Enzyme and Microbial Technology 43 (2008) 544–549

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Synthesis of ethyl-(3*R*,5*S*)-dihydroxy-6-benzyloxyhexanoates via diastereo- and enantioselective microbial reduction: Cloning and expression of ketoreductase III from *Acinetobacter* sp. SC 13874

Steven Goldberg, Zhiwei Guo, Steve Chen, Animesh Goswami, Ramesh N. Patel *

Chemistry Technology, Process Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute, New Brunswick, NJ 08903, United States

ARTICLE INFO

Article history:
Received 28 May 2008
Received in revised form 7 July 2008
Accepted 8 July 2008

Keywords:
Enantioselective diastereoselective reduction
Biocatalysis
Ketoreductase
Cloning and expression

ABSTRACT

Previously we have demonstrated the reduction of ethyl and *t*-butyl diketoesters **1** to the corresponding syn-(3*R*,5*S*)-dihydroxy esters **2a** by *Acinetobacter* sp. 13874. The *syn*-(3*R*,5*S*)-dihydroxy ester **2a** was obtained with an enantiomeric excess (e.e.) of 99% and a diastereomeric excess (de) of 63%. In this report, we identified a gene encoding desired ketoreductase III which catalyzed the diastereoselective reduction of diketoesters **1** to *syn*-(3*R*,5*S*)-dihydroxy esters **2a** and describe cloning and expression of ketoreductase III into *Escherichia coli*. Cells or extracts of recombinant *E. coli* efficiently reduced the diketoester **1** to the corresponding *syn*-(3*R*,5*S*)-dihydroxy ester **2a** in 99.3% yield, 100% e.e., and 99.8% de.

1. Introduction

Ketoreductases are present in various bacteria, yeast and fungi. Largely due to their high enantioselectivity, ketoreductases have been recognized and utilized as an important class of enzymes for biocatalytic applications in the chemical and pharmaceutical industries for the preparation of chiral alcohols [1–8]. Chiral β-hydroxy esters are useful intermediates for synthesizing bioactive chiral compounds. Biocatalytic reduction of ketoesters by ketoreductases offers an attractive route to optically pure β-hydroxy esters. Previously, we have shown that *Acinetobacter* sp. SC 13874 contains a ketoreductase capable of stereoselectively reducing 3,5-dioxo-6-(benzyloxy)hexanoic acid ethyl ester (**1**, Scheme 1) to its corresponding *syn*-diol (**2a**) [9,10]. In the present study, the desired ketoreductase has been purified to homogeneity and the gene encoding a ketoreductase from *Acinetobacter* sp. SC 13874 was identified and cloned into *Escherichia coli*. Cells or extracts of recombinant *E. coli* efficiently reduced the diketoester **1** to the corresponding *syn*-(3*R*,5*S*)-dihydroxy ester **2a**, a key intermediate required for the synthesis of **6**, a hydroxyl methyl glutaryl (HMG)-CoA reductase inhibitor and other statin-type cholesterol-lowering drugs such as Lipitor (atorvastatin) and Crestor (rosuvastatin). Several other enzymatic approaches to this key intermediate have been demonstrated [11,12].

2. Materials and methods

2.1. Chemicals and general methods

Chemicals were purchased from VWR and/or Aldrich.

2.2. Microorganisms

Acinetobacter sp. SC 13874 was grown in F7 medium, which contained 10 g malt extract, 10 g yeast extract, 1 g peptone and 20 g dextrose per litre of water, adjusted to pH 7 and autoclaved at 121 °C for 20 min. *Acinetobacter* sp. SC 13874 is maintained in the culture collection of the Bristol-Myers Squibb Pharmaceutical Research Institute as frozen vials stored at −70 °C. Vials (containing 1 mL culture) were prepared from cells grown on F7 medium.

2.3. Growth of *A. calcoaceticus* SC 13874

Acinetobacter sp. SC 13874 culture from a frozen vial (1 mL) was inoculated into 100 mL of F7 medium in a 500-mL flask and grown on a shaker at 28 °C and 200 rpm for 48 h. The entire stage I culture was transferred to a 4-L flask containing 1 L of F7 medium. The second stage was grown for 24 h under the same conditions. The second stage culture was used as an inoculum for growth of cultures in a 20-L fermentor containing 15-L of F7 medium. The culture was grown at 28 °C and 300 rpm with 1 vvm aeration for 24 h. After 24 h growth, the cells were harvested by centrifugation. The harvested cells were washed with 15 L of 0.1 M potassium phosphate buffer (pH 7) and centrifuged again to collect the cell paste (~450 g). The cells were stored at −70 °C until further use.

