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Development of a Practical, Biocatalytic Synthesis of *Tert*-Butyl (*R*)-3-Hydroxyl-5-Hexenoate: a Key Intermediate to Statin Side Chain

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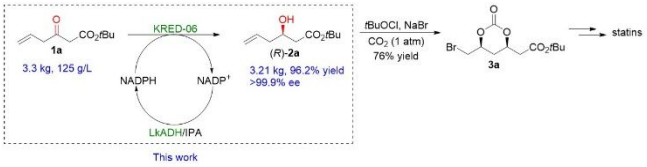


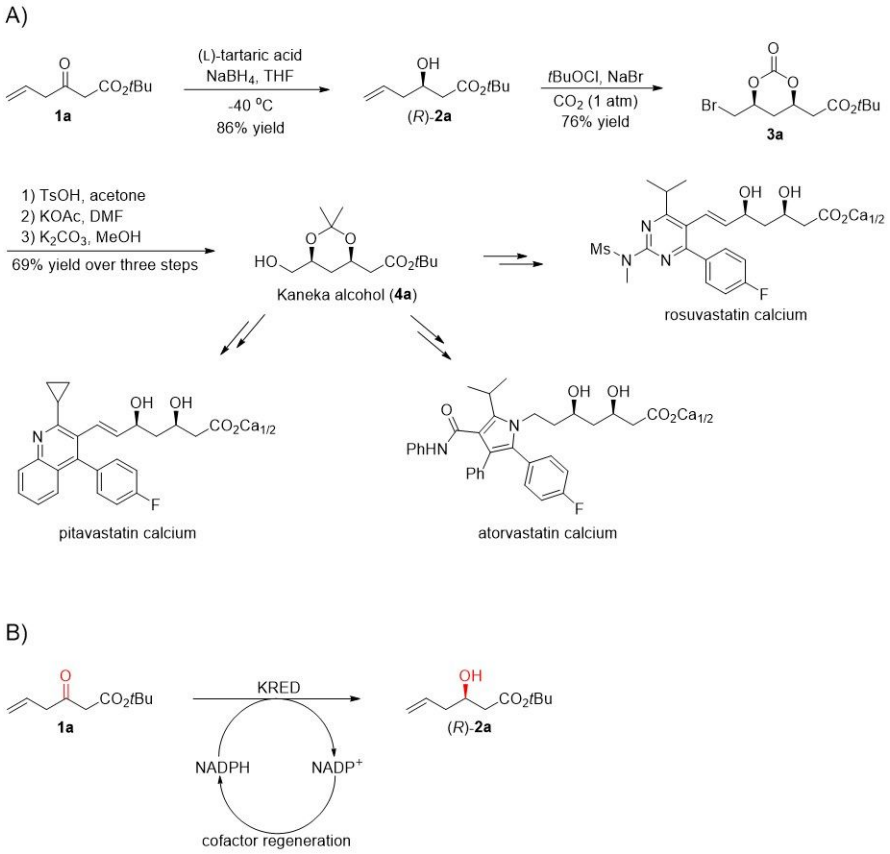
ABSTRACT: The HMG-CoA reductase inhibitors, statins, are one of the most effective and best-selling cholesterol-lowering drugs. The use of statins has greatly extended people's lives and improved the quality of their life. Development of a more efficient, stereoselective, and sustainable synthesis of statins is continuingly of utmost importance. In the present study, through screening of ketoreductases (KREDs) and reaction optimization, we have successfully performed a highly stereoselective reduction of ketoester **1a** catalyzed by KRED-06 at a pilot-plant-scale without the addition of exogenous $NADP^+$, generating 3.21 kg of enantiomerically pure *tert*-butyl (*R*)-3-hydroxyl-5-hexenoate ((*R*)-**2a**) (96.2% yield, >99.9% ee). This newly developed biocatalytic process alleviates the cryogenic conditions (-40 ℃) employed in our first-generation synthesis of (*R*)-**2a** using $NaBH_4$ and (L)-tartaric acid. Coupled with our previously established synthesis of bromocarbonate **3a** via a one-pot diastereoselective carboxylation/bromocyclization of (*R*)-**2a**, we have developed an innovative, practical synthesis route to statin side chain, possessing great potential to be implemented into industrial production of statins.

