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Tetrahedron
Tetrahedron 62 (2006) 901–905

A recombinant ketoreductase tool-box. Assessing the substrate selectivity and stereoselectivity toward the reduction of β-ketoesters

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Received 22 August 2005; revised 12 October 2005; accepted 14 October 2005

Available online 7 November 2005

Abstract—The substrate selectivity and stereoselectivity of a series of ketoreductases were evaluated toward the reduction of two sets of β-ketoesters. Both the structural variety at β-position and the substituent at α-position greatly affected the activity and stereoselectivity of these ketoreductases. For the first set of β-ketoesters, at least one ketoreductase was found that catalyzed the formation of either (D) or (L) enantiomer of β-hydroxyesters from each substrate with high optical purity, with the only exception of ethyl (D)-3-hydroxy-3-phenylpropionate. For the second set of β-ketoesters with α-substituents, the situation is more complex. More commonly, a ketoreductase was found that formed one of the four diastereomers in optically pure form, with only a few cases in which enzymes could be found that formed two or more of the diastereomers in high optical purity. The continued development of new, more diverse ketoreductases will create the capability to produce a wider range of single diastereomers of 2-substituted-3-hydroxy acids and their derivatives.

1. Introduction

With the advantages of environmentally benign reaction conditions, broad reaction scope, and high stereo- and regioselectivity, biocatalytic reductions of prochiral ketones offer significant potential in the synthesis of optically pure alcohols.[1] A biocatalytic reduction can be carried out using either whole cell systems[2] or isolated ketoreductases.[3] Since a whole cell may contain more than one ketoreductases, frequently with opposing stereoselectivities, not all whole-cell-mediated ketone reductions provide product chiral alcohols in high optical purity.[4] A straightforward approach to solve this problem is to carry out the reduction with an isolated ketoreductase in an in vitro reaction system. However, until recently the application of isolated keto-reductases to ketone reduction has been hampered by their limited availability.[3] In this context, we have developed an 'easy-to-use' ketoreductase tool-box consisting of more than 30 recombinant ketoreductases **KRED101–131** by genome mining and protein engineering, and have shown that these isolated recombinant ketoreductase enzymes efficiently catalyze the enantioselective reduction of a variety of substituted aryl ketones to optically pure aryl alcohols.[5]

Since optically pure β-hydroxy carboxylic acids and their derivatives are key building blocks in the synthesis of bioactive compounds,[6] many efforts have been made to develop effective methods for their synthesis in enantiomerically pure form.[7] Enzymatic reduction of β-ketoesters catalyzed by ketoreductases represents an attractive approach to enantiomerically pure β-hydroxy carboxylic acids and their derivatives. The β-ketoester starting materials are readily available in many cases, and the environmentally friendly reaction conditions are easily introduced in an industrial setting and can be scaled to commercial volumes.[3a,8] In this study, we report on the substrate selectivity and stereoselectivity of a recombinant ketoreductase tool-box by evaluating the reduction of a series of diverse β-ketoesters. These studies represent a systematic look at enzyme-catalyzed stereoselective ketone reduction, which will serve as a useful guideline for developing enzymatic processes for the production of optically pure β-hydroxy carboxylic acid esters and also guide the needs for future development of new ketoreductase enzymes.

2. Results and discussion

Two sets of β-ketoesters have been chosen as substrates to evaluate the activity and stereoselectivity of the

Keywords: Ketoreductase; Stereoselectivity; β-Ketoesters.
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0040–4020/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2005.10.044

ketoreductase tool-box, which comprises 31 recombinant ketoreductase enzymes **KRED101–131**. The first set of β-ketoesters **1–8** have diverse structure at the β-position, while the second ones **9–15** possess different substituents at the α-position as shown in Figure 1. The activity and stereoselectivity of the ketoreductases toward these two sets of β-ketoesters will be presented separately.

