was achieved. As a high racemisation tendency can be assumed for product **15**, our focus on this reaction parameter screening was on conversion but not on enantioselectivity.

None of the tested salts was able to increase the conversion. The most negative effect was observed in the presence of very chaotropic salts (LiSCN) as well as strong kosmotropic sulfates. A positive influence towards activity was found if we used PEG (88% conversion), glycerol (70% conversion, however, at 8% impurity), D-asparagine (D-ASN; 69% conversion) and β-cyclodextrin (69% conversion) as an additive. In addition, an increased conversion was observed with Tris buffer (pH 8.0, 63% conversion but 4% impurity) and 2-propanol as a co-solvent (66% conversion). Solvents with high log*P* values of 1.43 (*tert*-butyl methyl ether; TBME) and higher led to a loss of enzyme activity. An interesting observation is the dependence of activity on the absolute configuration of a chiral additive: in a solution saturated with D-ASN a conversion of 69% was achieved, which is much higher that than obtained if L-ASN is used as an additive (23% conversion). In general, a decrease in conversion was observed if salt additives were used, and organic water-immiscible solvents gave a strong decrease and even complete loss of activity.

As a further biotransformation, the reduction of cyclohexene derivative **12**, which bears an acetyl moiety as an electron-withdrawing group, in the presence of the enzyme NCR was studied (Figure 7). Whereas a 66% conversion was achieved under standard reaction conditions, the applied salts decreased the conversion to 37–63%. However, there is no clear tendency with regard to the type of ion. The conversion could be increased significantly in the presence of additives such as PEG (81% conversion), sorbitol (78% conversion), glycine (77%

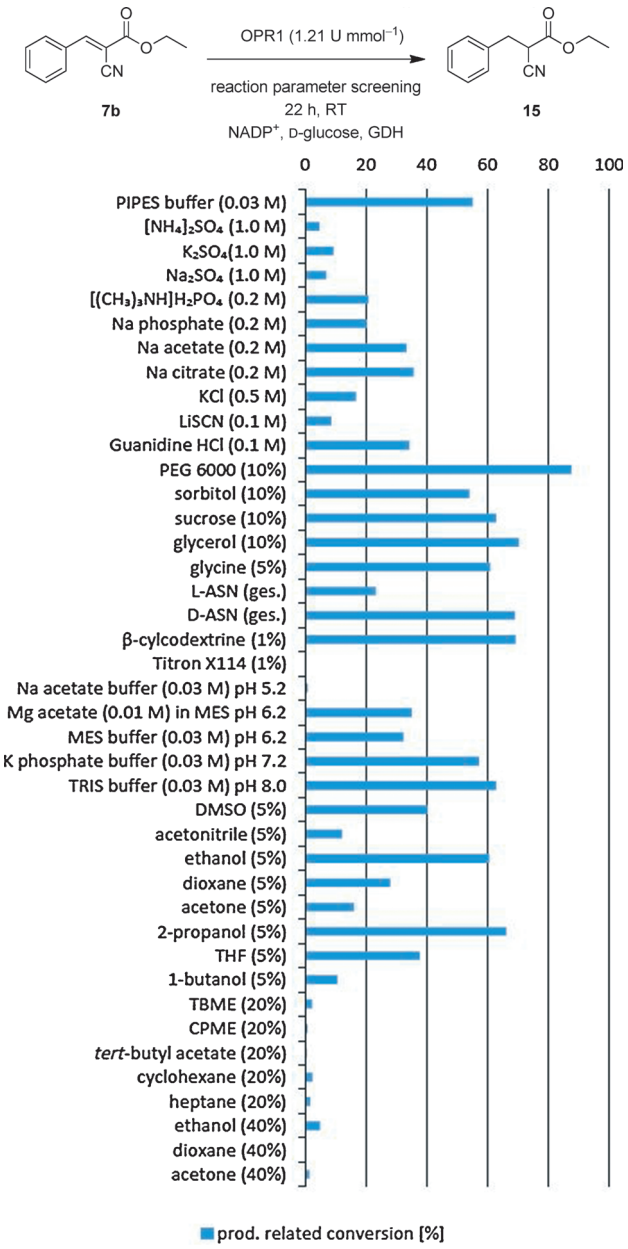


Figure 6. Reaction parameter screening results with substrate **7b** and OPR1.