KEYWORDS: asymmetric catalysis, ketoreductase, statin, *tert*-butyl (*R*)-3-hydroxyl-5-hexenoate, pilot-plant-scale synthesis

Introduction

Since their introduction to market in 1987, statins (Scheme 1A), the inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), have developed as one of the top selling families of drugs prescribed for lowering cholesterol, hence preventing cardiovascular diseases such as hypercholesterolemia, and coronary heart disease.[1-5] In the past several decades, millions of people have benefited from the use of statins in terms of both extending their lives and improving the quality of life.[1] Moreover, extensive researches in the field indicate that statins have many other pharmacological activities, including anti-inflammatory and anti-cancer activity, as well as anti-oxidative effects.[4, 6-9] Given their pharmaceutical importance, tremendous efforts have been devoted to the efficient and sustainable synthesis of statins.[4, 10-11] In particular, various methods have been developed to build the chiral *syn*-3,5-dihydroxy-6-heptenoic or heptanoic acid side chain, the common and pharmacologically important structural feature of statins.[4] Within this context, our group has longtime interests in the efficient and asymmetric synthesis of statins.[11-16] Recently, we have demonstrated a pilot-plant-scale synthesis of bromocarbonate **3a** via a one-pot diastereoselective carboxylation/bromocyclization of *tert*-butyl (*R*)-3-hydroxyl-5-hexenoate ((*R*)-**2a**) (Scheme 1A).[17] The bromocarbonate **3a** was transformed further to Kaneka alcohol **4a**, the common synthetic intermediate to statin molecules, in three steps with high yields. In our original synthetic route, enantiomerically pure (*R*)-**2a** was produced through the reduction of ketoester **1a** by $NaBH_4$ in the presence of (L)-tartaric acid at -40 ℃. Although effective, the cryogenic reaction conditions used present limitations. For industrial application, development of an alternative synthesis of (*R*)-**2a** under mild and sustainable conditions is highly wanted.

Ketoreductases (KREDs) are a class of enzymes that can reduce prochiral ketones to chiral alcohols using NADPH or NADH as the hydride donor.[18-23] Owing to their excellent stereoselectivity, broad substrate spectrum, environmental-friendliness, and good stability, KREDs have been developed as some of most widely used biocatalysts in the industrial synthesis of pharmaceuticals,[24-29] as exemplified in the synthesis of the key intermediates to atorvastatin,[24] montelukast,[25] and (*S*)-licarbazepine.[26] In fact, KREDs have become the first choice for the synthesis of chiral alcohols in major pharmaceutical companies nowadays. Herein, we report the development of a pilot-plant-scale synthesis of (*R*)-**2a** through a KRED-catalyzed reduction of **1a** under mild conditions (Scheme 1B).

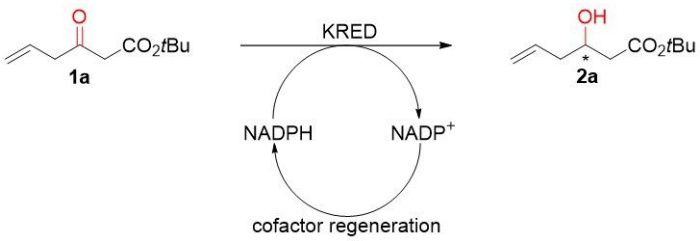


Scheme 1. A. New synthetic route to statins recently developed by our group.[17] B. KRED-catalyzed reduction of ketoester **1a** to alcohol (*R*)-**2a**.

Results and Discussion

Screening of ketoreductases. Our study commenced with searching for the most efficient and selective KRED catalyst. Hence, a small library of in-house preserved KREDs were screened for the reduction of **1a** with the glucose dehydrogenase (GDH)/glucose system employed to regenerate the cofactor NADPH. As shown in Table 1, most of the enzymes tested reduced **1a** to selectively yield (*S*)-**2a**. The desired (*R*)-**2a** was only selectively formed in KRED-03-, and KRED-06-catalyzed reduction reactions (Entry 3 and 6). Fortunately, KRED-06 was exceptionally effective and able to completely reduce **1a** to generate (*R*)-**2a** with >99% ee. When the GDH/glucose system is adopted to regenerate the cofactor NAD(P)H, in particular at high-substrate loading cases, bases, such as K_2CO_3 or NaOH have to be routinely added to maintain the reaction pH because of the formation of gluconic acid.[29] To alleviate this inconvenience, we examined the possibility of using LkADH,[30-31] an enzyme known for its ability to regenerate NADPH via the oxidation of *iso*-propanol (IPA) to acetone, for the cofactor regeneration. To our delight, excellent reaction conversion and stereoselectivity were achieved (Entry 12, Table 1). Therefore, LkADH/IPA was employed for NADPH regeneration in the following studies.

