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SHORT COMMUNICATION

Stereoselective introduction of two chiral centers by a single diketoreductase: an efficient biocatalytic route for the synthesis of statin side chains

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Abstract Statins, including atorvastatin (Lipitor®), are the top-selling drugs in the world. The biocatalytic production of chiral side chains of statin drugs is of great interest to academia and industry. Stereoselective double reduction of a β,δ-diketo ester catalyzed by a diketoreductase offers a simple and efficient route for the preparation of statin side chains. Comparison of different cofactor regeneration systems resulted in an easy and cost-effective process for this enzymatic reduction.

Keywords Biocatalysis · Chiral side chain · Diketoreductase · Oxidoreductase · Statin

Introduction

Statins, such as atorvastatin (Lipitor®), the top-selling cholesterol-lowering drugs in the world (Müller 2005; Thayer 2006), contain two chiral centers in the β,δ-dihydroxyheptanoate side chains. To pursue a practical biocatalytic process for asymmetric synthesis of statin side chains, a number of approaches have been explored. For example, a strain of *Lactobacillus brevis* was reported to convert δ-keto group of β,δ-diketo ester to δ-hydroxy product with *ee* value of 98.1% (Wolberg et al. 2000; Wolberg et al. 2001). 2-Deoxy-D-ribose-5-phosphate aldolase (DERA), on the other hand, showed the ability to introduce two hydroxyl groups by two sequential aldol reactions with high stereoselectivity ($ee > 99.9\%$, $de = 96.6\%$), and the biocatalytic process is under development (Barbas et al. 1990; Gijsen and Wong 1994; Greenberg et al. 2004). Meanwhile, an *Acinetobacter* species SC 13874 was found to reduce both β- and δ-carbonyl groups of ethyl-6-(benzyloxy)-3,5-dioxohexanoate (**1**) to its corresponding ethyl *3R,5S*-6-(benzyloxy)-3,5-dihydroxy hexanoate (**2**) with 99% *ee* and 63% *de* (Guo et al. 2006). Later, a ketoreductase responsible for the conversion was cloned from *Acinetobacter* sp. SC 13874 and expressed in *Escherichia coli* to show excellent stereoselectivity, and the study on this enzyme is being undertaken (Goldberg et al. 2008). In addition, nitrilase has been considered as another option for the synthesis of statin side chains, but there are several challenges in this method, such as the difficulty of synthesizing 3-hydroxyglutaronitrile and the problems associated with the isolation of the product (Bergeron et al. 2006). Therefore, development of a practical biocatalytic route for statin side chains is of great interest to both academia and industry.

We recently reported the cloning, expression and characterization of a novel diketoreductase (GenBank accession no. EU273886) from *Acinetobacter baylyi* ATCC 33305. This recombinant diketoreductase (rDKR) is able to stereoselectively reduce **1**–**2** with both *de* and *ee* greater than 99.5% (Wu et al. 2008, 2009). Compound **2** is a valuable intermediate in the synthesis of statin side chains (Scheme 1). Herein, we present an efficient and practical route for the preparation of statin side chains using whole cells containing over-expressed rDKR with optimized cofactor regeneration system. After purification of **2**, ethyl

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3R,*5S*-6-hydroxy-3,5-*O*-isopropylidene-3,5-dihydroxy-hexanoate (**6**) was obtained with high yield through hydrogenolysis of ethyl *3R*,*5S*-6-(benzyloxy)-3,5-*O*-isopropyli-dene-3,5-dihydroxyhexanoate (**5**) that was synthesized from **2** (Scheme 1).

Materials and methods

Instruments and materials

HPLC was performed on Shimadzu 2010A HT with UV detector. NMR spectra were recorded at 300 K on a Bruker AV-500 spectrometer (Bruker Optics, Germany). High resolution mass spectra (HRMS) were recorded on Agilent HPLC/ESI-Q-TOFMS spectrometer. Optical rotation was obtained on a Pekin-Elmer 431 Polarimeter. NADH, $NADP^+$ and IPTG (Isopropyl-β-D-thiogalactopyranoside) were purchased from Sigma (USA). The expression of rDKR in *E. coli* and activity assay were performed as described previously (Wu et al. 2008, 2009).

