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Identification of a Robust Carbonyl Reductase for Diastereoselectively Building *syn*-3,5-Dihydroxy Hexanoate: a Bulky Side Chain of Atorvastatin

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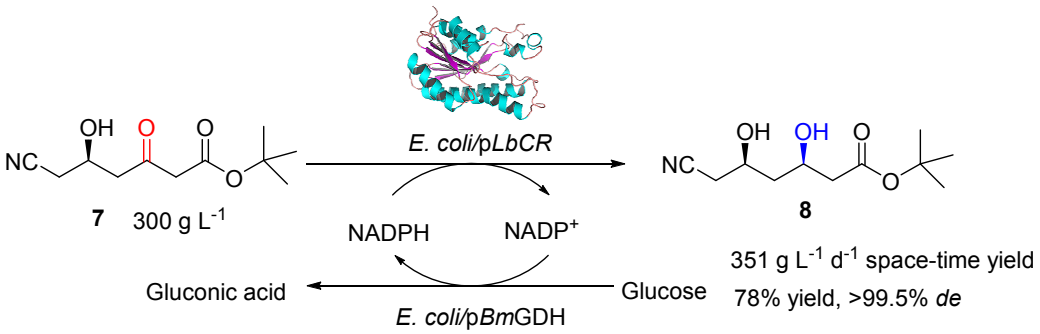


Abstract

t-Butyl 6-cyano-(3*R*,5*R*)-dihydroxyhexanoate is an advanced chiral precursor for the synthesis of the side chain pharmacophore of cholesterol-lowering drug Atorvastatin. Herein, a robust carbonyl reductase (*Lb*CR) was newly identified from *Lactobacillus brevis*, which displays high activity and excellent diastereoselectivity toward bulky *t*-butyl 6-cyano-(5*R*)-hydroxy-3-oxo-hexanoate (**7**). The engineered *Escherichia coli* cells haboring *Lb*CR and glucose dehydrogenase (for cofactor regeneration) were employed as biocatalysts for the asymmetric reduction of substrate **7**. As a result, as much as 300 g L^{-1} of water-insoluble substrate was completely converted to the corresponding chiral diol with >99.5% *de* in a space−time yield of 351 g L^{-1} d^{-1}, indicating a great potential of *Lb*CR for practical synthesis of the very bulky and *bi*-chiral 3,5-dihydroxy carboxylate side chain of best-selling statin drugs.

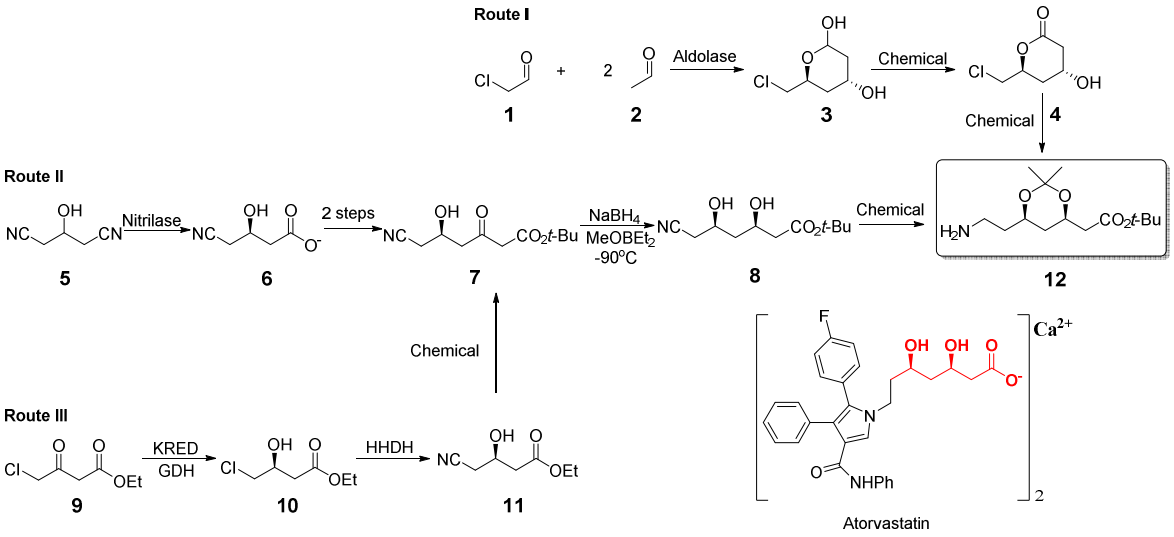
Keywords: asymmetric reduction; biocatalysis; carbonyl reductase; *t*-butyl 6-cyano-(3*R*,5*R*)-dihydroxyhexanoate; statin side chain; enzymatic catalysis

