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Exploiting Designed Oxidase−Peroxygenase Mutual Benefit System for Asymmetric Cascade Reactions

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Supporting Information

ABSTRACT: A unique P450 monooxygenase−peroxygenase mutual benefit system was designed as the core element in the construction of a biocatalytic cascade reaction sequence leading from 3-phenyl propionic acid to (*R*)-phenyl glycol. In this system, P450 monooxygenase (P450-BM3) and P450 peroxygenase ($OleT_{JE}$) not only function as catalysts for the crucial initial reactions, they also ensure an internal in situ H_2O_2 recycle mechanism that avoids its accumulation and thus prevents possible toxic effects. By directed evolution of P450-BM3 as the catalyst in the enantioselective epoxidation of the styrene-intermediate, formed from 3-phenyl propionic acid, and the epoxide hydrolase ANEH for final hydrolytic ring opening, (*R*)-phenyl glycol and 9 derivatives thereof were synthesized from the respective carboxylic acids in one-pot processes with high enantioselectivity.

Oxygen is a ubiquitous oxidant which exists in a variety of life processes, such as respiration, metabolism, and nutrition.[1] Inevitably, when it is used by an oxidase to perform an oxidative reaction, hydrogen peroxide (H_2O_2) will be generated in the redox process.[2−5] H_2O_2 is a potentially toxic agent for the cells, because it injures proteins and damages nucleic acids in many ways.[6] For example, it can inhibit cytoplasmic enzymes which use Fe^{2+} ion or [4Fe−4S] as cofactors[7−11] and induce the Fenton reaction with generation of DNA-damaging hydroxyl radicals.[12] To relieve oxidative stress caused by H_2O_2, organisms have developed versatile approaches to degrade this toxicant.[13,14] In these processes, H_2O_2 is decomposed into water and oxygen by catalase or it is utilized by peroxidases or peroxygenases as an oxidant to generate new oxidative products.[15,16] Thus, based on the activities of oxidases and peroxidases/peroxygenases, cascade reactions with high economic efficiency can be created. This is achieved upon combining oxygen-dependent oxidases with peroxidases/peroxygenases. In these reactions, the only oxidative source is oxygen (O_2), and H_2O_2 is produced slowly as a direct product or byproduct when oxygen is reduced by oxidases. Peroxidases/peroxygenases accept H_2O_2 to synthesize new compounds. The two types of enzymes can benefit from each other in what can be called "oxidase−peroxidase/peroxygenase mutual benefit systems" (OPMBS) (Figure 1).

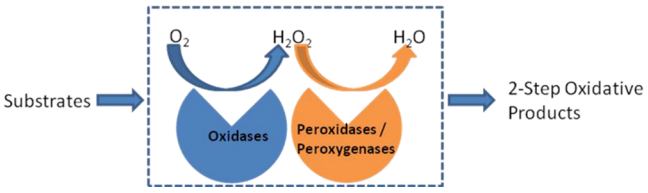

Figure 1. Concept of oxidase−peroxidase/peroxygenase mutual benefit systems.

In previous work, oxidases−peroxidases/peroxygenases fusion proteins or oxidases−peroxidases/peroxygenases in mixtures were reported, as in the synthesis of divanillin, lignin oligmers and α-ketoacids.[17−20] However, to the best of our knowledge, such systems have not been exploited to perform asymmetric synthesis, which would be of notable interest in synthetic organic chemistry. Moreover, most studies utilize external H_2O_2 to switch on the reaction, specifically when peroxidases/peroxygenases function in the first catalytic step, which may have significant disadvantages.

Here, we demonstrate a biocatalytic one-pot cascade reaction sequence in which the core steps are catalyzed by a unique oxidase−peroxygenase mutual benefit system, thereby enabling the enantioselective production of (*R*)-phenyl glycols from 3-phenyl propionic acid or derivatives thereof. In this approach, the initial 2-step oxidative reaction sequence is triggered by the peroxygenase without any external H_2O_2. Furthermore, all of the enzymes involved in this reaction can be used in their cell-free lysate form, which catalyzes the target reactions with high conversion and excellent enantioselectivity.

