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Biocatalytic Oxidative C–C Bond Formation Catalysed by the Berberine Bridge Enzyme: Optimal Reaction Conditions

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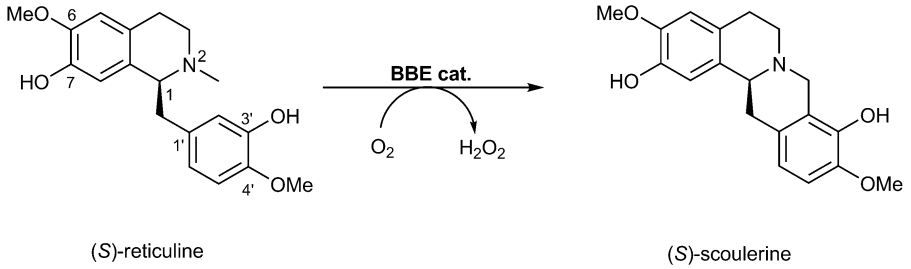
Abstract: Berberine bridge enzyme (BBE) catalyses the oxidative formation of an intramolecular C–C bond using (*S*)-reticuline as the natural substrate to form (*S*)-scoulerine as the product. To allow application of the enzyme on a preparative scale for the synthesis of novel optically pure berbine and isoquinoline derivatives, an organic solvent is required to solubilise the barely soluble substrates. It was shown that BBE tolerates a broad variety of organic co-solvents. Ideally the enzymatic enantioselective oxidative C–C bond formation can be performed in 70% $v v^{-1}$ toluene concentration, which allowed a soluble substrate concentration of at least 20 $g L^{-1}$. In addition, the enzyme works in a broad operational window concerning pH and temperature. High conversions can be reached between pH 8 and 11 and from 30 to 50 °C, respectively. The enantioselective oxidative C–C bond formation was demonstrated on a preparative scale (500 mg) in a kinetic resolution leading to optically pure products (>97% *ee*).

Keywords: alkaloids; biotransformations; C–C bond formation; enzyme catalysis; oxidation

Introduction

Employing biocatalysts for synthetic organic chemistry gains constantly increasing significance.[1–4] Focusing on C–C bond formation,[5] aldolases and transketolases,[6–9] hydroxynitrile lyases,[5,10–12] ThDP-dependent enzymes[5–15] as well as laccases and peroxidases[16–18] belong to the most commonly employed biocatalysts. Berberine bridge enzyme (BBE), first identified in 1963,[19,20] catalyses in nature an outstanding oxidative intramolecular C–C bond formation by bridging a phenol moiety to an *N*-methyl group of (*S*)-reticuline to yield (*S*)-scoulerine at the expense of molecular oxygen (Scheme 1). The enzyme contains a bicovalently bonded flavin responsible for the reaction.[21]

Recently, it could be shown that recombinant BBE from *Eschscholzia californica* (California poppy)[22,23] can be employed efficiently for the preparation of novel optically pure isoquinoline and berbine alkaloids *via* enantioselective oxidative C–C bond formation.[24] The enzyme displayed perfect enantioselectivi-



(*S*)-reticuline (*S*)-scoulerine

Scheme 1. Natural reaction catalysed by the berberine bridge enzyme (BBE).

ty, thus only a single substrate enantiomer of a racemate was converted. Unfortunately, potential substrates of BBE, isoquinolines, are only barely soluble in buffer at a useful preparative concentration (>50 mM); consequently an organic solvent is required to solubilise the substrate. Mainly lipases and proteases have been described to show a significant tolerance toward organic solvents beside a few other enzymes like alcohol dehydrogenases.[25] Nevertheless, the solvent tolerance can be improved, for instance, by protein engineering[26–31] or immobilisation.[32–36]

In this study we report in detail on the influence of the organic solvent, pH, temperature, and buffer on the conversion of the oxidative C–C bond formation catalysed by BBE from *Eschscholzia californi*ca.

