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Enzymatic enantioselective reduction of α-ketoesters by a thermostable 7α-hydroxysteroid dehydrogenase from *Bacteroides fragilis*

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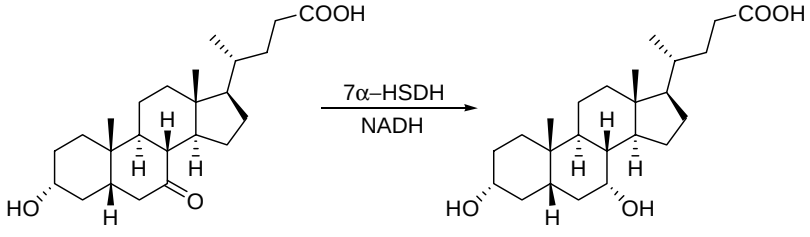
Abstract—A thermostable 7α-hydroxysteroid dehydrogenase (7-HSDH) from *Bacteroides fragilis* ATCC 25285 was cloned and over-expressed in *E. coli*, and its substrate specificity and stereoselectivity toward reduction of various ketones were examined. This alcohol dehydrogenase was active toward a series of aromatic and bulky aliphatic α-ketoesters. The substituents at the phenyl ring of aromatic α-ketoesters greatly affected the activity, but their effects on enantioselectivity were minimal. The synthetic application of this enzyme was then demonstrated through the preparation of a few α-hydroxy carboxylic acid esters of pharmaceutical interest.

1. Introduction

Optically pure α-hydroxy carboxylic acids and their derivatives are important intermediates in the synthesis of pharmaceuticals and other fine chemicals. Many approaches have been developed to obtain enantiomerically-enriched α-hydroxy carboxylic acid esters.[1] This includes (dynamic) kinetic resolution of racemic α-hydroxy esters,[2,3] hydrolysis of optically pure cyanohydrin, which in turn can be obtained via several asymmetric synthetic methods.[4–6] Another straightforward method to enantiomerically pure α-hydroxy carboxylic acid esters is the asymmetrical reduction of prochiral α-ketoesters that could be performed either chemically[7–10] or enzymatically.[1,11–13] Because of environmentally benign reaction conditions and unparallel selectivity, biocatalytic reduction has attracted more and more attention from both academia and industry. Recently, great efforts have been made to develop enzyme catalysts for the enantioselective reduction of ketones and varied levels of success have been achieved.[14–16] However, most research has been focused on the enantioselective reduction of aryl ketones and β-ketoesters.[17–20] Studies on enzymatic reduction of α-ketoesters have been only scarcely reported.[14,21,22] Especially biocatalytic reduction of aromatic α-ketoesters has been very limited and much less successful than that of their small counterparts.[23] Hydroxysteroid dehydrogenases normally reduce 3,7,12-oxo group of sterically bulky steroids in vivo.[24] These dehydrogenases belong to short-chain dehydrogenase family and their synthetic application has been largely unexplored.[25] Recently, a thermostable 7α-hydroxysteroid dehydrogenase from *Bacteroides fragilis* ATCC 25285 has been cloned,[26] and it reduces sterically demanding native substrate 7-keto-lithocholic acid to cheno-deoxycholic acid in gastrointestinal tract (Scheme 1).



Scheme 1.