Introduction

Statins are an important class of cholesterol-lowering drugs, which hinder the synthesis of cholesterol by inhibiting 3-hydroxy-3-methylglutaryl−CoA reductase, a rate-limiting enzyme in the biosynthesis of cholesterol. Among the statin family, Atorvastatin calcium, under the brand name Lipitor®, was the first drug to have annual sales exceeding 10 billion US dollars. Like all statins, Atorvastatin contains a *syn*-3,5-dihydroxy hexanoate side chain pharmacophore with two chiral centers (Scheme 1), which is required with >99.5% *ee* and >99.5% *de*.

In terms of sustainable synthesis, environmentally benign enzymatic catalysis has attracted increasing attention as an alternative to traditional chemical catalysis in the synthesis of chiral intermediates from cheap achiral materials[1]. Numerous elegant enzymatic strategies have been developed, such as the sequential aldol condensation catalyzed by 2-deoxyribose-5-phosphate aldolase (DERA) to give chiral intermediate **3** from the cheap bulk chemicals chloroacetaldehyde **1** and acetaldehyde **2** (Scheme 1, Route I)[2]. Desymmetrization of 3-hydroxyglutaronitrile **5** catalyzed by nitrilase affords chiral **6** (Route II)[3]. Asymmetric reduction of ethyl 4-chloroacetoacetate **9** accesses chiral alcohol **10** using ketoreductase (KRED)[4], and replacement of the chlorine by a cyano group using halohydrin dehalogenase (HHDH)[5] enables the synthesis of chiral **11** (Route III).

Scheme 1. Chemoenzymatic synthetic routes for Atorvastatin chiral intermediates. KRED: Ketoreductase; GDH: Glucose Dehydrogenase; HHDH: Halohydrin Dehalogenase.



Although these enzymatic protocols are economically viable and environmentally attractive, they have mainly been used to synthesize primary intermediates with a single stereocenter. Indeed, a major challenge is the formation of second stereocenter to give advanced intermediates in high diastereoselectivity and productivity, such as *t*-butyl 6-cyano-(3*R*,5*R*)-dihydroxyhexanoate **8**, a more advanced precursor for the manufacture of Atorvastatin. The homo-chiral intermediate **8** was traditionally synthesized by diastereoselective chemical reduction of **7**, derived from **11**, using $NaBH_4$ and Et_2BOMe[6]. To obtain high diastereoselectivity (>99.5% *de*), extremely low temperature (−90°C) and pyrophoric triethyl borane are necessary, resulting in extensive energy consumption and a substantial amount of waste formation. An

alternative chemical route was developed to replace the traditional reduction using chlororuthenium (II) arene/β-amino alcohol. However, diastereoselectivity was insufficient (80% *de*)[7]. Hence, it is highly desirable to develop green and sustainable processes for the synthesis of **8**.