Table 1. Initial screening of KREDs for the reduction of **1a**.[a]

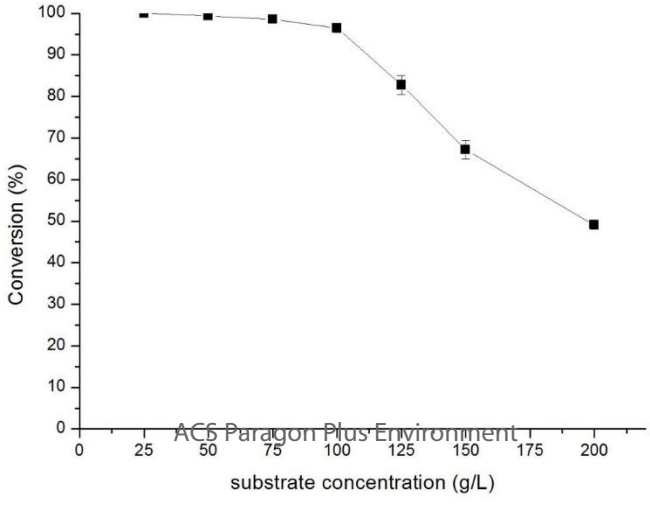


Entry	Enzyme	Conversion (%)[b]	Ee (%)[c]	Configuration[d]
1	KRED-01	36.0	2.5	*R*
2	KRED-02	82.7	88.7	*S*
3	KRED-03	>99	34.0	*R*

4	KRED-04	78.2	98.9	*S*
5	KRED-05	>99	>99	*S*
6	KRED-06	>99	>99	*R*
7	KRED-07	52.8	80.3	*S*
8	KRED-08	78.2	2.5	*S*
9	KRED-09	27.9	37.3	*S*
10	KRED-10	81.6	28.9	*S*
11	KRED-11	92.2	20.1	*S*
12[e]	KRED-06	>99	>99	*R*

[a]Reaction conditions (9 mL): **1a** (10 g/L), glucose (19.6 g/L), $NADP^+$ (0.0125 g/L), KRED (2.7 g/L), and GDH (1 g/L) in KP_i buffer (100 mM, pH 7.0). Reaction mixtures were incubated at 25 ℃ with 200 rpm shaking for 90 min. [b]The conversion was determined by GC-MS analysis. [c]The ee was determined by chiral HPLC analysis after benzoylation of the product alcohol. [d]The absolute configuration was assigned by comparing the optical rotation data of the product to that of the literature data. [e]LkADH and IPA were used for the regeneration of NADPH.

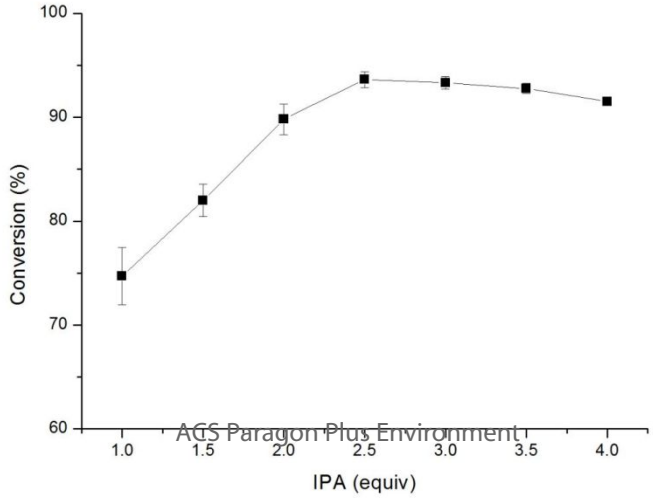
Effect of substrate concentration. With an effective KRED identified, we next proceeded to further modify this bioreduction process. Substrate loading was the first parameter we wanted to optimize. To make a biocatalytic process industrially viable, a substrate concentration of ≥100 g/L is normally required.[23] In the present study, a substrate concentration range of 20 g/L to 200



g/L of ketoester **1a** was tested (Figure 1). When 100 g/L or less of **1a** was used, >95% conversion was achieved. When 125 g/L of **1a** was subjected to the same reaction conditions, approximately 83% conversion was obtained. Further increase of the substrate loadings to 150 g/L and 200 g/L resulted in moderate reaction conversions (67% and 49%, respectively). Taking into consideration of both relatively high substrate loading and good reaction conversion, we chose the substrate concentration of 125 g/L for the remaining study of process optimization.

Figure 1. Effect of substrate concentration on the bioreduction of ketone **1a**. Reaction conditions (0.8 mL): **1a** (variable concentrations), IPA (2.0 equiv. relative to **1a**), $NADP^+$ (0.0125 g/L), KRED-06 (5 g/L), and LkADH (2.5 g/L) in KP_i buffer (100 mM, pH 7.0). Reaction mixtures were incubated at 25 ℃ with 200 rpm shaking for 12 h. Each data point represents the mean ± S.D. (range) of triplicate assays.

Effect of IPA equivalent. Efficient regeneration of NADPH plays a pivotal role in KRED-catalyzed reduction reactions. With this in mind, we next studied the effect of IPA, the sacrificial substrate used in our catalytic system. As seen in Figure 2, by increasing the amount of IPA from 1 equiv. to 2.5 equiv., the reaction conversion was improved from 75% to 94%. On the other hand,



using more than 2.5 equiv. of IPA did not improve the reaction conversion further. Hence, 2.5 equiv. of IPA was identified as the optimal amount.

