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Evaluation of substituent effects on activity and enantioselectivity in the enzymatic reduction of aryl ketones

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Abstract—The enzymatic reduction of a series of substituted aryl ketones catalyzed by 24 isolated recombinant ketoreductases was studied and the substituent effects on activity and enantioselectivity were evaluated. When comparing *p*- and *m*-substituted acetophenones, the substituent significantly affects the activity of some of the tested ketoreductases, while it has little effect on the activity of other ketoreductases. Most of the tested ketoreductases were highly enantioselective in the reduction of these aryl ketones. The electronic properties, steric factors, and the ability to form a hydrogen bond to the substituents at the *ortho*-position play a significant role in determining both the activity and enantioselectivity of the ketoreductase-catalyzed reductions. From an applicability point of view, both enantiomers of the product aryl alcohols could be prepared via reduction catalyzed by one or more of the ketoreductases in most cases.

1. Introduction

The four major catalytic procedures for the enantioselective reduction of ketones are (i) enantioselective hydride reduction, (ii) enantioselective hydrogenation, (iii) enantioselective transfer hydrogenation, and (iv) biocatalytic reduction. Among them, biocatalytic reduction of prochiral ketones shows great potential in terms of mild reaction conditions and unparalleled stereo- and regioselectivity. The biocatalytic reduction can be carried out using either whole cell systems or isolated ketoreductases.[1–3] Since more than one ketoreductases with opposite stereoselectivities may be present in the same cell, not all whole-cell-mediated ketone reductions provide product alcohols in high enantiomeric excess.[4] A straightforward approach to solve this problem is to carry out the reduction with an isolated ketoreductase in an in vitro system. However, the application of isolated ketoreductases to ketone reduction has been hampered by their limited availability. Recently, Stewart et al. have reported the overproduction of 18 key reductases from baker's yeast (*Saccharomyces cerevisiae*) in *E. coli* as GST-fusion proteins and evaluation of their activities and stereoselectivities toward the reduction of α- and β-ketoesters.[5] This has greatly expanded the scope of isolated ketoreductases and their application. To provide an 'easy-to-use' ketoreductase tool-box for organic synthesis, we have developed more than 20 new recombinant ketoreductases via genome mining and protein engineering,[6] and studied the activities and enantioselectivities of these isolated recombinant ketoreductase enzymes. Here we would like to report the results of the reduction of a series of substituted aryl ketones catalyzed by these ketoreductases (Scheme 1) and evaluate the subtituent effects on activity and enantioselectivity in the enzymatic reduction of these ketones. This would not only lead to further understanding of these ketoreductases and provide guidance for their application to the synthesis of optically pure alcohols, but also provide direction for the future development of new ketoreductase enzymes.

2. Results and discussion

If each aryl ketone were tested with each ketoreductase separately, the number of reactions that are needed to be carried out would be 360. Hence we put the substrates into three groups; **1**, **3**, **4**, **5**, **8**, **11**, and **13** in Group A; **9**, **10**, **12**, and **15** in Group B; **2**, **6**, **7**, and **14** in Group C. In each group, both enantiomers of the product alcohols and the substrate ketones were separated nicely by

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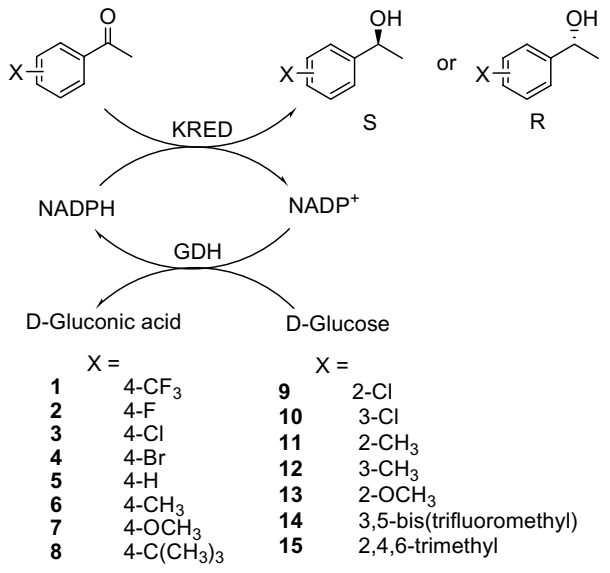

Scheme 1. Reduction of aryl ketones by ketoreductases with NADPH recycle system.