Results and Discussion

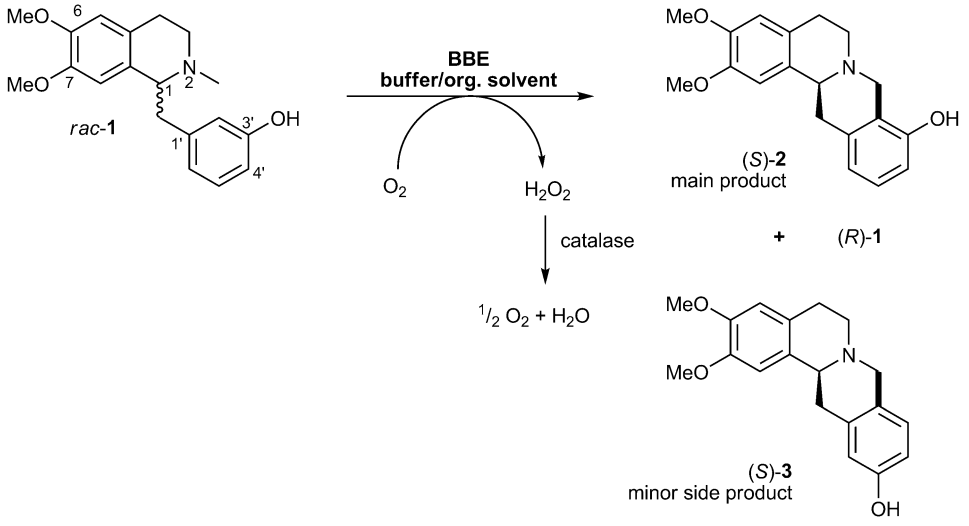
For studying the influence of organic solvents and other relevant parameters a racemic non-natural model substrate **1** (Scheme 2) was chosen.[22] The model substrate was designed considering the natural substrate reticuline as a template, but omitting the methoxy group of reticuline in the 4′-position and introducing a methoxy instead of the hydroxy group in position 7. Before starting with the organic solvents some basic pre-experiments were performed: hydrogen peroxide is formed as a side product, which might harm the enzyme. To test this, experiments were performed with and without catalase. In presence of catalase a 4-fold higher conversion (37 *vs.* 9.5%) was achieved than without catalase. Additional studies showed that $MgCl_2$ has a stabilising effect on the enzyme. For this reason catalase and $MgCl_2$ (10 mM) were added to the reaction mixtures.

As a first approach to evaluate the tolerance of the enzyme towards organic solvents various water miscible, water immiscible solvents and ionic liquids were tested at a concentration of 10% $v\,v^{-1}$ at 6.5 mM substrate concentration (Figure 1). In most tested water miscible and immiscible organic solvents high conversion was achieved. Low conversions were obtained in the presence of selected water miscible organic solvents (THF, pyridine), some immiscible solvents (CH_2Cl_2, EtOAc, $CHCl_3$) and one tested water immiscible ionic liquid [trioctylmethylammonium bis(trifluoromethylsulfonyl)imide = TOMAbisTMSI]. Moderate to high conversion was achieved employing water miscible ionic liquids (Ammoeng 102, T-2HMAMS, BMimAc, EMimAc).

Due to the promising tolerance of the enzyme towards organic solvents, the organic solvent concentration was increased to 20% $v\,v^{-1}$ (Figure 2). Since a number of common organic solvents were superior compared to ionic liquids, the ionic liquids were not tested further.

At 20% $v\,v^{-1}$ of co-solvent all water miscible co-solvents seemed to affect the enzyme, except for methanol and DMSO, whereby DMSO was the best. All water immiscible solvents tested led to perfect conversion for an enantioselective kinetic resolution (50%).

Consequently DMSO and toluene were chosen for further studies to reach even higher co-solvent concentrations. Toluene was chosen since it is less toxic than benzene and more commonly employed than diphenyl ether. On increasing the concentration of co-solvent up to 40% $v\,v^{-1}$, DMSO caused a significantly reduced conversion at 30% $v\,v^{-1}$ and complete loss of activity at 40% $v\,v^{-1}$ (Figure 3). In contrast, toluene still led to perfect conversion at 40% $v\,v^{-1}$.



Scheme 2. Enantioselective oxidative C–C bond formation of the non-natural racemic substrate **1** catalysed by berberine bridge enzyme (BBE).

