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One-Pot Conversion of Cinnamaldehyde to 2-Phenylethanol via a Biosynthetic Cascade Reaction

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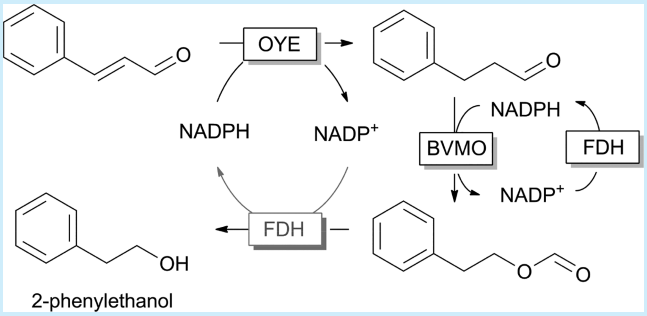
Supporting Information

ABSTRACT: A novel biosynthetic pathway for the production of natural 2-phenylethanol from cinnamaldehyde is reported. An ene-reductase (OYE)-mediated selective hydrogenation of cinnamaldehyde to hydrocinnamaldehyde is followed by a regioselective Baeyer−Villiger oxidation (BVMO) to produce the corresponding formate ester that either spontaneously hydrolyzes to 2-phenylethanol in water or is assisted by a formate dehydrogenase (FDH). This cascade reaction is performed in a one-pot fashion at ambient temperature and pressure. High selectivity and complete conversion were achieved.



2-Phenylethanol (2-PE) is an aromatic alcohol with a roselike aroma.[1] It is an important chemical used in the food and fragrance industry, with annual production of 2-PE estimated at more than 10000 tons.[2] Traditionally natural 2-PE is extracted and purified from flowers, specifically the hydrodistillation of rose petals.[3] This natural route, however, yields very low product recovery with very high cost implications. To meet the current global demands for 2-PE, most 2-PE is thus currently synthesized chemically. 2-PE can be chemically synthesized by a Grignard reaction from chlorobenzene[4] or via Friedel−Crafts alkylation of benzene.[5] Both of these chemical routes have several drawbacks including the use of hazardous or corrosive chemicals, difficult separation mixtures, and low selectivity. Alternatively, 2-PE production has been demonstrated via the catalytic hydrogenation of styrene oxide. Originally proven using Raney nickel as catalyst and hydrogen gas,[6] other nonpyrophoric catalysts have been developed.[7−9] Although the cost of chemical 2-PE is significantly lower than that of natural 2-PE, chemically synthesized 2-PE is limited in its use as an aroma compound in food, beverages, and cosmetics. Not only do these reactions rely on petrochemical feedstocks, but the formation of various side products, which at even very low concentrations can destroy the aroma of 2-PE.

The increased demand for natural products has seen the rapid development of biotechnological routes to 2-PE.[2,10−12] The US Food and Drug Administration and European legislation state that products from biotechnological (enzymatic or microbiological) processes can be classified as natural if the substrate used is of natural origin.[13,14] Yeasts such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* can convert L-phenylalanine via the Ehrlich pathway[15−17] to 2-PE via phenylpyruvate and phenylacetaldehyde when actively metabolizing cells are given L-Phe as the sole nitrogen source. The intermediates can, however, be overoxidized via endogenous dehydrogenases present in these yeasts. Additionally, 2-PE can also be further metabolized and degraded. 2-PE production is also eventually limited by its toxicity to growing cells. For the efficient production of 2-PE from L-Phe in situ product removal to avoid 2-PE toxicity is thus essential. Much research has been done on improving the productivity of this biological route, e.g., biphasic or in situ product removal[18−21] to overcome the toxicity of 2-PE and genetic engineering of yeasts strains to increase space−time yields (STY).[22] Engineered bacterial strains mimicking the Ehrlich pathway have also been created for the production of 2-PE from L-Phe[23−25] or from glucose by exploiting the shikimate pathway.[26−28] More recently, an *E. coli* strain coexpressing styrene monooxygenase (SMO), styrene oxide isomerase (SOI), and phenylacetaldehyde reductase (PAR) was shown to catalyze the hydration of styrene to 2-PE.[29] 2-PE production via this styrene pathway was also recently extended, enabling the conversion of L-Phe to styrene by introducing phenylalanine ammonia lyase (PAL) and phenylacrylic acid decarboxylase (PAD).[30] Similarly, an engineered styrene producing *E. coli* strain[31] was further modified through the introduction of SMO and SOI for 2-PE production from glucose.[32] Although product titers of ca. 2 g L^{-1} were reached, high glucose loading was required with yields of only 61 mg 2-PE g^{-1} glucose. 2-PE production from glucose could be significantly improved (ca. 5-fold) by utilizing two *E. coli* strains to couple L-Phe production from glucose and its further conversion to 2-PE.[33] Despite improved 2-PE titers, the system likewise required high glucose concentrations with L-Phe yields of only 60 mg g^{-1} glucose.

