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Asymmetric synthesis of *syn*-aryl-(2*S*,3*R*)-2-chloro-3-hydroxy esters *via* an engineered ketoreductase-catalyzed dynamic reductive kinetic resolution

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ABSTRACT

We report here a generic, green synthesis of 17 valuable *syn*-aryl-(2*S*,3*R*)-2–chloro-3–hydroxy esters (*syn*-(2*S*,3*R*)-**1**) in 73%-99% isolated yields along with 6.1:1–83:1 *dr* and 31%∼>99% *ee*, through dynamic reductive kinetic resolution of racemic aryl α–chloro β-keto esters (**2**) catalyzed by an engineered ketoreductase which was obtained *via* epPCR-based directed evolution. The hectogram scale synthesis of *syn*-(2*S*,3*R*)-**1b** at a substrate concentration of 120 g/L showcased the application potential of the biocatalytic method developed presently.

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Recent years have witnessed the growing applications of ketoreductase (KRED)-catalyzed dynamic reductive kinetic resolution (DYRKR) to the asymmetric synthesis of chiral alcohols containing two adjacent stereogenic centers [1–4]. In addition to the intrinsic merits of biocatalysis including sustainability and mild operation conditions [5,6], undoubtedly this increasing popularity is mainly ascribed to the capability of predominantly forming one of the four possible stereoisomeric products in one-step, with a maximum theoretical yield of 100% [1–4]. Within this context, our group very recently performed the first systematic synthesis of various *anti*-aryl-(2*S*,3*S*)-2–chloro-3–hydroxy esters (*anti*-(2*S*,3*S*)-**1**, Fig. 1) through ketoreductase LfSDR1-catalyzed DYRKR of racemic aryl α–chloro β-keto esters (**2**), and further realized the chemoenzymatic synthesis of the calcium channel blocker D-*cis*-diltiazem as wells as its structural analogs clentiazem and siratiazem [7].

Given the fact that not only *anti*-(2*S*,3*S*)-**1**, but also their diastereomers, *syn*-aryl-(2*S*,3*R*)-2–chloro-3–hydroxy esters (*syn*-(2*S*,3*R*)-**1**, Fig. 1) are valuable for the synthesis of pharmacologically important molecules, such as *N*-benzoyl-2*R*,3*S*-3-phenyl isoserine (**3**), the critical structure component of taxol [8], antiplatelet agent TA-993 (**4**) [9,10], and the cyclic nucleotide gated channel blocker L-*cis*-diltiazem (**5**) [11–13], it is desirable to develop a generic, stereoselective synthesis of *syn*-(2*S*,3*R*)-**1** *via* KRED-catalyzed DYRKR. To the best of our knowledge, the only relevant studies were ketoreductases YDL124w-, and CaADH-catalyzed highly stereoselective synthesis of *syn*-(2*S*,3*R*)-**1a** (Scheme 1), the synthetic precursor of **3** [14,15]. Notably, chemical reduction of racemic aryl α–chloro β-keto esters (**2**) catalyzed by chiral ruthenium complexes only furnished *syn*-(2*S*,3*R*)-**1** with poor-to-moderate diastereoselectivities (<9:1 *dr*) [16–18]. Herein, we report the enzyme identification, directed evolution, and application of the evolved variant to the asymmetric synthesis of a variety of *syn*-aryl-(2*S*,3*R*)-2–chloro-3–hydroxy esters *via* DYRKR.

In our previous study [7], we found that three ketoreductases, namely KRED-F42, KdoADH, and KRED-Bt could reduce the model substrate **2b** to furnish *syn*-(2*S*,3*R*)-**1b** as the major product (Table 1, entries 1–3). Nevertheless, the diastereoselectivities were still unsatisfactory (1.1:1–6.2:1 *dr*). Prompted by the report that *syn*-(2*S*,3*R*)-**1a** was generated in a highly stereoselective manner through YDL124w-catalyzed reduction of **2a** (Scheme 1) [14], we tested this enzyme for the reduction of **2b**. To our delight, the target (2*S*,3*R*)-**1b** was afforded in 98.4% conversion with 46:1 *dr* and 98.1% *ee* (Table 1, entry 4). The relative *syn*-configuration of thus formed (2*S*,3*R*)-**1b** was determined based on the $J_{2,3}$ value of 6.7 Hz, whereas the absolute configuration was initially assigned according to the optical rotation data and further unambiguously

