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Enantioselective Biocatalytic Reduction of 2*H*-1,4-Benzoxazines using Imine Reductases

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Supporting Information Placeholder

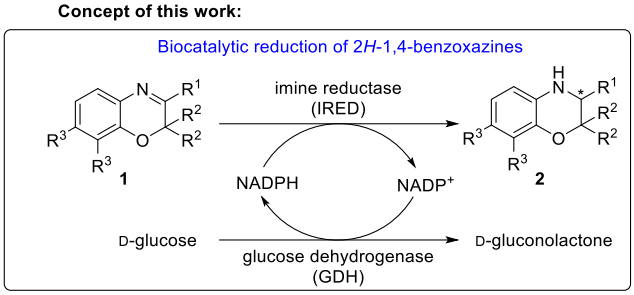
ABSTRACT: A biocatalytic reduction of 2*H*-1,4-benzoxazines using imine reductases is reported. This process enables a smooth and enantioselective synthesis of the resulting cyclic amines under mild conditions in aqueous media by means of a catalytic amount of the cofactor NADPH as hydride source as well as glucose as reducing agent used in stoichiometric amount for *in situ*-cofactor recycling. Several substrates were studied, and the 3,4-dihydro-2*H*-1,4-benzoxazines were obtained with up to 99% *ee*. In addition, the efficiency of this reduction process based on imine reductases as catalysts has been demonstrated for one 2*H*-1,4-benzoxazine on elevated lab scale running at a substrate loading of 10 g L^{-1} in the presence of a tailor-made whole cell-catalyst.

INTRODUCTION

Oxygen-containing heterocyclic amines like 3,4-dihydro-2*H*-1,4-benzoxazines **2** are important structural moieties in biological and pharmaceutical active compounds, representing promising antidepressants, calcium antagonists, anti-inflammatory, antibacterial, antimicrobial or antinociceptive agents.[1–8] Prominent examples for pharmaceuticals bearing 3,4-dihydro-2*H*-1,4-benzoxazines **2** as a structural motif are levofloxacin[8] or obscurinervidine.[7]

Accordingly, the synthesis of these heterocyclic amines gained broad interest and as such compounds often bear at least one stereogenic center, enantioselective routes for their preparation are desirable. In general, the corresponding cyclic imines are readily accessible molecules, thus serving as attractive starting materials. The reduction of these cyclic imines, namely the 2*H*-1,4-benzoxazines **1**, represents a straightforward approach toward 3,4-dihydro-2*H*-1,4-benzoxazines **2**. The asymmetric hydrogenation of 2*H*-1,4-benzoxazines **1** has been studied extensively in the last years using transition-metal catalyzed hydrogenation leading to, at least in part, high enantioselectivities.[9–16] Very recently, a metal-free hydrogenation of 2*H*-1,4-benzoxazines **1** using tris(pentafluorophenyl)borane has been reported giving, however, only moderate enantioselectivity.[17] Progress has also been made in the organocatalytic asymmetric transfer hydrogenation using Hantzsch ester as hydrogen source and chiral Brønsted acids as catalysts,[18–25] showing a broad substrate scope and high enantioselectivities. A limitation of this methodolology is the need for stoichiometric amount of Hantzsch ester due to the lack of an *in situ*-recycling system. A biomimetic transfer hydrogenation using dihydrophenanthridine as NAD(P)H mimic was established by Chen *et al.* allowing a recycling of the hydrogen source *via* a ruthenium catalyst.[26] Complementing these chemocatalytic methods for the reduction of 2*H*-1,4-benzoxazines **1**, biocatalysis could represent a promising alternative. An enzyme-catalyzed process would offer the possibility for a catalytic use of the cofactor NAD(P)H by using an enzymatic recycling system under mild conditions. Such recycling systems have already been proven to be suitable even in technical processes. Recently, for a range of cyclic imines as model substrates (without further heteroatoms) imine reductases (IREDs) have been demonstrated by various groups to be suitable biocatalysts for the enantioselective reduction of (sterically-demanding) cyclic imines.[27–37] However, the impact of additional heteroatoms in such cyclic imines is rarely explored although they can have a strong impact on reactivity. In continuation of our recent study on the reduction of sulfur-containing cyclic imines with imine reductases (IREDs) exemplified for 3-thiazolines as well as 2*H*-1,4-benzothiazines,[38] we became interested in studying the suitability of this technology toward the reduction of selected 2*H*-1,4-benzoxazines **1** as an alternative synthetic route to 3,4-dihydro-2*H*-1,4-benzoxazines **2** by means of a catalytic use of NADPH *via in situ*-cofactor recycling using a glucose dehydrogenase (GDH) (Scheme 1).

Scheme 1. Biocatalytic reduction of 2*H*-1,4-benzoxazines **1** using imine reductases and *in situ*-cofactor recycling by glucose dehydrogenase.



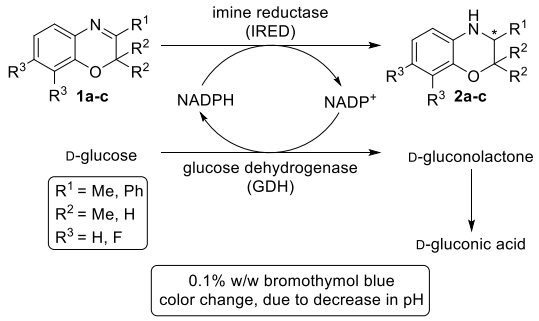
In the following we report our results on the development of such a biocatalytic reduction of selected 2*H*-1,4-benzoxazines **1** using imine reductases for the enantioselective synthesis of 3,4-dihydro-2*H*-1,4-benzoxazines **2** under mild conditions in aqueous media.