We propose a new synthetic route for natural 2-PE production from inexpensive and abundant cinnamaldehyde

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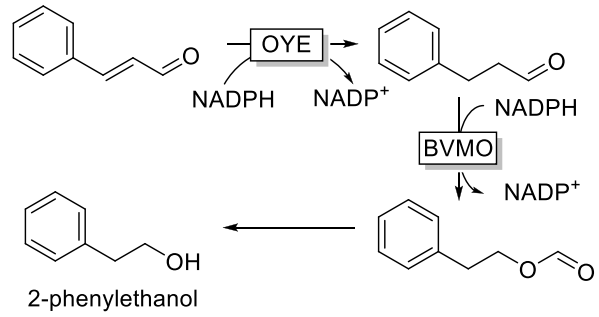
(Scheme 1) mediated via a biocatalytic cascade reaction utilizing a novel Baeyer–Villiger monooxygenase (BVMO).

Scheme 1. Biosynthetic Pathway of 2-Phenylethanol from Cinnamaldehyde via an Enzymatic Cascade Involving an Ene-Reductase from the OYE Family of Enzymes and a Baeyer–Villiger Monooxygenase and Water-Assisted Hydrolysis



Our previous investigations into BVMOs revealed $BVMO_{AFL838}$ from *Aspergillus flavus* to uniquely and preferentially produce formyl esters rather than fatty acids from aliphatic aldehydes.[34] Testing of $BVMO_{AFL838}$ and its orthologue from *A. oryzyae* ($BVMO_{AO}$) against hydrocinnamaldehyde gave exclusively the formyl ester which spontaneously hydrolyzes in aqueous solution to 2-phenylethanol. It has also been reported extensively in literature that ene-reductases (ERs) from the Old Yellow Enzyme (OYE) family can reduce the α,β-unsaturated double bond of cinnamaldehyde and its derivatives.[35−38] Indeed, screening of five recombinant ERs (Figure S3) revealed the ERs from *S. cerevisiae* ("classical" OYEs), also commonly referred to as OYE2 and OYE3, to rapidly reduce the activated C−C double bond of cinnamaldehyde. Further kinetic characterization revealed OYE3 to have approximately three times higher specific activity (V_{max}) for cinnamaldehyde than OYE2, and also a lower K_M (0.07 mM). Despite mild product inhibition, the catalytic efficiency of OYE3 was still higher than that of OYE2 and was therefore selected (Figure S5).