Biocatalytic asymmetric reduction provided a straightforward and environmentally attractive process for the synthesis of **8**. Although several ketoreductases for the asymmetric synthesis of **8** have been reported[8], most of them showed insufficient activity towards non-natural substrate **7** at high substrate loading. Only an engineered KRED gave 98% conversion at 300 g L^{-1} substrate concentration but with only 0.84 s^{-1} mM^{-1} of catalytic efficiency (k_{cat}/K_m)[9]. Therefore, it is still highly desirable to develop more robust biocatalysts to reduce the difficult-to-reduce ketone **7**. Herein, we describe a newly discovered carbonyl reductase from *Lactobacillus brevis* (*Lb*CR) capable of efficiently reducing **7** to **8** on a preparative scale.

Results and discussion

Screening of recombinant reductases

Initially, carbonyl reductases *Rh*CR[10] and *Cp*AR2[11] possessing high activity towards ethyl 8-chloro-6-oxooctanoate **S16** were trialed in the reduction of target substrate **7**, which is structurally similar to **S16**. However, none was found to be active against **7**. Subsequently, a ketoreductase toolbox containing 100 enzymes developed by our laboratory was evaluated. As a result, 17 enzymes displayed activities for substrate **7** (Figure 1), however, activities of majority of the enzymes

were extremely low (<10% relative activity compared with *Lb*CR, Table S1). To our delight, *Lb*CR (GenBank accession no.: NC_008497.1) displayed a relatively high activity toward the difficult-to-reduce ketone **7**, and was thus chosen for further investigation.

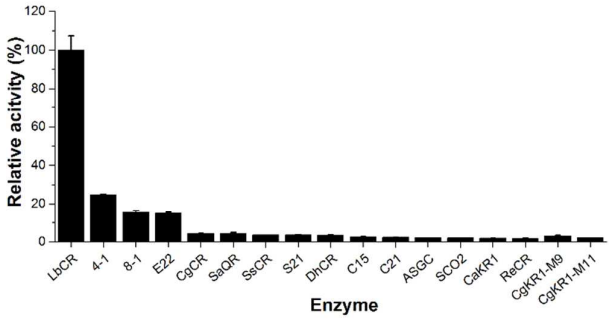


Figure 1. The screening results of a home reductase library for reduction of a bulky substrate **7**.

A multiple sequence alignment shown in Figure S1 revealed that the amino acids of *Lb*CR have merely 46% and 49% identities respectively with the reported *Lb*ADH from *Lactobacillus brevis* (GenBank accession no.: CAD66648.1)[12] and *Lk*ADH from *Lactobacillus kefir* (GenBank accession no.: WP_054768785.1)[13]. *Lb*ADH and *Lk*ADH exhibited exclusively catalytic activities toward the C-5 carbonyl group of *tert*-butyl 6-chloro-3,5-dioxohexanoate (CDOH). However, only *Lb*CR showed a significnat activity toward the C-3 keto group of substrate **7,** indicating its unique feature of which is distinct from majority of well-known ketoreductases.

Characterization of *Lb*CR

The intrinsic catalytic properties of *Lb*CR were then investigated using the enzyme purified by nickel-affinity chromatography. A single band with an apparent molecular size of 29 kDa corresponds to *Lb*CR (Figure S2); this confirms the calculated molecular mass of 26 kDa derived from the amino acid sequence. The specific activity of the pure protein was 24.8 U mg^{-1} protein under standard conditions.

The optimum temperature and pH were determined to be 40°C (Figure S3B) and 6.0 (Figure S3A), respectively. The half-life of *Lb*CR was 2 h at 30°C and 1.2 min at 40°C (Figure S3C). The effect of various metal ions and EDTA on the activity of *Lb*CR were also investigated. The results (Table S2) showed that EDTA and most of the tested metal ions had no significant effect on the activity of *Lb*CR except Cu^{2+}, which inhibited the activity strongly.

The kinetic parameters of *Lb*CR were also determined (Table S3). The K_M and k_{cat} of *Lb*CR toward substrate **7** are 0.58 mM and 24.6 s^{-1}, while those toward NADPH are 0.026 mM and 25.5 s^{-1}. Compared with other reported ketoreductases (Table 1), *Lb*CR afforded the lowest K_m and the highest catalytic efficiency constant k_{cat}/K_M (42.4 s^{-1} mM^{-1}), indicating a high binding affinity with ketoester **7**.