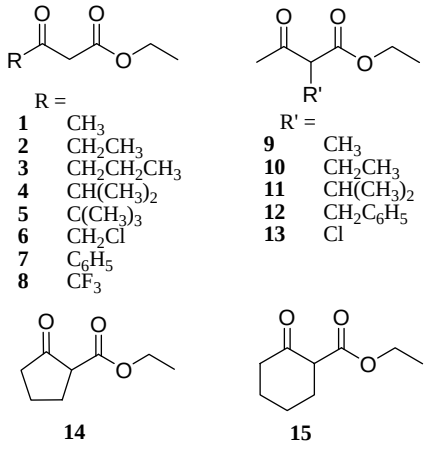
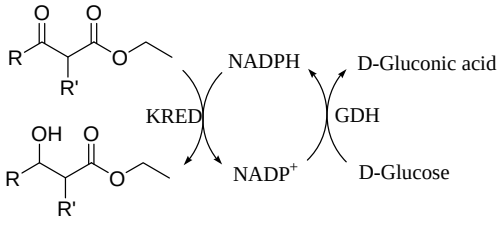


Figure 1. β-Ketoesters **1–15**.

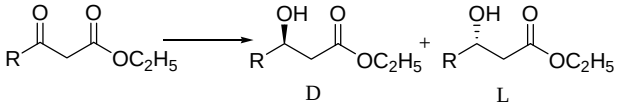


Scheme 1. Reduction of β-ketoesters catalyzed by ketoreductases with NADPH recycle system.

The activities of the ketoreductases toward the reduction of β-ketoesters **1–8** were determined by spectrophotometrically measuring the oxidation of NADPH at 340 nm at room temperature. The relative activity of **KRED101** in the reduction of ethyl 3-oxobutyrate **1** was defined as 100. The enantioselectivity of the ketoreductase-catalyzed reduction of β-ketoesters **1–8** were studied using an NADPH recycle system as shown in Scheme 1.

The selected data of the relative activity and enantioselectivity for the reduction of β-ketoesters **1–8** are presented together in Table 1. From Table 1 it can be seen that most of the ketoreductases effectively catalyzed the reduction of ethyl 3-oxobutyrate **1**. When the chain length of the β-ketoesters increases or become branched (from **1**→**2**→**3**→**4**→**5**), three major trends were observed for the ketoreductase activities. For example, the activity of **KRED108** followed a descending order from **1**→**2**→**3**→**4**→**5** as shown in Figure 2. Several other ketoreductases, **KRED102**,**103**,**106**, and **KRED107**, followed the same decreasing order. This may be due to the alkyl group at β-position becoming more bulky from **1** to **5**. An interesting trend was observed for **KRED112** and **KRED118**, which showed an overall decrease in activity from **1**→**2**→**3**→**4**→**5**, but with an unexpected increase in activity for substrate **3** (**KRED112** is shown in Fig. 2 as an example). **KRED114**,**121**,**123**, and **KRED130** did not show significant activity change across the series of β-ketoesters **1–5** (**KRED114** is shown in Fig. 2 as an example). Interestingly, **KRED101** showed the highest activity toward the reduction of ethyl 4,4-dimethyl-3-oxo-pentanoate **5**. For the reduction of ethyl 4-chloro-3-oxo-butyrate **6** and ethyl 4,4,4-trifluoro-3-oxo-butyrate **8**, most of the ketoreductases were efficient catalyst, while only a few ketoreductases were effective in the reduction of ethyl benzoylacetate **7**.

Table 1. The relative activity and enantioselectivity of the ketoreductases toward the reduction of β-ketoesters **1–8**