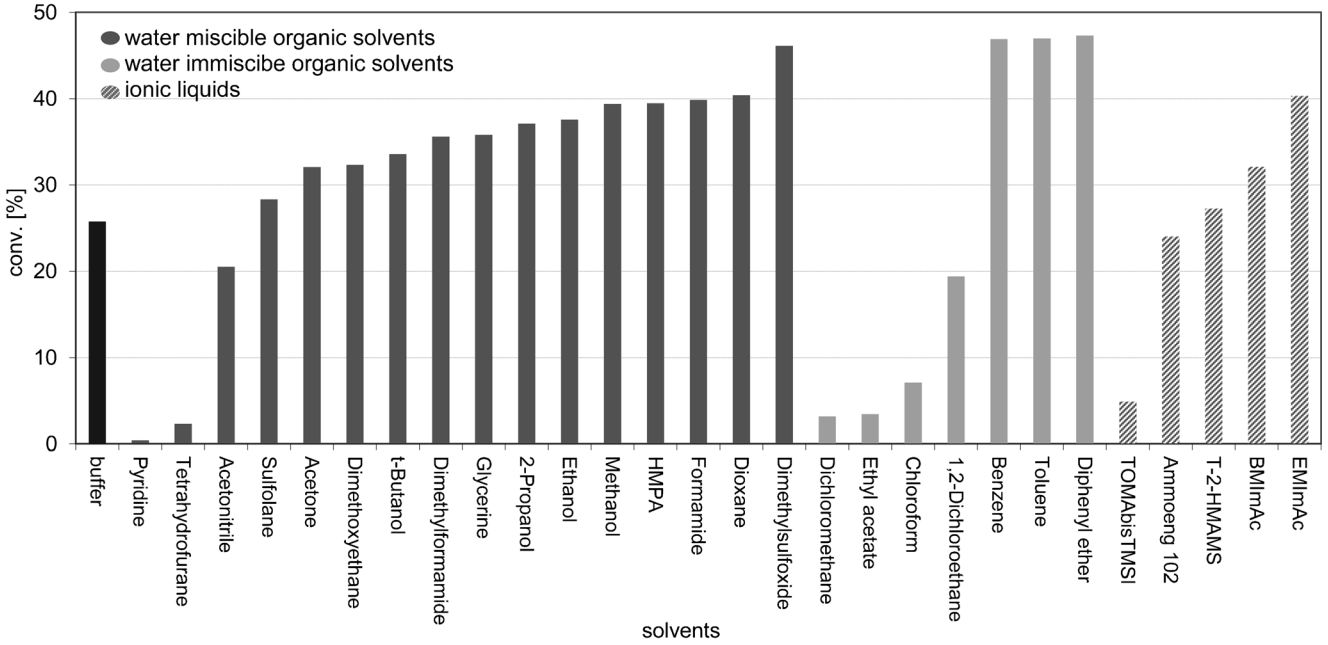


Figure 1. Conversion in the presence of various solvents 10% (vv^{-1}). *Reaction conditions:* 2 gL^{-1} substrate **1** (6.5 mM), BBE (0.0017 mM), Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9, 10% vv^{-1} organic solvent, 5 gL^{-1} crude catalase, 4 h, 40 °C; TOMA-bisTMSI: trioctylmethylammonium bis(trifluoromethylsulfonyl)imide, T-2-HMAMS: tris-(2-hydroxyethyl)-methylammonium methyl sulfate, BMimAc: 1-butyl-3-methylimidazolium acetate, EMimAc: 1-ethyl-3-methylimidazolium acetate.