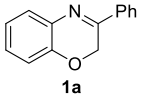
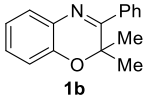
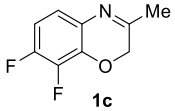
RESULTS AND DISCUSSION

In our initial investigations, we tested if imine reductases are suitable biocatalysts for the reduction of 2*H*-1,4-benzoxazines **1**. Toward this end, we prepared two 2*H*-1,4-benzoxazines **1a** and **1b**, bearing a phenyl group as substituent at R^1 and methyl groups or hydrogen atoms as substituents at R^2 as well as one 2*H*-1,4-benzoxazine **1c**, containing a methyl group at R^1, hydrogen atoms at R^2 and fluoro atoms at R^3. The latter compound represents an intermediate for the synthesis of levofloxacin. We screened these

substrates against a set of 31 IREDs (see Table S1),[34,35,32] which had been prepared in a recombinant form by means of overexpression in *Escherichia coli* (*E. coli*) BL21(DE3). As in our previous study a colorimetric pH shift assay[39] was used as fast screening methodology. The assay is based on a color change of bromothymol blue, due to a shift of the pH, caused by consumption of the substrate and *in situ*-cofactor-regeneration by glucose dehydrogenase (GDH) leading to a formation of gluconic acid through oxidation of D-glucose to gluconolactone and subsequent spontaneous hydrolysis of the lactone (Table 1). Color changes from green to yellow, thus indicating a successful reaction, were observed for all 2*H*-1,4-benzoxazines **1a-c** with a range of imine reductases (used as cell crude extracts). Moreover, both negative controls showed no color change in all cases, indicating that the glucose dehydrogenase being utilized for this screening and all other proteins expressed by the *E. coli* strain show no side-activity toward reduction of the 2*H*-1,4-benzoxazines **1a**, **1b** and **1c**.

Table 1. Colorimetric pH shift assay as initial screening toward reduction of 2*H*-1,4-benzoxazines **1a-c** using imine reductases as biocatalysts



substrate	positive IREDs	without IRED	crude extract without IRED expression
 **1a**	4 of 31	negative	negative
 **1b**	3 of 31	negative	negative
 **1c**	4 of 31	negative	negative

Examined imine reductases are literature-known enzymes (see Table S1);[34,35,32] negative means that no color change was observed.

With these hints for the most promising IREDs in hand, we subsequently performed the spectrophotometric determination of the enzyme activities. Toward this end, the consumption of the cofactor NADPH was observed spectrophotometrically at 340 nm in the initial 60 seconds of the reaction and the enzyme activity calculated. We were pleased to observe moderate to high activities for all three tested 2*H*-1,4-benzoxazines **1a-c** in combination with a range of imine reductases (Figure 1). It turned out, that the imine reductases show the highest activities toward cyclic imine **1b**, which is up to 10-fold higher, in comparison to substrates **1a** and **1c**. Striking about this result is that **1b** is the most sterically demanding substrate in comparison to the other 2*H*-1,4-benzoxazines tested. Regarding the cyclic imine **1a**, IRED12 showed the highest activity, whereas IRED05 and IRED27 show only moderate activity. The activity of the four most promising IREDs toward the intermediate of levofloxacin **1c** is, however, only moderate, which indicates that for achieving high conversions a higher biocatalyst loading for reduction of **1c** might be necessary in comparison to substrates **1a** and **1b** (Figure 1).

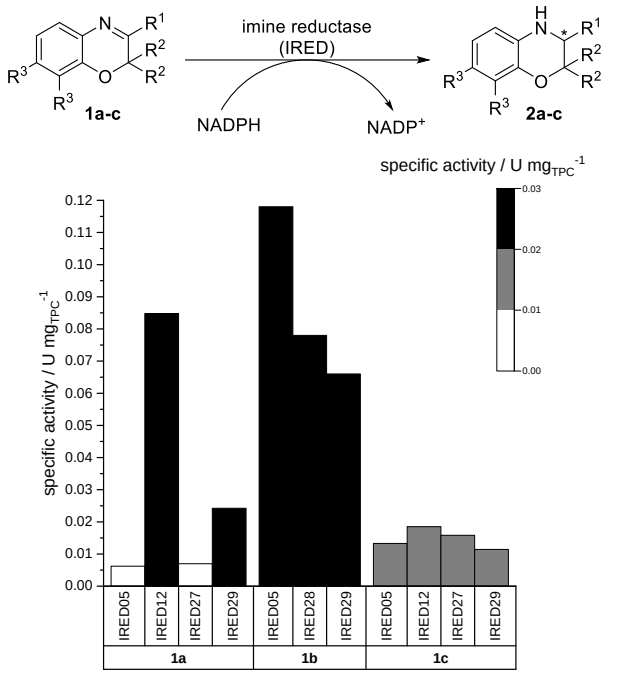
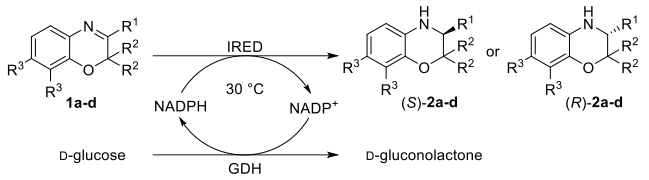


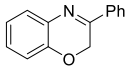
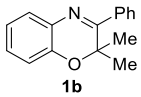
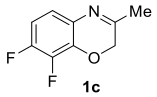
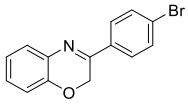
Figure 1. Specific activities of imine reductases towards reduction of 2*H*-1,4-benzoxazines **1a-c**. Examined imine reductases are literature-known enzymes[34,35,32]; for reasons of better readability IREDs are numbered and the corresponding names of the original strains and the literature are given in Table S1; total protein concentration (TPC) as cell crude extract was used.

