Enantioselective Synthesis of (*R*)-2-Arylpropanenitriles Catalysed by Ene-Reductases in Aqueous Media and in Biphasic Ionic Liquid–Water Systems

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Introduction

A recent perspective,[1] outlined by the medicinal chemistry subgroup of the American Chemical Society's Green Chemistry Institute Pharmaceutical Roundtable, has highlighted the advantages of using biocatalysed synthetic methods, not only for the optimisation of sustainable drug manufacturing processes but also at the medicinal chemistry level. Typical features of enzyme-mediated transformations are: 1) exquisite chemo-, regio-, and stereoselectivity, which decreases the number of reaction steps required and enables high stereoisomeric purity values; 2) mild reaction conditions, which usually avoid extensive heating and high pressure; 3) high synthetic potential; and 4) the renewability and biodegradability of enzymes themselves.

A possible approach to promote the use of biocatalysis, either in the early stage of drug development or during the optimization of the process route, is to enrich the enzyme toolbox available to synthetic chemists to perform classical organic transformations. For example, the stereoselective reduction of suitably substituted C=C bonds is one of the best methods to introduce stereogenic centres in the synthesis of chiral compounds. The first approach to the enzyme-mediated variant of this reduction was based on the exploitation of the resting cells of various microorganisms, particularly those of baker's yeast (BY; *Saccharomyces cerevisiae*).[2] The enzymes responsible for this reaction (including those present in BY) are called ene-reductases (ERs; EC 1.6.99.1), and most of them belong to the well-known family of old yellow enzymes (OYEs),[3] which are characterised from a biochemical viewpoint since the 1930s, but only recently studied in detail for their use in preparative biocatalysis applications.[4]

These enzymes can reduce suitably substituted alkenes with high conversion and excellent enantiomeric purities that are activated towards this bioreduction by one or two electron-withdrawing groups (EWGs). The effects due to the stereochemistry of the double bond as well as due to the steric and electronic properties of the activating EWGs and of other substituents are currently under investigation[5] so as to define the limits and the potential of this kind of enzyme-mediated reaction.

With this aim, we investigated the capability of ERs to reduce C=C bonds bearing a nitrile moiety as an EWG, which affords valuable enantiopure building blocks for synthetic applications. The relevance of this study lies mainly in the fact that the nitrile functional group is a pharmacophore in many biologically active compounds[6] and a versatile precursor of other functionalities such as carboxylic acids, amides, aldehydes, ketones, and amines. A significant interest exists in the optimisation of highly selective synthetic methods to prepare or manipulate nitrile derivatives,[7] especially the chiral ones.[8]

Herein, we report on the optimisation of the enzyme-mediated stereoselective reduction of suitably substituted unsaturated nitriles, which represents a novelty in the field of biocatalysis because ERs have been rarely reported (see the next section) to be active in the conversion of unsaturated nitriles with no other EWGs on the C=C bond.

This enzymatic method is also an improvement in organic synthesis. Despite recent advances in the metal-catalysed stereoselective hydrogenation, the development of effective

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methods in this area for the reductions of unsaturated nitriles is still a challenge because of their intrinsic low reactivity[9] and the linearity of the CN group. Owing to their electronic structure, nitriles prefer end-on coordination to metal ions, which is not suitable for the hydrogenation of a conjugated double bond.[10] Only a few reports exist in the literature of this field,[9,10] and in some cases, an additional functionality, such as a carbonyl or an alcohol group, was required to enable the coordination of the substrate to the metal of the catalyst.[11] Furthermore, the chemoselection between the olefinic double bond and the nitrile group in hydrogenation poses additional problems.

Results and Discussion

The few data found in the literature indicated that the presence of the CN moiety as the sole activating group of the double bond is not enough to promote reduction, except the α-methylene nitrile derivative **1a**. Specifically, BY was shown to catalyse the conversion of 2-phenyl-2-propenenitrile (**1a**) into the *R* enantiomer of the corresponding reduced product with high *ee* and 64% yield in the presence of petroleum ether.[12] No further investigation was performed on this class of compounds.

