VIP Biocatalytic C–C Coupling

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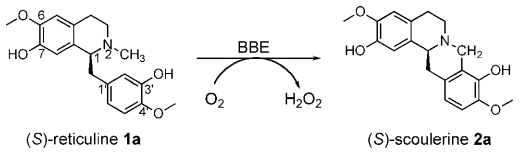
Biocatalytic Enantioselective Oxidative C–C Coupling by Aerobic C–H Activation**

*Joerg H. Schrittwieser, Verena Resch, Johann H. Sattler, Wolf-Dieter Lienhart, Katharina Durchschein, Andreas Winkler, Karl Gruber, Peter Macheroux, and Wolfgang Kroutil**

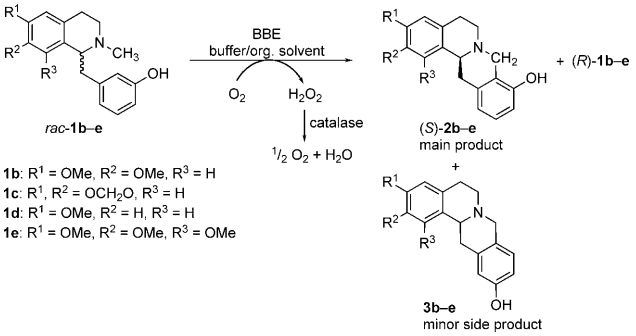
Reactions that form carbon–carbon bonds are the basis for organic chemistry, setting up the carbon framework of organic molecules. In particular, the formation of C–C bonds by organo- or metal-catalyzed activation of C–H bonds[1] has recently obtained increased attention.[2] However, to the best of our knowledge, no biocatalytic oxidative C–C bond-forming reaction has yet been exploited for synthetic purposes. To date, biocatalysis only offers a limited number of enzymes for synthetic C–C bond formation,[3] such as aldolases,[4] transketolases[4d,5] and hydroxynitrile lyases,[3,6] and thiamine diphosphate (ThDP)-dependent enzymes.[3,7] Other enzymatic C–C bond-forming reactions have just started to be investigated.[8] All of these enzymes are either lyases or transferases, but not redox enzymes.[9]

In contrast, the berberine bridge enzyme (BBE) [EC 1.21.3.3] is a redox enzyme that converts (*S*)-reticuline **1a** as the natural substrate with a 1-benzyl-1,2,3,4-tetrahydroisoquinoline backbone to (*S*)-scoulerine **2a**, a berbine derivative. The transformation occurs through an intramolecular C–C coupling by activation of the methyl group attached to the tertiary nitrogen atom at the expense of molecular oxygen (Scheme 1).[10] The enzyme is found in plants, mainly from the poppy family, where it plays a central role in the biosynthesis of benzophenantridine alkaloids.[11,12] Only recently, BBE from *Eschscholzia californica* (California poppy) could be expressed efficiently in *Pichia pastoris*[13] to give a sufficient quantity of the enzyme for crystallization and investigation of the mechanism.[10,14] Thus, BBE has been thoroughly investigated regarding its biochemical properties and catalytic mechanism, while its potential as a biocatalyst for preparative transformation of non-natural substrates has not been explored yet. A major concern referred to the question whether non-natural substrates could be transformed at all, since plant enzymes may have strict substrate specificity. Furthermore, no information on the enantioselectivity of the catalyst for non-natural substrates was available. The biochemical investigations of BBE were all performed on a microgram[10] scale and at low substrate concentration (0.5 mM),[15] both of which have to be significantly increased for preparative applications.



Scheme 1. Natural reaction of BBE. Formation of the "berberine bridge" by oxidative C–C coupling at the expense of molecular oxygen.