KRED	**1** (CH_3)	**2** (CH_2CH_3)	**3** ($CH_2CH_2CH_3$)	**4** ($CH(CH_3)_2$)	**5** ($C(CH_3)_3$)	**6** (CH_2Cl)	**7** (C_6H_5)	**8** (CF_3)
101	37[a] (100)[b]	−65 (154)	75 (120)	−99 (170)	−>99 (277)	−>99 (394)	−61 (94)	−88 (71)
102	>99 (51)	>99 (16)	—[c]	—	—	99 (226)	—	87 (9)
103	>99 (97)	>99 (13)	—	—	—	99 (311)	—	96 (9)
106	>99 (67)	>99 (24)	—	—	—	97 (353)	—	98 (4)
107	−>99 (60)	−>99 (21)	−>99 (7)	−>99 (3)	−>99 (1)	−>99 (271)	−54 (3)	−97 (6)
108	>99 (354)	>99 (114)	95 (34)	—	—	98 (406)	66 (29)	83 (77)
110	>99 (13)	—	—	—	—	−8 (14)	99 (10)	—
112	84 (361)	23 (210)	96 (355)	−89 (127)	−>99 (29)	−9 (516)	−68 (93)	−75 (237)
113	89 (318)	52 (60)	97 (296)	−87 (79)	−99 (29)	−89 (24)	−67 (60)	−65 (203)
114	37 (43)	−56 (51)	−6 (61)	−73 (45)	−99 (54)	−63 (263)	73 (174)	−46 (27)
118	>99 (630)	>99 (516)	>99 (617)	>99 (369)	99 (13)	97 (396)	−77 (136)	91 (224)
121	−23 (30)	−90 (47)	−72 (46)	−>99 (20)	−>99 (71)	−82 (164)	80 (189)	−91 (49)
123	−22 (17)	−78 (27)	−62 (51)	−97 (41)	−>99 (17)	−93 (174)	—	−89 (29)
128	96 (200)	98 (223)	>99 (149)	65 (16)	—	79 (137)	—	64 (63)
130	70 (20)	0 (14)	−88 (20)	−99 (16)	−>99 (7)	−8 (294)	—	−90 (81)
131	−52 (16)	−91 (34)	−98 (41)	−>99 (16)	—	−94 (404)	—	35 (337)

[a] ee%, the positive ee value indicates that L-enantiomer is the major product, while the negative ee value indicates that D-configuration is the major enantiomer.
[b] The initial reaction rate was measured by the procedure described in the Section 4, the relative activity of **KRED101** in the reduction of ethyl 3-oxobutyrate **1** was defined as 100 and its specific activity was 88 nmol min^{-1} mg^{-1}.
[c] The conversion of the reduction was less than 20% after 24 h, the ee value and relative activity was thus not reported.

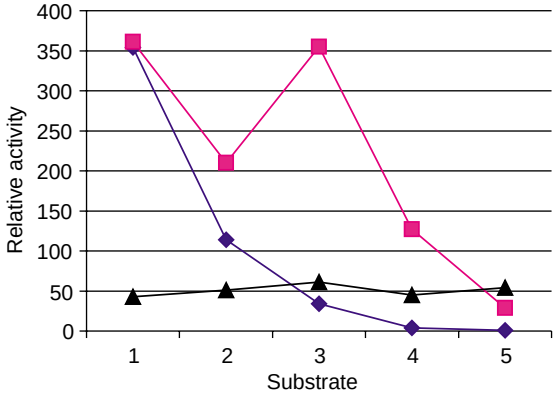


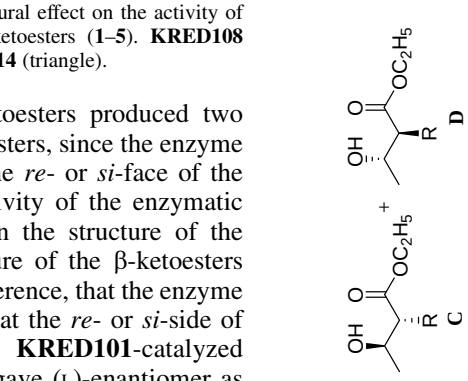
Figure 2. Three major patterns for the structural effect on the activity of ketoreductases toward the reduction of β-ketoesters (**1**–**5**). **KRED108** (diamond), **KRED112** (square), and **KRED114** (triangle).

