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

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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.
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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 ketoreductases 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

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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.

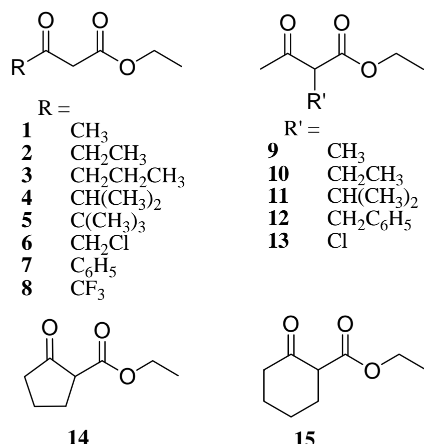
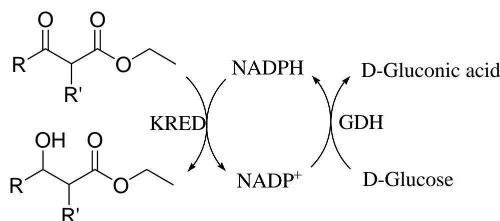


Figure 1. β-Ketoesters **1–15**.



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.

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