chiral GC analysis and identified by comparing the retention time with the standards. Therefore, each group of ketones was reacted with each of the ketoreductases with the number of reactions performed being reduced to 72. This greatly enhanced the efficiency of studying these ketoreductases. The reduction was carried out with an NADPH recycle system (D-glucose dehydrogenase and D-glucose) as shown in Scheme 1. The conversion and enantiomeric excess (ee) of the product alcohol were measured by chiral GC analysis at several time intervals. The absolute configurations of the product alcohols were determined by comparison of the chiral GC data with those in the literature.[7] The relative reaction rate of the ketone reduction was defined as 1000 times the conversion per hour under the tested reaction conditions, and the data are summarized in Table 1. In the table, a relative rate greater than 1000 means the conversion reached 100% in less than 1 h. For a relative rate of 10, the reaction conversion reached 24% in 24 h. For a relative rate of less than 1, trace of product alcohol was detected by GC analysis after 24 h, while zero means no product alcohol was detected after 24 h. All the tested ketoreductases showed no activity toward the reduction of 2′,4′,6′-trimethylacetophenone, so it is not listed in the table.

For some ketoreductases (e.g., KRED101, KRED111, KRED113, KRED114, and KRED115), all the tested ketones, except for 2′,4′,6′-trimethylacetophenone, were suitable substrates. The remaining ketoreductases showed low activity toward some of the tested substituted acetophenones. When comparing substrates **1**–**8**, **10**, and **12** (the *p*- and *m*-substituted acetophenone), the type of substituent significantly affected the activity of some of the tested ketoreductases, while the activity of some other ketoreductases showed little change for substrates **1**–**8**, **10**, and **12**. For example, Figure 1 shows the correlation between the relative activity of KRED123 toward substrates **1**–**8**, **10**, and **12** and the Hammett σ value, with $\rho = 1.826$ implying that the electron-withdrawing character of the substituent enhances the activity of KRED123.[8,9] The deviation of 4′-trifluoromethyl- and 4′-*tert*-butylacetophenone with lower activity (diamond) may be due to their steric factors. Similar tendencies are found for ketoreductases KRED101, KRED113, KRED124, and KRED125. The correlation between the relative activity of KRED111 toward substrates **1**–**8**, **10**, and **12** and the Hammett σ value is plotted in Figure 2. The nearly flat line ($\rho = 0.0911$) indicates that the substituent exerts little effect on the activity of KRED111, except a *tert*-butyl substituent, which showed lower activity because of its bigger size. A similarly small ρ value was observed for ketoreductases KRED114, KRED115, and KRED128. This implies that the rate-determining step might be different for different enzymes. Three *o*-substituted acetophenones were also examined. For 2′-chloroacetophenone, all the ketoreductases except KRED104 showed high activity, while the activity of most ketoreductases toward 2′-methylacetophenone were low or even not active at all. Interestingly, 2′-methoxyacetophenone also had much higher activities than its methyl counterpart in some cases. This suggests that the electronic-withdrawing character of chloro group and the ability of the oxygen atom in methoxy group to form a hydrogen bond enhance the activity of these ketoreductases, although steric factors associated with the *ortho*-position substituent may have an opposite impact on this activity. Since (*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethanol is a key pharmaceutical intermediate, 3′,5′-bis(trifluoromethyl)acetophenone was tested with these ketoreductases. Several ketoreductases were active for this substrate.

The enantiomeric excess of the product alcohols are summarized in Table 2. It can be seen that most of the tested ketoreductases were highly enantioselective in the reduction of these aryl ketones. A few exceptions were ketoreductases KRED101, KRED113, and KRED128. It is interesting that KRED101 and KRED113 showed much higher enantioselectivities in the reduction of 2′-methoxyacetophenone than in the reduction of 2′-methylacetophenone, while in some other cases (KRED111, KRED114, KRED115, and KRED121) the reduction of 2′-methoxyacetophenone was less enantioselective than its methyl counterpart. This might be due to the ability of the oxygen atom in the methoxy group to form a hydrogen bond with the enzyme. Most of the tested ketoreductases catalyzed the reduction of aryl ketones to give the (*S*)-enantiomer of the alcohol as the major product. A few exceptions were KRED101, KRED107, and KRED113. In some cases, the substituent also affected the configuration of the major product alcohol. For example, the reduction of 3′,5′-bis(trifluoromethyl)acetophenone catalyzed by KRED101 and KRED113 afforded the (*S*)-enantiomer of the alcohol in greater than 99% ee, while (*R*)-enantiomers were obtained for other substrates. Similar results were obtained for the reduction of 3′,5′-bis(trifluoromethyl)acetophenone by KRED126 and 2′-methoxyacetophenone by KRED111, KRED114, KRED115, KRED121, and KRED123.

