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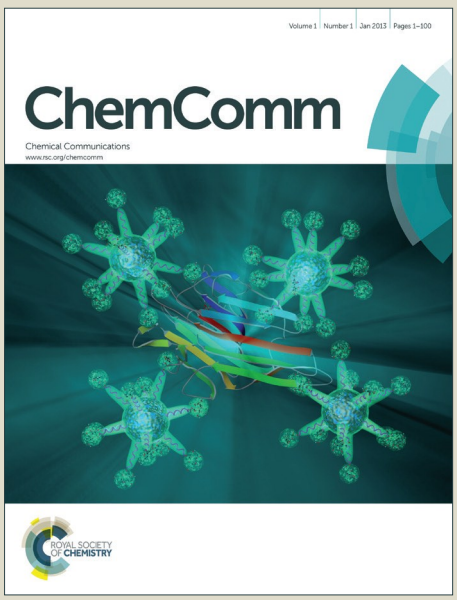

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Switchable asymmetric bio-epoxidation of α,β-unsaturated ketones

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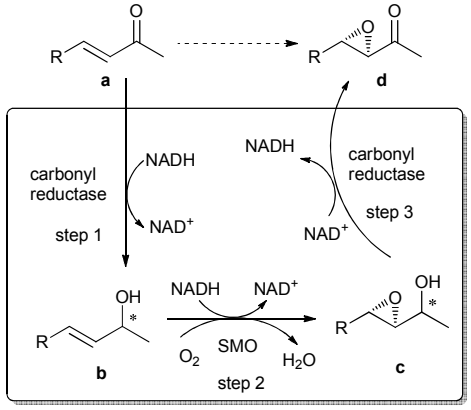
Efficient asymmetric bio-epoxidation of electron-deficient α,β-unsaturated ketones was realized via a tandem reduction-epoxidation-dehydrogenation cascade, which proceeds in a switchable manner to afford either chiral epoxy ketones or allylic epoxy alcohols with up to >99% yield and >99%ee.

The asymmetric epoxidation of α,β-unsaturated ketones generates enantiomerically enriched α,β-epoxy ketones, which serve as extremely versatile intermediates with further functionalization[1-3]. Besides the powerful ring opening reactions on the epoxide moiety, the ketone moiety can also undergo various transformations such as the addition of various nucleophiles, reductive amination, Baeyer-Villiger oxidation and Wittig olefination etc[3]. Therefore, chiral α,β-epoxy ketones have been applied in the synthesis of a wide range of natural products and biologically active compounds, such as Leukotriene precursors, Diltiazem and Taxol[3]. During the past decades, a broad range of stereoselective metal- and organo-catalytic strategies have been developed with varied success[4, 5]. However, due to the synthetic challenge posed by the electron-deficient nature of the carbon-carbon double bond, there still lacks of general method for the asymmetric epoxidation of simple α,β-unsaturated ketones[6-11]. On the other hand, biocatalytic approaches have been well recognized as a great alternative to classic synthetic routes, especially with the ever-growing demand on environmentally benign catalysts and processes. Bio-epoxidation uses molecular oxygen as an oxidant under mild reaction conditions, and often delivers outstanding stereoselectivity[12]. However, in contrast to general or electron-rich olefins, biocatalytic methods on the asymmetric bio-epoxidation of the challenging electron-deficient olefins for synthetic purpose are scarce. Although the multifunctional cytochrome P450s are known to catalyze the epoxidation of inert C=C double bonds including aromatic hydrocarbons for the bioactivation and detoxication of xenobiotic chemicals, yet in the majority of the cases, the products are reactive entities with short half-lives, or metabolite mixtures of physiological concentrations [13, 14].



Scheme 1. Proposed enzymatic cascade for the asymmetric epoxidation of α, β-unsaturated ketones.