Figure 2. Effect of concentration of IPA on the reaction conversion. Reaction conditions (0.8 mL): **1a** (125 g/L), IPA (variable concentrations), $NADP^+$ (0.0125 g/L), KRED-06 (5 g/L), and LkADH (2.5 g/L) in KP_i buffer (100 mM, pH 7.0). Reaction mixtures were incubated at 25 ℃ with 200 rpm shaking for 12 h. Each data point represents the mean ± S.D. (range) of triplicate assays.

Effect of reaction temperature and pH. Temperature is another important parameter for biocatalytic reactions. On one hand, high temperature usually means fast reaction rate, which is beneficial for an efficient industrial process. On the other hand, unlike chemo-catalysts, biocatalysts are usually not able to withstand high temperature conditions. Therefore, it is essential to find out the optimal operating temperature for a given biocatalytic process. Firstly, our study of temperature on the reaction conversion suggested this bioreduction reaction could occur readily at the temperature range of 25 to 50 ℃ (Figure 3), with 30 ℃ giving a slightly better conversion. Secondly, in the temperature-tolerant experiments (Figure 4), our biocatalysts (KRED-06 and LkADH) retained more than 94% activity after being incubated at 30 ℃ for 24 h. In comparison, the biocatalysts lost about 22% and 70% activity after being incubated at 35 ℃ and 40 ℃ for 24 h, respectively. Collectively, 30 ℃ was identified as the optimal reaction temperature.

Next, the effect of pH on the reaction conversion was examined as well. Apparently, this biocatalytic reaction functioned well at a pH range of 5.0 to 9.0, with pH 7.0 giving the best results (Figure 5).

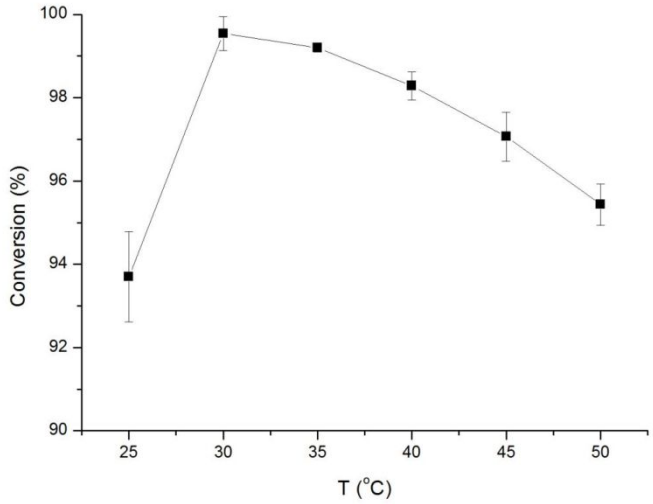


Figure 3. Effect of temperature on the reaction conversion. Reaction conditions (0.8 mL): **1a** (125 g/L), IPA (2.5 equiv. relative to **1a**), $NADP^+$ (0.0125 g/L), KRED-06 (5 g/L), and LkADH (2.5 g/L) in KP_i buffer (100 mM, pH 7.0). Reaction mixtures were incubated at different temperatures with 200 rpm shaking for 12 h. Each data point represents the mean ± S.D. (range) of triplicate assays.

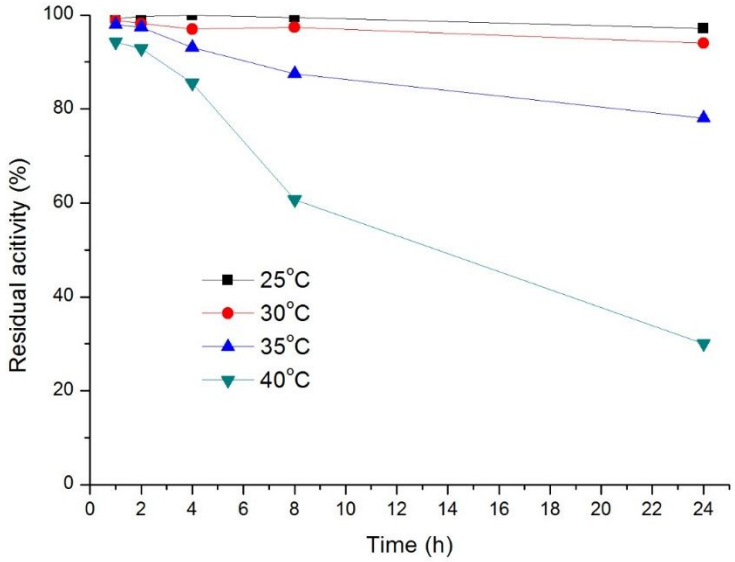


Figure 4. Activities of KRED-06/LkADH to thermal treatment at 25 ℃, 30 ℃, 35 ℃, and 40 ℃. The initial activity of KRED-06/LkADH without thermal treatment was defined as 100%. Reaction conditions: the crude enzyme extracts of KRED-06 and LkADH were incubated at temperatures of 25 ℃, 30 ℃, 35 ℃, and 40 ℃ for different periods prior to assaying activity.