Encouraged by the promising enzyme activities toward reduction of 2*H*-1,4-benzoxazines **1a-c**, we performed biotransformations on 0.5 mL scale at substrate concentrations of 20 mM of 2*H*-1,4-benzoxazines **1a-c**, using the IREDs that were prioritized according to the colorimetric pH shift assay and spectrophotometric activity assay. For *in situ*-cofactor-regeneration a glucose dehydrogenase from *Bacillus subtilis*[38,40,41] as well as D-glucose as co-substrate was used (Table 2). Under non-optimized reaction conditions, we observed moderate to quantitative conversions (9 - > 99%) with perfect product selectivity toward reduction of the cyclic imines **1a-c** (Table 2, entries 1-3). Unfortunately, no conversion was observed for the biocatalytic reduction of 2*H*-1,4-benzoxazine **1d**, bearing a 4-bromophenyl substituent at the prochiral carbon center (R^1), even at an elevated biocatalyst loading of 10 mg mL^{-1} IRED cell crude extract in combination with five different IREDs (Table 2, entry 4). We assume, that the benzoxazine **1d** does not fit into the active site of the enzyme and cannot be reduced consequently by the IREDs tested. Negative controls of biotransformations of substrates **1a-c** without IRED showed no conversion, indicating that the GDH has no side-activity towards imine reduction (which is in contrast to analogous reductions of different imines as reported by Roth *et al.*[42]). A relative low biocatalyst loading of 0.6 mg mL^{-1} or 1.2 mg mL^{-1} total protein concentration of cell crude extract was sufficient to reach moderate to high conver-

sions for the cyclic imines **1a-c**, being in accordance with the determined enzyme activities (Figure 1 and Table 2). Accordingly, imine reductases represent valuable biocatalyst for the reduction of 2*H*-1,4-benzoxazines **1a-c**.

Table 2. Biocatalytic reduction of 2*H*-1,4-benzoxazines **1a-d** using imine reductases (IREDs)



entry	substrate	time [h]	IRED[a]	conv [%][b]	*ee* [%][c]
1	 **1a**	15	IRED05[d]	19	52 (*R*)
		15	IRED12[d]	> 99	99 (*S*)
		15	IRED29[d]	29	81 (*S*)
2	 **1b**	8	IRED05[e]	82	99 (*R*)
		24	IRED20[e]	9	62 (*S*)
		8	IRED28[e]	71	99 (*R*)
		8	IRED29[e]	31	99 (*R*)
3	 **1c**	19.5	IRED05[e]	21	72 (*R*)
		19.5	IRED12[e]	84	26 (*R*)
		19.5	IRED27[e]	26	60 (*R*)
		19.5	IRED29[e]	20	71 (*R*)
4	 **1d**	24	IRED05[f]	0	-
		24	IRED07[f]	0	-
		24	IRED25[f]	0	-
		24	IRED27[f]	0	-
		24	IRED29[f]	0	-

[a]Examined imine reductases are literature-known enzymes[34,35,32]; for reasons of better readability IREDs are numbered and the corresponding names of the original strains are given in Table S1; IRED05 *Cupriavidus sp. HPC(L)*, IRED12 *Nocardia cyriacigeorgica GUH-2*, IRED20 *Streptomyces viridochromogenes*, IRED27 *Mesorhizobium sp. L48C026A00*, IRED28 *Aeromonas veronii*, IRED29 *Aeromonas veronii*; [b]determined by SFC-HPLC *via* comparison of substrate and product integral; [c]Enantiomeric excess was determined by means of SFC-HPLC. Absolute configuration for **2a** was determined *via* specific rotation, comparison to literature and absolute configuration of **2b** and **2c** was assigned in analogy by chiral SFC-HPLC; [d]0.6 mg mL^{-1}; [e]1.2 mg mL^{-1}; [f]10 mg mL^{-1} (total protein concentration of IRED crude cell extract is given in footnote d and e, for expression see SDS-PAGE Figure S1)

Moreover, we were pleased to find that IREDs catalyze the reduction of 2*H*-1,4-benzoxazines **1a-b** with moderate to high enantioselectivites (52-99% *ee*) and it is noteworthy that enantiocomplementary product formation was observed, thus providing an access to both enantiomers of the 3,4-dihydro-2*H*-1,4-benzoxazines **2a-b**. Excellent enantioselectivity was observed towards (*S*)-**2a** (99% *ee*) and (*R*)-**2b** (99% *ee*), whereas only moderate enantioselectivitiy could be observed towards the complementary enantiomers (*R*)-**2a** (52% *ee*) and (*R*)-**2b** (62% *ee*) (Table 2, entries 1-2). In contrast, enantioselectivity of IREDs towards reduction of 2*H*-1,4-benzoxazine **1c** is only low to high (26-72% *ee*). Furthermore, all tested IREDs only provided an access towards the (*R*)-enantiomer of **2c** (Table 2, entry 3) and not the desired (*S*)-enantiomer for the synthesis of levofloxacin. For the imine reductases IRED12[34] and IRED29[35], we observed switches in the enantiopreference depending on the substituents of the substrates **1a-c**. For both IREDs in combination with substrate **1a**, containing a phenyl group at R^1 and hydrogen atoms at R^2 and R^3, formation of the (*S*)-enantiomer is favored (Table 2, entry 1). However, when using IRED12[34] for the reduction of 2*H*-1,4-benzoxazine **1c** the (*R*)-enantiomer is formed with 26% *ee* (Table 2, entry 3). We observed the same tendency when using IRED29[35] as biocatalyst: Substrates **1b** and **1c** were reduced to the corresponding (*R*)-enantiomers with good to high enantioselectivities (Table 2, entries 2-3). It might be possible that the substrates allow different interactions to amino acids in the active site of the IRED and are therefore reduced with such different enantioselectivites. In our future work, we will investigate these observations by means of docking studies in order to understand if different interactions in the active site of the enzyme are responsible for switches in the enantiopreference.