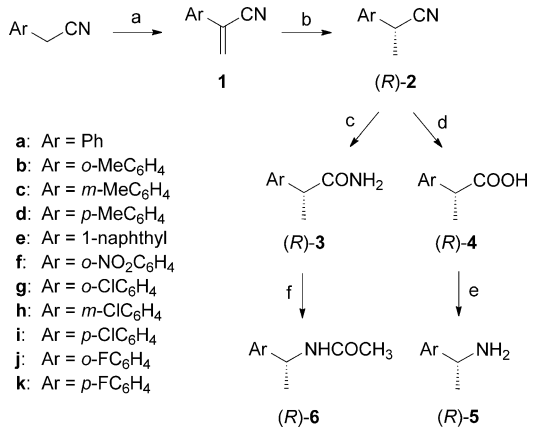
In other reports involving isolated ERs of the OYE family, pentaerythritol tetranitrate reductase and OYE1 were described to be unable to reduce *trans*-cinnamonitrile and estrogen-binding protein EBP1 did not reduce crotonitrile.[13] In 2008,[14] a range of commercially available NAD(P)H-dependent ERs were used to reduce various (*Z*)-*p*-substituted phenyl butenenitriles to the corresponding *R* enantiomers with good to high enantioselectivities (70–99% *ee*). A known pharmaceutical synthon (6-chloro-5-methylspiro[1*H*-indene-1,4′-piperidine]-3-carbonitrile) was also submitted to the same reaction, although the absolute configuration of the product was not determined. Two contemporary reports have described the possibility of reducing the C=C bond of α,β-unsaturated nitriles by ERs with an ester group present as a substituent at the olefinic carbon atom in the β position.[15,16]

With the aim of exploring the biocatalysed synthesis of chiral nitriles, we prepared α-methylene nitrile derivatives **1a–k** and submitted them to the biocatalysed reduction. We envisaged the possibility of exploiting their enantioselective reduction to create a benzylic stereogenic centre, bearing a methyl group in this position, for the preparation of chiral building blocks to be used in the synthesis of active pharmaceutical ingredients.

Synthesis of the substrates

α-Methylene nitrile **1a–k** were prepared through the reaction of the arylacetonitrile with paraformaldehyde, potassium carbonate, and tetra-*n*-butylammonium iodide in toluene at 80 °C (Scheme 1).

As for the *para*-substituted derivatives, the isolation of the desired compound was made more difficult in some cases because of the occurrence of a dimerisation process, which likely involves a [2+2] cycloaddition of the methylenic double bonds of the two molecules of acrylonitrile.



Scheme 1. Synthesis and bioreduction of nitriles **1a–k**, and synthetic manipulation of the products. Reagents and conditions: a) $(CH_2O)_n$, toluene, K_2CO_3, nBu_4NI; b) ER reduction; c) H_2O_2, Na_2CO_3, H_2O; d) HCl (37%), reflux; e) diphenylphosphoryl azide, toluene, 80 °C; then HCl (10%), 60 °C; f) *N*-bromosuccinimide, 1,8-diazabicyclo[5.4.0]undec-7-ene, MeOH, reflux.

The reference racemic propanenitriles **2a–k** were obtained either by the hydrogenation of the methylenic parent compound on Pd/C or by alkylation of the starting arylacetonitrile with sodium hydride and methyl iodide in THF.

BY and OYE1–3 bioconversions in aqueous systems

The α-methylene nitrile derivatives **1a–k** were submitted to the bioreduction with whole-cells BY in aqueous medium by adsorbing the substrate on a hydrophobic resin (polystyrene XAD1180N) to ensure its slow release in the aqueous phase, which prevented toxic and inhibitory effects on the cells. Parallel experiments were performed with OYE1–3 in aqueous medium with DMSO as a cosolvent. In view of designing a scalable and convenient process, we were not interested in performing the reactions with stoichiometric amounts of the expensive NADPH cofactor required by the enzyme as a source of hydride; this problem was circumvented by using glucose dehydrogenase (GDH) as a regeneration enzyme and glucose as a cheap cosubstrate. This cofactor regeneration system enables the use of the cheaper oxidised form of the cofactor ($NADP^+$) only in a catalytic amount. The results of all the bioreduction experiments in aqueous systems are compared in Table 1.