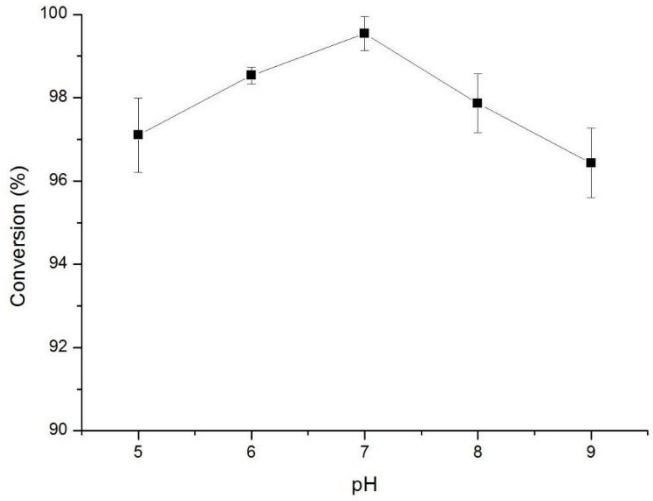


Figure 5. Effect of pH on the reaction conversion. Reaction conditions (0.8 mL): **1a** (125 g/L), IPA (2.5 equiv. relative to **1a**), $NADP^+$ (0.0125 g/L), KRED-06 (5 g/L), and LkADH (2.5 g/L) in different buffers, including 100 mM sodium citrate buffer (pH 5.0 or 6.0), 100 mM KP_i buffer (pH 7.0 or 8.0), and 100 mM glycine buffer (pH 9.0). Reaction mixtures were incubated at 30 ℃ with 200 rpm shaking for 12 h. Each data point represents the mean ± S.D. (range) of triplicate assays.

Effect of biocatalyst and cofactor loadings. The amounts of biocatalysts and cofactor used largely determine the production cost of a biocatalytic process. Regarding the former aspect, when the KRED-06 loading was reduced from 5 g/L to 4 g/L, the reaction was still able to go to completion (Figure 6). More significantly, our study of the $NADP^+$ loading showed that without the addition of exogenous $NADP^+$, the bioreduction reaction was able to go to completion, although a longer reaction time was required compared to those reactions running in the presence of exogenous $NADP^+$ (Figure 7). These results suggested the endogenous $NADP^+$ present in the crude enzyme extracts was sufficient to drive the biotransformation to completion, which would greatly reduce the production cost of this biocatalytic process.

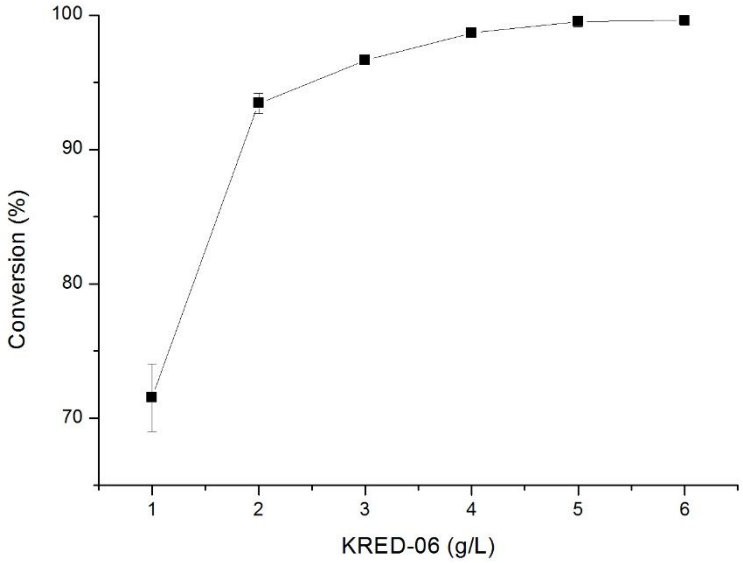


Figure 6. Effect of biocatalyst loading on the reaction conversion. Reaction conditions (0.8 mL): **1a** (125 g/L), IPA (2.5 equiv. relative to **1a**), $NADP^+$ (0.0125 g/L), KRED-06 (variable concentrations), and LkADH (50% w/w relative to KRED-06) in KP_i buffer (100 mM, pH 7.0). Reaction mixtures were incubated at 30 ℃ with 200 rpm shaking for 12 h. Each data point represents the mean ± S.D. (range) of triplicate assays.