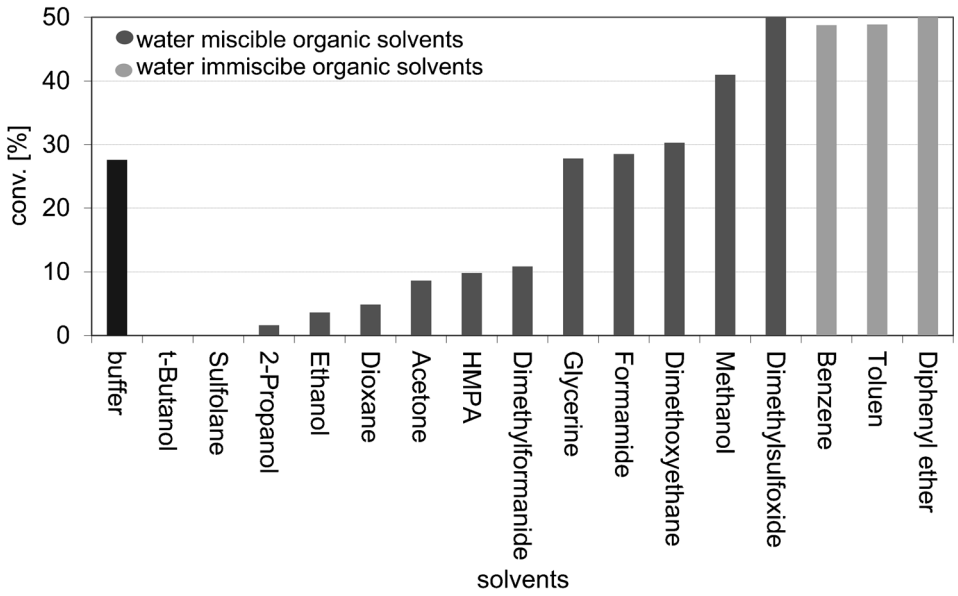


Figure 2. Conversion in the presence of 20% vv^{-1} organic solvents. *Reaction conditions:* 2 gL^{-1} substrate **1** (6.5 mM), 0.1 gL^{-1} BBE, Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9, 20% vv^{-1} organic solvent, 5 gL^{-1} crude catalase, 4 h, 40 °C.

Encouraged by these results, the concentration of toluene was further increased up to 100% (Figure 4). For comparison the related experiments were performed in the presence of benzene. These experiments were already performed at a substrate concentration of 13 mM, thus the concentration was doubled compared to previous studies.

Product was detected at solvent concentrations up to 99% vv^{-1}. Just in toluene alone no conversion was detected. This experiment was performed with freeze-dried enzyme; additional tests ensured that the freeze-dried enzyme is active.

In the case of benzene full conversion (50%) was achieved at a solvent concentration of 40 to 60% vv^{-1}, while in the case of toluene also at 80% vv^{-1}

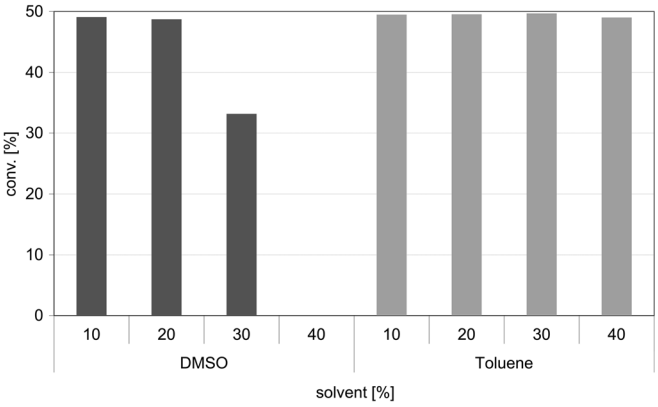


Figure 3. Comparison of DMSO and toluene at solvent concentrations from 10 to 40% vv^{-1}. *Reaction conditions:* 2 gL^{-1} substrate **1** (6.5 mM), BBE (0.0017 mM), Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9, co-solvent, 5 gL^{-1} crude catalase, 4 h, 40 °C.

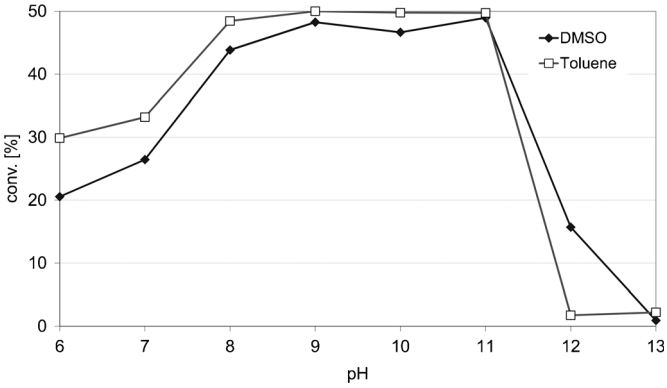


Figure 5. Conversion at varied pH values ranging from 6 to 13. *Reaction conditions:* 2 gL^{-1} substrate **1** (6.5 mM), BBE (0.0017 mM), Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 6–13, co-solvent: DMSO 10% vv^{-1}, toluene 70% vv^{-1}, 5 gL^{-1} crude catalase, 4 h, 40 °C.