Table 1. Kinetic parameters of *Lb*CR and other reported enzymes toward substrate **7**.

Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	Ref.
KRED[a]	10.9 ± 2.3	9.12 ± 0.78	0.84	9
*Ca*AKR	1.91	3.64	1.91	8h

*Kl*AKR	4.4	11.6	2.64	8g
*Kl*AKR[b]	3.4	42.2	12.4	8i
*Lb*CR	0.58 ± 0.13	24.6 ± 6.7	42.4	This work

[a] KRED was synthesized according to SEQ ID No. 316, which was used for the preparation of *t*-butyl 6-cyano-(3*R*,5*R*)-dihydroxyhexanoate at 300 g L^{-1}. [b] *Kl*AKR mutant (Y295W/W296L).

In order to understand the high binding affinity of *Lb*CR toward **7**, we performed homology modeling and docking calculation. As shown in Figure 2, *Lb*CR displays a typical "Rossmann-fold" of short-chain dehydrogenases/reductases (SDRs) family, while *Kl*AKR and KRED have a classical $(\alpha/\beta)_8$ barrel of the aldo-keto reductases (AKRs). According to the proposed catalytic mechanism of SDR, the conserved Y158 and S145 form hydrogen bonds with the carbonyl oxygen atom of the substrate and a hydrogen from NADPH attacks the carbonyl carbon atom of the substrate at the *si*-face to generate corresponding (*R*)-alcohol. The orientation of substrate **7** docked in the substrate-binding pocket of *Lb*CR showed that the *t*-Bu of the substrate was surrounded by more hydrophobic residues (V96, L152, A155, A201, I197 and V198) than the corresponding residues of *Kl*AKR and KRED, and the CN-group pointed toward Q247, facilitating the binding of substrate. The substrate binding pocket of *Lb*CR (Figure 2D) exhibited apparently different geometry from *Kl*AKR (Figure 2E) and KRED (Figure 2F). A tunnel through *Lb*CR was buried inside, and the size of this tunnel was more suitable to accommodate **7** than the other enzymes. The special geometry and polarity of the substrate binding pocket of *Lb*CR may contribute to its high binding affinity toward **7**.