Styrene monooxygenase (SMO) is an NADH-dependent epoxygenase that catalyzes the asymmetric bio-epoxidation of styrene in the upper catabolic pathway of styrene degradation, affording (*S*)-styrene epoxide with >99% ee[15, 16]. Further investigation has expanded its substrate spectrum to styrene derivatives and analogues[17-21], as well as aliphatic olefins [22]. In our study on SMO-catalyzed reactions, (E)-4-phenylbut-3-en-2-ol (**1b**) has appeared as a good substrate for SMO with excellent activity and enantioselectivity, albeit with low diastereoselectivity[23]. The results inspired us to establish a "formal" asymmetric epoxidation of α,β-unsaturated ketones, which would involve a tandem three-step enzymatic cascade using SMO coupled with a carbonyl reductase coexpressed in recombinant *E. coli* (Scheme 1). The bio-epoxidation would take place after the reduction of the carbonyl group; then the resulting product undergoes dehydrogenation to re-generate the carbonyl group to yield the desired product **d**. At the

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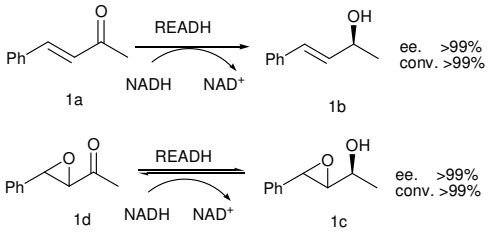
meantime, the dehydrogenation process would partly supply the reduced co-factor to the first two steps, provided that the carbonyl reductase uses the same cofactor as SMO (Scheme 1).

We also noticed that this envisaged system would involve a secondary allylic epoxy alcohol intermediate **c** with three contiguous stereocenters, which is among the most valuable intermediates in organic synthesis due to the reactive oxirane moiety that can be opened with various nucleophiles in a stereoselective manner. The secondary allylic epoxy alcohols can be generated using Sharpless kinetic resolution, but often with low conversion if high product ee is expected, and with limited substrate spectrum[24-26]. The series work by the Walsh group circumvents some of the problems by designing tandem reactions that involve the enantioselective addition to unsaturated ketones or aldehydes followed by a diastereoselective epoxidation[27-29]. Here, we envisioned the possibility of obtaining optically pure c using the same cascade shown in Scheme 1 by blocking step 3 with excessive supply of NADH to provide an efficient method for the synthesis of this class of epoxy alcohol as well.

To realize this strategy, a suitable NADH-dependent carbonyl reductase that can catalyze both step 1 and step 3 is a prerequisite. Two model substrates 4-phenylbut-3-en-2-one (**1a**) and racemic 3,4-epoxy-4-phenyl-butan-2-one (**1d**) with a concentration of 1 g/l were used to evaluate the performance of carbonyl reductases in the presence of NADH. The alcohol dehydrogenase from *Rhodococcus erythropolis* DSM 43297 (READH)[30] was found to catalyze the reduction of both **1a** and **1d** with excellent stereoselectivity and activity, affording single enantiomer of the alcohols with complete conversion within 1 h. Then the dehydrogenation of **1c** to **1d**, i.e. step 3, was evaluated in the presence of excessive NAD^+. The reaction appeared thermodynamically limited and quickly reached equilibrium after a substrate conversion of 5-7%. The results indicated that the reverse reaction of step 3 was much more favored than the forward reaction. However, based on the design of the 3-step system, it is reasonable to expect that the chemical equilibrium could be driven toward the synthesis of ketone **1d** when step 1 and 2 take place to continuously deplete the reduced cofactor. Therefore, we considered READH as a suitable enzyme to construct the 3-step system together with SMO.