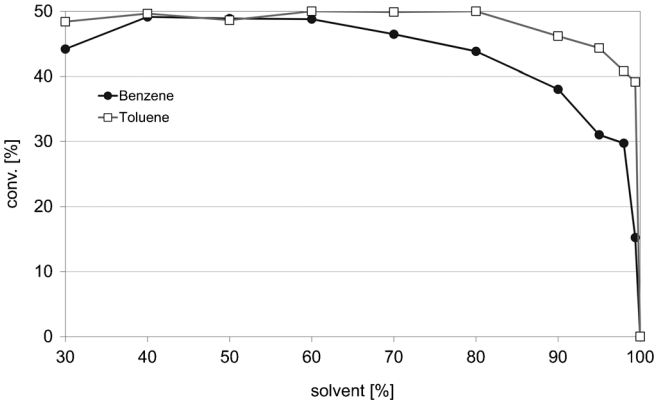


Figure 4. Conversion in the presence of toluene and benzene reaching from 30% to 100% (vv^{-1}). *Reaction conditions:* 4 gL^{-1} substrate **1** (13 mM), BBE (0.0017 mM), Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9, co-solvent, 5 gL^{-1} crude catalase, 4 h, 40 °C.

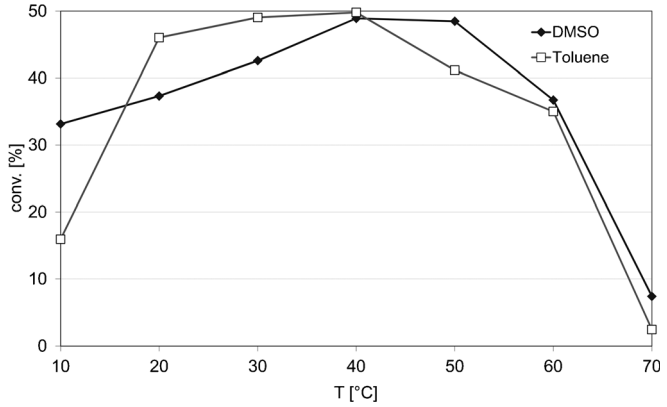


Figure 6. Conversion at varied temperature. *Reaction conditions:* 2 gL^{-1} substrate **1** (6.5 mM), BBE (0.0017 mM), Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9, co-solvent: DMSO 10% vv^{-1}, toluene 70% vv^{-1}, 5 gL^{-1} crude catalase, 4 h.

perfect conversion was found. A toluene concentration of 70% vv^{-1} was considered as ideal for an optimal performance.

In addition to the type of co-solvent also the optimal pH of the reaction medium had to be elucidated. In the literature the pH optimum for the aqueous buffer is reported to be pH 9.[20] Testing toluene in a bi-phasic system and DMSO for comparison in a mono-phasic system revealed that the pH optimum was actually rather broad ranging from pH 8 to pH 11 (Figure 5). At pH 12 only in the case of DMSO (10% vv^{-1}) was a significant conversion detected, while in the case of toluene almost no conversion was observed.

Four different buffer salts (Tris-HCl, phosphate buffer, Ches-HCl and triethylamine-HCl) were evalutated at varied buffer concentration (10–50 mM, 2 gL^{-1} substrate); all buffer systems led to similar results. Thus, neither the buffer salt nor the salt concentration had any significant effect on the conversion (data not shown).

The published assays for the BBE were performed at 37 °C[37,38] and 45 °C.[39] This was also the reason why all studies described here until now were performed at 40 °C as a compromise. To get a clear picture for the temperature optimum, the conversion was determined at temperatures between 10 °C and 70 °C employing toluene as co-solvent but also DMSO for comparison.

High conversion was reached over a broad temperature range between 30–50 °C (Figure 6). Nevertheless, highest conversion was achieved at 40 °C in the cases of DMSO and toluene, respectively.