To gain sufficient quantities of a non-natural model substrate that can easily be prepared, racemic tetrahydroisoquinoline *rac*-**1b** (Scheme 2), which lacks the methoxy group of reticuline in the 4′-position and possesses a methoxy instead of the hydroxy group in position 7 of the benzylisoquinoline backbone, was prepared in five steps with 40% overall yield (see the Supporting Information). As a first test, reductive rate measurements were performed, which clearly indicated that the non-natural substrate *rac*-**1b** was accepted



Scheme 2. Biocatalytic enantioselective oxidative C–C coupling of non-natural substrates by BBE led to optically pure (*R*)-**2b–e** and (*S*)-**2b–e** as the main products by kinetic resolution.

[*] J. H. Schrittwieser,[+] V. Resch,[+] J. H. Sattler, W.-D. Lienhart, K. Durchschein, Prof. Dr. W. Kroutil
Department of Chemistry, Organic and Bioorganic Chemistry
University of Graz, Heinrichstrasse 28, 8010 Graz (Austria)
Fax: (+43) 316-380-9840
E-mail: wolfgang.kroutil@uni-graz.at
Dr. A. Winkler, Prof. Dr. P. Macheroux
Institute of Biochemistry, Graz University of Technology
Petersgasse 12, 8010 Graz (Austria)
Prof. Dr. K. Gruber
Institute of Molecular Biosciences, University of Graz
Humboldtstraße 50/III, 8010 Graz (Austria)
[+] These authors contributed equally to this work.

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by purified BBE at a promising rate of $k_{red} = 2\ s^{-1}$. A first preparative transformation (8 mg) under nonoptimized conditions allowed to identify the product of the transformation as **2b** by comparison of the MS spectrum with literature values.[16]

Subsequently, substrate *rac*-**1b** was used for further optimization studies. It quickly became clear that as soon as a higher substrate concentration was used and higher conversions were achieved, the hydrogen peroxide formed as byproduct inhibited or degraded the enzyme; therefore it was necessary to add catalase to disproportionate the formed hydrogen peroxide to water and molecular oxygen.[17]

Since substrate *rac*-**1b** was barely soluble in buffer, various water-miscible as well as water-immiscible organic solvents were tested for substrate solubilization. A substrate concentration of 2 $g\,L^{-1}$ (7 mM) was chosen for the studies of organic cosolvents as a first step to increase the substrate concentration compared to the biochemical studies. Testing the solvents at 10% v/v, BBE showed an unexpectedly high tolerance toward a broad range of organic solvents (Figure 1).

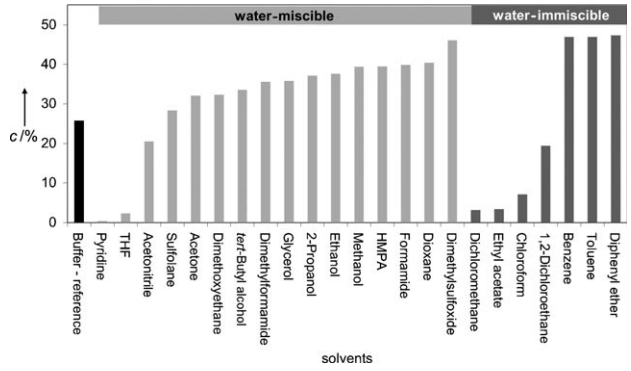

Figure 1. Conversion *c* of substrate *rac*-**1b** by BBE in the presence of organic solvents (10% v/v) in buffer.

For instance, dimethyl sulfoxide (DMSO) led to almost 50% conversion, which was the best value obtained for all water-miscible organic solvents tested. Nevertheless, also dioxane, formamide, methanol, ethanol, and even hexamethyl phosphoric triamide (HMPA) were accepted; methanol was already used in previous biochemical studies to solubilize the natural substrate (*S*)-reticuline. On the other hand, tetrahydrofuran (THF) led to low conversion. Similar low conversions were achieved by employing some water-immiscible solvents like dichloromethane or ethyl acetate. Best results were obtained by employing toluene, benzene, or diphenyl ether in a two-phase system. Toluene was chosen for further studies because of its lower toxicity compared to benzene and because of an easier work-up procedure compared to the water-miscible organic solvents. Testing the tolerance of BBE toward increasing concentrations of toluene showed that employing 80% v/v of toluene still led to 50% conversion within 24 h at 4 $g\,L^{-1}$ substrate concentration and 0.1 $g\,L^{-1}$ BBE (Figure 2). Even at 99% v/v toluene the enzyme was still remarkably active; however, in dry toluene no conversion could be detected. The latter experiment was