The catalytic system introduced here is composed of two P450 enzymes, P450-BM3 (a P450 monoxygenase)[21] and $OleT_{JE}$ (a P450 peroxygenase)[22] for the first and second steps, respectively (Scheme 1). The final step in the overall cascade reaction sequence is the selective hydrolytic ring-opening of the respective epoxide catalyzed by the epoxide hydrolase from

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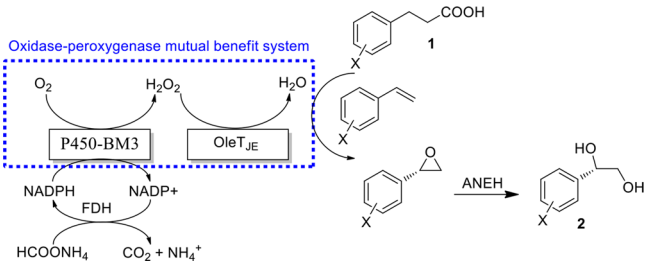
Scheme 1. Designed Cascade Sequence Leading from 3-Phenyl Propionic Acid to the Asymmetric Production of (*R*)-Phenyl Glycol Based on an Oxidase−Peroxygenase Mutual Benefit System

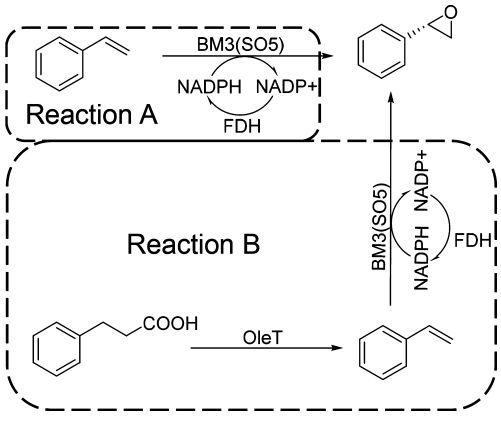


Aspergillus niger (ANEH).[23] To regenerate the cofactor NADPH needed for P450-BM3 catalysis, a formate dehydrogenase mutant (FDH) from *Candida bodinii* was applied.[24] Notice that this cascade starts from 3-phenyl propionic acid and is therefore different from the elegant one-pot sequence starting from styrene, catalyzed by styrene monooxygenase in combination with the epoxide hydrolase from *Sphingomonas* sp. HXN-200, as reported by Li et al.[25]

The first step in the overall sequence in which chirality is created is the P450-BM3 catalyzed epoxidation of styrene. In this model reaction, WT P450-BM3 leads to the formation of (*R*)-styrene oxide with an enantioselectivity of only 20% ee.[26] In order to enhance (*R*)-selectivity, we applied a special form of iterative saturation mutagenesis at the active site (CAST/ISM)[27] which we had used previously to improve and to reverse the enantioselectivity of P450-BM3-catalyzed sulfoxidation of thiochroman-4-one.[28] Briefly, on the basis of earlier experience, we first screened a small previous mutant library and used the best (*S*)-selective mutants with high activity (56% and 63% ee) as templates for further focused ISM employing highly reduced amino acid alphabets. After 3 cycles, the best mutant, SO5, improved (*R*)-selectivity to ee = 96% (Table S2).

In order to confirm the first two steps of the overall cascade reaction sequence, purified $OleT_{JE}$ was mixed with the purified SO5 variant, and tested in the model reaction using 3-phenyl propionic acid as the starting substrate (Scheme 2). The reaction was monitored by gas chromatography (GC). As shown in Figure 2, styrene oxide was produced successfully

Scheme 2. In Reaction A, P450-BM3 Mutant SO5 Transforms Styrene into Styrene Oxide, while the OleT-Catalyzed Reaction B Supplies the Necessary Styrene from 3-Phenyl Propionic Acid



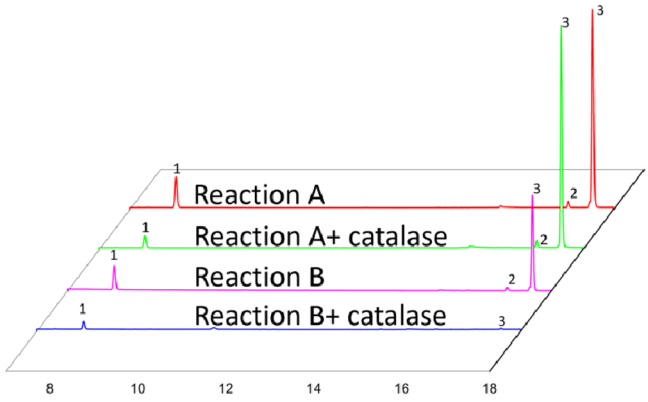


Figure 2. Different responses to the influence of catalase in Reaction A and Reaction B as measured by GC. Peak 1, styrene; peak 2, (*S*)-styrene oxide; peak 3, (*R*)-styrene oxide.

with high conversion, demonstrating that $OleT_{JE}$ was indeed activated successfully to switch on the cascade reaction. To confirm that the reaction was switched on by H_2O_2, a control reaction with catalase was performed in the desaturase transformation (Reaction B). As expected, the reaction was inhibited almost completely, essentially no styrene epoxide being detected in the GC run (Figure 2.).