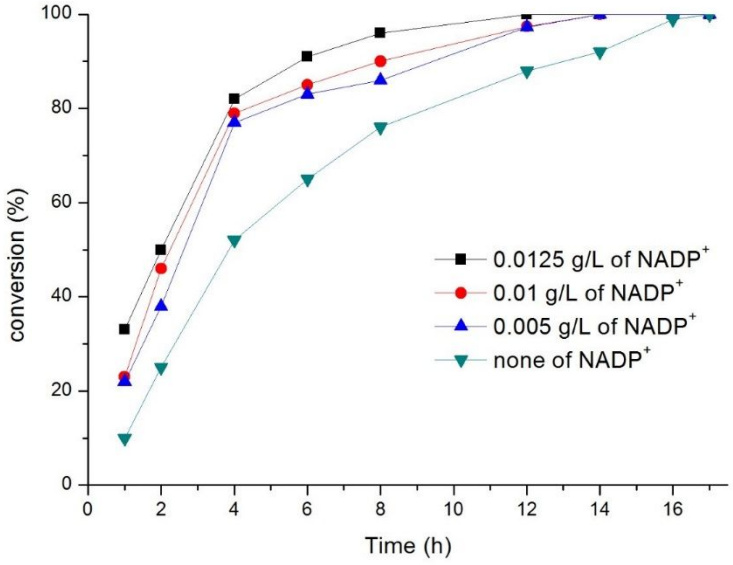
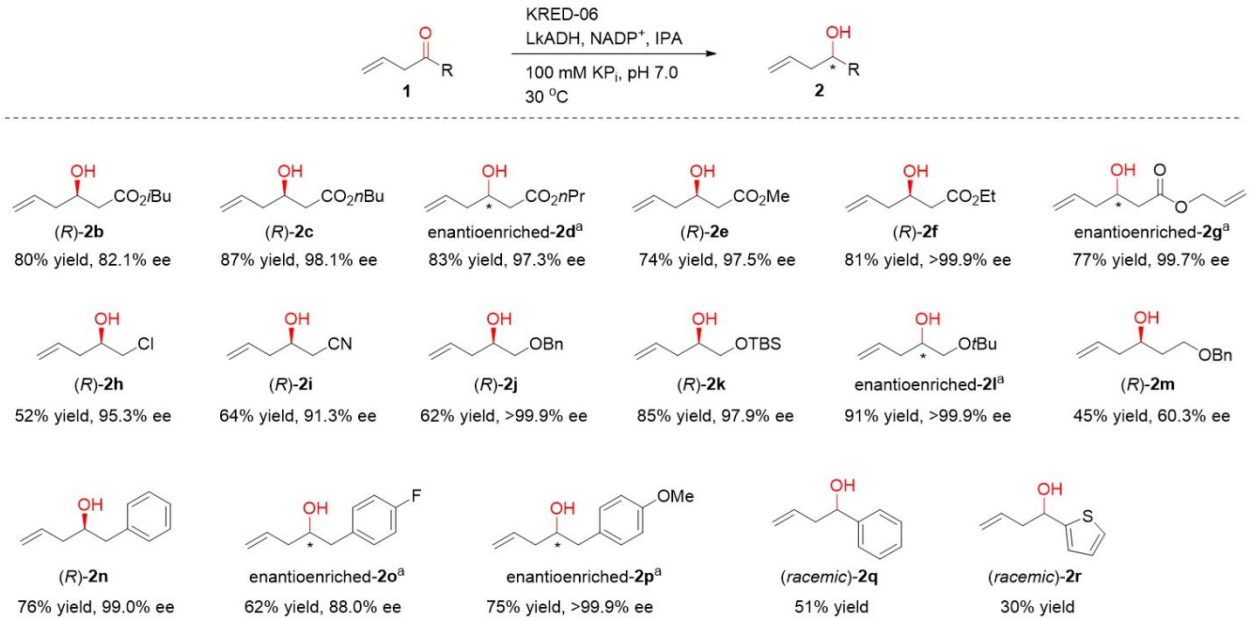


Figure 7. Effect of cofactor loading on the reaction conversion. Reaction conditions (0.8 mL): **1a** (125 g/L), IPA (2.5 equiv. relative to **1a**), $NADP^+$ (variable concentrations), KRED-06 (4 g/L), and LkADH (2 g/L) in KP_i buffer (100 mM, pH 7.0). Reaction mixtures were incubated at 30 ℃ with 200 rpm shaking for 1-17 h.