Keywords: α-Ketoesters; Dehydrogenase; Enzyme.
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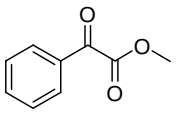
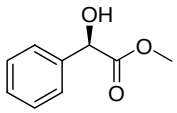
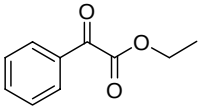
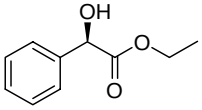
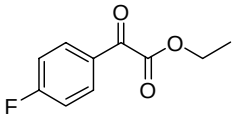
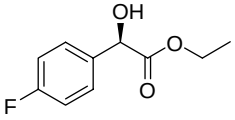
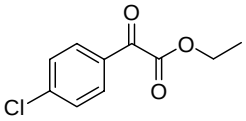
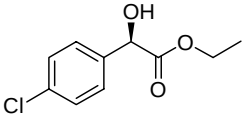
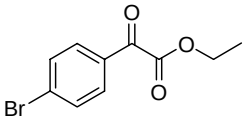
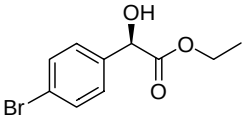
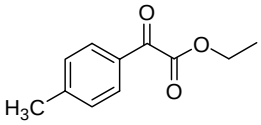
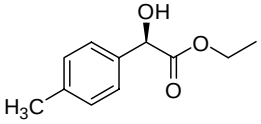
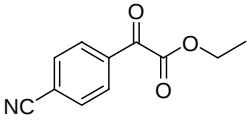
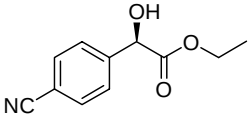
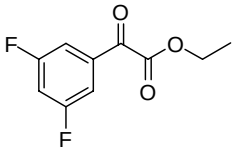
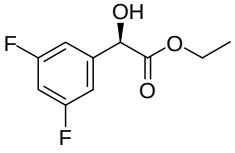
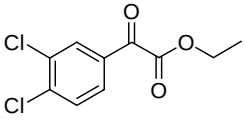
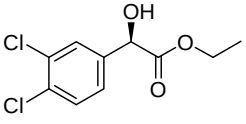
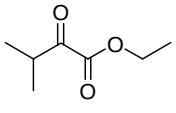
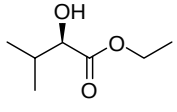
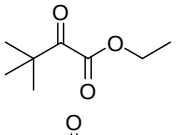
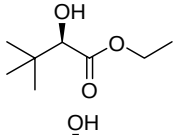
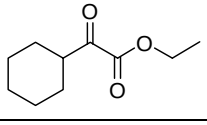
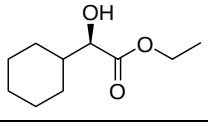
We reasoned that this enzyme might be useful in the reduction of bulky ketones such as aromatic α-ketoesters. Therefore, we have cloned and over-expressed this 7α-hydroxysteroid dehydrogease in *E. coli* and examined its substrate specificity and stereoselectivity toward reduction of various ketones including aromatic and aliphatic α-ketoesters. This NADH-dependent alcohol dehydrogenase was found to be active toward a series of

Table 1. The activity and enantioselectivity of 7α-hydroxysteroid dehydrogenase toward various α-ketoesters

Entry	α-Ketoester	Relative activity[a]	Product[b]	ee (%)[c]
1		100		98
2		354		>99
3		421		95
4		209		99
5		163		99
6		85		99
7		788		99
8		364		98
9		172		99
10		22		97
11		63		>99
12		823		>99

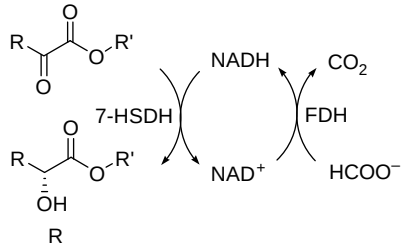
[a] The relative activity for methyl benzoylformate was defined as 100.
[b] The absolute configuration was determined by comparison with authentic standards, or the reported optical rotation.
[c] The ee value was determined by chiral HPLC or GC analysis.

aromatic α-ketoesters and aliphatic α-ketoester with bulky groups such as *tert*-butyl and cyclohexyl groups. The substituents at the phenyl ring of aromatic α-ketoesters greatly affected the enzyme activity, but exerted less effect on enantioselectivity of the reduction. This enzyme was then applied to the synthesis of a few pharmaceutical important α-hydroxy carboxylic acid esters.

2. Results and discussion

The 7α-hydroxysteroid dehydrogenase gene from *B. fragilis* ATCC 25285 was cloned and expressed in *E. coli* and the recombinant enzyme was purified sequentially by heat treatment, PEI treatment and fractional ammonium sulfate precipitation of cell-free extract (see Section 4). The obtained 7α-hydroxysteroid dehydrogenase was assayed for activity toward various ketones by spectrophotometrically measuring the oxidation of NADH at 340 nm at room temperature. The results are presented in Table 1. Surprisingly, while this dehydrogenase showed almost no activity toward a series of acetophenone derivatives and β-ketoesters (data not shown), it was very active for the reduction of aromatic and bulky aliphatic α-ketoesters using NADH as co-factor. This suggested that a carboxylic group adjacent to the carbonyl group might be necessary for being the substrate of 7α-hydroxysteroid dehydrogenase from *B. fragilis* ATCC 25285. From Table 1 it can be seen that the substituent at *para*-position of phenyl ring of aromatic α-ketoesters greatly affected the activity. For example, the fluoro- and cyano-group at *para*-position showed higher activity than ethyl benzoylformate, while chloro-, bromo- and methyl-substituents decreased the activity. The ester group also exerted some effects on the enzyme activity with ethyl ester being more active than methyl counterpart. Among the tested aliphatic α-ketoesters, ethyl 2-cyclohexyl-2-oxoacetate showed highest activity. Interestingly, this alcohol dehydrogenase was more active for the reduction of ethyl 3,3-dimethyl-2-oxo-butyrate than that of less bulky ethyl 3-methyl-2-oxo-butyrate, and showed almost no activity for the reduction of ethyl pyruvate (data not shown). Thus 7α-hydroxysteroid dehydrogenase from *B. fragilis* ATCC 25285 took more sterically demanding carbonyl compounds as substrates. This is probably due to the spacious active site cavity for its native substrate 7-ketolithocholic acid. The steric crowdness of the bulky non-native substrates might fit better to the enzyme's active site for the hydride transfer from NADH to the carbonyl group of substrates, while the less bulky substrates failed to accomplish this hydride transfer.