conversion), L-ASN (74% conversion) and D-ASN (76% conversion). Phosphate buffer (pH 7.2, 76% conversion) or Tris buffer (pH 8.0, 74% conversion) are good alternatives to PIPES. Notably, with the exception of 1-butanol and the ethers TBME and cyclopentyl methyl ether (CPME), the enzyme NCR shows a sufficient stability towards many organic solvents. For example, in the presence of 5% DMSO a still high conversion of 70% was observed, and the use of ethanol (67% conversion) as well as THF, acetonitrile, dioxane, 2-propanol, acetone, heptane and cyclohexane (51–56% conversion) is also tolerated by the enzyme. In summary, there is no clear correlation between log*P* and enzyme activity. In terms of a synergetic effect of beneficial reaction parameters, the combination of phosphate buffer and PEG showed a high conversion of 81% and only

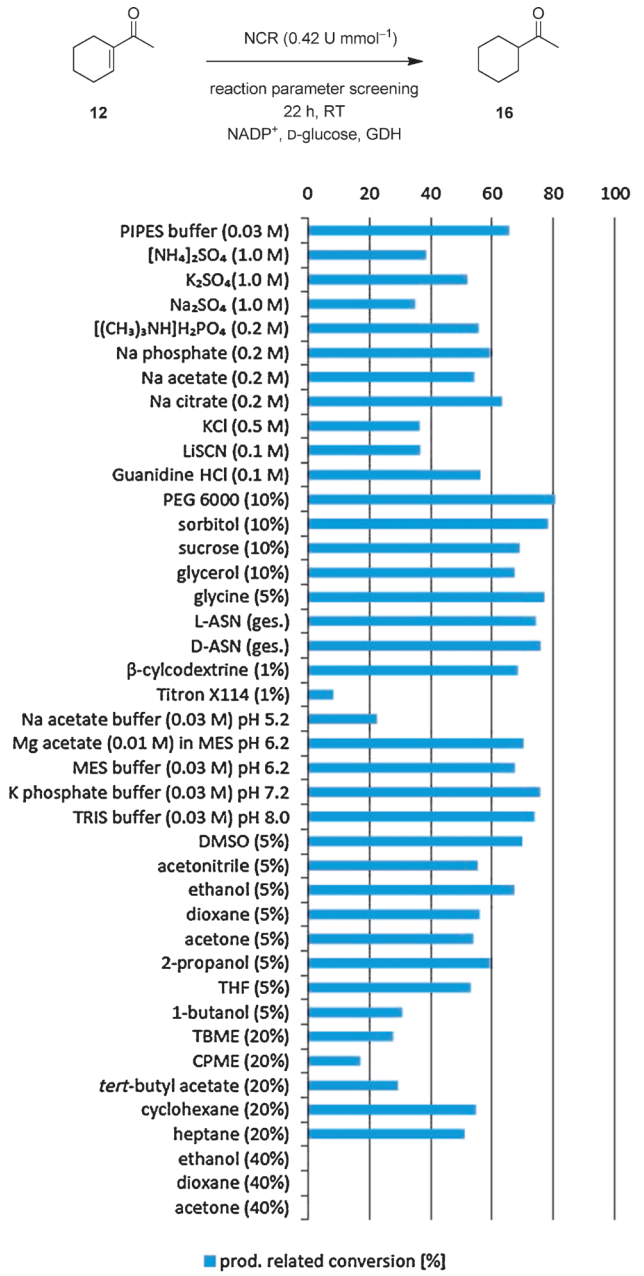


Figure 7. Reaction parameter screening results with substrate **12** and NCR.

a minimal loss in activity in the presence of additional amounts of cyclohexane and DMSO (74 and 76% conversion, respectively; Supporting Information).