Reduction of the first set of β-ketoesters produced two enantiomers (L and D) of β-hydroxyesters, since the enzyme delivered the hydride either from the *re*- or *si*-face of the prochiral ketone. The enantioselectivity of the enzymatic reduction was clearly dependent on the structure of the β-ketoesters. In some cases, structure of the β-ketoesters also affected the stereochemical preference, that the enzyme delivered the hydride preferentially at the *re*- or *si*-side of the carbonyl group. For example, **KRED101**-catalyzed reductions of β-ketoesters **1** and **3** gave (L)-enantiomer as the major product, resulting from the hydrogen transfer occurring at the *re*-face of the carbonyl group; while (D)-enantiomer was obtained as the major product in the **KRED101**-catalyzed reduction of β-ketoesters **2**, **4**, and **5**, indicating that the hydrogen transfer occurred at the *si*-face of the carbonyl group. Similar structure-induced inversion of configuration was observed for **KRED112**,**113**,**114**, and **KRED130**. From the results, it can be found that both enantiomers of β-hydroxyesters were obtained in high optical purity by one or more ketoreductases for each of the tested β-ketoesters, the only exception was ethyl (D)-3-hydroxy-3-phenylpropionate. The breadth of scope offered by ketoreductases demonstrates that such a group of enzymes can serve as a useful tool-box for the synthesis of optically pure β-hydroxyesters.

The selected data for the relative activity and stereoselectivity of the ketoreductase-catalyzed reduction of α-substituted-β-ketoesters **9**–**15** are presented in Table 2. When the size of the alkyl group at the α-position of ethyl 3-oxo-butyrate increased (from **9**→**10**→**11**), the activity of most ketoreductases showed a decreasing trend. The benzyl group at the α-position **12** had an unexpectedly diverse effect on the enzyme activity when compared with the introduction of an α-methyl group **9**. Most of the ketoreductases showed high activity toward the reduction of ethyl 2-chloro-3-oxo-butyrate **13**. Both ethyl 2-oxo-cyclopentanecarbonate **14** and ethyl 2-oxo-cyclohexane-carbonate **15** were good substrates for most of the tested ketoreductases, although the activities toward these two substrates were significantly varied.

The reduction of the second set of β-ketoesters generated two chiral carbon atoms, with the possible formation of four diastereomers (Table 2). In many cases, a single

Table 2. Stereoselectivity of ketoreductase-catalyzed reduction of ethyl 2-substituted-3-oxo-butyrate



KRED	**9** (CH_3)	**10** (CH_2CH_3)	**11** ($CH(CH_3)_2$)	**12** (CH_2Ph)	**13** (Cl)	**14**	**15**
102	100/0/0/0[a] (116)[b]	100/0/0/0 (81)	3.3/0/96.7/0 (13)	95.6/0/4.4/0 (37)	100/0/0/0 (191)	97.7/2.3/0/0 (76)	100/0/0/0 (133)
103	100/0/0/0 (130)	95.5/0/4.5/0 (19)	3.4/0/96.6/0 (10)	95.3/0/4.7/0 (4)	100/0/0/0 (180)	98.0/1.1/0/0.9 (43)	100/0/0/0 (33)
106	100/0/0/0 (186)	100/0/0/0 (49)	4.5/0/95.5/0 (13)	94.2/0/5.8/0 (29)	100/0/0/0 (404)	99.0/1.0/0/0 (126)	100/0/0/0 (191)
107	3.0/0/92.1/4.9 (198)	0/18.5/57.8/23.7 (36)	28.6/12.0/59.4/0 (7)	80.6/0/19.4/0 (13)	—[c]	28.0/72/0/0 (96)	71.2/19.7/0/9.1 (153)
111	19.1/10.6/21.3/49.0 (64)	8.7/14.0/60.8/16.5 (41)	1.0/66.6/3.1/29.2 (23)	15.6/0/84.4/0 (67)	100/0/0/0 (301)	2.6/13.1/39.3/45.0 (44)	8.9/35.1/32.1/23.9 (451)
113	56.4/2.7/26.0/14.8 (393)	4.1/26.9/5.0/64.0 (121)	—	34.8/0/64.1/1.1 (281)	100/0/0/0 (443)	0/24.2/0/75.8 (167)	0.5/32.1/0.3/66.1 (397)
114	16.7/2.7/71.0/9.7 (183)	15.3/5.4/70.5/8.8 (194)	0.9/35.3/6.7/57.1 (23)	5.8/0/94.0/0.2 (206)	100/0/0/0 (257)	2.9/12.8/42.5/41.8 (53)	9.2/27.5/33.4/30.0 (633)
115	17.9/10.7/49.8/21.5 (94)	9.1/13.9/59.6/17.4 (66)	0.7/63.6/1.6/34.1 (33)	5.4/0/94.2/0.4 (124)	95.5/2.0/2.5/0 (327)	2.6/13.2/39.4/44.8 (77)	10.4/31.9/29.3/28.3 (580)
117	89.0/0/11.0/0 (41)	10.4/0/89.6/0 (43)	—	65.3/0/34.7/0 (6)	100/0/0/0 (464)	100/0/0/0 (207)	100/0/0/0 (36)
118	72.0/0/28.0/0 (653)	10.4/3.0/85.6/1.0 (601)	—	9.3/0/90.7/0 (114)	100/0/0/0 (487)	100/0/0/0 (354)	95.9/0/4.1/0 (324)
121	13.5/4.0/70.7/11.8 (104)	7.6/5.6/78.9/7.9 (99)	0/44.8/9.0/46.2 (18)	7.9/0/92.1/0 (140)	100/0/0/0 (1414)	1.1/19.4/10.6/68.9 (36)	3.8/36.2/7.7/52.3 (557)
129	—	—	—	—	100/0/0/0 (27)	—	5.0/95.0/0/0 (54)
131	49.5/39.3/11.1/0 (16)	73.9/2.3/23.8/0 (13)	—	4.0/0/96.0/0 (21)	100/0/0/0 (129)	19.8/80.2/0/0 (40)	42.6/57.4/0/0 (574)