* Corresponding author. Tel.: +1 732 227 6213; fax: +1 732 227 3994.
E-mail address: patelr@bms.com (R.N. Patel).

0141-0229/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2008.07.005

2.4. Analytical methods

Analytical HPLC methods were performed with various gradients of solvent A (0.05% TFA in water:methanol 80:20) and solvent B (0.05% TFA in acetonitrile:methanol 80:20) at ambient temperature with UV detection at 220 nm except where otherwise indicated. HPLC method 1 was performed with a Phenomenex Luna Phenyl-Hexyl column (5 μm, 150 mm × 4.6 mm) with a flow rate of 1 mL/min. Chiral methods 2-3 were performed with a chiral column as indicated with a flow rate of 0.5 mL/min. Some modification of these HPLC methods was also used during the study for better separation or faster analysis.

Method-1 was used for achiral analysis of the ethyl esters with a gradient from 0% to 100% solvent B over 15 min, and the retention times were 9.1 min for *anti*-**3**, 9.3 min for *syn*-**2**, 10.1 min for 5-OH-**4**, 9.8 min for 3-OH-**5** and 11.9 min (broad) for diketoester-**1**.

Method-2 was used for chiral analysis of the ethyl esters and was performed on a Chiralcel OD-RH column (150 mm × 4.6 mm) using an isocratic composition of A:B 90:10 for 60 min. The retention times were 17.1 min for *anti*-3*R*,5*R*-**3a**, 21.7 min for *anti*-3*S*,5*S*-**3b**, 24.6 min for *syn*-3*S*,5*R*-**2b**, 32.3 min for *syn*-3*R*,5*S*-**2a**, 36.4 min for 3*R*-OH-**5a**, 38.3 min for 3*S*-OH-**5b**, 41.2 min for 5*R*-OH-**4b** and 43.5 min for 5*S*-OH-**4a**. Diketoester **1** gave a broad peak beginning at 58 min.

Method-3 was used for chiral analysis of the ethyl monohydroxy esters and was performed on a Chiralpak AD-RH column (150 mm × 4.6 mm) with a gradient of 50% to 70% B over 30 min. The retention times of the ethyl monohydroxy esters were 8.3 min for 5*S*-OH-**4a**, 10.0 min for 5*R*-OH-**4b**, 12.1 min for 3*R*-OH-**5a** and 13.5 min for 3*S*-OH-**5b**.

2.5. Synthesis of various compounds

Ethyl 3,5-diketo-6-benzyloxyhexanoate (**1**), racemic ethyl *syn*-3,5-dihydroxy-6-benzyloxyhexanoate (**2**), racemic ethyl *anti*-3,5-dihydroxy-6-benzyloxyhexanoate (**3**), racemic ethyl 3-keto-5-hydroxy-6-benzyloxyhexanoate (**4**) and ethyl 3-hydroxy-5-keto-6-benzyloxyhexanoate (**5**) were synthesized as described previously [10]. Microbial reduction of ethyl diketoester **1** and isolation and purification of the dihydroxy products *syn*-(3*R*,5*S*)-**2a** and *anti*-(3*S*,5*S*)-**2b** was carried out as described earlier [10].

2.6. Reduction of ethyl 3,5-diketo-6-benzyloxyhexanoate (1) by Acinetobacter sp. SC 13874 and general procedure for microbial reduction in a small flask

Acinetobacter sp. SC 13874 cells (2 g wet weight) were suspended in 10 mL of 0.1 M potassium phosphate buffer (pH 7) in a 50-mL flask. Glucose (750 mg) was added. Diketoester **1** (20 μL) was added. The biotransformation was conducted by shaking at 200 rpm at 28 °C. After 20 h, a 1-mL sample was withdrawn from the biotransformation mixture. The sample was extracted with ethyl acetate (4 mL). Solvent was removed from the ethyl acetate extract, and the residue was dissolved in acetonitrile–methanol (1 mL, 1:1), filtered and analyzed by HPLC.

2.7. Preparation of cell-free extract

Cell extracts of *Acinetobacter* sp. SC 13874 (50 g) were prepared and ketoreductase I, ketoreductase II and ketoreductase III were purified as described previously [10].