Table 1. Relative reaction rate of aryl ketone reductions catalyzed by the ketoreductases[a]

KRED	**1** (4′-CF_3)	**2** (4′-F)	**3** (4′-Cl)	**4** (4′-Br)	**5** (4′-H)	**6** (4′-CH_3)	**7** (4′-OCH_3)	**8** (4′-$C(CH_3)_3$)	**9** (2′-Cl)	**10** (3′-Cl)	**11** (2′-CH_3)	**12** (3′-CH_3)	**13** (2′-OCH_3)	**14** (3′,5′-$(CF_3)_2$)
101	460	138	250	290	380	116	28	24	>1000	>1000	30	850	600	159
102	60	1	25	38	2	1	<1[b]	7	182	42	<1	15	50	2
103	5	<1	3	<1	<1	<1	<1	0[b]	892	55	20	20	100	1
104	6	<1	4	4	<1	<1	<1	0	<1	36	<1	14	<1	1
105	24	8	28	15	11	5	<1	0	>1000	57	70	19	246	1
106	<1	<1	<1	<1	<1	<1	<1	0	143	58	0	24	5	<1
107	>1000	516	>1000	>1000	142	570	61	97	20	172	0	62	0	8
108	7	14	28	14	22	<1	3	0	50	159	0	76	12	<1
109	<1	<1	<1	<1	<1	<1	<1	<1	76	26	<1	10	5	<1
110	<1	<1	2	<1	<1	0	0	0	96	36	0	14	13	<1
111	468	730	532	582	495	678	562	57	>1000	977	37	366	125	789
113	140	80	96	109	172	56	12	13	>1000	>1000	16	714	585	164
114	190	461	170	190	144	626	446	117	899	>1000	18	307	119	862
115	519	753	571	628	520	681	513	72	982	918	64	257	238	831
116	6	8	25	14	18	2	4	0	46	120	0	21	14	3
117	6	6	30	20	27	2	5	0	55	140	0	23	23	3
120	16	32	55	40	49	7	11	0	93	181	0	122	42	3
121	672	23	550	734	352	38	17	130	547	906	5	77	32	682
123	458	573	606	623	351	145	42	34	958	893	2	296	63	792
124	274	100	438	389	50	10	33	10	39	179	0	30	0	3
125	650	6	167	256	14	10	2	70	153	94	4	25	5	4
126	14	1	5	6	1	1	<1	<1	34	42	0	15	1	21
127	8	1	4	5	1	0	0	<1	30	36	0	14	1	2
128	>1000	735	>1000	>1000	470	620	123	338	401	933	<1	186	<1	85

[a] The relative reaction rate was defined as $1000 \times c\%/h$, $c\%/h$ was the conversion per hour.

[b] <1 means trace of product alcohol being detected by GC in 24 h, while 0 means no product alcohol being detected by GC in 24 h.

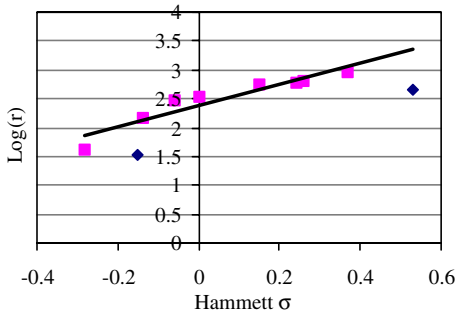

Figure 1. The correlation between the relative activity of KRED123 toward substrates (**1**–**8**, **10**, and **12**) and the Hammett σ value.

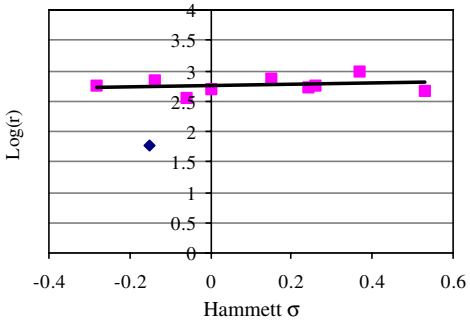

Figure 2. The correlation between the relative activity of KRED111 toward substrates **1**–**8**, **10**, and **12** and the Hammett σ value.