[a] The data were presented as the ratio of diastereomers (**A**/**B**/**C**/**D**).
[b] The initial reaction rate was measured by the procedure described in the Section 4, the relative activity of **KRED101** in the reduction of ethyl 3-oxobutyrate **1** was defined as 100 and its specific activity was 88 nmol min^{-1} mg^{-1}.
[c] The conversion was less than 20% after 20 h, so the ratio and relative activity are not reported.

diastereomer was obtained with very high diastereoselectivity. Mechanistically, the high diastereoselectivity results from the acidity of the α-proton in the β-ketoester, allowing the epimerization of the α-carbon of the β-ketoester starting material, but not the β-hydroxyester product, under the reaction conditions. Especially for ethyl 2-chloro-3-oxo-butyrate **13**, almost every ketoreductase showed high diastereoselectivity. In most cases, diastereomer **A** was obtained. A few exceptions were **KRED102**- and **KRED103**-catalyzed reduction of ethyl 2-isopropyl-3-oxo-butyrate **11**, in which diastereomer **C** was obtained with selectivity greater than 96%. Although the other diastereomers were also obtained as the major product in other cases, the diastereoselectivity was not as high, ranging from 40–95% in most cases. This indicates that while this current group of ketoreductases show high stereoselectivity and good diversity in terms of the substrate acceptance toward the reduction of 2-substituted-3-ketoesters, further efforts are required to develop ketoreductases with high and diverse diastereoselectivities, that is, for the production of diastereomers **B**, **C**, and **D**.

3. Conclusion

The substrate selectivity and stereoselectivity of a series of ketoreductases have been studied toward the reduction of two sets of β-ketoesters. Both the structural variety at β-position and the substituent at α-position greatly affect the activity and stereoselectivity of these reductions. For the synthetic purposes, the ability to prepare each stereoisomer of β-hydroxyester building blocks in homochiral form is important. For the first set of β-ketoesters, our ketoreductase collection has been demonstrated to be a useful tool-box for this goal. For the second set of β-ketoesters with α-substituent, one of the four diastereomers was produced in optically pure form, although diastereomer **A** was obtained in most cases. We are continuing efforts to develop new ketoreductases with differing diastereoselectivity in the reduction of α-substituted-β-ketoesters.