The enantioselectivity for reduction of various aromatic and aliphatic α-ketoesters catalyzed by 7α-hydroxysteroid dehydrogenase from *B. fragilis* was evaluated using NADH as co-factor, which was regenerated with a recycling system comprising formate dehydrogenase and sodium formate (Scheme 2). The results are summarized in Table 1. From the results it can be seen that both aromatic and aliphatic α-ketoesters were reduced to the (*R*)-enantiomer of the corresponding α-hydroxy carboxylic acid esters in high enantioselectivity with up to >99% ee. The substituent on phenyl ring of aromatic α-ketoesters had minimal effect on the enzyme enantioselectivity.



Scheme 2. Reduction of α-ketoesters catalyzed by 7α-hydroxysteroid dehydrogenase from *B. fragilis*.

Many of the α-hydroxy carboxylic acid esters listed in Table 1 are important intermediates in the synthesis of many pharmaceuticals. For example, optically active 2-hydroxy-3-methylbutyrate is an important chiral synthon in the preparation of a potent, selective and cell-penetrable inhibitor of caspase 3.[27] (*R*)-2-Hydroxy-3,3-dimethylbutyrate is a key component P3 of thrombin inhibitor identified by Merck.[28] Optically pure 3,5-difluoromandelate has recently been used to synthesize amino alcohol dipeptides designed to inhibit β-amyloid peptide (Aβ) formation, which is related to Azheimer's desease.[29,30] The results in Table 1 showed that 7α-hydroxysteroid dehydrogenase from *B. fragilis* ATCC 25285 had synthetic potential for the preparation of these α-hydroxyesters in optically pure form. Therefore, the reductions of ethyl 3-methyl-2-oxobutyrate, ethyl 3,3-dimethyl-2-oxobutyrate and ethyl (3,5-diflurophenyl)-glyoxylate were performed in 1 mmol scale. Ethyl (*R*)-2-hydroxy-3-methylbutyrate, ethyl (*R*)-2-hydroxy-3,3-dimethylbutyrate and ethyl (*R*)-2-hydroxy-2-(3,5-diflurophenyl)acetate were indeed obtained in isolated yields of 85–94%. The enantiomeric purities of the product α-hydroxyesters were from 97 up to >99%.

3. Conclusion

A thermostable recombinant 7α-hydroxysteroid dehydrogenase from *B. fragilis* ATCC 25285 was produced by overexpression of the 7-HSDH gene in *E. coli*. This alcohol dehydrogenase catalyzed the reduction of aromatic and sterically demanding aliphatic α-ketoesters to the corresponding α-hydroxyesters in essentially optically pure form. The synthetic application of 7α-hydroxysteroid dehydrogenase was then demonstrated by the preparation of ethyl (*R*)-2-hydroxy-3-methylbutyrate, ethyl (*R*)-2-hydroxy-3,3-dimethylbutyrate and ethyl (*R*)-2-hydroxy-2-(3,5-diflurophenyl)acetate.