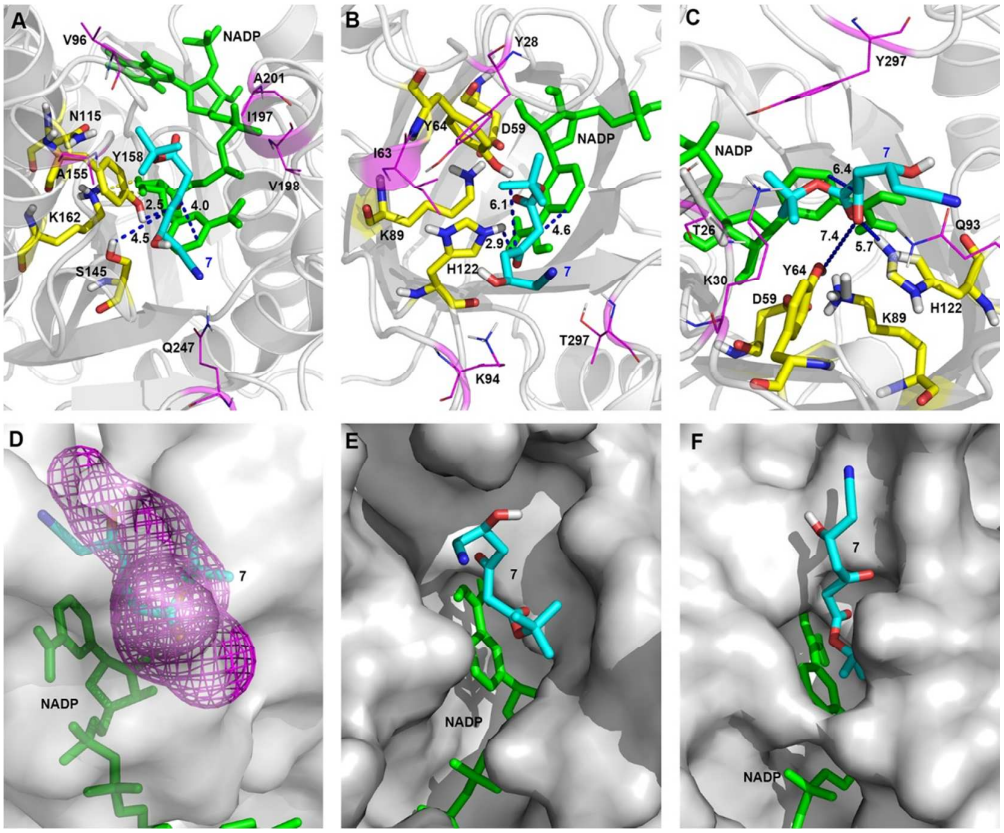


Figure 2. Docking of substrate **7** into the active sites of *Lb*CR, *Kl*AKR and KRED. (A, D) *Lb*CR; (B, E) *Kl*AKR; (C, F) KRED. Interactions were highlighted by blue dashed lines. The active sites, $NADP^+$ and **7** were shown as yellow sticks, green sticks and cyan sticks. Other residues surrounding the substrate were shown as magenta lines. Tunnels of *Lb*CR calculated by Caver 3.0 were shown as magenta mesh.

Substrate specificity

The substrate specificity of *Lb*CR was investigated using various ketoesters and ketones (Table S4). As shown in Figure 3, *Lb*CR displayed higher activity toward substrates bearing long side chains adjacent to the carbonyl function group (**S2**, **S3**, **S9**, and **S13**–**15**). In addition, *Lb*CR showed excellent activity towards chlorinated

compounds (**S11**, **S16** and **S18**), probably because chlorine leads to a higher electrophilicity of the carbonyl group. *Lb*CR showed higher activity for aryl ketoesters compared with alkyl ketoesters, and gave the highest activity towards **S20**, the important precursor of angiotensin-converting enzymes (ACE) inhibitors[14].