Furthermore, we also performed the reaction parameter screening for the following substrate/enzyme combinations: (i) the reduction of **5** with Ene-114, (ii) the reduction of **7c** with Ene-107 and (iii) the reduction of **7c** with Ene-114. Detailed data are given in the Supporting Information. Briefly, if the ene reductase Ene-114 was used for the reduction of α-phenyl acrylic acid (**5**) as a substrate under standard conditions, a conversion of 32% (with 2% impurity) and an excellent enantioselectivity of >99% *ee* were obtained. A positive impact on activity was found for the additives sodium sulfate (47% conversion, however, at 12% impurity; >99% *ee*) and glycine (49% conversion, 11% impurity). The use of MES buffer with (58% conversion, 19% impurity, >99% *ee*) and without magnesium acetate (55% conversion, 10% impurity, >99% *ee*) was also beneficial. In terms of enantiospecificity, we found that Ene-114 and GOx-ER both have the same enantiopreference, thus forming the *R* products.

In the reduction of **7c** with Ene-107 under standard reaction conditions, a conversion of 55% was observed, which was only increased if PEG was used as an additive (68% conversion).

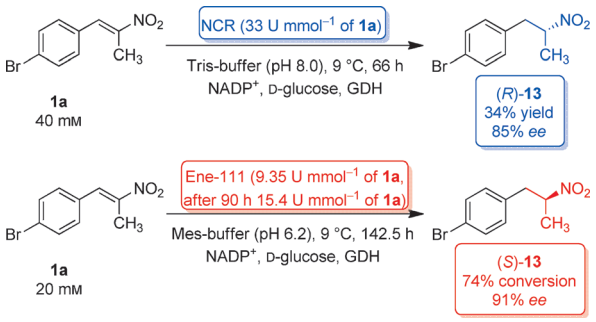
In contrast, the activity decreased in the presence of organic solvents. Interestingly, if we compare L-ASN and D-ASN as additives, we found a lower reactivity in the presence of L-ASN (7% conversion) compared to that with D-ASN (56% conversion) in analogy to the reduction of **7a** with OPR1.

If the enzyme Ene-114 was used for the reduction of **7c**, 32% conversion under standard reaction conditions was observed. As additives, the presence of PEG (39% conversion) and D-ASN (35% conversion) had a positive influence on activity. The study revealed no other organic solvent that led to an increased conversion, but the enzyme Ene-114 showed sufficient stability towards ethanol, DMSO and 2-propanol with 29, 27 and 31% conversion, respectively.

Process development

After we had identified suitable reaction parameters in the reaction parameter screening for each of the prioritised biotransformations, we decided to perform the reductions of **1a**, **4**, **7b** and **12** at an increased preparative scale of 30 mL under the optimised reaction conditions. Enzymatic reactions conducted at such an increased lab scale give an insight into their robustness under preparative process conditions and are, therefore, of value in further scale-up work.

To start with the synthesis of nitroalkane (*R*)-**13**, which has a stereogenic centre in the C–H acidic α-position, and a substrate concentration of 40 mM (10 g L^{-1}) was chosen in combination with the use of the ene reductase NCR as biocatalyst and Tris buffer with pH 8.0, which was the most suitable in the screening. In our previous study on the enzymatic reduction of this type of nitroalkanes, a decrease of the reaction temperature was beneficial to improve the enantioselectivity (probably caused by the suppression of racemisation).[3b] Accordingly, in this study (on a 30 mL scale) we performed the reduction at 9 °C (Scheme 1). The resulting product (*R*)-**13** was isolated in

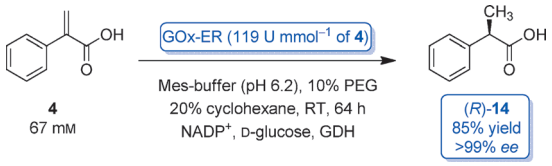


Scheme 1. Biocatalytic reductions of **1a** on a preparative scale.

34% yield with a high enantioselectivity of 85% *ee*, which is the highest enantioselectivity reported so far for the reduction of this substrate.