H_2O_2 is usually particularly toxic for enzymes when it accumulates.[29,30] To verify whether it can inhibit the activity of variant SO5, an enzyme assay was designed with purified enzyme (Reaction A, Scheme 2/Figure 2). The results indicate that the addition of catalase actually increases the SO5-activity by about 10% (Figure 2). Since H_2O_2 usually results from the uncoupling pathway, we measured the uncoupling efficiency by comparing the consumption of NADPH with the production of styrene oxide in reaction A, which was found to account for 43% of the result. Therefore, it can be concluded that in the SO5-catalyzed epoxidation, H_2O_2 causes enzyme stress. Consequently, combining $OleT_{JE}$ with mutant SO5 not only enables the cascade reaction, it also relieves oxidative stress originating from H_2O_2. This is the reason why we call the overall technique "oxidase−peroxygenase mutual benefit system".

In order to synthesize enantiopure phenyl glycols, we cloned ANEH, following SO5, under the same T7 promoter, and expressed them in a single strain. $OleT_{JE}$ was expressed separately, then both of them were subjected to sonification, and the respective supernatants were lyophilized into cell-free powders. The overall cascade reaction sequence was performed with these powders as biocatalysts, specifically by adjusting the ratio of the SO5-ANEH powder and the $OleT_{JE}$ powder. When the ratio of SO5-ANEH to $OleT_{JE}$ was adjusted to 10 g/L: 20 g/L (enzymes' concentration ratio is 5.6 μM: 4.5 μM), the conversion of 3-phenyl propionic acid to (*R*)-phenyl glycol amounted to only 31% with an ee-value of 97%. Since $OleT_{JE}$ is known to favor high salt concentrations,[31] 300 mM of NaCl were added to the system, which increased conversion to >92%. With the aim of testing the substrate scope of our catalytic system, 9 different 3-phenyl propionic acid derivatives were tested. As summarized in Table 1, generally excellent results were obtained, exceptions being the two *ortho*-substituted derivatives.

In summary, the designed "oxidase−peroxygenase mutual benefit system" described herein constitutes a synthetically attractive way to combine a monooxidase with a peroxygenase,

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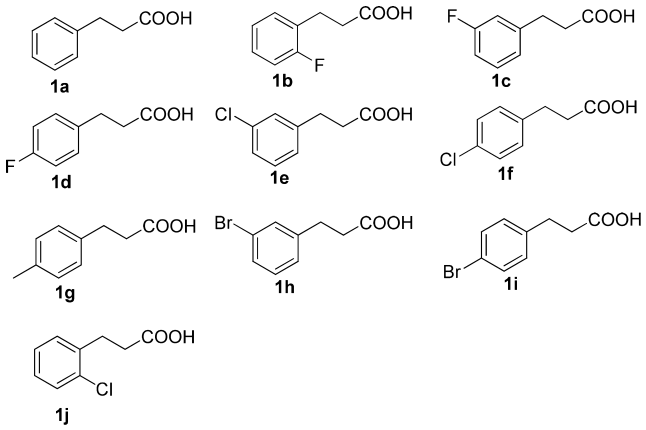
Table 1. Transformation of 3-Phenyl Propionic Acid and Derivatives Thereof Using the Designed Oxidase–Peroxygenase Mutual Benefit System



substrate	conv. (%)	%-ee(*R*) of diol **2**
1a	92	97
1b	74	59
1c	93	98
1d	76	92
1e	94	98
1f	95	95
1g	79	95
1h	97	93
1i	88	91
1j	36	25

[a]Standard reaction condition: 50 mM PBS buffer (pH = 8.0), ammonium formate (100 mM), NADP+ (0.8 mM), NaCl (300 mM), substrate (5 mM), $OleT_{JE}$ (20 mg/mL), BM3-ANEH (10 mg/mL); total volume: 500 μL.

thereby avoiding the destructive use of toxic H_2O_2. The concept extends the utility of biocatalytic cascade reactions considerably.[5,31−33] The results also shed light on the mechanism of the involved processes. We expect that our study will accelerate more research based on this approach for accessing useful products in an ecologically and economically viable manner.[2,16−19,32−35]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b01939.

Experimental materials and procedure details concerning mutagenesis, screening, plasmid construction and reaction details (PDF)

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Notes

The authors declare no competing financial interest.

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