The enantioselectivity of the bioreduction was 99% for most substrates, by using either BY or isolated ERs. These value decreased slightly in the reaction of halogen-substituted derivatives and OYE1–3, except in the case of *o*-Cl and *o*-F compounds. Enantioselectivity decreased considerably with the *o*-NO_2 derivative **1f**, which gave a racemic material upon reacting with OYE2 and OYE3, and only modest values of enantioselectivity (50–72%) were obtained with OYE1 and BY.

Similar conversion values (60–90%) were obtained by using either BY or OYE1–3 with Ph, *p*-Me, and *m*-Cl derivatives **1a**, **d**, and **h**. As for other biotransformations, the highest conversion

Table 1. Results of the biocatalysed reductions of substrates **1a–k** in aqueous systems.

Substrate	Aryl group	BY		OYE1		OYE2		OYE3	
		c[a] [%]	ee[b] [%]	c[c] [%]	ee[b] [%]	c[c] [%]	ee[b] [%]	c[c] [%]	ee[b] [%]
1a	phenyl	52	99 (*R*)	65	99 (*R*)	72	99 (*R*)	65	99 (*R*)
1b	*o*-tolyl	95	99 (*R*)	99	99 (*R*)	52	99 (*R*)	17	99 (*R*)
1c	*m*-tolyl	90	98 (*R*)	52	99 (*R*)	52	99 (*R*)	94	99 (*R*)
1d	*p*-tolyl	90	95 (*R*)	91	99 (*R*)	95	99 (*R*)	94	99 (*R*)
1e	1-naphthyl	82	99 (*R*)	65	99 (*R*)	49	99 (*R*)	5	–
1f	*o*-nitrophenyl	31	72 (*R*)	96	50 (*R*)	94	*rac*	5	*rac*
1g	*o*-chlorophenyl	74	99 (*R*)	96	99 (*R*)	18	99 (*R*)	24	99 (*R*)
1h	*m*-chlorophenyl	65	82 (*R*)	66	82 (*R*)	69	90 (*R*)	66	90 (*R*)
1i	*p*-chlorophenyl	2	–	9	89[d]	5	90[d]	8	88[d]
1j	*o*-fluorophenyl	81	99 (*R*)[e]	15	99 (*R*)[e]	15	99 (*R*)[e]	10	99 (*R*)[e]
1k	*p*-fluorophenyl	100	99 (*R*)	47	88 (*R*)	47	64 (*R*)	45	88 (*R*)

[a] Conversion values calculated by GC analysis of the crude mixture after 72 h of reaction; [b] The *ee* values calculated by GC or HPLC analysis on a chiral stationary phase; [c] Conversion values calculated by GC analysis of the crude mixture after 24 h of reaction; [d] The absolute configuration could not be determined because the saturated nitrile derivative could not be isolated; [e] The *R* configuration was attributed by analogy to the sign of the optical rotation value of the nitrile derivative.

values were generally obtained with BY and the lowest with OYE3, except for the *m*-Me derivative **1c** for which approximately 90% conversion values were obtained with both BY and OYE3.

BY and OYE1–3 bioconversions in biphasic ionic liquid–water systems

The low solubility of these arylacrylonitriles in aqueous medium prompted us to investigate the possibility of performing the bioreductions with BY in a biphasic system composed of water and an immiscible ionic liquid (IL).

Over the past decade, a large number of applications of ILs in biocatalysed reactions (free enzymes and whole cells)[17] have been discussed in the literature, mostly dealing with the so-called second generation ILs,[18] that is, those containing weakly coordinating anions, such as PF_6^- and Tf_2^-, which are characterised by high water and air stability. Furthermore, third generation ILs have been introduced, with structures containing biodegradable or readily available ions, such as natural bases, amino acids, and natural carboxylic acids.[18] The evolution of ILs is represented by deep eutectic solvents and mixtures of solid salts (e.g., choline chloride) and hydrogen bond donors (e.g., urea), which are cheaper and more biodegradable than ILs and do not require purification in their production process.[19]