Pilot-plant-scale synthesis. After practice at gram- and hundred-gram-scale, the above-optimized reaction conditions were eventually applied to the synthesis of (*R*)-**2a** at a pilot-plant-scale. Specifically, **1a** (3.3 kg, 125 g/L, 90% GC purity), IPA (3.42 L, 2.5 equiv.), crude enzyme extracts of KRED-06 (4 g/L), and crude enzyme extracts of LkADH (2 g/L) in KP_i buffer (23 L, 100 mM, pH 7.0) were stirred in a reactor at 30 ℃. After completion of the reaction as monitored by GC, the pH was adjusted to 3~4 by the addition of 4 N HCl. Celite was added and the mixture was stirred for 10 min prior to filtration. The filter cake was extracted with EtOAc for three times, and this combined organic solution was then used to extract the aqueous filtrate from the first filtration. The combined EtOAc solution was dried, and concentrated *in vacuo* to give (*R*)-**2a** (3.21 kg, 96.2% yield) with a GC purity of 80%, as well as an enantiomeric purity of >99.9% ee. Without further purification, thus obtained (*R*)-**2a** can be directly used in our reported synthesis of bromocarbonate **3a** via a one-pot diastereoselective carboxylation/bromocyclization sequence.[17]

Biocatalytic synthesis of homoallylic alcohols 2. To further show its synthetic potential, the currently developed biocatalytic system was applied to the synthesis of various homoallylic alcohols **2** (Scheme 2). Firstly, ketoesters **1c**-**1g** were all efficiently reduced by KRED-06 to give the desired alcohols in 74%-to-87% isolated yields and excellent enantiomeric purity (all >97% ee). Ketoester **1b** containing an *iso*-butyl group was reduced in a less stereoselective manner, yielding the corresponding alcohol (*R*)-**2b** with 82% ee. Functional groups, such as chloro group (**1h**), cyano group (**1i**), and ether moieties (**1j**-**1l**) were well tolerated under our bioreduction conditions, with the corresponding homoallylic alcohols **2h**-**2l** being produced in moderate-to-excellent yields and excellent enantiomeric purity (all >91% ee). Interestingly, the enantiomeric purity of alcohol (*R*)-**2m** bearing a benzyl ether moiety was only 60% ee, much lower than that of its shorter-carbon-chain counterpart (*R*)-**2j** (>99% ee), suggesting the chain length of the substrates

might affect the binding modes in the KRED-06-catalyzed reduction reactions. Similarly, ketones with a benzyl group attaching to the carbonyl group (**1n**-**1p**) were all reduced by KRED-06 in a highly stereoselective manner, affording product alcohols with ≥88% ee. In stark contrast, substrates with an aryl group directly attaching to the carbonyl group (**1q** and **1r**) were reduced by the enzyme in a completely non-stereoselective fashion, generating racemic products **2q** and **2r**.



Scheme 2. KRED-06-catalyzed stereoselective synthesis of enantioenriched homoallylic alcohols **2**. [a]The absolute configuration was not assigned.

Conclusions

In summary, through screening a small library of in-house preserved KREDs, KRED-06 was identified as an efficient and selective biocatalyst capable of reducing ketoester **1a** to generate *tert*-butyl (*R*)-3-hydroxyl-5-hexenoate ((*R*)-**2a**). After further reaction optimization, this biocatalytic process was successfully performed at a pilot-plant-scale without the addition of exogenous $NADP^+$, producing 3.21 kg of enantiomerically pure (*R*)-**2a** (96.2% yield, >99.9% ee).

Compared to our first-generation synthesis of (*R*)-**2a** using $NaBH_4$ and (L)-tartaric acid under cryogenic conditions (-40 ℃), the currently developed biocatalytic approach features mild reaction conditions, environmental friendliness, and outstanding stereoselectivity, therefore possessing great potential to be implemented into industrial production of statins.

EXPERIMENTAL SECTION

General Methods. Unless otherwise specified, all reagents and solvent were purchased from commercial sources and used as received. ^{1}H (400 MHz) and ^{13}C (101 MHz) NMR were recorded on a Bruker Avance 400 spectrometer in $CDCl_3$ using tetramethylsilane (TMS) as internal standards. Products were purified by flash column chromatography on silica gel purchased from Qingdao Haiyang Chemical Co., Ltd. Optical rotations were measured by a Rudolph AUTOPOL I Automatic Polarimeter. HRMS were recorded on a Bruker micrOTOF spectrometer. The compounds (**2a-i**, **2k-l**, **2p**) were benzoylated and their enantiomeric excess was determined by HPLC with Chiralpak IC column (25 cm × 4.6 mm × 5 μm) and Chiralpak AD-H column (25 cm × 4.6 mm × 5 μm). The enantiomeric excess of compounds (**2j**, **2m-o**, **2q-r**) was determined by HPLC with Chiralpak OJ column (25 cm × 4.6 mm × 5 μm) and Chiralpak OD-H column (25 cm × 4.6 mm × 5 μm).