Inspired by the positive results for the IRED-catalyzed reduction of 2*H*-1,4-benzoxazines **1a-c**, we started a process development demonstrated by the enantioselective reduction of **1a** using IRED12[34] as biocatalyst (Figure 3). In analogy to our previous work[27,38,40], a novel *E. coli* whole cell-catalyst was designed: This tailor-made whole cell-catalyst is overexpressing IRED12 and a glucose dehydrogenase from *Bacillus subtilis* for *in situ*-regeneration of the cofactor NADPH on different plasmids. The experiment on preparative scale (40 mL) was performed at a substrate concentration of 50 mM, corresponding to a substrate loading of 10 g L^{-1}, and a remarkably low biocatalyst loading of 0.1 g mmol^{-1} lyophilized whole-cell catalyst at 30 °C and the pH was adjusted at 7 using a TitrinoLine® apparatus. We were pleased to find that the reaction also proceeds smoothly on preparative scale, giving quantitative conversion after 26 hours of reaction time and excellent enantioselectivity of 99% for the (*S*)-enantiomer. After work-up the desired product (*S*)-**2a** was isolated in 71% yield (Figure 2). The results of the preparative scale biotransformation also underline the applicability and the value of this biocatalytic technology.

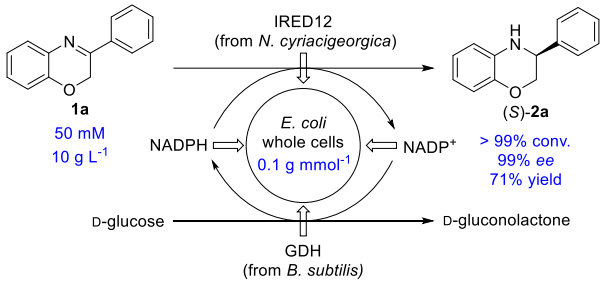


Figure 2. Biocatalytic Reduction of 1a on 10 g L^{-1} scale (40 mL) using lyophilized whole cells

CONCLUSION

In conclusion, an enantioselective biocatalytic reduction of 2*H*-1,4-benzoxazines, exemplified for selected examples, has been developed using imine reductases as catalysts and D-glucose as reducing agent in the presence of a glucose dehydrogenase as further enzyme for *in situ*-cofactor recycling. This technology turned out as a smooth and enantioselective reduction for three of in total four tested 2*H*-1,4-benzoxazines under mild conditions, leading to the resulting 3,4-dihydro-2*H*-1,4-benzoxazines **2** with enantioselectivities of up to 99% *ee*. We also demonstrated the efficiency of this biocatalytic method exemplified for one 2*H*-1,4-benzoxazine in an

initial process development on elevated lab scale (40 mL) at a substrate loading of 10 g L^{-1}, giving quantitative conversion and 99% *ee* for the (*S*)-enantiomer in the presence of a tailor-made whole cell catalyst.

EXPERIMENTAL PROCEDURES

General Information. Commercially available reagents (*Acros, Alfa Aesar, Merck, VWR, Acros, Fisher Scientific, TCI*) were used as received. Solvents were used in high-grade purity or purified by distillation prior to use. NMR spectra were recorded on Bruker Advance III 500 or Bruker Advance III 500HD at a frequence of 500 MHz (^{1}H) or 126 MHz (^{13}C). The chemical shift δ is given in ppm and referenced to the corresponding solvent signal ($CDCl_3$). Coupling constants (*J*) are given in Hz. Column Chromatography was performed using Biotage „Isolera One" flash chromatography system with cyclohexane/ethyl acetate mixtures. Chiral SFC-HPLC analysis was performed using the LC2000 SFC-HPLC system from *Jasco* (Easton, USA) with the HPLC columns Chiralpak® OJ-H and Chiralpak® OB-H from *Daicel* (Tokyo, Japan).

Recombinant Expression of IREDs. Imine reductases are recombinantly expressed in *E. coli* BL21(DE3), according to a literature-known procedure.[38]

Expression of IREDs for the colorimetric pH shift assay (according to ref[38]). All 31 imine reductases as well as the empty pET-22b(+) vector are expressed simultaneously in 2 mL deep-well plates. 1.5 mL autoinduction medium (AI medium prepared from TB medium, containing 2 g L^{-1} lactose, 0.5 g L^{-1} D-glucose), containing 100 µg mL^{-1} carbenicillin, is added to every well. The wells are directly inoculated from agar plates (fourfold for each IRED) and incubated for 3 h at 37 °C and 1350 rpm in Heidolph Titramax 1000 incubator. The production of recombinant protein is induced over night at 20 °C and 1350 rpm. The cells are harvested by centrifugation at 4000 rpm and 4 °C for 30 minutes and cell pellets are stored at -20 °C. The cell pellets are resuspended in lysis-buffer (250 µL) (50 mM potassium phosphate buffer (KP_i) pH 7 containing 2 mg mL^{-1} lysozyme, 0.04 mg mL^{-1} DNAseI) and incubated for 1 h at 37 °C and 1350 rpm in Heidolph Titramax 1000 incubator, frozen at -20 °C for 30 min and then incubated again at 37 °C and 1350 rpm for 30 min. After centrifugation at 4000x g for 30 min at 4 °C, the cell crude extract is obtained as supernatant, which is directly used in the colorimetric pH shift assay.

Expression of IREDs for biotransformations (according to ref[38]). A preculture (10 mL lysogeny broth (LB) medium, containing 100 µg mL^{-1} of carbenicillin) of *E. coli* BL21(DE3) carrying the recombinant plasmid with the IRED gene is cultivated over night at 37 °C. The main culture (300 mL terrific broth (TB) medium, containing 100 µg mL^{-1} of carbenicillin) is inoculated with the preculture to a final concentration of 1%. The production of recombinant protein is induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration 0.5 mM) at an OD_{600} between 0.4 und 0.6. Cultures are shaken at 25 °C for 20 h and 140 rpm and harvested by centrifugation at 4000x g and 4 °C for 30 min. Cell pellets are stored at -20 °C. Cells are resuspended in 3 mL 50 mM KP_i pH 7 per g wet cell weight. Cell disruption is performed by sonification (Bandelin Sonopuls HD 2070) with 3x 120 s burst (5 cycles, 20% energy) and 120 s intervals and both steps being carried out on ice. After centrifugation at 21 000x g for 5 min at 4 °C, cell debris is removed and the supernatant is centrifugated again at 21 000x g for 25 min at 4 °C. The crude extract is obtained as supernatant, whereas inclusion bodies are obtained as pellets. The protein concentration is determined using the Bradford assay against BSA as a concentration standard. The supernatant is used for biotransformations.