Thus, as an initial proof-of-principle, an in vivo cascade was constructed in *E. coli*, with simultaneous recombinant expression of OYE3 and $BVMO_{AO}$. The pET-Duet-1 vector containing the open reading frames of both biocatalysts were transformed into *E. coli* BL21(DE3) and grown for 12 h at 25 °C, after which 10 mM cinnamaldehyde was introduced to the growing culture and conversion determined after 2 and 24 h. Despite complete conversion of the cinnamaldehyde after 2 h, only ca. 2.7 mM 2-PE was observed, which increased to ca. 4.2 mM after 24 h (Figure S1). Although 2-PE was obtained, the low yields with various side products formed, such as the corresponding alcohols from cinnamaldehyde and hydrocinnamaldehyde, could be attributed to the action of endogenous enzymes of *E. coli* such as alcohol dehydrogenases (ADHs). Despite many of these reactions being reversible, phenacetaldehyde and benzyl alcohol were also observed, suggesting the endogenous ADHs are also able to convert the desired 2-PE to the corresponding aldehyde and further conversion by the BVMO. Biotransformations under non-growing conditions in only buffer were also evaluated. As both of these enzymes require NADPH as cofactor, the reaction mixtures were supplemented with 100 mM glucose and glycerol to allow cofactor recycling via *E. coli* central metabolism. Similar low conversion and high side-product formation were observed.

In an effort to avoid side-product formation and increase 2-PE yields, we decided to change to an in vitro system using purified biocatalysts. Reaction mixtures (1 mL) contained 2 μM of ER and BVMO and 1 U of purified glucose dehydrogenase (*Bm*GDH) with 100 mM glucose for cofactor regeneration. Disappointingly low concentrations of 2-PE were again observed (<2 mM) but with side product formation drastically reduced. Examination of the time-course analysis of the intermediates of the cascade revealed the ER-mediated reduction step to proceed rapidly, with the complete reduction of the cinnamaldehyde within 1−2 h and the rate-limiting step the autohydrolysis of the phenethyl formate to 2-PE (Figure 1.

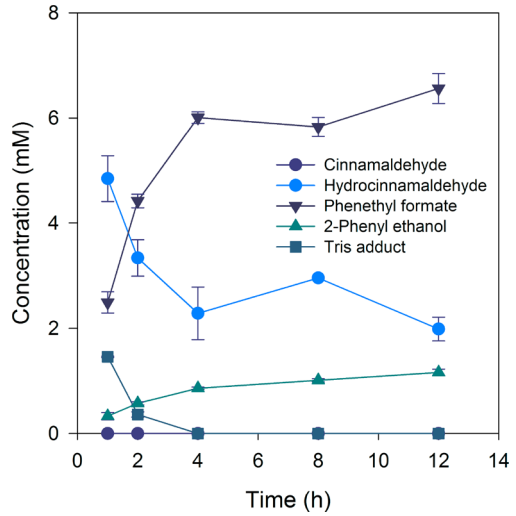


Figure 1. Time course of the conversion of cinnamaldehyde to 2-phenylethanol via the biocatalytic cascade reaction using glucose dehydrogenase for cofactor regeneration. Conditions: 50 mM Tris-HCl buffer (pH 8), [*Sc*OYE3] = 2 μM, [$BVMO_{AO}$] = 2 μM, [*Bm*GDH] = 1 U mL^{-1}, [glucose] = 100 mM, [$NADP^+$] = 0.3 mM, [cinnamaldehyde] = 10 mM, T = 25 °C, shaking = 200 rpm.

A Tris adduct, formed as a Schiff base with hydrocinnamaldehyde, was also observed in the earlier stages of the reaction. This reversible reaction, occurring at higher pH values,[39] decreased the initial effective hydrocinnamaldehyde concentration, potentially alleviating the observed substrate inhibition of $BVMO_{AO}$ with hydrocinnamaldehyde (Figure S6).

Overall, the reaction leveled off after only 4 h, with no further conversion of hydrocinnamaldehyde by the BVMO nor autohydrolysis of the already produced phenethyl formate. Evaluation of the pH after 24 h of biotransformation revealed significant acidification of the reaction (pH < 4).