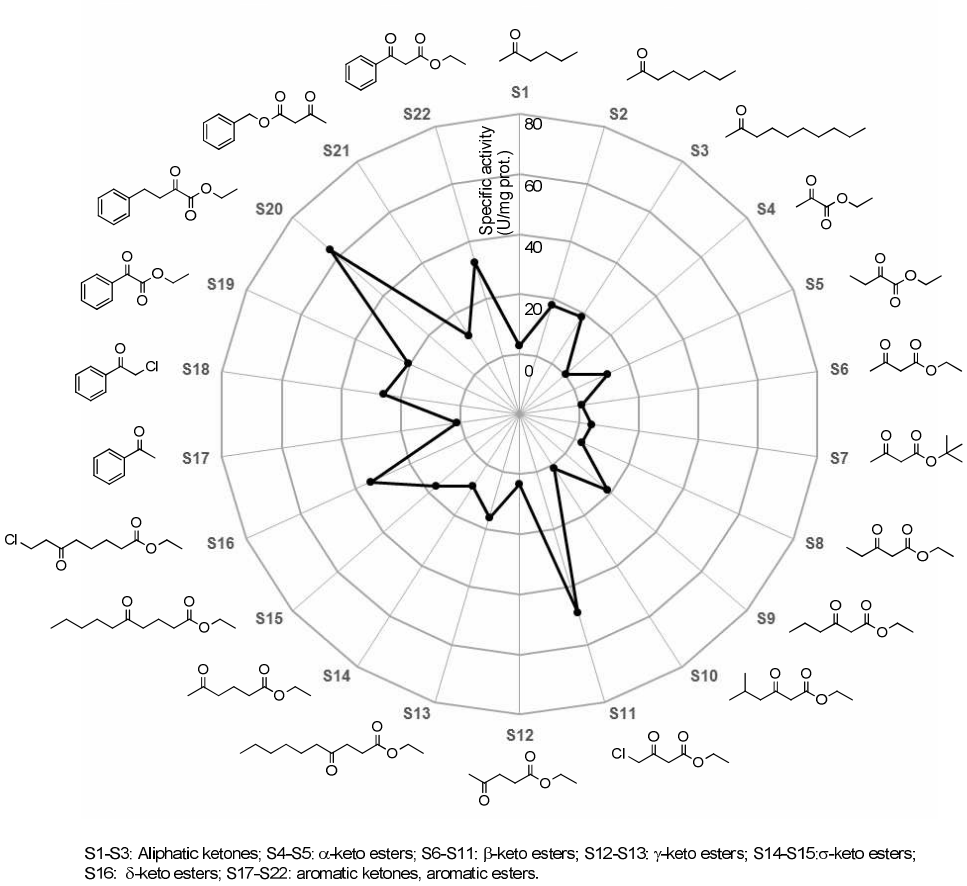


Figure 3. Substrate profile of *Lb*CR.

To explore the substrate scope of this new enzyme, the kinetic parameters of *Lb*CR towards several representative substrates were measured. As shown in Table 2, *Lb*CR exhibited markedly high catalytic efficiency towards aliphatic esters (**S13**, **S15**, **S16**)

and ketones (**S3**) with long chains, mainly attributing to their lower K_m values. However, *Lb*CR showed higher K_M values towards aliphatic esters with short chains.

Table 2. Kinetic parameters of *Lb*CR toward several ketones and ketoesters.

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
S2	0.56 ± 0.02	16.2 ± 0.2	28.9
S3	0.09 ± 0.01	12.3 ± 0.3	136.7
S5	13.1 ± 1.5	92.4 ± 2.9	40.0
S7	5.5 ± 1.3	14.3 ± 1.3	2.6
S9	1.2 ± 0.1	22.4 ± 0.7	18.6
S11	0.31 ± 0.06	49.7 ± 4.3	160.5
S13	0.075 ± 0.030	15.6 ± 1.0	207.4
S15	0.035 ± 0.008	13.4 ± 0.5	382.6
S16	0.136 ± 0.020	25.7 ± 0.9	189.3
S18	0.22 ± 0.10	26.1 ± 4.2	118.6
S19	1.2 ± 0.2	21.0 ± 1.0	17.5
S20	0.26 ± 0.03	56.6 ± 1.2	217.5
S22	1.4 ± 0.3	42.5 ± 2.6	30.4

The substrates **S5** and **S15** were docked into the active site of *Lb*CR to understand the substrate specificity. As shown in Figure 4, the catalytic residue Y158 was proximal to the carbonyl oxygen of substrates **7** and **S15**, but a bit distal to **S5**. Otherwise, the substrate tunnels seemed too large for **S5** with short chain, and several substrate molecules may bind in the active site simultaneously, leading to non-productive binding and lowering the binding affinity.