Construction and preparation of whole cell-catalyst (in analogy to ref.[38]). The whole-cell catalyst was constructed as a two-plasmid-system, containing the gene for the glucose dehydrogenase from *Bacillus subtilis* in a pACYCDuet-1 vector[38,27,40] and the gene for the imine reductase from *Nocardia cyriacigeorgica GUH-2* in a pET-22b(+) vector.[34] A preculture of *E. coli* BL21(DE3) carrying the two recombinant plasmids (10 mL LB medium, containing 80 µg mL^{-1} of carbenicillin and 28 µg mL^{-1} of chloramphenicol) is cultivated over-night at 37 °C and 140 rpm. The main culture (600 mL TB medium containing 80 µg mL^{-1} of carbenicillin and 28 µg mL^{-1} of chloramphenicol) is inoculated with the starting culture to a final concentration of 1%. At an OD_{600} between 0.4 und 0.6, the production of recombinant protein is induced by addition of IPTG (0.5 mM final concentration). Cultures are shaken at 25 °C for 20 h and harvested by centrifugation (4000x g for 30 min at 4 °C). The cells are lyophilized from a 50% cell suspension in water and the resulting lyophilized cells are stored in a freezer at -20 °C.

Colorimetric pH shift assay (as described in ref.[38], which was adapted from ref.[39]). The screening of IREDs for reduction of 2*H*-1,4-benzoxazines is performed in 10 mM KP_i pH 7.4 (+ 0.01% w/w bromothymol blue (BTB)), containing 20 mM D-glucose, 10 mM substrate **1a-c** (100 mM stock in methanol or dimethyl sulfoxide), 10 µL GDH, 10 or 40 µL IRED (10 µL for screening of **1a-c** and 40 µL for **1d**) and 0.1 mM NADPH. Two different negative controls were also performed. One negative control comprising everything except IRED crude extract (without IRED) and one with cell crude extract of an empty pET-22b(+) vector (cell crude extract without IRED expression).

Spectrophotometric activity assay (as described in ref.[38]). For the determination of the specific activity, the spectrophotometric activity is determined by measuring the consumption of NADPH spectrophotometrically at 340 nm for 60 seconds at the Tecan Reader Spark 10M (*Tecan Trading AG*, Switzerland) in 96 well microtiterplates, containing 2*H*-1,4-benzoxazines **1a-c** (0.5 mM), 4% MeOH as a co-solvent in KP_i buffer (100 mM, pH 7), IRED crude extract and NADPH (0.36 mM).

Synthesis of 2*H*-1,4-benzoxazines 1a-c.

3-phenyl-2*H*-1,4-benzoxazine (1a) (according to ref.[43]). 2-aminophenole (2.00 g, 18.3 mmol) and potassium carbonate (3.80 g, 27.5 mmol) are added to acetone (500 mL). The suspension is stirred for 3 h at room temperature. Potassium carbonate (3.80 g, 27.5 mmol) and 2-bromoacetophenone (3.64 g, 18.3 mmol) are added and the mixture is stirred under refluxing for 6 h. The solid is filtered off, washed with acetone and the solvent evaporated *in vacuo*. Cold, distilled water (200 mL) and diethylether (300 mL) are added to the residue, phases are separated, the organic phase is washed with HCl (1.5 M) and distilled water. The organic phase is dried over magnesium sulfate and the solvent is evaporated *in vacuo*. **1a** (1.49 g, 7.13 mmol, 39%) is obtained after flash column chromatography (1-10% ethyl acetate in cyclohexane) as yellowish solid. **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 7.96–7.90 (m, 2H), 7.50–7.46 (m, 3H), 7.44 (dd, J = 7.7, 1.6 Hz, 1H), 7.15 (td, J = 7.7, 1.6 Hz, 1H), 7.03 (td, J = 7.6, 1.4 Hz, 1H), 6.92 (dd, J = 8.0, 1.3 Hz, 1H), 5.08 (s, 2H). **^{13}C{1H} NMR** (126 MHz, $CDCl_3$) δ (ppm): 158.9, 146.5, 135.6, 133.9, 131.3, 128.9, 128.8, 128.0, 126.6, 122.5, 115.7, 63.1. **MS (ESI)** m/z calculated for $C_{14}H_{12}NO$ $[M+H]^+$: 210.0, found: 210.08. The analytical data corresponds with literature data.[43,18] **chiral SFC-HPLC**: Chiralpak® OJ-H (supercritical CO_2:EtOH (Et_2NH) = 85:15 (0.015), 1 mL min^{-1}, 20 °C, 10 MPa, 210 nm, t_R = 37.9 min.

2-bromo-2-methyl-1-phenylpropan-1-one (according to ref.[44]). Isobutyrophenone (20.0 mL, 135 mmol) und aluminium chloride (0.50 g, 3.70 mmol) are disssolved in dry diethylether (150 mL). Bromine (7.20 mL, 140 mmol) is added slowly over 30 min. The mixture is washed with brine (2x100 mL), the organic phase is

dried over magnesium sulfate and the solvent is evaporated *in vacuo*. The resulting colorless oil (26.7 g, 117 mmol, 86%) is directly used for the synthesis of **1b**, without further purification.