Glucose dehydrogenase is known to form gluconic acid during cofactor recycling,[40] lowering the pH after prolonged reactions to below the operational levels for many biocatalysts. However, considering the concentrations of the substrates and intermediates utilizing NADPH during the biotransformation, this atypically fast and drastic acidification could be attributed to the uncoupling of the OYE. OYEs are known to also readily reduce molecular oxygen,[35] leading to the formation of reactive oxygen species and the depletion of glucose (and thus excessive gluconic acid production) even in the absence of

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substrate. Incubation of phenethyl formate in aqueous buffers at different pH values also showed a significant pH dependence of the rate of hydrolysis, with significantly lower rates observed at neutral pH values and almost none at pH 6 (Figure S7). The cascade was again tested with the buffering capacity increased (200 mM Tris, pH 8). Nearly complete conversion of the cinnamaldehyde to 2-PE was observed after only 8 h, with only trace amounts of intermediates (with the exception of phenethyl formate) observed after 4 h (Figure 2). Complete

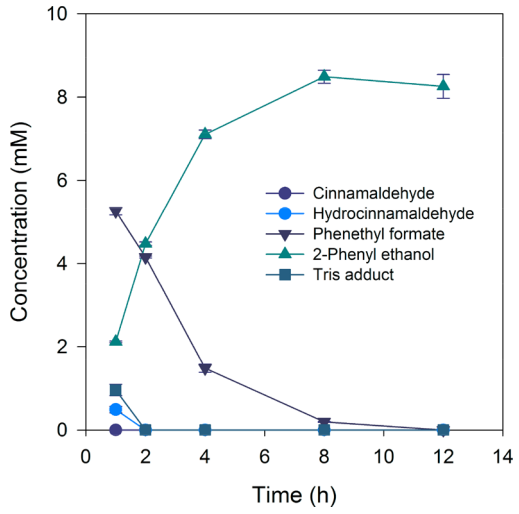


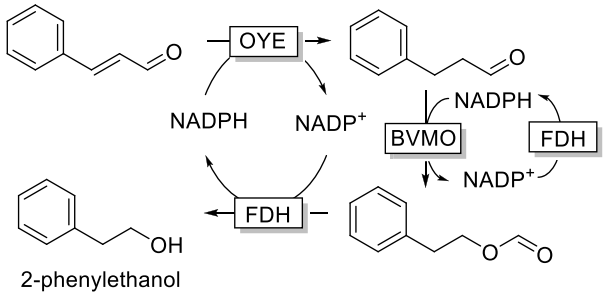
Figure 2. Time course of the conversion of cinnamaldehyde to 2-phenylethanol via the biocatalytic cascade reaction using glucose dehydrogenase for cofactor regeneration under higher buffer concentrations. Conditions: 200 mM Tris–HCl buffer (pH 8), [*Sc*OYE3] = 2 μM, [$BVMO_{AO}$] = 2 μM, [*Bm*GDH] = 1 U mL^{-1}, [glucose] = 100 mM, [$NADP^+$] = 0.3 mM, [cinnamaldehyde] = 10 mM, T = 25 °C, shaking = 200 rpm.

conversion was obtained after 12 h, but surprisingly, benzyl alcohol was again observed as a minor byproduct (Figure S4). As no ADHs are present and none of the enzymes have been found to possess the ability to oxidize 2-PE, the manner for benzyl alcohol formation is currently unknown.

To avoid the constraint of exceedingly high buffer concentrations, we decided to replace the GDH with a formate dehydrogenase (FDH). FDH is a common cofactor regenerating enzyme utilizing formate to regenerate NAD^+ to NADH with only CO_2 as byproduct. Wild-type FDH, however, typically only accepts NAD^+ and not its phosphorylated counterpart $NADP^+$.[41] Two mutants of the FDH from *Candida boidini* (*Cb*FDH) have been described in the literature with the ability to also accept $NADP^+$. These two mutant *Cb*FDHs (designated as CbFDH_P1[42] and P2[43]) were thus created through site-directed mutagenesis for this study. Higher turnover frequencies (TOFs) were observed with *Cb*FDH_P2 under the tested conditions and were selected for NADPH regeneration. Moreover, FDHs have previously been demonstrated to accept formate esters as alternative substrates. This oxidative ester cleavage also yields terminal alcohols and CO_2 instead of formic acid.[44,45] Phenethyl formate was tested against *Cb*FDH (and the $NADP^+$-specific mutant P2), which proved to be accepted as a substrate with oxidative hydrolysis to 2-PE, albeit at very low reaction rates.