The ketoreductases with the highest activity and enantioselectivity for the reduction of each tested aryl ketone are listed in Table 3 with their relative reaction rates and ee values. With an initial relative rate greater than 100, the conversion of the reduction usually reaches 100% in 24 h. In order to possess preparative applicability, high activity and enantioselectivity are desirable. It can be seen from Table 3 that, for most of the tested aryl ketones, both enantiomers of the product alcohols could be prepared via the reduction catalyzed by one or more of the ketoreductases. A few exceptions were the (*S*)-enantiomer of 4′-fluorophenylethanol and the (*R*)-enantiomer of 2′-chloro-, 3′-chloro-, 2′-methyl-, 3′-methyl-, and 3′,5′-bis(trifluoromethyl)phenylethanol. While many ketoreductases efficiently catalyzed aryl ketone reduction to give the (*S*)-enantiomer in enantiomerically pure form, most of the aryl ketone reductions to the enantiomerically pure (*R*)-enantiomer were catalyzed by KRED107. This suggests that more effort should be made to develop new ketoreductases for the production of enantiomerically pure alcohols with an (*R*)-configuration, especially those with substituents on the *meta*- and *ortho*-position of the benzene ring.

3. Conclusion

A new method has been developed to measure the activity and enantioselectivity of multiple substrates in one reaction by chiral GC analysis. This method could be useful for the highthrough-put screening against multiple substrates during the development of new ketoreductase mediated chiral reductions. The enzymatic reductions of a series of substituted aryl ketones were studied using 24 isolated recombinant ketoreductases and the substituent effects on activity and enantioselectivity were evaluated. When comparing *p*- and *m*-substituted acetophenones, the electronic character of the substituent significantly affects the activity of some of the tested ketoreductases (the correlation fits the Hammett equation), while it has little effect on the activity of some other ketoreductases. Most of the tested ketoreductases were highly enantioselective in the reduction of these aryl ketones. The electronic properties, steric factors, and the ability to form a hydrogen bond to the substituent at the *ortho*-position play a significant role in determining both the activity and enantioselectivity of the ketoreductase-catalyzed reductions, although no general correlation suitable for every enzyme has been found. These results will not only provide guidance for the application of these ketoreductases to the preparation of enantiomerically pure aryl alcohols, but also point out future directions for developing new ketoreductases.

4. Experimental

The chiral GC analysis was performed on a Hewlett Packard series II plus gas chromatograph equipped with, EPC, split/splitless injector, FID detector, and 25 m × 0.25 mm CP-Chirasil-Dex CB chiral capillary column. All the ketoreductases were purified recombinant enzymes, which were developed by genome mining and protein engineering, and are commercially available from BioCatalytics, Inc.[6] All the ketones were purchased from Aldrich and the racemic alcohol standards prepared by the reduction of the corresponding ketones with sodium borohydride in ethanol.

4.1. Grouping of the ketones

Chiral GC analysis was performed under exactly the same conditions for each ketone and the corresponding racemic alcohols. The ketones were grouped according to retention time with no overlap. Then each ketone and the racemic corresponding alcohol in each group were mixed together and subjected to chiral GC analysis to confirm that there was no overlap for all peaks. The GC conditions were as follows: column head pressure: 30 psi; injector temperature; 250 °C; detector temperature: 250 °C; column temperature: at initial temperature 120 °C for 2 min, increasing to 150 °C at the rate of 1 °C/min, then at 150 °C for 5 min. The aryl ketones were divided into three groups; **1**, **3**, **4**, **5**, **8**, **11**, and **13** in Group A; **9**, **10**, **12**, and **15** in Group B; **2**, **6**, **7**, and **14** in Group C.

4.2. Enzymatic reduction of the ketones

General procedure for the ketone reduction using an NADPH recycle system was as follows (Group A): 24 mg of D-glucose, 4 mg of D-glucose dehydrogenase, 4 mg of NADPH, 4 mg of ketoreductase, and 25 μL of each ketone solution in DMSO (0.25 M) were mixed in

Table 2. Enantiomeric excess of aryl ketone reductions catalyzed by the ketoreductases[a]