Having identified optimised reaction conditions, a time study was performed at 20 gL^{-1} (65 mM) substrate concentration to clarify when the reaction

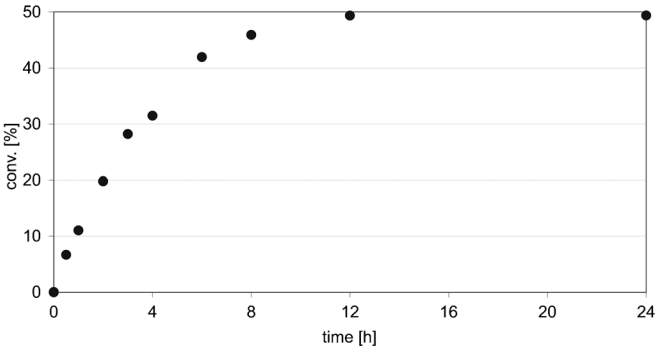


Figure 7. Time course of enantioselective oxidative C–C bond formation of substrate **1** catalysed by BBE. *Reaction conditions:* 20 g L^{-1} substrate 1 (65 mM), BBE (0.017 mM), Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9, 70% v v^{-1} toluene, 5 g L^{-1} crude catalase, 4 h.

reaches full conversion (50%) (Figure 7). For this study the enzyme concentration was increased to 0.017 mM to reach completion within a reasonable time.

Using the optimised conditions the reaction reached 49% conversion within 12 h. The space time yield is therefore 10 g L^{-1} d^{-1}. For the turnover number a value of 1849 was calculated.

Performing a preparative transformation, 500 mg of substrate **1** (65 mM) were shaken for 24 h under optimised conditions leading to 50% conversion. Work-up of the reaction yielded 249 mg (50%) of optically pure (*R*)-**1** (*ee* > 97%) and 207 mg (42%) of optically pure (*S*)-**2** (*ee* > 97%) besides 8% of regioisomer **3**.

Conclusions

Berberine bridge enzyme (BBE) was shown to tolerate organic solvents, especially toluene, DMSO, benzene and diphenyl ether. Ideally, the enzymatic enantioselective oxidative C–C bond formation was performed at 70% v v^{-1} toluene concentration, which allowed a substrate concentration of 20 g L^{-1}. Its excellent stability in the presence of organic solvents renders the enzyme suitable for application in organic synthesis to prepare non-natural optically pure alkaloids. Additionally, the enzyme works in a broad operational window concerning pH and temperature. High conversions were obtained between pH 8 and 11 and from 30 to 50 °C, respectively, with still good activity remaining at 60 °C.

The reaction was demonstrated successfully on a preparative scale (500 mg) leading to optically pure products (*ee* > 99%).

Experimental Section

General Remarks

Substrate **1** was synthesised as previously described.[22] For the expression of BBE from *Eschscholzia californica* in *Pichia pastoris* and its purification see Supporting Information.

Analytics

Determination of conversion: Conversions were measured by HPLC: Shimadzu, Communication Bus Module CBM-20 A, column oven CTO-20 AC, degasser DGU-20 A5, liquid chromatograph LC-20 AD, auto sampler SIL-20 AC, diode array detector SPD-M20, C18 column (Phenomenex, LUNA C18, 0.46 cm × 25 cm, 5 μm). Eluent: acetonitrile/methanol/ammonium formate (30 mM, pH 2.8) 15:18:67 (isocratic), flow rate: 0.5 mL min^{-1}, detection wavelength: 280 nm. Retention times: **3**: 10.0 min, **1**: 13.2 min, **2**: 16.6 min.

Determination of optical purity: The HPLC system described above was equipped with a chiral column (Chiralcel OJ from Daicel Chemical Industries, 0.46 cm × 25 cm, Lot. No.: OJ00CE-NK006). Eluent: *n*-heptane (0.1% formic acid)/2-propanol (0.1% formic acid) 70:30, flow rate: 0.5 mL min^{-1} (isocratic), detection wavelength: 280 nm. Retention times substrate **1**: (*S*): 14.2 min, (*R*):18.8 min

For chiral analysis of product **2** the same system as mentioned above was used but employing as chiral phase Chiralpak AD from Daicel Chemical Industries (0.46 cm × 25 cm, Lot. No.: AD00CE-AB081). Eluent: *n*-heptane/2-propanol 70:30, flow rate: 0.5 mL min^{-1}, detection wavelength: 280 nm. Retention times: 18.4 min (*S*), 11.0 min (*R*).