2,2-dimethyl-3-phenyl-2*H*-1,4-benzoxazine (1b) (according to ref.[43]). 2-aminophenole (3.00 g, 27.6 mmol) and potassium carbonate (3.80 g, 27.5 mmol) are added to acetone (100 mL). The suspension is stirred for 3 h at room temperature. Potassium carbonate (6.00 g, 41.5 mmol) and 2-bromo-2-methyl-1-phenylpropan-1-one (synthesis is described above) (4.60 ml, 27.6 mmol) are added and the mixture is stirred under refluxing for 6 h. The solid is filtered off, washed with acetone and the solvent evaporated *in vacuo*. Cold, distilled water (200 mL) and diethylether (300 mL) are added to the residue, phases are separated, the organic phase is washed with HCl (1.5 M) and distilled water. The organic phase is dried over magnesium sulfate and the solvent is evaporated *in vacuo*. **1b** (530 mg, 2.5 mmol, 9%) is obtained after flash column chromatography (20-70% ethyl acetate in cyclohexane) as colorless solid. **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 7.59 (dd, J = 7.0, 2.8 Hz, 2H), 7.42 (m, 4H), 7.17 (td, J = 7.7, 1.5 Hz, 1H), 7.00 (td, J = 7.7, 1.5 Hz, 1H), 6.90 (dd, J = 8.0, 1.3 Hz, 1H), 1.59 (s, 6H). **^{13}C{1H} NMR** (126 MHz, $CDCl_3$) δ (ppm): 167.5, 145.9, 137.8, 133.6, 129.7, 129.0, 128.5, 127.9, 127.5, 122.0, 116.5, 74.7, 25.6. **MS (ESI)** m/z calculated for $C_{16}H_{15}NO$ $[M+H]^+$: 238.1, found: 238.1. The analytical data corresponds with literature data.[43] **chiral SFC-HPLC**: Chiralpak® OJ-H (supercritical CO_2:EtOH (Et_2NH) = 90:10 (0.01), 1 mL min^{-1}, 20 °C, 10 MPa, 210 nm, t_R = 24.3 min.

1-(2,3-difluoro-6-nitrophenoxy)propan-2-one (according to ref.[45]). 2,3-difluoro-6-nitrophenole (2.12 g, 12.1 mmol) is dissolved in toluene (4 mL). Chloracetone (1.94 mL, 24.2 mmol), potassium bromide (173 mg, 1.45 mmol), sodium bicarbonate (1.12 g, 13.3 mmol) and tributylmethylammoniumchloride (75% w/w in dH_2O, 0.26 mmol) are added and the mixture is stirred for 6 h at 65 °C. Chloracetone (0.49 mL, 6.04 mmol) is added and the mixture is stirred for 18 h at 65 °C. Distilled water (4 mL) is added and the pH is adjusted to 6.5-7. The phases are separated, the organic phase is mixed with sodium chloride solution (5%, 8 mL) and stirred for 10 min at 55-60 °C. The phases are separated, the organic phase is dried over magnesium sulfate and the solvent is evaporated *in vacuo*. 1-(2,3-Difluoro-6-nitrophenoxy)propan-2-one (2.53 g, 10.9 mmol, 91%) is obtained as yellow solid. **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 7.73 (ddd, J = 9.4 Hz, J_{FH} = 5.2, 2.4 Hz, 1H), 7.07–7.03 (m, 1H), 4.80 (d, J_{FH} = 1.3 Hz, 2H), 2.31 (s, 3H). **^{13}C{1H} NMR** (126 MHz, $CDCl_3$) δ (ppm): 203.0, 154.1 (dd, J_{FC} = 259.6, 11.5 Hz), 144.7 (dd, J_{FC} = 252.9, 14.5 Hz), 142.6 (dd, J_{FC} = 10.9, 2.9 Hz), 140.0, 120.8 (dd, J_{FC} = 9.0, 4.0 Hz), 111.8 (d, J_{FC} = 19.2 Hz), 78.2 (d, J_{FC} = 5.3 Hz), 26.5. **MS (ESI)** m/z calculated for $C_9H_7F_2NO_4Na$ $[M+Na]^+$: 254.1, found: 254.0. The analytical data corresponds with literature data.[45]

7,8-difluoro-3-methyl-2*H*-benzoxazine (1c) (according to ref.[44]). 1-(2,3-Difluoro-6-nitrophenoxy)propan-2-one (synthesis described above) (540 mg, 2.33 mmol) is diluted in THF/H_2O (1:1, 20 mL), sodiumhypophosphite-monohydrate (1.36 g, 12.8 mmol) and palladium on activated carbon (10 wt%, 30.9 mg, 0.29 mol) are added and the mixture is stirred for 15 min at room temperature. The suspension is filtered and washed with THF/H_2O (1:1). The filtrate is diluted with dH_2O, extracted with diethylether, the organic phase is dried over magnesium sulfate and the solvent is evaporated *in vacuo*. **1c** (194 mg, 1.32 mmol, 57%) is obtained after flash-column chromatography (5-40% ethyl acetate in cyclohexane) as brownish oil. **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 7.67 (ddd, J = 9.4 Hz, J_{FH} = 5.2, 2.4 Hz, 1H), 6.99 (ddd, J = 9.3 Hz, J_{FH} = 8.7, 7.2 Hz, 1H), 4.80 (d, J_{FH} = 1.3 Hz, 2H), 2.32 (s, 3H). **^{13}C{1H} NMR** (126 MHz, $CDCl_3$) δ (ppm): 202.9, 154.0 (dd, J_{FC} =259.6, 11.5 Hz), 144.7 (dd, J_{FC} =252.9, 14.5 Hz), 142.5 (dd, J_{FC} = 10.9, 2.9 Hz), 139.9, 120.7 (dd, J_{FC} = 9.0, 4.0 Hz), 111.7 (d, J_{FC} = 19.2 Hz), 78.1 (d, J_{FC} = 5.3 Hz), 26.4. The analytical data corresponds with literature data.[13] **chiral HPLC**: Chiralpak® OJ-H (*n*-hexane:2-propanol = 95:5, 0.8 mL min^{-1}, 30 °C, 10 MPa, 210 nm, t_R = 50.8 min.[45]

Synthesis of *rac*-3,4-dihydro-2*H*-1,4-benzoxazines 2a-d as reference compounds.