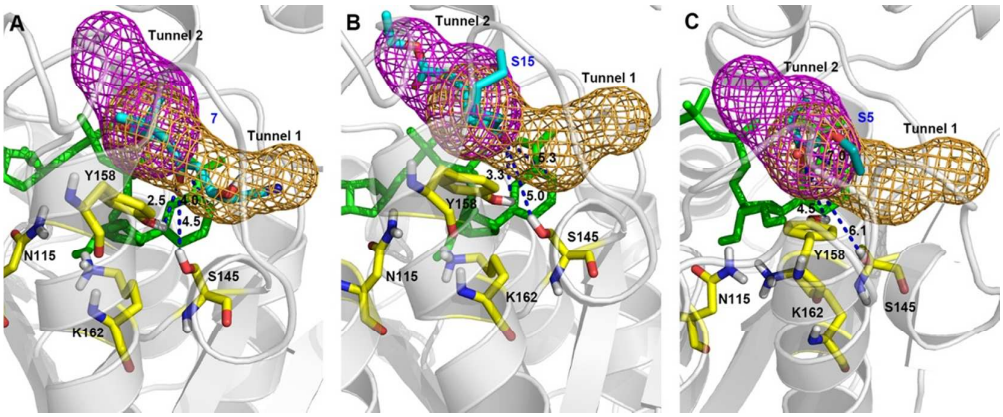


Figure 4. Docking of substrates **7**, **S15** and **S5** into the active sites of *Lb*CR. The active sites, $NADP^+$ and substrate were shown as yellow sticks, green sticks and cyan sticks. Tunnels calculated by Caver 3.0 were shown as magenta mesh and orange mesh.

In general, *Lb*CR is able to accept a broad range of substrates, particularly bulky substrates, suggesting its great potential as a biocatalyst for the asymmetric reduction of carbonyl compounds.

Preparative Synthesis of 8

Finally, to demonstrate the utility of *Lb*CR for practical applications, the asymmetric reduction of **7** was performed on a gram scale using lyophilized cells of *E. coli*/p*Lb*CR coupled with glucose dehydrogenase (GDH) for the regeneration of NADPH. Initial experiments were performed in 10 mL of potassium phosphate buffer (100 mM, pH 6.0) with a substrate concentration of 23 g L^{-1}. The pH of the reaction mixture was maintained at pH 6.0 by titrating 2 M K_2CO_3. It was found that the reaction was complete within 1 h with >99% conversion (Table 3, entry 1). When the

substrate loading was raised to 100 g L^{-1} without changing any of the other conditions, the reaction was completed smoothly within 3 h (entry 2). Moreover, the substrate was almost totally converted in 4 h at the same substrate loading when the amount of external addition of $NADP^+$ was reduced from 0.5 mM to 0.1 mM (entry 3). This prompted us to increase the substrate concentration further to 200 g L^{-1}, resulting in >99% conversion and a productivity of 522 g L^{-1} d^{-1} (entry 4). The reaction proceeded smoothly, even with a substrate concentration of 300 g L^{-1} (35.5% v/v). After a reaction time of 16 h (entry 5), the product was harvested in >99.5% *de* (Figure S6B) and 351 g L^{-1} d^{-1}. These results indicate that *Lb*CR is able to tolerate high substrate concentrations, making it promising for practical applications.

Table 3. Asymmetric reduction of **7** with lyophilized *E. coli c*ells of *Lb*CR and *Bm*GDH[a].

Entry	Substrate loading (g L^{-1})	Cell loading (g L^{-1})	$NADP^+$ (mM)	Time (h)	Conv. (%)[b]	*de* (%)[b]
1	23	16	0.5	1	>99	>99.5
2	100	16	0.5	3	>99	>99.5
3	100	16	0.1	4	>99	>99.5
4	200	16	0.1	8	>99	>99.5
5	300	16	0.1	16	>99	>99.5

[a] Reaction conditions: substrate **7** (1.22–3.66 g substrate), glucose (1.2 equiv.), lyophilized *E. coli*/p*Lb*CR (0.16 g), lyophilized *E. coli*/p*Bm*GDH (0.055 g), $NADP^+$ (0.1–0.5 mM), potassium phosphate buffer (100 mM, pH 6.0) to 10 mL at 30°C, and pH was kept at 6.0 with 2 M K_2CO_3.

[b] Determined by HPLC.

Conclusions

In conclusion, we have identified a new carbonyl reductase, *Lb*CR, from *Lactobacillus brevis*, which displayed very high specific activity, diastereoselectivity, and substrate tolerance in the asymmetric synthesis of *t*-butyl 6-cyano-(3*R*,5*R*)-dihydroxyhexanoate **8**. As much as 300 g L^{-1} of substrate **7** was reduced to give diastereomerically pure *t*-butyl 6-cyano-(3*R*,5*R*)-dihydroxyhexanoate **8** in >99.5% *de* and 351 g L^{-1} d^{-1} under the optimized conditions. The enzyme should therefore be suitable for the synthesis of an advanced statin side chain intermediate. Further studies to improve the catalytic activity and stability by protein engineering are now in progress to further reduce the enzyme loading, and make it a more efficient tool for industrial manufacturing.