The set-up of biphasic systems involving immiscible ILs has been highlighted as a promising research area in whole-cell biotransformations[20] because the non-invasive effects of hydrophobic ILs on cellular membranes make them superior to many organic solvents. With substrates and products that are poorly soluble in water, as in our case, the IL can act as an effective substrate reservoir and in situ extracting agent.[21] Howarth et al. reported the first example of a whole-cell bioreduction in ILs in which immobilised BY was used as a catalyst to reduce a set of ketones in a [BMIM][PF_6]–water mixture (BMIM = 1-butyl-3-methylimidazolium hexafluorophosphate).[22] An increase in conversion (from 44 to 80%) and enantioselectivity (from 62 to 84%) values was obtained in the reduction of 4-chloroacetoacetate to (*S*)-4-chloro-3-hydroxybutanoate in 20 vol% [BMIM][PF_6] in the presence of BY.[21]

Moran et al. described the consecutive reduction of C=C and C=O bonds of (*Z*)-3-halo-4-phenyl-3-buten-2-one mediated by various microorganisms in the [BMIM][PF_6]–water biphasic system, which gave the corresponding halohydrins with better diastereoselectivity and enantioselectivity than did the reduction in pure water.[23]

On the basis of these literature data, we decided to investigate our BY reactions in a biphasic 1:10 [BMIM][PF_6]–water system: the experimental results are presented in Figure 1a and b. The conversion and *ee* values of these reactions were generally higher than those obtained with OYE1–3-mediated reductions in water with DMSO as a cosolvent and similar to the values obtained by using BY with the use of resin. However, the work-up and product recovery from the biphasic IL–water system was easier and more effective than the extraction of the organic products from the resins, and thus isolation yields increased.

In the case of the *o*-Cl derivative **1g**, we experimented with the use of the biphasic IL–water system in the biotransformation with OYE1–3. It has been reported in the literature that the *Lactobacillus brevis* alcohol dehydrogenase-catalysed enantioselective reduction of 2-octanone in a biphasic system composed of an aqueous buffer and [BMIM][Tf_2N],[24] with the enzyme and the cofactor dissolved in the aqueous phase and physically separated from the substrates and products mainly in the IL phase, showed higher reaction rates than that in the aqueous buffer/methyl *tert*-butyl ether biphasic system. For substrate **1g**, we performed the OYE-mediated reductions in biphasic IL–water systems with either [BMIM][PF_6] or [BMIM][Tf_2N]. The latter is used in the only literature example describing the use of ILs with isolated enzymes.[24] The corresponding experimental data are presented in Figure 1c and d. Notably, in the case of [BMIM][PF_6], the biphasic medium had a beneficial effect on the conversion values obtained in the biocatalysed reductions with isolated OYEs, most likely owing to a better dissolution of the substrate.

To the best of our knowledge, this study represents the first application of isolated ERs in the IL–water system; it clearly demonstrates that these enzymes are compatible with hydrophobic ILs and that the method is efficient and advantageous in terms of yields, selectivity, and work-up practicality.

Absolute configurations of the products

The absolute configuration of the Ph, *p*-Me, 1-naphthyl, and *m*-Cl saturated nitriles **2a**, **d**, **e**, and **h** has been reported in the literature(see the Supporting Information). The mild and high yield conversion of (+)-*p*-F-**2k** and (+)-*o*-Cl-**2g** into (*R*)-amides