3-phenyl-3,4-dihydro-2*H*-benzoxazine (2a). 1a (300 mg, 1.43 mmol) is dissolved in dichloromethane (5 mL). Hantzsch Ester (507 mg, 2.00 mmol) and diphenylphosphate (18.0 mg, 0.07 mmol) are added and the mixture is stirred for 24 h at 40 °C under argon atmosphere. The solvent is evaporated *in vacuo*. **2a** (266 mg, 1.26 mmol, 88%) is obtained after flash-column chromatography (1-10% ethyl acetate in cyclohexane) as colorless oil. **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 7.43–7.33 (m, 5H), 6.87–6.80 (m, 2H), 6.70 (ddd, J = 17.5, 7.7, 1.6 Hz, 2H), 4.52 (dd, J = 8.6, 3.0 Hz, 1H), 4.29 (dd, J = 10.6, 3.0 Hz, 1H), 4.01 (dd, J = 10.7, 8.6 Hz, 1H). **^{13}C{1H} NMR** (126 MHz, $CDCl_3$) δ (ppm): 142.9, 138.5, 133.3, 128.2, 127.7, 126.6, 120.9, 118.3, 116.0, 114.8, 70.4, 53.6. **MS (ESI)** m/z calculated for $C_{14}H_{14}NO$ $[M+H]^+$: 212.1, found: 212.0. The analytical data corresponds with literature data.[18] **chiral SFC-HPLC**: Chiralpak® OJ-H (supercritical CO_2:EtOH (Et_2NH) = 85:15 (0.015), 1 mL min^{-1}, 20 °C, 10 MPa, 210 nm, t_R (*S*) = 28.7 min, t_R (*R*) = 30.6 min.

2,2-dimethyl-3,4-dihydro-3-phenyl-2*H*-1,4-benzoxazine (2b) (according to ref.[44]). 1b (50.0 mg, 0.24 mmol) is diluted in THF/H_2O (1:1, 10 mL), sodiumhypophosphite-monohydrate (76.0 mg, 0.70 mmol) and palladium on activated carbon (10% w/w, 10.0 mg) are added and the mixture is stirred for 24 h at room temperature. The suspension is filtered and washed with THF/H_2O (1:1). The filtrate is diluted with dH_2O, extracted with diethylether, the organic phase is dried over magnesium sulfate and the solvent is evaporated *in vacuo*. **2b** (34.0 mg, 0.14 mmol, 59%) is obtained after flash-column chromatography as brownish oil. **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 7.43 (m, 2H), 7.34 (m, 3H), 6.84 (dd, J = 7.9, 1.3 Hz, 1H), 6.80 (td, J = 7.6, 1.4 Hz, 1H), 6.71 (td, J = 7.6, 1.5 Hz, 1H), 6.65 (dd, J = 7.7, 1.5 Hz, 1H), 4.21 (s, 1H), 3.99 (s, 1H), 1.31 (s, 3H), 1.17 (s, 3H). **^{13}C{1H} NMR** (126 MHz, $CDCl_3$) δ (ppm): 142.6, 140.1, 133.3, 128.5, 128.4, 128.2, 121.1, 119.1, 117.1, 114.8, 62.8, 26.4, 20.5. **MS (ESI)** m/z calculated for: $C_{16}H_{17}NO$ $[M+H]^+$: 240.1, found: 240.0. The analytical data corresponds with literature data.[44] **chiral SFC-HPLC**: Chiralpak® OJ-H (supercritical CO_2:EtOH (Et_2NH) = 90:10 (0.01), 1 mL min^{-1}, 20 °C, 10 MPa, 210 nm, t_R (*S*) = 30.0 min, t_R (*R*) = 40.3 min.

7,8-difluoro-3-methyl-3,4-dihydro-2*H*-benzoxazine (2c) (according to ref.[45]). 1-(2,3-Difluoro-6-nitrophenoxy)propan-2-one (1.85 g, 8.00 mol) is dissolved in methanol (200 mL) and palladium on activated carbon (800 mg, 7.52 mmol 10%wt) is added. The mixture is stirred under hydrogen atmosphere (20 bar) in a high-pressure autoclave for 6 h at room temperature. The suspension is filtered, washed with diethylether and the solvent is evaporated *in vacuo*. **2c** (1.36 g, 7.35 mmol, 92%) is obtained as colorless oil after flash-column chromatography (100% ethyl acetate). **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 6.55 (ddd, J = 10.0 Hz, J_{FH} = 9.0, 7.8 Hz, 1H), 6.25 (ddd, J = 9.0 Hz, J_{FH} = 4.7, 2.3 Hz, 1H), 4.28 (dd, J = 10.5, 2.8 Hz, 1H), 3.78 (dd, J = 10.4, 8.2 Hz, 1H), 3.61 (s, 1H), 3.53–3.47 (m, 1H), 1.19 (d, J = 6.4 Hz, 3H). **^{13}C{1H} NMR** (126 MHz, $CDCl_3$) δ (ppm): 144.6 (dd, J_{FC} = 236.9, 10.8 Hz), 140.8 (dd, J_{FC} = 244.2, 15.7 Hz), 133.4 (dd, J_{FC} = 10.6, 2.8 Hz), 131.1 (t, J_{FC} = 2.5 Hz), 108.6 (dd, J_{FC} = 7.4, 3.8 Hz), 107.9 (d, J_{FC} = 18.3 Hz), 71.1, 45.0, 17.6. **MS (ESI)** m/z calculated for $C_9H_{10}F_2NO$ $[M+H]^+$: 186.06, found: 186.0. The analytical data corresponds with literature data.[45] **chiral HPLC**: Chiralpak® OJ-H (*n*-hexane:2-propanol = 95:5, 0.8 mL min^{-1}, 30 °C, 10 MPa, 210 nm, t_R (*R*) = 24.5 min, t_R (*S*) = 26.3 min.[45]