To obtain the opposite enantiomer (*S*)-**13**, we reduced **1a** with Ene-111 under the optimised reaction conditions (MES buffer pH 6.2) at a substrate concentration of 20 mM (5 g L^{-1}). In addition, the reaction was performed at a low temperature of 9 °C to obtain the desired nitroalkane (*S*)-**13** with 74% conversion and excellent enantioselectivity of 91% *ee* (Scheme 1). Thus, if we used Ene-111, we also could overcome the literature-known limitation[3b] of the enzyme GOx-ER, which shows a decreased enantioselectivity for *para*-substituted α-methylated nitroalkenes. Accordingly, Ene-111 is a promising catalyst for the highly *S*-enantioselective reduction of **1a** and its combination with the *R*-enantioselective ene reductase NCR provides a stereo-complementary set of catalysts for the biocatalytic reduction of **1a** and related nitroalkenes to lead to high enantioselectivities of the resulting nitroalkanes. As these products can be subsequently converted chemically into the corresponding amines, both the *R* and *S* enantiomers of such types of amines are accessible through such a chemo-enzymatic pathway.

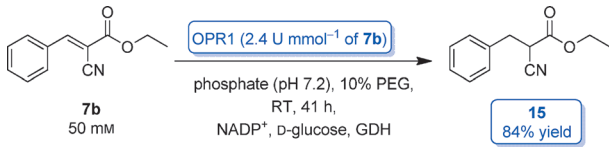
For the biocatalytic reduction of α-phenyl acrylate **4** the enzyme GOx-ER, which was identified to be suitable for this transformation in the screening, was used. If we performed the reduction at a substrate concentration of 67 mM (10 g L^{-1}) we were pleased to find that the product (*R*)-**14** was formed with complete conversion and an excellent enantioselectivity of >99% *ee* (Scheme 2). After work-up, the desired product (*R*)-**14** was obtained in 85% yield in an enantiomerically pure form.



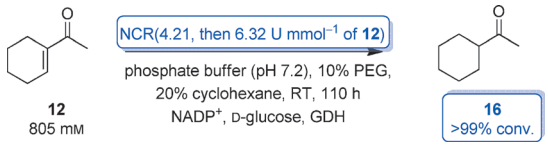
Scheme 2. Biocatalytic reduction of **4** on a preparative scale.

In addition, we chose the reduction of **7b** as a further reaction of interest for the preparative biotransformations. If we conducted this biotransformation using the ene reductase OPR1 as a preferred enzyme at a substrate concentration of 50 mM (10 g L^{-1}) we found a quantitative formation of the α-cyano ester **15**, which was subsequently isolated in 84% yield (Scheme 3).

As we were also interested in the impact of substrate loading as well as water-immiscible co-solvents, the biocatalytic reduction of enone **12** in the presence of the ene reductase NCR and 20% cyclohexane as a co-solvent was performed at an elevated substrate concentration of 805 mM (100 g L^{-1}; Scheme 4). Notably, this reaction proceeded with complete conversion, which underlines the compatibility of the ene reductase NCR towards both a large solvent volume and high substrate loading.



Scheme 3. Biocatalytic reduction of **7b** on a preparative scale.



Scheme 4. Biocatalytic reduction of **12** on a preparative scale.

Conclusions

We successfully screened 23 recombinant ene reductases for the C=C bond reduction of 21 activated alkenes as substrates, which comprised nitroalkenes, carboxylic acids and esters, α-cyano cinnamic acid derivatives, a range of enones and an α,β-unsaturated boronic acid. We identified enzymes with high enantioselectivity for numerous alkene substrates and further investigated the influence of 41 parameters for the selected biotransformations prioritised after the initial screening. After we had identified suitable reaction parameters for each of the chosen enzymes and model reactions, for example, additives that increase the activity and/or the enantioselectivity, we were able to perform a range of biocatalytic C=C reductions of several substrates from different compound classes (namely, **1a**, **4**, **7b** and **12**) on an increased laboratory scale successfully. Therein, substrate loading was varied between 5 and 100 g L^{-1}. For example, the synthesis of both enantiomers of an α-methylated nitroalkane was achieved with 85% *ee* (for (*R*)-**13**) and 91% *ee* (for the opposite (*S*)-**13**), which are the highest reported values for the biocatalytic reduction of this substrate so far. In addition, the reduction of an alkene (**12**) at a high substrate concentration (805 mM, 100 g L^{-1}) and in the presence of a water-immiscible co-solvent (cyclohexane, 20%) was demonstrated, which underlines that ene reductases (in this case NCR) can tolerate both high substrate loading and the presence of organic solvents. Thus, this study further evidences the suitability of ene reductases as efficient (bio)catalysts in the reduction of a range of activated alkenes from different compound classes and accordingly broadens the knowledge on the potential of ene reductases as catalysts in organic synthesis.