Although it would be advantageous to have a redox-balanced cascade reaction, the proposed cascade reaction requires twice the molar equivalents of reduced cofactor (Scheme 2). Additional formate was thus included as cosubstrate to redox

Scheme 2. Biosynthetic Pathway of 2-Phenylethanol from Cinnamaldehyde via an Enzymatic Cascade Involving an Ene-Reductase from the OYE Family of Enzymes and a Baeyer–Villiger Monooxygenase with Formate Dehydrogenase Mediated Cofactor Regeneration and Oxidative Cleavage of Phenethyl Formate



balance the cascade. As the observed specific activity of *Cb*FDH is much lower than that of *Bm*GDH, reactions were constrained and contained only 0.2 U of *Cb*FDH. Cofactor regeneration now became the limiting factor, as cinnamaldehyde was only completely converted after 4 h, and significant amounts of intermediates other than phenethyl formate were observed at 8 h. Nearly complete conversion to 2-PE was, however, still observed after 12 h (Figure 3) and no acidification was observed, allowing for the cascade reaction to proceed in a low concentration buffer.

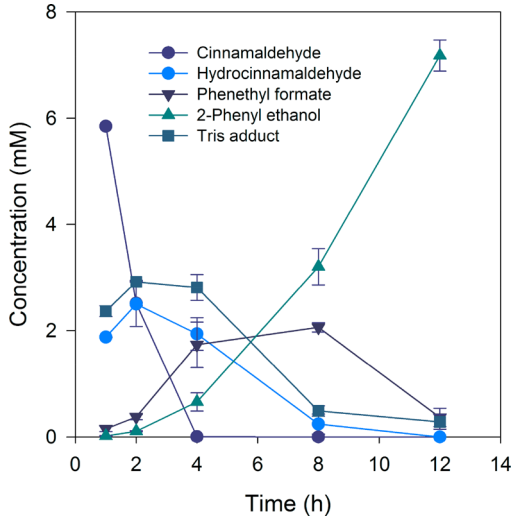


Figure 3. Time course of the conversion of cinnamaldehyde to 2-phenylethanol via the biocatalytic cascade reaction using formate dehydrogenase for cofactor regeneration. Conditions: 50 mM Tris-HCl buffer (pH 8), [*Sc*OYE3] = 2 μM, [$BVMO_{AO}$] = 2 μM, [*Cb*FDH_P2] = 0.2 U mL^{-1}, [formate] = 50 mM, [$NADP^+$] = 0.3 mM, [cinnamaldehyde] = 10 mM, t = 25 °C, shaking = 200 rpm.

In summary, we report here a novel biocatalytic one-pot cascade reaction for the conversion of cinnamaldehyde to 2-phenylethanol. This cascade allows for the conversion of cheap and abundant cinnamon to natural rose flavor, a high-value fine chemical in the food and fragrance industry. Total turnover numbers, with respect to either *Sc*OYE3 or $BVMO_{AO}$, of more

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than 4000 were routinely obtained, and space time yields of between 0.07 and 0.09 g L^{-1} h^{-1} (aqueous phase) were achieved in these initial unoptimized proof-of-principle experiments. Upon complete conversion, isolated yields of at least 60% (6 mM 2-PE) were typically obtained.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b02611.

Biocatalyst production and purification, experimental procedures, characterization data, and GC−MS spectra of products and intermediates (PDF)

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Notes

The authors declare no competing financial interest.

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