4. Experimental

The chiral HPLC analysis was performed on an Agilent 1100 series high-performance liquid chromatography system with (*S*,*S*)-Whelk-O 1 column (25 cm×4.6 mm, Regis Technologies Inc.). The chiral GC analysis was performed on a Hewlett Packard 5890 series II plus gas chromatograph equipped with autosampler, EPC, split/splitless injector, FID detector and CP-Chirasil-Dex CB chiral capillary column (25 m×0.25 mm) (Table 2). The 7α-hydroxysteroid dehydrogenase activities toward the reduction of α-ketoesters (Table 1) were assayed using

Table 2. Details of chiral HPLC and GC analysis

α-Hydroxyester	Method[a]	Retention time (min)	
		t_R	t_S
Methyl 2-hydroxy-2-phenylacetate	A	10.2	11.0
Ethyl 2-hydroxy-2-phenylacetate	A	9.8	10.7
Ethyl 2-hydroxy-2-(4-fluorophenyl)acetate	A	8.4	8.9
Ethyl 2-hydroxy-2-(4-chlorophenyl)acetate	A	8.9	9.5
Ethyl 2-hydroxy-2-(4-bromophenyl)acetate	A	9.4	10.0
Ethyl 2-hydroxy-2-(4-methylphenyl)acetate	A	11.6	12.3
Ethyl 2-hydroxy-2-(4-cyanophenyl)acetate	A	21.6	22.6
Ethyl 2-hydroxy-2-(3,5-diflurophenyl)acetate	B	9.2	9.7
Ethyl 2-hydroxy-2-(3,4-dichlorophenyl)acetate	A	8.8	9.6
Ethyl 2-hydroxy-3-methylbutyrate	C	22.0	22.3
Ethyl 2-hydroxy-3,3-dimethylbutyrate	D	11.2	11.8
Ethyl 2-hydroxy-2-cyclohexylacetate	E	15.6	15.9

[a] (A) HPLC, flow rate 1.0 ml/min, hexane/isopropanol (0.1% HOAc)=95:5; (B) HPLC, flow rate 1.0 ml/min, hexane/isopropanol (0.1% HOAc)=99:1; (C) GC, 60 °C for 2 min, 1 °C/min to 90 °C, 90 °C for 5 min; (D) GC, 80 °C for 2 min, 1 °C/min to 110 °C, 110 °C for 5 min; (E) GC, 90 °C for 2 min, 1 °C/min to 120 °C, 120 °C for 5 min, α-hydroxyl group was acylated as trifluoroacetate.

SpectraMax M2 microplate reader (Molecular Devices). Methyl phenylglyoxylate, ethyl phenylglyoxylate, ethyl (4-cyanophenyl)glyoxylate, ethyl (3,4-dichlorophenyl)glyoxylate, ethyl (3,5-difluorophenyl)glyoxylate, and ethyl 3-methyl-2-oxobutyrate were purchased from Aldrich or Acros. All the other α-ketoesters were prepared by Friedel–Crafts acylation of substituted benzene with ethyl oxalyl chloride in the presence of anhydrous $AlCl_3$,[31] or reaction of diethyl oxalate with the corresponding Grignard reagents.[10] The racemic α-hydroxyester standards were prepared by reduction of α-ketoesters with sodium borohydride. Methyl and ethyl (*S*)-mandelate were purchased from Aldrich. (*R*) or (*S*) enantiomers of other α-hydroxyesters were prepared by following the literature methods.[28,32,33]

4.1. Gene expression and purification of 7α-hydroxysteroid dehydrogenase

Plasmid pBPC-1 (from James P. Coleman) containing 7-HSDH gene from *B. fragilis* ATCC 25285 was used as template for PCR amplification. The PCR fragment was cloned into pTXB1 expression vector at the *Nde* I and *Bam*H I sites to give JS2.2 and the cloned 7-HSDH gene was confirmed by DNA sequencing. The plasmid JS2.2 was transformed into Rosetta2(DE3)pLysS for expression. Overnight culture (20 ml) was diluted into 1 l of LB media containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol and propagated until OD_{595} reached 0.6–1.0 at 37 °C. The cells were then induced with 0.1 mM of IPTG and continuing grown at 30 °C for 5 h. The cells were harvested and lysed in 10 mM of potassium phosphate (pH 7.0) by homogenizer. The cell-free extract was heat-treated in a water-bath for 30 min at 55–60 °C and centrifuged at 20,000*g* for 30 min. The heat-treated lysate was then mixed with equal volume of PEI solution (0.25% polyethyleneimine MW 40–60 K, 6% NaCl, 100 mM Borax, pH 7.4) to remove lipids.[34] The PEI-treated supernatant was precipitated with 45% ammonium sulfate. The resulting precipitate was collected after centrifugation and dissolved in potassium phosphate buffer (10 mM, pH 7.0). The lysate was dialysed by gel filtration into potassium phosphate buffer (10 mM, pH 7.0), and then lyophilized as powder.