Representative Procedure for Solvent Study

A stock solution of substrate *rac*-**1** in dichloromethane (2 mg mL^{-1}) was prepared and aliquots containing 1 mg of substrate were transferred into glass vials (4 mL). Dichloromethane was removed under constant air flow. The substrate was redissolved in the appropriate organic solvent (50 μL). For the addition of the enzyme a stock solution of BBE (0.1 mg mL^{-1}) and catalase (0.05 mg mL^{-1}) was prepared in Tris-HCl buffer (50 mM, pH 9, 10 mM $MgCl_2$). After adding the enzyme stock solution (450 μL) to the substrate in organic solvent (final concentration: substrate 2 g L^{-1}, BBE 0.1 g L^{-1}, catalase 5 g L^{-1}), the glass vials were closed with screw caps containing sealing gaskets and samples were shaken in an Incubator Mini Shaker (VWR, rotary, orbit 3 mm) with 200 rpm at 40 °C for four hours. The reaction was extracted with ethyl acetate (3 × 300 μL) and the combined organic layers were dried (Na_2SO_4). The organic solvents were removed under constant air flow and the residue was redissolved in methanol (1 mL, Roth, Rotisolv HPLC grade, Lot. P717.1) for HPLC analysis. This procedure was repeated with 20% v v^{-1} (100 μL) for selected co-solvents.

Influence of Catalase

The experiments were performed similar as described above. Substrate concentration: 8 g L^{-1}, BBE amount:

0.1 $g L^{-1}$, catalase amount: 5 $g L^{-1}$, reaction time: 24 h, co-solvent: methanol 10% $v v^{-1}$, buffer: Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH of the buffer: 9, reaction temperature: 40 °C. The work-up was performed as described above.

pH Study

The experiments were performed similar as described above. A Tris-HCl buffer (50 mM + $MgCl_2$ 10 mM) with pH values from 6 to 13 was used (the pH was measured before addition of solvent). As co-solvent 10% $v v^{-1}$ of DMSO and toluene, respectively, was employed. Conditions: 2 $g L^{-1}$ of substrate, BBE: 0.1 $g L^{-1}$, Catalase: 5 $g L^{-1}$, reaction temperature: 40 °C, reaction time: 4 h. Before extraction with ethyl acetate (3 × 300 μL), 500 μL of 500 mM Tris-HCl buffer pH 9 was added to minimise different extraction behaviour at different pH values. After extraction the work-up was performed as described above.

Time Study

The experiments were performed using 20 $g L^{-1}$ of substrate in 70% $v v^{-1}$ toluene and 30% $v v^{-1}$ Tris-HCl buffer, 1 $g L^{-1}$ of BBE and 5 $g L^{-1}$ catalase in a total volume of 0.5 mL. Samples were taken at 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h. Work-up and analysis were performed as described above.

Preparative Transformation

Substrate **1** (500 mg, 1.6 mmol, final concentration: 20 $g L^{-1}$ = 65 mM) was shaken in a mixture of toluene 70 $v v^{-1}$ (17.5 mL) and buffer (7.5 mL, Tris-HCl, 50 mM, pH 9, 10 mM $MgCl_2$) with BBE (1.5 mL enzyme solution, final concentration: 1 $g L^{-1}$ = 0.017 mM) and catalase (125 mg crude preparation). The mixture was shaken in a light-shielded round bottom flask (50 mL) in an Incubator Mini Shaker (VWR, rotary, orbit 3 mm) at 200 rpm and 40 °C for 24 h. Reaction work-up: the phases were separated separation followed by extraction of the aqueous phase with ethyl acetate (3 × 10 mL). The combined organic phases were dried (Na_2SO_4) and the organic solvents were removed under reduced pressure. The crude product was purified by silica gel chromatography (silica gel 60, 0.040–0.063 mm, Merck, Lot.: 1.09385.9025; eluent: CH_2Cl_2/MeOH/NH_4OH 97:2:1)

For analytical details, preparation of enzyme and product characterization see Supporting Information.

Acknowledgements

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