Experimental Section

Initial ene reductase screening in 2 mL deep-well plates

Ene reductase crude cell extracts were dissolved, pipetted into 2 mL deep-well (DW) plates (1 mg of OPR1, OYE2.6, XenB, NCR, DBVPG, OYE3, BL21(DE3); 0.5 mg of Ene-101 to Ene-116) and lyophilised. For the ene reductase screening, an aqueous solution of various components (475 µL; pH 7.0, 0.1 M phosphate buffer,

0.25 M D-glucose, 0.34 mM NADPH, 2.5 mg/100 mL glucose dehydrogenase CDX901) and the corresponding substrate (0.5 mg; dissolved in 25 μL of a suitable solvent) were added, and the plates were incubated at RT on a plate-shaker at 750 U/min. After 24 h, acetonitrile (0.5–1.0 mL) was added (for substrates analysed by HPLC), and the conversion was determined by HPLC, or the mixtures were extracted into ethyl acetate (0.5 mL), and the product-related conversion was determined by GC (for details, see the Supporting Information). The product-related conversion was defined as the ratio of the amount of product to the sum of the amounts of (remaining) substrate, (formed) product and all side products.

Reaction parameter screening

In a 2 mL glass tube, a mixture of D-glucose (0.3 mmol), an aqueous solution of the corresponding additive (1.41 mL), $NADP^+$ (0.5 μmol; dissolved in 10 μL H_2O), GDH CDX901 (36 μg; dissolved in 10 μL H_2O), the corresponding enzyme (dissolved in 20 μL H_2O) and substrate (dissolved in 50 μL of a suitable solvent) was incubated in an overhead shaker for 22 h at RT. The substrate/enzyme ratio was adjusted to a product-related conversion between 30 and 60%. The reaction mixture was extracted into ethyl acetate and analysed by GC or HPLC, for details, see the Supporting Information). The product-related conversion (abbreviation: prod. related conversion) was defined as the ratio of the amount of product to the sum of the amounts of (remaining) substrate, (formed) product and all side products.

Conversion of 1a on a preparative scale

(*E*)-1-Bromo-4-(2-nitroprop-1-enyl)benzene (**1a**, 300 mg, 1.24 mmol) was mixed with 2-propanol (1 mL) for 5 min. Tris buffer (27 mL, 0.03 M, pH 8.0) and D-glucose monohydrate (3.0 g, 15 mmol) were added, and the resulting mixture was cooled to 9 °C. Afterwards $NADP^+$ (6.0 mg, 8.1 μmol), glucose dehydrogenase (CDX901, 6.0 mg) and NCR (240 mg; enzyme activity: 0.17 U/mg of enzyme sample, determined spectrophotometrically using *trans*-2-hexenal as a substrate) were added. The mixture was stirred at 9 °C, and the pH was kept constant by titration of 1.0 M NaOH solution. After 66 h, the mixture was decanted from the yellow solid, and the solution was stirred with 5 g Dicalite and 30 mL ethyl acetate. After filtration over Dicalite, the phases were separated and the aqueous phase was extracted with ethyl acetate twice more. The combined organic layers were dried over magnesium sulfate, and the solvent was evaporated. The product (*R*)-**13** was obtained in 34% yield and 85% *ee*. The same protocol was used for the reduction of (*E*)-1-bromo-4-(2-nitroprop-1-enyl)benzene (150 mg, 0.62 mmol) with Ene-111 (20 mg; enzyme activity: 0.29 U/mg of enzyme sample, determined spectrophotometrically using *trans*-2-hexenal as a substrate) in MES buffer (0.03 M, pH 6.2, 0.01 M magnesium acetate, 5% glycine). After 90 h additional $NADP^+$ (6.0 mg, 8.1 μmol), GDH CDX901 (6.0 mg) and Ene-111 (13.0 mg) were added. The overall reaction time was 142.5 h. The product (*S*)-**13** was obtained as a crude mixture with 74% conversion and 94% *ee*. The absolute configuration was determined by comparison with data reported previously.[3b]