Experimental

General Information

t-Butyl 6-cyano-(3*R*,5*R*)-dihydroxyhexanoate and *t*-butyl 6-cyano-(3*S*,5*R*)-dihydroxy hexanoate were obtained from J&K (Shanghai, China). **S13** and **S15** were obtained from Xiamen Bestally Biotechnology Co., Ltd. (Fujian, China). All others chemicals and reagents were purchased from commercial suppliers. *E. coli* DH5α and *E. coli* BL21 (DE3) were used as the cloning and expression hosts, respectively. Both cells were incubated in Luria-Bertani (LB) medium. ^{1}H NMR was

measured on a Bruker Avance 400 MHz spectrometer using tetramethylsilane (TMS) as internal standard. Optical rotation measurements were taken on a Rudolph Research Analytical Autopol I automatic polarimeter at 25°C.

Expression of ketoredutase in *E. coli*

E. coli BL21/pET28a−*Lb*CR and other recombinant cells were cultivated in Luria−Bertani (LB) medium containing 50 mg mL^{-1} kanamycin at 37°C and 180 rpm until $OD_{600\ nm}$ reached 0.6–0.8, then IPTG was added to the culture (final concentration 0.2 mM) to induce enzyme expression at 16°C for further 20–24 h. Cells were harvested by centrifugation (7000 × *g*, 10 min, 4°C) and washed with normal saline.

Enzyme assay

The reductase activity was assayed at 30°C by monitoring the decrease in the absorbance of NADPH at 340 nm on a UV-spectrophotometer (Beckman DU730). The standard assay mixture (1 mL) was composed of 970 μL potassium phosphate buffer (100 mM, pH 6.0), 10 μL *t*-butyl 6-cyano-(5*R*)-hydroxy-3-oxohexanoate (200 mM), 10 μL NADPH (10 mM), and 10 μL enzyme with an appropriate concentration. One unit of enzyme activity (U) was defined as the amount of enzyme that can catalyze the oxidation of 1 μmol NADPH per minute at above conditions.

Asymmetric bioreduction of ketoester 7

The reaction mixture (10 mL) was prepared by dissolving substrate **7** (1−13.2 mmol, 0.28−3.66 g), glucose (1.2 equiv. of substrate), lyophilized cells of *Lb*CR (10 kU, 0.16 g), lyophilized cells of GDH (10 kU, 0.055 g), 5% DMSO (v/v) and $NADP^+$ (1−5 μmol, 0.1−0.5 mM) in potassium phosphate buffer (100 mM, pH 6.0). The pH value of the mixture was monitored and adjusted to 6.0 using 2 M K_2CO_3 during the reaction. Products were extracted twice with ethyl acetate. The combined organic layers were washed twice with brine solution, dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum, giving crude **8** as yellow oil. The crude product was purified by column chromatography eluting with a mixture of ethyl acetate and petroleum ether (1:5) to provide pure **8 (**2.4 g, HPLC purity, 92%**)**. $[\alpha]_D^{25} = -41.3$ (*c*=1.0, $CHCl_3$), >99.5% *de*[8g, 9]. ^{1}H NMR ($CDCl_3$, 400 MHz), δ/ppm: 1.47 (s, 9H), 1.71~1.75 (m, 2H), 2.42-2.44 (m, 2H), 2.54-2.56 (m, 2H), 3.71 (brs, 2H), 4.18-4.22 (m, 1H), 4.26-4.31 (m, 1H).

ASSOCIATED CONTENT

Supporting Information

Complete experimental procedures and characterization of the enzymes and new products; NMR spectra and HPLC chromatograms.

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Notes

The authors declare no competing financial interest.

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