3-(4-bromophenyl)-3,4-dihydro-2*H*-1,4-benzoxazine (2d) (according to ref.[18]). 3-(4-Bromphenyl)-2*H*-1,4-benzoxazin (0.20 g, 0.70 mmol) is dissolved in dichloromethane (3 mL). Hantzsch Ester (0.25 g, 1.00 mmol) and diphenylphosphate (8.00 mg, 5 mol%) are added and the mixture is stirred for 24 h at 40 °C under argon atmosphere. The solvent is evaporated *in vacuo*. **2d** (159 mg, 0.05 mmol, 79%) is obtained after flash-column chromatography (5-20% ethyl acetate in cyclohexane) as colorless solid. **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 7.57–7.45 (m, 2H), 7.31–7.27 (m, 2H), 6.88–6.78 (m, 2H), 6.75–6.65 (m, 2H), 4.49 (d, *J* = 8.4 Hz, 1H), 4.29 – 4.22 (m, 1H), 3.96 (t, *J* = 9.3 Hz, 1H). **^{13}C{1H} NMR** (126 MHz, $CDCl_3$) δ (ppm): 143.9, 138.7, 133.9, 132.4, 129.2, 122.6, 122.0, 119.6, 117.1, 115.8, 71.1, 54.1. **MS (ESI)** m/z calculated for $C_{14}H_{13}BrNO$ $[M+H]^+$: 289.0, found: 290.1. The analytical data corresponds with literature data.[46] **chiral SFC-HPLC**: Chiralpak® OB-H (supercritical CO_2:EtOH (Et_2NH) = 80:20 (0.02), 1 mL min^{-1}, 20 °C, 10 MPa, 210 nm, t_R = 20.3 min, t_R = 27.8 min.

General Procedure: Biotransformations of 2*H*-1,4-benzoxazines using IREDs. Biotransformations of *2H*-1,4-benzoxazines **1a-c** are performed on 0.5 mL scale at 30 °C and 850 rpm in 100 mM KP_i buffer pH 7, containing 4% v/v of DMSO (in case of **1a**, **1b** and **1d**) or methanol (in case of **1c**) as co-solvent, 40 mM D-glucose, 20 mM *2H*-1,4-benzoxazine **1a-d**, 0.6 mg mL^{-1} (in case of substrate **1a**) or 1.2 mg mL^{-1} (in case of substrates **1b-c**) or 10 mg mL^{-1} (in case of **1d**) IRED crude extract, 12 U (in case of 0.6 mg mL^{-1} IRED), 24 U (in case of 1.2 mg mL^{-1} IRED) or 250 U (in case of 10 mg mL^{-1} IRED) of GDH and 0.1 mM $NADP^+$. After 8, 15, 19.5 or 24 h, the reaction is stopped by adding 10 µL of 32% NaOH solution and 300 µL of dichloromethane. Phase separation is promoted by centrifugation. The conversion and the enantiomeric excess are determined by analyzing the organic phase by SFC-HPLC (HPLC methods are described in Experimental procedures for each substrate and product).

Preparative scale biotransformation of 1a. The preparative scale biotransformation (40 mL) is performed from a 50 mM concentration of **1a**, 5 mg mL^{-1} of lyophilized whole-cell catalyst (which is prepared from a 10 mg mL^{-1} of cell suspension in 50 mM KP_i buffer pH 7), 120 mM of D-glucose, 0.1 mM of $NADP^+$ and 10% v/v of DMSO as cosolvent in distilled water. The flask is equipped with the titration device and pH electrode of a pH stat apparatus and stirred at 30°C for 26 h. By addition of aqueous NaOH solution, the pH is kept stable at pH 7. The reaction is stopped by adding 2 mL of 32% NaOH solution and 50 mL of dichloromethane. Phase separation is promoted by centrifugation. The organic phase is dried over magnesium sulfate. The conversion (> 99%) and the enantiomeric excess (99%) are determined by analyzing the organic phase by means of chiral SFC-HPLC (Chiralpak® OJ-H (supercritical CO_2:EtOH (Et_2NH) = 85:15 (0.015), 1 mL min^{-1}, 20 °C, 10 MPa, 210 nm). The crude product is purified by flash column chromatography (2-40% ethyl acetate in cyclohexane). (*S*)-3-phenyl-3,4-dihydro-2*H*-benzoxazine ((*S*)-**2a**) (300.8 mg, 1.42 mmol, 71%) is obtained as a yellowish oil with an isolated yield of 71%, a purity of 98% (determined by ^{1}H NMR spectroscopy) and 99% *ee*. The ^{1}H and ^{13}C NMR spectra of this experiment are shown in the SI. **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 7.42–7.31 (m, 5H), 6.87–6.80 (m, 2H), 6.73–6.67 (m, 2H), 4.52 (dd, *J* = 8.6, 3.0 Hz, 1H), 4.29 (dd, *J* = 10.6, 3.0 Hz, 1H), 4.01 (dd, *J* = 10.6, 8.6 Hz, 1H). **^{13}C{1H} NMR** (126 MHz, $CDCl_3$) δ (ppm): 143.7, 139.3, 134.0, 129.0, 128.5, 127.3, 121.6, 119.1, 116.7, 115.5, 71.1, 54.4. The analytical data corresponds with literature data.[18] $[\alpha]^{20}_D$ = + 148 (c 1,0, $CHCl_3$), literature: $[\alpha]^{20}_D$ = - 118.1 (c 1,0, $CHCl_3$) (98% *ee* for the (*R*)-enantiomer)[18]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Information about original IRED strains, SDS-PAGE, HPLC chromatograms, ^{1}H and ^{13}C NMR spectra (PDF).

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The manuscript was written through contributions of all authors.

Notes

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Biocatalytic reduction of 2*H*-1,4-benzoxazines