Cloning, Expression (Shake-flask), and crude enzyme extracts (CEE) preparation. KRED-06 was cloned into the NdeI and BamHI restriction sites of pET-24b. The rest of ketoreductases in this study were prepared as we previously described.[32-34]

An approximately 12 h culture of *E. coli* BL21 (DE3) cells freshly transformed with the appropriate plasmid and grown in LB medium supplemented with either kanamycin (50 μg/mL) or ampicillin (100 μg/mL) was diluted 1:100 into 0.5 L of the same medium in a 2 L flask. The

culture was shaking at 37 °C until the optical density at 600 nm reached 0.6-0.8, then the flask was placed in an ice/water bath for ca. 30 min before the addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 100 μM. The culture was shaking for an additional 14-16 h at 18 ºC. The cells were collected by centrifugation, and then resuspended in an appropriate amount of KP_i buffer (100 mM, pH 7.0) to make a 15% w/v suspension. The cells were lysed by sonication on ice and debris was removed by centrifugation at 37,000 x g for 30 min at 4 °C. This collected supernatant (crude enzyme extracts, CEE) was used as the biocatalyst. The quantification of the crude enzyme extracts was following the common practice for KREDs.[35-38] Briefly, a portion of the crude enzyme extracts was lyophilized and weighed. Next, this obtained information was used as the reference for the calculation of the biocatalyst loading (g/L).

Expression of KRED-06 using 7 L fermenter. An approximately 12 h culture of *E. coli* BL21 (DE3) cells freshly transformed with pET-24b-KRED-06 and grown in LB medium supplemented with kanamycin (50 μg/mL) was inoculated with an inoculation concentration of 1% (v/v) into a 7 L fermenter containing 5 L of a sterilized and modified LB medium (20 g/L tryptone, 10 g/L Yeast Extract, 10 g/L NaCl, 1.5 g/L glycerol) with kanamycin (100 mg). The fermentation process was first carried out at 30 °C for approximately 9 h with an aeration of 0.5 vvm (air volume/culture volume/minute). The agitation rate was occasionally adjusted to maintain the dissolved oxygen concentration above 30%. Then, the temperature was lowered to 25 °C and the pH was auto-controlled as 6.85 by the addition of ammonia and phosphoric acid. In addition, glycerol was added at the rate of 10 mL/h. At this point, 100 mg of IPTG was added to induce the expression. The agitation rate was auto-adjusted to maintain the dissolved oxygen concentration above 35%. After 14 h of induction, recombinant *E. coli* cells were harvested as described above, and stored at −80 °C until further use.

KRED-06-catalyzed synthesis of (*R*)-2a at a pilot-plant-scale. The reaction mixture containing **1a** (3.3 kg, 125 g/L, 90% GC purity), IPA (3.42 L, 2.5 equiv.), crude enzyme extracts of KRED-06 (4 g/L), crude enzyme extracts of LkADH (2 g/L), and KP_i buffer (23 L, 100 mM, pH 7.0) in a 50 L reactor was stirred at 30 ℃. After completion of the reaction as monitored by GC, the pH was adjusted to 3~4 by the addition of 4 N HCl. Celite was added and the mixture was stirred for 10 min prior to filtration. The filter cake was extracted with EtOAc for three times and this combined organic solution was then used to extract the aqueous filtrate from the first filtration. The combined EtOAc solution was dried, and concentrated *in vacuo* to give (*R*)-**2a** (3.21 kg, 96.2% yield) with a GC purity of 80%, as well as an enantiomeric purity of >99.9% ee. Without further purification, thus obtained (*R*)-**2a** can be directly used in our reported synthesis of bromocarbonate **3a** via a one-pot diastereoselective carboxylation/bromocyclization sequence. A small portion of this product was subjected to vacuum distillation (58-63 ℃/1-2 mm Hg) to afford (*R*)-**2a** for analytical purpose. Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.83-5.69 (m, 1H), 5.06 (d, J = 6.4 Hz, 1H), 5.02 (s, 1H), 3.97 (dd, J = 5.9, 2.9 Hz, 1H), 3.18 (s, 1H), 2.40-2.14 (m, 4H), 1.39 (s, 9H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 172.2, 134.1, 117.8, 81.1, 67.4, 41.6, 40.9, 28.0. HRMS(ESI) m/z $[M+Na]^+$ calcd for $C_{10}H_{18}O_3Na$ 209.1148, found 209.1144. $[\alpha]^{20}_D$ -26.9 (c 1.14, $CHCl_3$). lit.$[\alpha]^{20}_D$ -24.3 (c 1.0, $CHCl_3$).[17] HPLC (Chiracel® IC, Hexane **:** Isopropanol = 90 **:** 10, Flow rate = 1.0 mL/min, λ = 254 nm, 25 ℃) : t_1 = 4.9 min (major enantiomer), t_2 = 6.5 min. (> 99.9% ee)

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Experimental details including enzyme information, synthesis of substrates, enzymatic reactions, product characterization. NMR spectra and chiral-HPLC spectra are provided as well.

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Notes

The authors declare no competing financial interest.

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