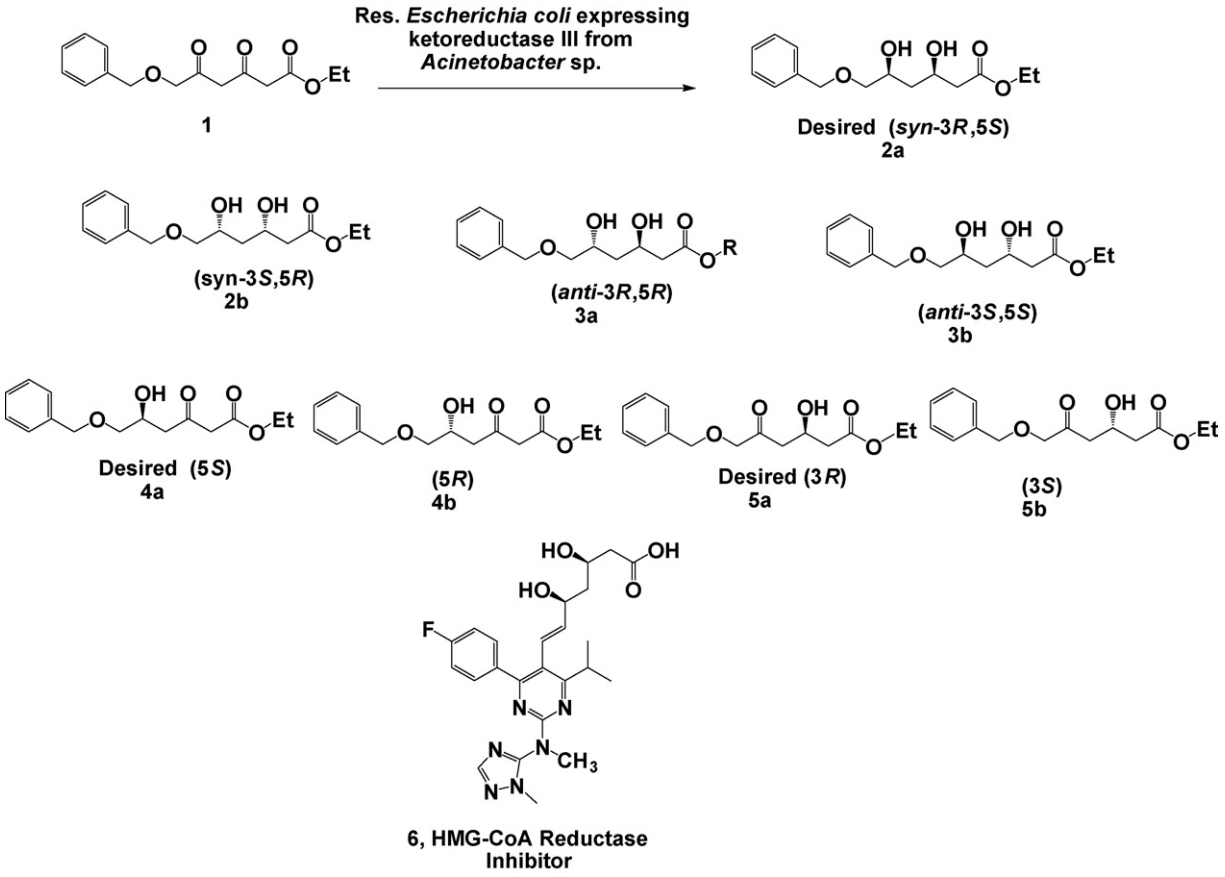
2.8. Enzyme assay

Enzyme assays were performed using a mixture containing 2.5 mM diketone **1** dissolved in ethanol, 0.5 mM NAD^+, 2 units of formate dehydrogenase, 200 mM sodium formate, 0.1–2 mg enzyme solution and 0.1 M potassium phosphate buffer (pH 6.0) in a final volume of 0.5 mL. All assay components were prepared freshly. The reaction was initiated by addition of the enzyme, and terminated by adding 0.5 ml ethanol to the reaction mixture after incubating at 28 °C and 200 rpm for 18 h. After vortexing and centrifugation in a microfuge for 5 min, the resulting supernatant was subjected to HPLC analysis. Over 18 h, under the conditions described, product formation was linear. Enzyme activity was expressed as product formation as μg/(h mg) protein.

2.9. Cloning and expression of ketoreductase III

2.9.1. N-terminal and internal sequence of ketoreductase III

The purified ketoreductase III was blotted on to a PVDF-membrane, stained with Coumassie blue and the protein band was excised from the membrane. The excised



Scheme 1. Reduction of diketoester.