Scheme 2. The bioreductions of **1a** and **1d** catalyzed with READH

Then we examined the background biotransformation of **1a** using the resting cells of *E. coli* carrying the empty vector to probe any side-reactions, since resting cells instead of free enzymes are well established to better realize the catalytic activity of SMO. The results showed that **1a** was converted to 4-phenylbutan-2-one (**1e**) with a yield of 30% within 10 h, which was apparently originated from the reduction of C=C bond. *E. coli* is known to produce ene-reductases, and several of them have been characterized[31, 32]. N-acetyl maleimide reductase (*Nem*A) is one of the well-known *E. coli* ene-reductases that can reduce a spectrum of activated alkenes[33]. It belongs to the Old Yellow Enzyme family and is not essential to cell growth[34]. Therefore, we performed the knockout of the *nemA* gene from *E. coli* BL21 (DE3) using the *λ* Red recombination technology[35]. The resulting strain of *E. coli* BL21Δ*nemA* showed normal growth rate, but its ene-reductase activity toward **1a** decreased significantly to yield 2% of the undesired product **1e** within 10 h. Attempts to knockout other potential ene-reductase genes, such as the enoyl-ACP dehydrogenase gene (*fab*I)[34], were not successful, because quite a few are essential genes related to cell growth. We reckoned that the side-reaction would be further eliminated when the ketone reduction and epoxidation took place to compete for the cofactor. Therefore, the strain of *E. coli* BL21Δ*nemA* was applied in subsequent studies without further modification.

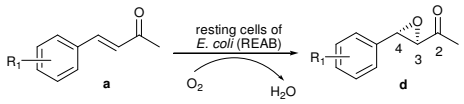
At the same time, to develop the catalytic system, the coding sequences of SMO and READH were amplified and engineered into the commercially available vector pRSFDuet-1^T designed to coexpress two target proteins. To achieve better reactivity, two expression constructs, namely ABRE and REAB, were made where the two coding sequences were located at different cassettes of the vector. They were transformed into *E. coli* BL21Δ*nemA*, resulting in *E. coli* (ABRE) and *E. coli* (REAB), respectively. Both of the recombinant strains realized soluble co-expression of READH and StyAB, and the resting cells were applied in the biotransformation of substrate **1a**. The three-step tandem reaction was successfully achieved with either strain, resulting in the complete conversion of **1a** to the epoxy ketone (3*R*,4*S*)-**1d** within a reaction time of 1-h and 2-h for the recombinant *E. coli* (REAB) and *E. coli* (ABRE) system, respectively. The product was achieved as a single enantiomer due to the extraordinary stereoselectivity of SMO. Thus, the asymmetric bio-epoxidation of α,β-unsaturated ketones was first realized for synthetic purpose.

It's worth noting that the separate reaction of **1c** to **1d** did not occur in the presence of either *E. coli* cells carrying the empty vector or the *E. coli* (REAB) system. The formation of **1d** was only observed when **1a** or **1b** was used as the substrate, which indicated that the depletion of NADH is critical to trigger the conversion of **1c** to **1d**.

We then extended the cascade reaction system of *E. coli* (REAB) to a panel of α, β-unsaturated ketones (Table 1). The nitro-substituted 13a appeared to be a non-substrate for SMO, most probably due to the strong electron withdrawing nature of the substituent (entry 13). Other than that, complete conversion was achieved for all the substrates **2a**-**12a** within 2-h reaction, resulting in the expected (3*R*,4*S*)-epoxy ketones as the major product. Seven of the substrates achieved high HPLC yields of >99%, while the other five yielded the intermediate epoxy alcohols c as a minor product. Single enantiomers (>99% ee) were achieved for the majority of the substrates, while three para-substituted substrates yielded products with 85-96% ee (entries 7, 10 & 12, Table 1), indicating relaxed stereoselectivity of SMO toward para-substituted 4-phenylbut-3-en-2-one. As the major product, the epoxy ketones could be easily purified by flash chromatography on silica gel, and the

isolated yield was 59% for **8d**, and ranged from 85-95% for the others.

Table 1 Asymmetric epoxidation of α,β -unsaturated ketones with the resting cells of *E. coli* (REAB)[a].