Reduction of 1 by rDKR with 2-propanol as co-substrate

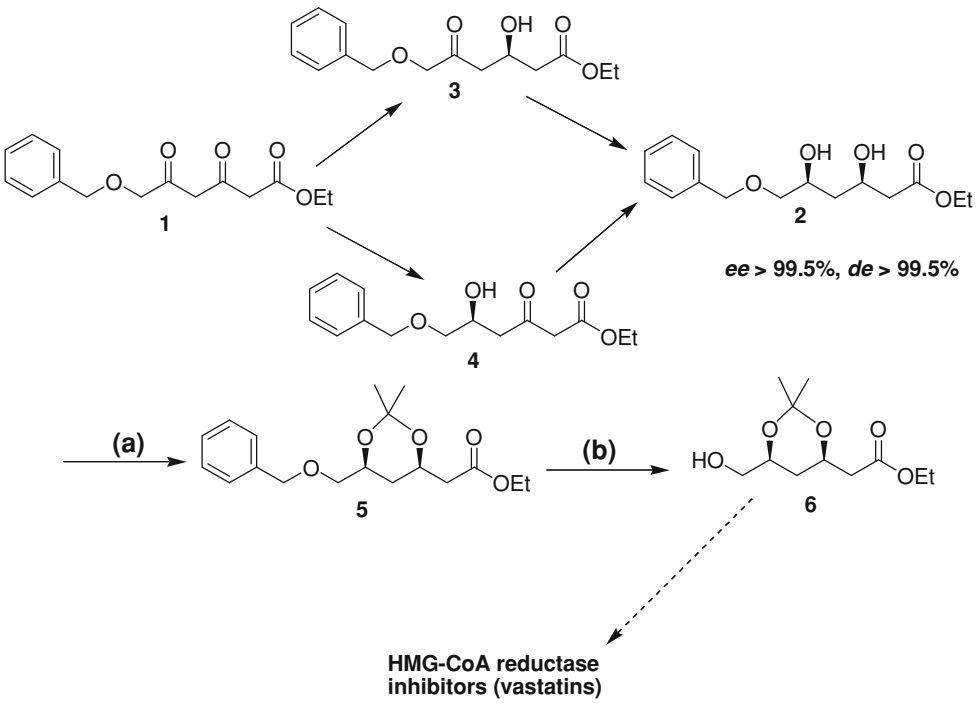
The reaction catalyzed using crude rDKR contained 1 g/L of **1**, 0.5 mM $NADP^+$, 2-propanol ranging from 1 to 5%, 0.7 U crude rDKR and 0.1 M potassium phosphate buffer (pH 6.0) in a final volume of 0.5 ml. When whole cells were used, reaction mixtures contained various concentrations of **1** ranging from 1 to 15 g/L, 0.5 mM $NADP^+$, 5% 2-propanol, 308 U rDKR in recombinant cells and 0.1 M potassium phosphate buffer (pH 6.0) in a final volume of 100 ml. All reactions were incubated at 25°C and 200 rpm for 18 h. Samples were taken and analyzed by HPLC (Wu et al. 2008, 2009; Liu et al. 2008).

Reduction of 1 by rDKR with glucose as co-substrate

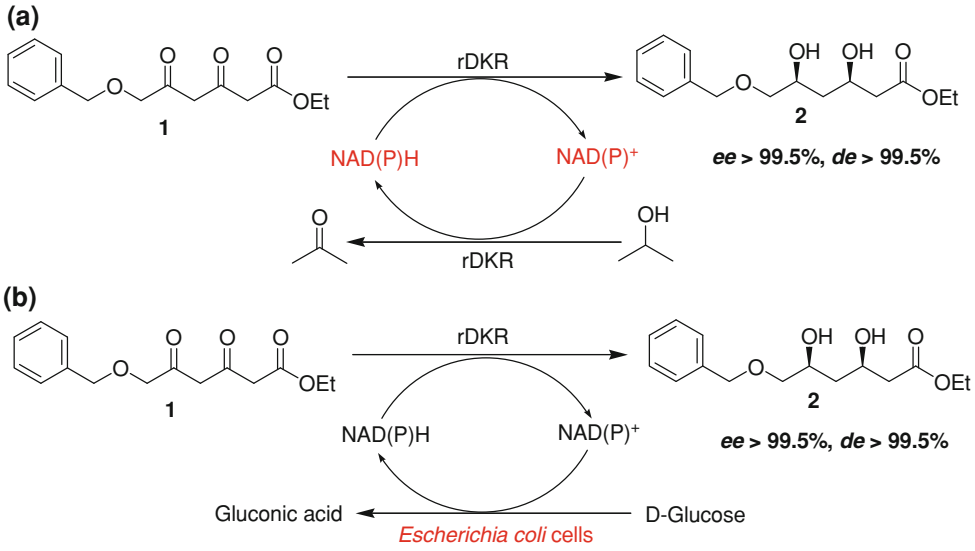
Thirty grams of glucose and 1 g of **1** dissolved in 10 ml ethanol were added to rDKR fermentation broth. The reaction was incubated at 25°C and 200 rpm. Each 0.5 ml of sample was taken and analyzed by HPLC. The reaction mixture was extracted by ethyl acetate for three times. Chromatography on silica gel (ethyl acetate/petroleum ether 10:90 v/v) gave **2** as a white powder.

Preparation of 5

The preparation of **5** was performed according to the literature (Bode et al. 2002). 2, 2-dimethoxypropane (20.8 g, 200 mmol) and a catalytic amount of camphorsulfonic acid at room temperature were added to a solution of **2** (5.64 g, 20 mmol) in acetone (50 ml). After stirring for 4.5 h, the volatiles were evaporated. The residue was dissolved in ethyl acetate and washed with saturated $NaHCO_3$ and NaCl solution, and dried over Na_2SO_4. Chromatography on silica gel (ethyl acetate/petroleum ether 1:10 v/v) gave **5** (6.02 g, 93.5% yield) as a colorless oil.