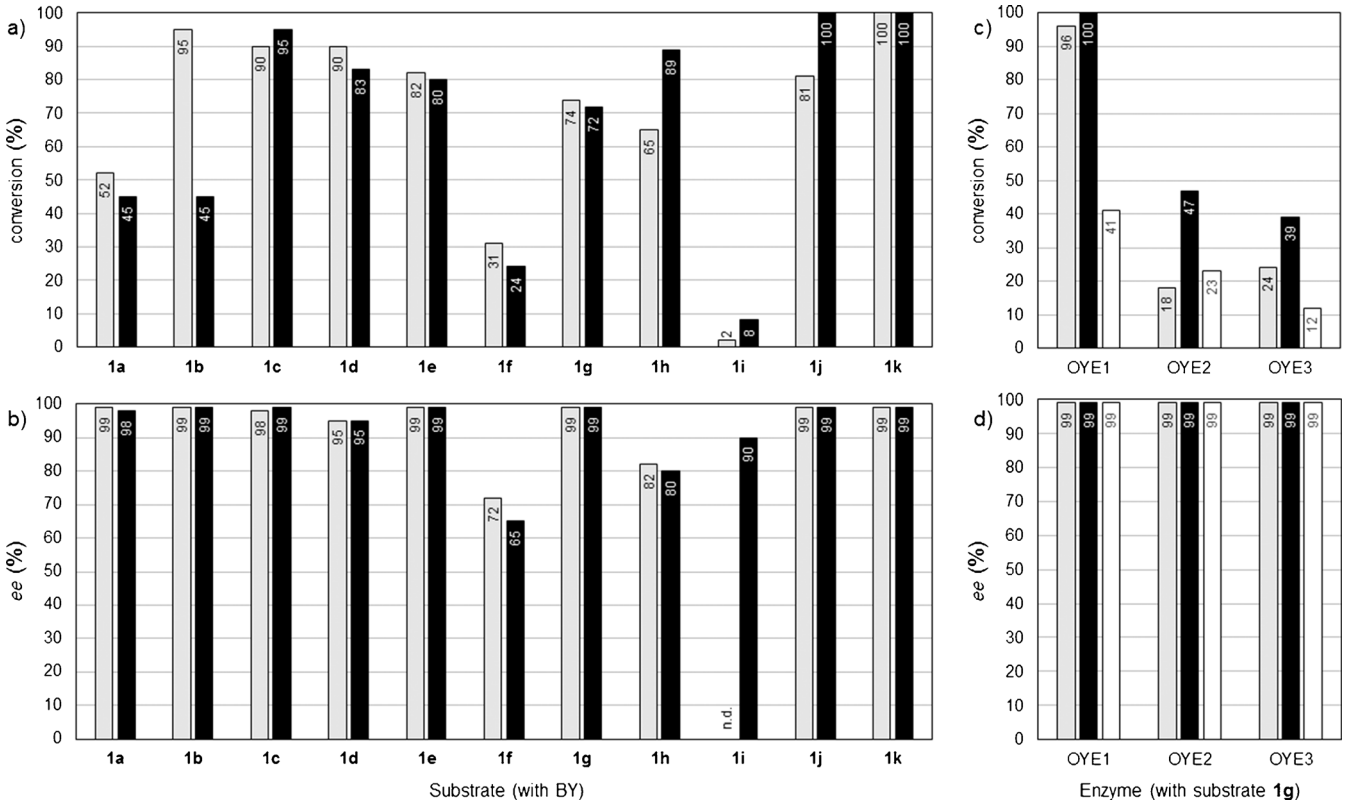


Figure 1. Results of the biocatalysed reductions in biphasic IL–water systems (■: [BMIM][PF_4], □: [BMIM][Tf_2N]) compared with those obtained in aqueous systems (▒): a) conversion and b) *ee* values for the BY-mediated reductions of substrates **1a**–**k** after 72 h; c) conversion and d) *ee* values for the ER-mediated reductions of substrate **1g** after 24 h.

3k and **g** (Scheme 1) was performed through the reaction with hydrogen peroxide in 10% aqueous solution of Na_2CO_3. (+)-*o*-Me-**2b** and (+)-*m*-Me-**2c** were hydrolysed to afford the corresponding carboxylic acid (*R*)-**4** by heating to reflux for 5 h in HCl (37%). A two-step method involving first the transformation of the CN group of (−)-*o*-NO_2-**2f** into the carboxylic acid function and then the reaction with diphenylphosphoryl azide and triethylamine in toluene at 80 °C followed by treatment with diluted HCl at 60 °C afforded the corresponding (*R*)-amine **5**. (+)-*o*-Me-**2b** was converted into the derivative (*R*)-**6** by reacting the corresponding amide with *N*-bromosuccinimide and 1,8-diazabicyclo[5.4.0]undec-7-ene in methanol heated to reflux.

Synthetic manipulation of arylpropanenitriles

As pointed out in the previous section, in most cases the enantiomerically enriched arylpropanenitriles had to be manipulated to determine their absolute configuration by chemical correlation. This process thus demonstrated the synthetic potential of these chiral derivatives. Arylpropanenitriles (*R*)-**2** themselves can be used for the diffusion of the nitrile pharmacophore in pharmaceutical applications. The synthetic versatility of the nitrile moiety can be exploited for the conversion of arylpropanenitriles (*R*)-**2** into amides (*R*)-**3** and amines (*R*)-**5** (Scheme 1) under the same reaction conditions used for the assignment of the absolute configuration of some of the reduced products of this work.