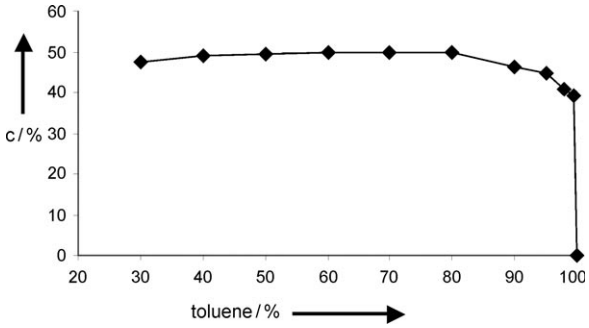

Figure 2. Enzymatic transformation of substrate *rac*-**1b** in the presence of increasing amounts of toluene (%v/v).

performed by suspending freeze-dried BBE in toluene; it was ensured that the enzyme was not damaged by freeze-drying: reactions with rehydrated enzyme showed full activity.

Experiments to chemically synthesize **2b** as reference material by Pictet–Spengler[18] reaction from N-demethylated **1b** and formaldehyde gave a mixture of the racemic regioisomers **2b**/**3b** in a 40:60 ratio according to GC-MS analysis. The products were isolated in low yields of 30% and 15%, respectively. Obviously, the enzymatic reaction is unique not only concerning the catalyzed transformation, but also with respect to regioselectivity. Nevertheless, the regioisomer **3b** was also found as a minor side-product of the enzymatic transformation in the solvent study. The formation of **3b** varied depending on 1) the type of solvent used and 2) solvent concentration. For instance, product **2b** and regioisomer **3b** were formed in a ratio of 96:4 when employing toluene (99.4% v/v), while when using acetonitrile (10% v/v) a ratio of 1:1 was obtained.[17]

In addition to the identification of a suitable cosolvent, other reaction parameters were optimized such as buffer salt and buffer concentration, pH value, temperature, shaking celerity, and the amount of catalase. The most suitable buffer was found to be 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) at pH 9 containing 10 mM $MgCl_2$. Reactions were ideally performed in the dark[19] by employing catalase, purified BBE (1 $g\,L^{-1}$), and a two-phase toluene/buffer mixture 70:30 v/v for a substrate concentration of 20 $g\,L^{-1}$. Employing these conditions, the transformation of *rac*-**1b** stops at 50% conversion after 12 h as shown in a time study. This corresponds to a space–time yield of 20 $g\,L^{-1}\,d^{-1}$ and an apparent turnover number of 1850.

The conversions obtained for the transformation of the racemic substrate *rac*-**1b** never exceeded 50%, which already indicated that the enzyme might possess excellent enantioselectivity. Indeed, the analysis of the reactions by HPLC on a chiral phase showed that only one single enantiomer was transformed while the other one remained untouched, consequently leading to an optically pure product. Thus, BBE catalyzed the kinetic resolution of *rac*-**1b** to give optically pure products (*S*)-**2b** and (*R*)-**1b** both in >97% *ee* as determined by HPLC, which corresponds to an enantioselectivity $E > 200$.

To test whether other racemic benzylisoquinoline derivatives would be transformed as well, substrates **1c–e** were

synthesized and subjected to BBE-catalyzed ring closure. Substrate *rac*-**1c** bears a methylene bridge between the two oxygen atoms at the isoquinoline part, *rac*-**1d** possesses only one methoxy and *rac*-**1e** three methoxy groups. All three benzylisoquinolines turned out to be good substrates that were enantioselectively transformed into the corresponding berbine derivative by oxidative C–C coupling. Most of the obtained products have never been described before, neither in optically pure nor racemic form. Compounds **1c** and **2c** have been described in racemic form only, and product **2b** has previously been described only after isolation of 5 mg of this compound (common name: manibacanine) from the stem bark of *Anila canelilla*.[16] Thus, the biocatalytic C–C coupling using BBE allowed to access novel optically pure alkaloids.