4.2. Activity assay of 7α-hydroxysteroid dehydrogenase

The activity of 7α-hydroxysteroid dehydrogenase toward the reduction of α-ketoesters (Table 1) was determined by spectrophotometrically measuring the oxidation of NADH at 340 nm ($\varepsilon=6.22\ mM^{-1}\ cm^{-1}$) in the presence of excess α-ketoesters. The activity was measured at room temperature in 96-well plate, in which each well contained α-ketoester (6.25 mM), NADH (0.25 mM) in potassium phosphate buffer (100 mM, pH 7.0, 190 μl). The reaction was initiated by the addition of 7α-hydroxysteroid dehydrogenase (10 μl solution containing 18 μg of enzyme). The specific activity was defined as the number of micromolar of NADH converted in 1 min by 1 mg of enzyme ($\mu mol\ min^{-1}\ mg^{-1}$). The specific activity for methyl benzoylformate was 0.32 $\mu mol\ min^{-1}\ mg^{-1}$ and its relative activity was defined as 100.

4.3. Enantioselectivity of reduction of α-ketoesters catalyzed by 7α-hydroxysteroid dehydrogenase

The enantioselectivity of the enzymatic reduction of α-ketoesters was studied using an NADH recycle system. The general procedure was as follows: sodium formate (3.4 mg), formate dehydrogenase (0.4 mg), NADH (0.4 mg), 7α-hydroxysteroid dehydrogenase (0.2 mg) and α-ketoester solution in DMSO (50 μl, 0.25 M) were mixed in a potassium phosphate buffer (1 ml, 100 mM, pH 7.0) and the mixture was shaken overnight at room temperature. The mixture was extracted with methyl *tert*-butyl ether (1 ml). The organic extract was dried over anhydrous sodium sulfate and was subjected to chiral HPLC or GC analysis to determine the enantiomeric excess. The absolute configuration of product α-hydroxyesters was identified by comparing the chiral HPLC or GC data with the standard samples, or by comparing the optical rotation of the product alcohols with the literature data.

4.4. Preparation of ethyl (*R*)-2-hydroxy-3-methylbutyrate, ethyl (*R*)-2-hydroxy-3,3-dimethylbutyrate, and ethyl (*R*)-2-hydroxy-2-(3,5-difluorophenyl)acetate

Sodium formate (4 mmol), formate dehydrogenase (40 mg), NADH (10 mg), 7α-hydroxysteroid dehydrogenase (10 mg) and ethyl 3-dimethyl-2-oxo-butyrate (1 mmol) were mixed in a potassium phosphate buffer (50 ml, 100 mM, pH 7.0) and the mixture was stirred at room temperature. After 24 h, GC analysis indicated that reduction was complete. The reaction mixture was extracted with ethyl ether (30 ml×2). The organic extract was dried over anhydrous Na_2SO_4 and removal of the solvent gave ethyl (*R*)-2-hydroxy-3-methylbutyrate as clear oil (124 mg, 85% yield). ^1H and ^{13}C NMR ($CDCl_3$) were in accordance with literature data.[35] $[\alpha]_D^{22}$ −9.4 (*c* 1.0, $CHCl_3$); lit.[35] $[\alpha]_D^{25}$ −10.5 (*c* 0.5, $CHCl_3$).

Similar procedures were followed for the preparation of other two α-hydroxyesters. Ethyl (*R*)-2-hydroxy-3,3-dimethylbutyrate (145 mg, 91% yield). ^1H and ^{13}C NMR ($CDCl_3$) were in accordance with literature data.[28] $[\alpha]_D^{22}$

−31.3 (*c* 1.0, $CHCl_3$); lit.[36] $[\alpha]_D^{22}$ +27.7 (*c* 3.4, $CHCl_3$) for (*S*)-enantiomer. Ethyl (*R*)-2-hydroxy-2-(3,5-difluorophenyl)acetate (203 mg, 94% yield). ^1H NMR (400 MHz, $CDCl_3$) δ ppm 1.27 (t, 3H, J=7.2 Hz), 3.58 (s, 1H), 4.21–4.34 (m, 2H), 5.15 (s, 1H), 6.78 (m, 1H), 7.03 (m, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ ppm 14.4, 63.2, 72.2, 104.1 (t, $^2J_{C-F}$=25 Hz), 109.8 (d, $^2J_{C-F}$=19 Hz), 109.9 (d, $^2J_{C-F}$=19 Hz), 142.5, 163.3 (d, $^1J_{C-F}$=247 Hz), 163.4 (d, $^1J_{C-F}$=247 Hz), 173.0. $[\alpha]_D^{22}$ −81.2 (*c* 1.0, $CHCl_3$).

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