The enantioselective synthesis of (*R*)-2-arylpropanamides, such as reparixin (Figure 2),[25] is a relevant achievement in preparative organic chemistry because these derivatives are useful in the prevention and treatment of tissue damage due to the exacerbate recruitment of polymorphonuclear neutro-

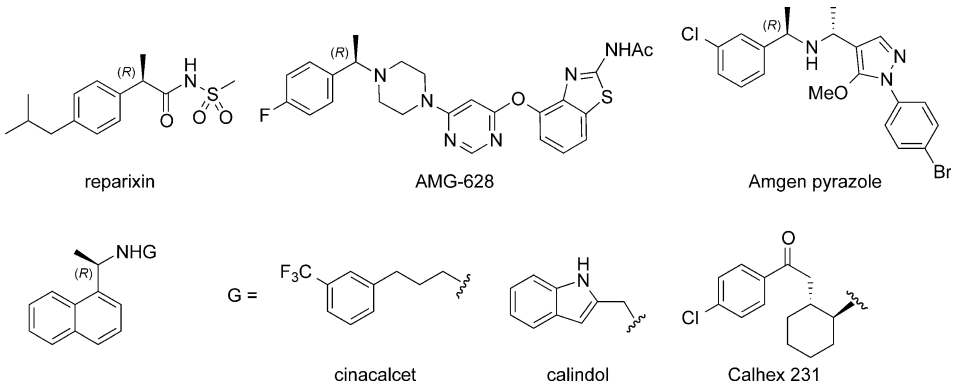


Figure 2. Chiral active pharmaceutical ingredients containing (*R*)-amide and amine moieties.

phils (leukocytes) at the inflammatory sites. They are used in the treatment of psoriasis, ulcerative colitis, glomerulonephritis, acute respiratory insufficiency, and rheumatoid arthritis.[26]

The preparation of (*R*)-amines **5** through the Curtius rearrangement of carboxylic acids (*R*)-**4** with loss of one carbon atom and retention of configuration is a valuable method because of the widespread diffusion of the structural unit of (*R*)-2-arylethanamine as the key motif in many drug candidates.[27] The relevant examples of chiral amino drugs for which the most bioactive enantiomer is the one showing the *R* configuration are illustrated in Figure 2. The drug candidate AMG 628[28] is a transient receptor potential antagonist used for the treatment of chronic pain, and cinacalcet hydrochloride[29] (Sensipar Amgen) is the first calcimimetic agent approved by the Food and Drug Administration for the treatment of secondary hyperparathyroidism. Other candidates such as calindol,[30] Calhex 231,[31] NPS R-467,[32] and Amgen pyrazole[33] (Figure 2) with potent and selective activity on the parathyroid calcium receptor are under pharmacokinetic studies. A series of small molecules developed as effective antivirals against severe acute respiratory syndrome[34] are (*R*)-2-naphthylethanamine derivatives.

The biological significance of enantiopure (*R*)-arylethanamines prompted us to demonstrate the versatility and the applicability of our method by converting (*R*)-arylethanamines into the corresponding amines, not only the *o*-NO_2 derivative (*R*)-**2 f** (for the configuration assignment) but also phenyl and 1-naphthyl derivatives (*R*)-**2 a** and (*R*)-**2 e**. Amines (*R*)-**5 a**, **e**, and **f** were finally prepared from the arylacetonitrile by using a four-step sequence in satisfactory yields and high enantiomeric purity.

Conclusions

The development of a catalytic stereoselective synthesis of enantiopure (*R*)-arylpropanenitriles and the corresponding amides and arylethanamines through an efficient and environmentally friendly method not involving toxic or hazardous reagents and with minimal use of organic solvents offers enormous promise for optimising new routes to prepare a wide range of pharmaceutical products. The nitrile moiety, which is necessary to act as an activating electron-withdrawing group in the bioreduction step, can be subsequently submitted to valuable chemical manipulations, which gives access to various derivatives.