Entry	R	Yield (%)[b]	ee (%)[c]
1	H (**1a**)	>99 (92)	>99
2	*o*-F (**2a**)	>99 (90)	>99
3	*m*-F (**3a**)	>99 (95)	>99
4	*p*-F (**4a**)	>99 (93)	>99
5	*o*-Cl (**5a**)	>99 (90)	>99
6	*m*-Cl (**6a**)	92 (86)	>99
7	*p*-Cl (**7a**)	95 (88)	96
8	*o*-Br (**8a**)	67 (59)	>99
9	*m*-Br (**9a**)	93 (85)	>99
10	*p*-Br (**10a**)	97 (91)	92
11	*m*-Me (**11a**)	>99 (93)	>99
12	*p*-Me (**12a**)	>99 (95)	85
13	*p*-NO_2 (**13a**)	0[d]	-

[a] The reactions were performed for 2 h at 30°C in 10 ml of potassium phosphate buffer (100 mM, pH 7.0) containing recombinant *E. coli* cells (0.4 g, cell dry weight) and substrate (10 mg) with shaking at 240 rpm. [b] HPLC yield and isolated yield (in brackets). The conversions of all the substrates were >99%, and the by-products were the corresponding epoxy alcohols 6c-10c. [c] Determined via chiral HPLC analysis. [d] 4-(4'-nitrophenyl)butan-2-ol achieved with >99% HPLC yield.

In addition, the biotransformation of several analogues of **1a** with varied aromatic rings was also attempted. (E)-4-(thiophen-2-yl)but-3-en-2-one, (E)-4-(pyridin-2-yl)but-3-en-2-one, (E)-4-(furan-3-yl)but-3-en-2-one yielded product **b**, but not **d**, while (E)-4-(thiophen-3-yl)but-3-en-2-one and (E)-4-(naphthalen-2-yl)but-3-en-2-one yielded product **d** with 5% and 10% yield, respectively, according to the HPLC traces. The results were most probably due to the limited substrate spectrum of SMO, which might be improved by the protein engineering of SMO or the introduction of other functional epoxygenases in the future.

To achieve the allylic epoxy alcohol intermediate c as the major product, we intended to block step 3 in Scheme 1 by excessive supply of NADH. Taking advantage of the ability of READH to catalyze the dehydrogenation of isopropanol to regenerate NADH[30], we tested the effect of isopropanol supplementation to the product composition. The results showed that with the increase of isopropanol, the main product was literally switched from **1d** to **1c** (Fig. 1). When the molar ratio of isopropanol to substrate reached above 20:1 (1% v/v of isopropanol in the reaction buffer), the allylic epoxy alcohol (2*S*,3*S*,4*S*)-**1c** was successfully obtained as the only product (Fig. 1). The reaction system could well tolerate up to 15% (v/v) isopropanol, but the efficiency started to decline when 20% (v/v) isopropanol (molar ratio 400:1) was applied (98% yield).

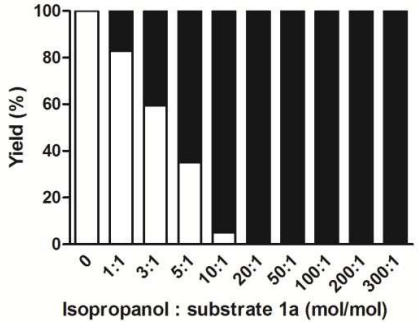
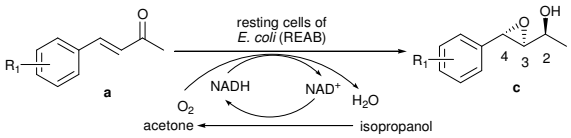


Figure 1. Change of product composition by the addition of isopropanol. HPLC yields of 1d (white) and 1c (black) are shown.