4. Experimental

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 25 m×0.25 mm CP-Chirasil-Dex CB chiral capillary column. The ketoreductase activities toward the reduction of β-ketoesters (Tables 1 and 2) were assayed using SpectraMax M2 microplate reader (Molecular Devices). All the ketoreductases were purified recombinant enzymes, which were developed by genome mining and protein engineering, and are commercially available from Bio-Catalytics, Inc. All the β-ketoesters were purchased from Aldrich and the β-hydroxyester standards were prepared by following the literature procedures.[9–18]

4.1. Activity assay of the ketoreductases

The activities of the ketoreductases toward the reduction of β-ketoesters (Tables 1 and 2) were determined by spectrophotometrically measuring the oxidation of NADPH at 340 nm ($\varepsilon=6.22\ mM^{-1}\ cm^{-1}$) in the presence of excess β-ketoesters. The activity was measured at room temperature in 96-well plate, in which each well contained β-ketoester (6.25 mM), NADPH (0.25 mM) in potassium phosphate buffer (100 mM, pH 7.0, 180 μl). The reaction was initiated by the addition of the ketoreductase (20 μl solution containing 2.5–100 μg of enzyme). The specific activity was defined as the number of nmol of NADPH converted in one minute by 1 mg of enzyme ($nmol\ min^{-1}\ mg^{-1}$).

4.2. Stereoselectivity of the enzymatic reduction of β-ketoesters

The stereoselectivity of the enzymatic reduction of β-ketoesters was studied using an NADPH recycle system. The general procedure was as follows: D-glucose (4 mg), D-glucose dehydrogenase (0.5 mg), NADPH (0.5 mg), ketoreductase (0.5 mg) and β-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 at 25 °C overnight. 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 GC analysis. The absolute configuration of ethyl 3-hydroxybutyrate, ethyl 3-hydroxypentanoate were determined by comparison of the chiral GC data with that in the literature.[19] The absolute configuration of other β-hydroxyesters were identified by comparing the chiral GC data with the standard samples. The racemate and enantiomers or diastereomers of the β-hydroxyesters were prepared by sodium borohydride reduction or following the literature methods: ethyl 3-hydroxyhexanoate,[9] ethyl 3-hydroxy-4-methylpentanoate,[10] ethyl 4-chloro-3-hydroxybutyrate,[9a,11] ethyl 3-hydroxy-3-phenylpropionate,[12] ethyl 3-hydroxy-4,4,4-trifluorobutyrate[13] ethyl 3-hydroxy-2-methylbutyrate,[9a,14] ethyl 2-ethyl-3-hydroxybutyrate,[9a,14a] ethyl 2-isopropyl-3-hydroxybutyrate,[14a,15] ethyl 2-benzyl-3-hydroxybutyrate,[16] ethyl 2-chloro-3-hydroxybutyrate,[17] ethyl 2-hydroxycyclopentanecarboxylate[18] and ethyl 2-hydroxycyclohexanecarboxylate.[18]

4.3. Preparative scale reduction of ethyl 4,4-dimethyl-3-oxo-pentanoate and absolute configuration determination of ethyl 4,4-dimethyl-3-hydroxy-pentanoate

D-Glucose (400 mg), D-glucose dehydrogenase (5 mg), NADPH (5 mg), **KRED101** (5 mg) and ethyl 4,4-dimethyl-3-oxo-pentanoate (200 mg) were mixed in a potassium phosphate buffer (50 ml, 100 mM, pH 7.0) and the mixture was stirred at room temperature with pH being controlled by 0.5 M sodium hydroxide solution. After 24 h, GC analysis indicated that reduction was complete. The reaction mixture was extracted with MTBE (30 ml×2). The organic extract was dried over anhydrous Na_2SO_4 and removal of the solvent gave product β-hydroxyester as clear oil (185 mg, 92% yield). ^1H and ^{13}C NMR ($CDCl_3$) are in accordance with literature data.[20] The ester was then hydrolyzed in H_2O with 1.5 equiv of NaOH solution to give (D)-4,4-dimethyl-3-hydroxy-pentanoic acid. ^1H and ^{13}C NMR ($CDCl_3$) are in accordance with literature data.[21] $[\alpha]_D^{22}$ −53.4 (*c* 1.0, $CHCl_3$); lit.[21] $[\alpha]_D^{25}$ 53.0 (*c* 1.0, $CHCl_3$) for L-enantiomer.

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