This enzyme-mediated method gives the possibility of creating a benzylic stereogenic centre by reduction of a C=C bond conjugated to a CN moiety, a transformation which is difficult to accomplish through metal catalysis. In this case, biocatalysed reductions represent a complementary activity to metal-catalysed hydrogenation: 1) the nitrile moiety can establish hydrogen bonds in the active site of the enzyme to promote the substrate binding; 2) the bioreduction is completely chemoselective towards the reduction of the C=C bond.

In this case, the use of whole-cell systems does not induce any undesired side reaction and has the advantage of employing the cofactor regeneration system of the cells. The operational solution to load the substrate onto a hydrophobic resin or to use biphasic ionic liquid–water systems improves the work-up method and the recovery of the reduced product.

Ionic liquids were combined with isolated old yellow enzymes, which afforded good results in terms of conversion and enantioselectivity in the case of [BMIM][PF_6] (BMIM = 1-butyl-3-methylimidazolium hexafluorophosphate). The ionic liquid phase represents a good reservoir that makes water-insoluble products gradually available to the reaction catalysed by these reducing enzymes.

Experimental Section

Sources of strains and enzymes

Either fresh or freeze-dried commercial BY (*S. cerevisiae*) was used for whole-cell biotransformations. OYEs (OYE1 from *Saccharomyces pastorianus* and OYE2 and OYE3 from *S. cerevisiae*) and GDH (from *Bacillus megaterium*) were overexpressed in *Escherichia coli* BL21 (DE3). Detailed methods are reported in the Supporting Information.

OYE-mediated bioreduction in aqueous system

General procedure: The substrate (5 μmol) dissolved in DMSO (10 μL) was added to a KP_i buffer solution (1.0 mL, 50 mM, pH 7.0) containing glucose (20 mM), $NADP^+$ (0.1 mM), GDH (4 U mL^{-1}), and the required OYE (40 μg mL^{-1}). The mixture was incubated for 24 h in an orbital shaker (160 rpm, 30 °C). The solution was extracted with EtOAc (2 × 250 μL), centrifuging after each extraction (15 000 *g*, 1.5 min), and the combined organic solutions were dried over anhydrous Na_2SO_4.

BY fermentation in aqueous system

General procedure: A mixture of BY (100 g) and D-glucose (40 g) in tap water (800 mL) was prepared. After stirring at 30 °C for 10 min, the nitrile derivative (3.0 g) adsorbed on a hydrophobic resin (60 g; polystyrene XAD1180N) was added in one portion. The mixture was kept under stirring for 72 h at RT and then filtered on a cotton plug. The collected mass was washed repeatedly with water to remove most of the cells. The resin was then collected and extracted twice in sequence with acetone (200 mL) and EtOAc (200 mL). The organic phase was concentrated to $^1/_3$ of its volume, washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The resulting mixture of products was then separated by using column chromatography in hexane with increasing amounts of EtOAc.

OYE-mediated bioreduction in the biphasic IL–water system

General procedure: The substrate (50 mg) dissolved in [BMIM][PF_6] or [BMIM][Tf_2N] (1500 μL) was added to a KP_i buffer solution (15 mL, 50 mM, pH 7.0) containing glucose (4 equiv. with respect to the substrate, ≈250 mg), $NADP^+$ (0.05 mM), GDH (4 U mL^{-1}), and the required OYE (40 μg mL^{-1}). The mixture was incubated for 24 h in an orbital shaker (160 rpm, 30 °C). The separated IL phase was extracted with *i*Pr_2O (3 × 5 mL), centrifuging after each extraction (3000 *g*, 5 min), and then the combined organic solutions were dried over Na_2SO_4 and concentrated under reduced pressure.

BY fermentation in the biphasic IL–water system

General procedure: The substrate (300 mg) dissolved in [BMIM][PF_6] (6 mL) was added to a mixture of BY (30 g) and D-glucose (10 g) in tap water (300 mL) at 35 °C. The mixture was stirred in an orbital shaker (220 rpm, 30 °C) for 1–4 days. After separation of the phases, the aqueous solution was washed with CH_2Cl_2 and the organic extract was added to the IL phase to recover all the IL and products in the organic phase. The latter was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was washed with iPr_2O (4×3 mL) to separate the IL, and then the combined ethereal extract was dried over Na_2SO_4 and concentrated under reduced pressure. The resulting mixture of products was then separated by using column chromatography in hexane with increasing amounts of EtOAc.

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