Conversion of 4 on a preparative scale

2-Phenylacrylic acid (**4**, 300 mg, 2.02 mmol) was suspended in MES buffer (pH 6.2, 30 mM, 10% PEG, 21 mL) and D-glucose monohydrate (3.0 g, 15 mmol), $NADP^+$ (6.0 mg, 8.1 μmol), glucose dehydrogenase (CDX901, 6.0 mg) and GOx-ER (600 mg; enzyme activity: 0.4 U mg^{-1} of enzyme sample, determined spectrophotometrically using *trans*-2-hexenal as a substrate) and cyclohexane (6.0 mL) were added. The mixture was stirred at RT, and the pH was kept constant by continuous titration of NaOH (1.0 M). After 64 h, the reaction mixture was acidified to pH 1, Dicalite (5 g) and ethyl acetate (30 mL) were added and the mixture was filtered over Dicalite. The phases were separated, and the aqueous phase was extracted into ethyl acetate twice more. The combined organic layers were dried over magnesium sulfate, and the solvent was evaporated. The product (*R*)-**14** was obtained in 85% yield (93% purity) and >99% *ee*. The absolute configuration was determined to be *R* by comparison of the optical rotation of −57° with data reported previously.[21]

Conversion of 7b on a preparative scale

(*E*)-Ethyl 2-cyano-3-phenylacrylate (**7b**, 300 mg, 1.5 mmol) and D-glucose monohydrate (3.0 g, 15 mmol) were dissolved in phosphate buffer (pH 7.2, 30 mM, 10% PEG, 27.0 mL). 2-Propanol (0.5 mL), $NADP^+$ (6.0 mg, 8.1 μmol), glucose dehydrogenase (CDX901, 6.0 mg) and OPR1 (60 mg; enzyme activity: 0.06 U mg^{-1} of enzyme sample, determined spectrophotometrically using *trans*-2-hexenal as a substrate) were added. The mixture was stirred at RT, and the pH was kept constant by continuous titration of NaOH (1.0 M). After 41 h, the reaction mixture was acidified to pH 1, Dicalite (5 g) and ethyl acetate (30 mL) were added, and the mixture was filtered over Dicalite. The phases were separated, and the aqueous phase was extracted with ethyl acetate twice more. The combined organic layers were dried over magnesium sulfate, and the solvent was evaporated. The product **15** was obtained in 84% yield.

Conversion of 12 on a preparative scale

D-Glucose monohydrate (6.0 g, 30 mmol) was dissolved in phosphate buffer (pH 7.2, 30 mM, 10% PEG, 21 mL), and $NADP^+$ (30.0 mg, 40 μmol), glucose dehydrogenase (CDX901, 30 mg) and NCR (600 mg; enzyme activity: 0.17 U mg^{-1} of enzyme sample, determined spectrophotometrically using *trans*-2-hexenal as a substrate) were added. In addition, 1-(cyclohex-1-en-1-yl)ethanone (**12**, 3.0 g, 24.2 mmol) was dissolved in cyclohexane (6 mL) and added to the reaction mixture. The mixture was stirred at RT, and the pH was kept constant by continuous titration of NaOH (1.0 M). After 91 h, additional $NADP^+$ (30.0 mg, 40 μmol), glucose dehydrogenase (CDX901, 30 mg) and NCR (300 mg) were added. After an overall reaction time of 110 h, Dicalite (5 g) and ethyl acetate (30 mL) were added, and the mixture was filtered over Dicalite. The phases were separated, and the aqueous phase was extracted with ethyl acetate twice more. The combined organic layers were dried over magnesium sulfate, and the solvent was evaporated at 800 mbar. The conversion and yield were calculated from NMR spectroscopic data, and accordingly **16** was formed with >99% (product-related) conversion and in 99% yield (97% purity).

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Keywords: alkenes · asymmetric catalysis · biotransformations · enzyme catalysis · reduction

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