The established system was then applied to the transformation of substrates **2a**-**12a** in the presence of 2.5% (v/v) isopropanol (Table 2). The reactions were traced until complete conversion was reached for each substrate. Meta-substituted substrates required relatively short reaction time, while the ortho- substituted substrates (entries 2, 5 & 8, Table 2) appeared to have lower reactivity than their para- and meta- counterparts. Substrates **1a**-**4a** and **11a**-**12a** yielded the desired chiral epoxy alcohols as the only product (>99% HPLC yield). However, with the efficient supply of NADH, the by-products of 4-arylbutan-2-ones (**5e**-**10e**) and saturated 4-arylbutan-2-ols (**5f**-**10f**) were detected for six of the substrates as a result of the residual ene-reductase activity of *E. coli* BL21Δ*nemA*. Nevertheless, the epoxy alcohols still appeared as the major products with HPLC yields of 55-94% (entries 5-10, Table 2).

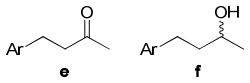
Table 2 Asymmetric epoxidation of α,β-unsaturated ketones with the resting cells of *E. coli* (REAB) in the presence of isopropanol[a].



Entry	R	Time (h)	Yield (%)[b]	ee (%)[c]	*dr*(erythro: threo)[c]
1	H (**1a**)	0.5	>99 (95)	>99	>99:1
2	*o*-F (**2a**)	1	>99 (92)	>99	>99:1
3	*m*-F (**3a**)	0.5	>99 (94)	>99	>99:1
4	*p*-F (**4a**)	1	>99 (94)	>99	>99:1
5	*o*-Cl (**5a**)	5	80 (73)	>99	>99:1
6	*m*-Cl (**6a**)	2	94 (87)	>99	>99:1
7	*p*-Cl (**7a**)	3	93 (81)	>99	98:2
8	*o*-Br (**8a**)	5	55 (50)	>99	>99:1
9	*m*-Br (**9a**)	2	93 (86)	>99	>99:1
10	*p*-Br (**10a**)	5	83 (77)	>99	98:2
11	*m*-Me (**11a**)	1	>99 (93)	>99	>99:1
12	*p*-Me (**12a**)	2	>99 (95)	>99	93:7

[a] The reaction was performed at 30°C in 10 ml of potassium phosphate buffer (0.1 M, pH 7.0) containing recombinant E.

coli cells (0.4 g, cell dry weight), 2.5% (v/v) isopropanol and substrate (10 mg) with shaking at 240 rpm. [b] HPLC yield and isolated yield (in brackets). The conversions of all the substrates were >99%. The by-products were substituted 4-arylbutan-2-ones (**5e**-**10e**) and saturated 4-arylbutan-2-ols (**5f**-**10f**). [c] Determined via chiral HPLC analysis.



Scheme 3. Structures of by-products **e** and **f**

The optical purities of the allylic epoxy alcohols were generally excellent, which reflected the stereoselectivity of both SMO and READH. The majority of the allylic epoxy alcohols were optically pure (>99% ee, >99:1 dr), and diastereoisomers were only detected for three para-substituted substrates (entries 7, 10 & 12, Table 2) with diastereoisomeric ratios ranging from 93:7-98:2. The results were in accordance with those listed in Table 1, indicating relaxed selectivity of SMO toward the same three substrates, while READH showed absolute selectivity toward all the tested substrates.

In conclusion, we have developed an concise enzymatic cascade to realize the "formal" asymmetric epoxidation of α,β-unsaturated ketones with excellent stereoselectivity. The reaction system circumvents the difficulty in the direct bio-epoxidation of electron-deficient olefins, and provides an efficient and convenient method to synthesize either optically pure allylic epoxy ketones or epoxy alcohols using recombinant *E. coli* co-expressing READH and SMO. The majority of the chiral epoxy ketones achieved in this work have not been reported using chemical approaches, which suggests the significance of the biocatalytic approach to this group of intermediates. Considering the variety of carbonyl reductases and epoxidases available, it is expectable that this approach would achieve a much broader substrate spectrum or complementary stereoselectivity with further investigations.

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