Scheme 1 Biocatalytic synthesis of statin side chain. Compound **2** was obtained by rDKR with both *de* and *ee* values greater than 99.5%. **a** 2, 2-dimethoxypropane, cat. camphorsulfonic acid, acetone, 25°C, 7 h, 93.5%; **b** 20 atm H_2, cat. 10% Pd/C, ethanol, 25°C, 18 h, 100%



Scheme 2 Different cofactor regeneration systems for the biocatalytic process by rDKR. **a** 2-Propanol was used to regenerate cofactors by rDKR; **b** glucose was used for cofactor regeneration

Preparation of **6**

The preparation of **6** was conducted according to reported method (Sun et al. 2007). Pd/C (10%, 0.35 g) was added to a solution of **5** (3.64 g, 11.3 mmol) in ethanol (100 ml) contained in a 250 ml autoclave. The autoclave was filled with H_2 (20 atm). After stirring for 18 h at room temperature, the catalyst was filtered and the solvent was evaporated. The residue was a colorless oil **6** (2.62 g, 100% yield).

Results and discussion

Previously, formate dehydrogenase (FDH) regeneration system was used to regenerate NADH over the course of conversion of **1**, 93% of transformation yield was observed within 3 h with the appearance of intermediates *R*-ethyl 6-(benzyloxy)-3-hydroxy-5-oxohexanoate (**3**) and *S*-ethyl 6-(benzyloxy)-5-hydroxy-3-oxohexanoate (**4**) (Wu et al. 2008, 2009). However, the requirement of FDH for NADH regeneration in the biocatalytic reaction makes the process costly and complicated. To overcome such shortcomings, we took advantage of the dual cofactor specificity exhibited by rDKR (Wu et al. 2008, 2009) to explore the possibility of utilizing 2-propanol as a co-solvent for the substrate and as a co-substrate for the regeneration of NAD(P)H (Inoue et al. 2005; Findrik et al. 2005). At the beginning, only <5% conversion of **1** was obtained when crude rDKR was utilized as a catalyst to reduce **1** with 2-propanol (1–5% in volume) mediated NADPH regeneration system (data not shown). This low conversion was likely due to the instability of rDKR against organic solvents. Then, a different approach with recombinant *E. coli* whole cells containing over-expressed rDKR was taken to conduct the conversion (Scheme 2a) in the presence of 2-propanol and $NADP^+$, resulting in high conversion within 3 h by resting cell suspensions (Supplementary Fig. 1). In order to maximize the catalytic capacity of this biocatalyst, different substrate concentrations were evaluated for this cofactor regeneration system, and we found that higher conversion rates showed at relatively lower substrate concentrations, and more accumulation of monohydroxy intermediates was observed at early phase of the reaction. Nevertheless, at 10 g/L substrate concentration, the conversion was finally reached to 93.5% (Supplementary Table 1).

To further facilitate the cofactor regeneration system and to simplify the biocatalytic process, an in situ reduction of **1** by whole cells containing over-expressed rDKR was investigated for the preparation of **2** since such system does not require exogenous addition of cofactors. Recombinant *E. coli* cells were grown in a flask, and subsequently induced by IPTG for the expression of rDKR. After 14 h, cell mass was reached to approximately 10%. Thereafter, glucose and substrate **1** dissolved in ethanol were directly added to the fermentation broth for the biocatalytic conversion. Given the fact that *E. coli* cells can simultaneously regenerate both NADH and NADPH by glycolysis and endogenous membrane-bound glucose dehydrogenase and glucose-6-phosphate dehydrogenase (Scheme 2b), the cells had enough capacity to regenerate the cofactors by serving glucose as co-substrate. Thus, the biocatalytic process was optimized to a conversion yield of 95% in 4 h without additional cofactors (Supplementary Fig. 2).

Since **6** is a more advanced intermediate in the synthesis of statins, we conducted two more chemical steps to demonstrate the feasibility of our approach for making the side chains. After extraction and purification of **2** from the biocatalytic reaction, acid-catalyzed protection reaction was carried out to protect the hydroxyl groups to form **5**.

Subsequently, hydrogenolysis of **5** yielded desired **6** (Bode et al. 2002; Sun et al. 2007). Compared to *tert*-butyl *3R*,*5S*-6-hydroxy-3,5-*O*-isopropylidene-3,5-dihydroxyhexanoate (Sun et al. 2007), compound **6** showed similar value of optical rotation.

Conclusion

We have investigated two different cofactor regeneration systems to optimize the biocatalytic process for rDKR reduction. The results have demonstrated that rDKR can be utilized as an effective biocatalyst for the preparation of statin side chains. At present, we are expending the utility of this unique biocatalyst for other chiral intermediates.

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