To demonstrate the applicability of the enzyme on a preparative scale, all four non-natural substrates (**1b–e**) were transformed on a 500 mg scale. All substrates could be fully resolved within 24 h and the products of the kinetic resolution (*R*)-**1b–e** and (*S*)-**2b–e** could be isolated with good to excellent yield and excellent optical purity (Table 1).

Table 1: Preparative oxidative C–C coupling employing BBE.[a]

Subst.	c[b] [%]	(*R*)-**1** [mg (%)]	*ee* (**1**)[c] [%]	(*S*)-**2** [mg (%)]	*ee* (**2**)[c] [%]	E[d]
1b	50	249 (50)	>97	207 (42)	>97	>200
1c	50	231 (46)	>97	155 (31)	>97	>200
1d	50	181 (36)	>97	177 (36)	>97	>200
1e	50	237 (47)	>97	194 (39)	>97	>200

[a] Reactions were performed in the dark in toluene/buffer 70:30, pH 9, at a substrate concentration of 20 g L^{-1}, 1 g L^{-1} BBE, 0.05 g L^{-1} catalase, 40 °C, 24 h. [b] Conversion was measured by HPLC on an achiral C18 phase. Depending on the substrate converted, 4–10% of the regioisomer **3b–e** were formed: **1b**: 8%, **1c**: 7%, **1d**: 4%, **1e**: 10%. [c] Enantiomeric excess was measured by HPLC on a chiral phase. [d] *E* value determined from the *ee* of the substrate and product.[20]

Benzylisoquinolines and berbines are two closely related families of alkaloids[21] that show a broad range of biological activities. For instance, 1-benzyl-1,2,3,4-tetrahydroisoquinolines have been found to act antispasmodic[22] or hypotensive.[23] Berbines possess many biological effects such as analgesic, sedative, tranquilizing, hypnotic, antihypertensive, hypo-locomotion, and muscle relaxation activity;[24] l-chloroscoulerine is expected to enable a novel treatment of schizophrenia.[25]

Chemical asymmetric synthesis of benzylisoquinoline and berbine alkaloids by various different strategies has been reported, in general requiring many steps and therefore resulting in limited overall yields.[26] Amongst the published procedures only few catalytic processes are found that involve metal-catalyzed asymmetric hydrogenation,[27] intramolecular allylic amination,[28] and metal- or organocatalyzed asymmetric alkylation reactions.[29] Despite the impressive progress in these areas, optically pure compounds (*ee* > 99%) were rarely obtained. The concept presented herein allows a novel approach and provides access to optically pure benzylisoquinoline and berbine alkaloids.

In summary, the berberine bridge enzyme from California poppy was employed for a highly enantioselective biocatalytic oxidative C–C coupling reaction to prepare optically pure berbine derivatives as well as optically pure tetrahydrobenzylisoquinolines. The described reaction was successfully performed on a 500 mg scale only requiring molecular oxygen as oxidant and mild reaction conditions, thus it represents a step towards cleaner and more selective organic transformations that expand the scope of C–C bond formation.[30]

Experimental Section

Representative preparative C–C coupling: Substrate **1b** (500 mg, 1.6 mmol, final concentration: 20 g L^{-1} = 65 mM) was dissolved in toluene (17.5 mL) and buffer (7.5 mL, Tris-HCl, 10 mM, pH 9, 10 mM $MgCl_2$) containing 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. The reaction was stopped by phase 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) to give 207 mg of (*S*)-**1b** (42% yield, > 97% *ee*) and 249 mg (*S*)-**2b** (49% yield, > 97% *ee*) (for full characterization (NMR spectra, HPLC data, optical rotation, HRMS, and CD spectra) see the Supporting Information).

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