KRED	**1** (4′-CF_3)	**2** (4′-F)	**3** (4′-Cl)	**4** (4′-Br)	**5** (4′-H)	**6** (4′-CH_3)	**7** (4′-OCH_3)	**8** (4′-$C(CH_3)_3$)	**9** (2′-Cl)	**10** (3′-Cl)	**11** (2′-CH_3)	**12** (3′-CH_3)	**13** (2′-OCH_3)	**14** (3′,5′-$(CF_3)_2$)
101	14	−18	−10	−20	−14	−42	−40	12	−18	−65	−69	−88	−>99	>99
102	>99	—[b]	>99	>99	—	—	—	—	>99	>99	—	>99	>99	—
103	—	—	—	—	—	—	—	—	>99	>99	>99	>99	>99	—
104	—	—	—	—	—	—	—	—	—	>99	—	>99	—	—
105	>99	—	>99	>99	>99	—	—	—	>99	>99	>99	>99	>99	—
106	—	—	—	—	—	—	—	—	>99	>99	—	>99	—	—
107	−>99	−>99	−>99	−>99	−>99	−>99	−>99	−>99	0	−86	—	−78	—	—
108	—	>99	>99	>99	>99	—	—	—	>99	>99	—	>99	>99	—
109	—	—	—	—	—	—	—	—	>99	>99	—	>99	—	—
110	—	—	—	—	—	—	—	—	>99	>99	—	>99	>99	—
111	91	90	98	98	85	>99	>99	>99	90	40	98	−30	−48	>99
113	−28	−16	−28	−30	0	−44	−50	12	32	−64	−50	−84	−>99	>99
114	91	84	98	98	90	88	>99	>99	83	84	77	34	−50	>99
115	90	82	98	98	85	84	>99	99	89	45	75	−23	−50	>99
116	—	—	>99	>99	>99	—	—	—	>99	>99	—	>99	>99	—
117	—	—	>99	>99	>99	—	—	—	>99	>99	—	>99	>99	—
120	>99	>99	>99	>99	>99	—	>99	—	>99	>99	—	>99	>99	0
121	90	99	99	99	99	99	>99	99	76	94	—	72	−42	>99
123	93	95	99	99	88	77	89	>99	82	58	—	−26	−34	>99
124	>99	>99	>99	>99	>99	>99	>99	>99	>99	>99	—	>99	—	—
125	>99	—	>99	>99	>99	>99	—	>99	>99	98	—	>99	—	—
126	>99	—	—	—	—	—	—	—	>99	>99	—	>99	—	−92
127	—	—	—	—	—	—	—	—	>99	>99	—	>99	—	—
128	>99	16	40	56	60	86	10	>99	84	82	—	78	—	90

[a] The positive ee value means (*S*)-enantiomer being the major product, while negative ee value means (*R*)-enantiomer being the major product.
[b] The conversion was less than 25% after 24 h, so the ee value was not very meaningful and thus not listed.

Table 3. Most efficient ketoreductase for the reduction of each ketone to (*S*)- or (*R*)-alcohol

Ketone	(*S*)-Enantiomer KRED (rate, ee)	(*R*)-Enantiomer KRED (rate, ee)
1. 4′-CF_3	128 (>1000, >99)[a]	107 (>1000, >99)
	125 (650, >99)	
2. 4′-F	123 (573, 95)	107 (516, >99)
3. 4′-Cl	123 (606, 99)	107 (>1000, >99)
	124 (436, >99)	
4. 4′-Br	121 (734, 99)	107 (>1000, >99)
	123 (623, 99)	
	124 (389, >99)	
5. 4′-H	121 (352, 99)	107 (142, >99)
6. 4′-CH_3	111 (678, >99)	107 (570, >99)
7. 4′-OCH_3	111 (562, >99)	107 (61, >99)
	115 (513, >99)	
	114 (446, >99)	
8. 4′-$C(CH_3)_3$	128 (338, >99)	107 (97, >99)
	121 (130, 99)	
	114 (117, >99)	
9. 2′-Cl	105 (>1000, >99)	101 (>1000, 18)
	103 (892, >99)	
10. 3′-Cl	120 (181, >99)	107 (172, 86)
	124 (179, >99)	
11. 2′-CH_3	105 (70, >99)	101 (30, 69)
12. 3′-CH_3	120 (122, >99)	101 (850, 88)
13. 2′-OCH_3	105 (246, >99)	101 (600, >99)
		113 (585, >99)
14. 3′,5′-$(CF_3)_2$	114 (862, >99)	126 (21, 92)
	115 (831, >99)	
	123 (792, >99)	
	111 (789, >99)	
	121 (682, >99)	

[a] The numbers in the parenthesis are the relative rate (rate) and enantiomeric excesses (ee), respectively.

5 mL of potassium phosphate buffer (100 mM, pH 6.75) and the mixture was shaken at 25 °C. A 100 μL of sample was taken at 1, 5, and 24 h interval. The sample was extracted with ethyl acetate. The organic extract was dried over anhydrous sodium sulfate and was subjected to chiral GC analysis. For Groups B and C, the amounts of D-glucose, D-glucose dehydrogenase, NADPH, ketoreductase, and reaction volume were adjusted accordingly to the same reactant ratio and concentration.

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