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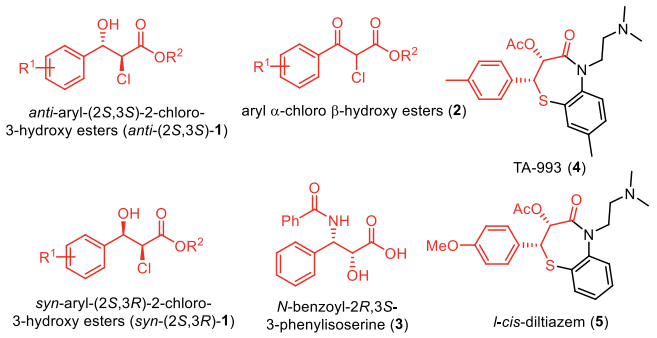
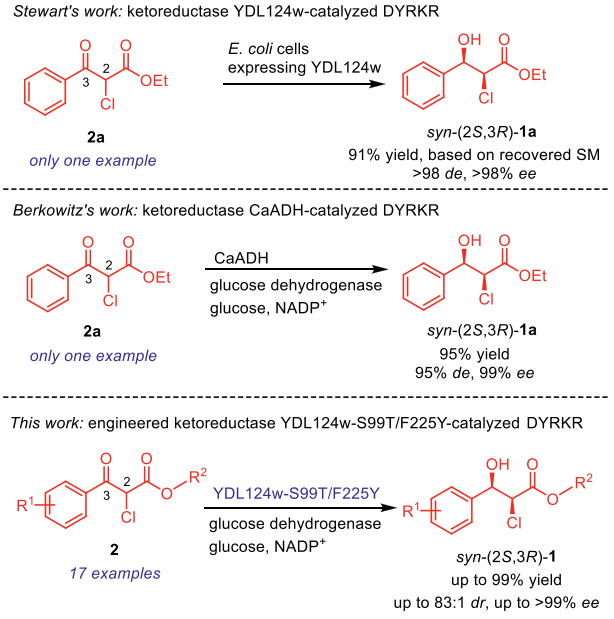


Fig. 1. Structures of *anti*-aryl-(2*S*,3*S*)-2–chloro-3–hydroxy esters (*anti*-(2*S*,3*S*)-**1**), aryl α–chloro β-keto esters (**2**), *syn*-aryl-(2*S*,3*R*)-2–chloro-3–hydroxy esters (*syn*-(2*S*,3*R*)-**1**), *N*-benzoyl-2*R*,3*S*-3-phenyl isoserine (**3**), TA-993 (**4**), and L-*cis*-diltiazem (**5**).



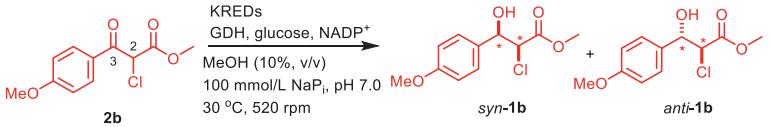
Scheme 1. Stereoselective synthesis of *syn*-(2*S*,3*R*)-**1** through ketoreductase-catalyzed DYRKR of aryl α–chloro β-keto esters (**2**).

confirmed by X-ray crystallography (Fig. S1 in Supporting information). Next, we purified the C-terminal-His_6-tagged YDL124w and determined its specific activity towards **2b** as 0.086 U/mg (Table 2). Given the moderate catalytic activity, we decided to engineer the enzyme first prior to examining the substrate scope of this biocatalytic reaction [19–21].

As a crystal structure of YDL124w is not available, we opted to invoke the error-prone PCR (epPCR) approach. Specifically, by employing the wild-type (WT) YDL124w as the template, epPCR was conducted with 0.3 mmol/L Mn^{2+} resulting in an average mutation rate of 1–3 amino acid substitutions. Upon screening roughly 36,000 colonies using activity assays performed with cell-free extracts of mutant enzymes, an enzyme variant with about 20-fold increased activity towards substrate **2b** was discovered. Sequence determination indicated this variant carried two mutations: S99T/F225Y. Hence, it was named as YDL124w-S99T/F225Y. The effect of different pH conditions ranging from 5.0 to 8.0, on the specific activity of the purified YDL124w-S99T/F225Y was examined, with pH 6.5 being identified as the optimal one (Fig. S2 in Supporting information). Within the temperatures between 20 °C and 50 °C tested, YDL124w-S99T/F225Y showed the highest specific activity at 30 °C (Fig. S3 in Supporting information). Intriguingly, assays on purified YDL124w-S99T/F225Y under optimal conditions suggested this enzyme only possessed 1.9-fold higher specific activity towards **2b** relative to WT (Table 2). Furthermore, we also compared the soluble expression levels of WT and YDL124w-S99T/F225Y using the SDS-page analysis. As seen in Fig. S4 (Supporting information), this evolved enzyme variant was better solubly expressed than WT. Therefore, both the improved specific activity and the enhanced expression likely contributed to the aforementioned 20-fold increased activity in the lysate-based assays. In addition to **2b**, we also determined the specific activity of WT and YDL124w-S99T/F225Y toward another two α–chloro β-keto esters **2a** and **2c**, because the bioreduction products of them, namely *syn*-(2*S*,3*R*)-**1a** and *syn*-(2*S*,3*R*)-**1c**, are critical synthetic intermediates to *N*-benzoyl-2*R*,3*S*-3-phenyl isoserine (**3**) and TA-993 (**4**), respectively. Compared with WT, YDL124w-S99T/F225Y exhibited 1.4-fold elevated specific activity towards both **2a** and **2c** (Table 2).

With an improved enzyme variant YDL124w-S99T/F225Y in hand, 17 chemically-prepared aryl α–chloro β-keto esters **2** were deployed for examining its synthesis versatility at a preparative-scale (0.6 mmol) (Scheme 2). Firstly, the current bioreduction of substrates equipped with a *para*-substituent at the phenyl ring (**2b**, **2c**, **2e**-**2j**) or substrate without a substituent (**2d**), all occurred with high levels of stereoselectivity, regardless of the substituent's electronic property. The corresponding products *syn*-

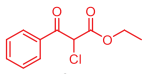
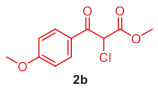
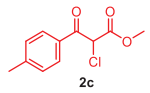
Table 1
KRED-catalyzed stereoselective reduction of α–chloro β-keto ester **2b**.[a]



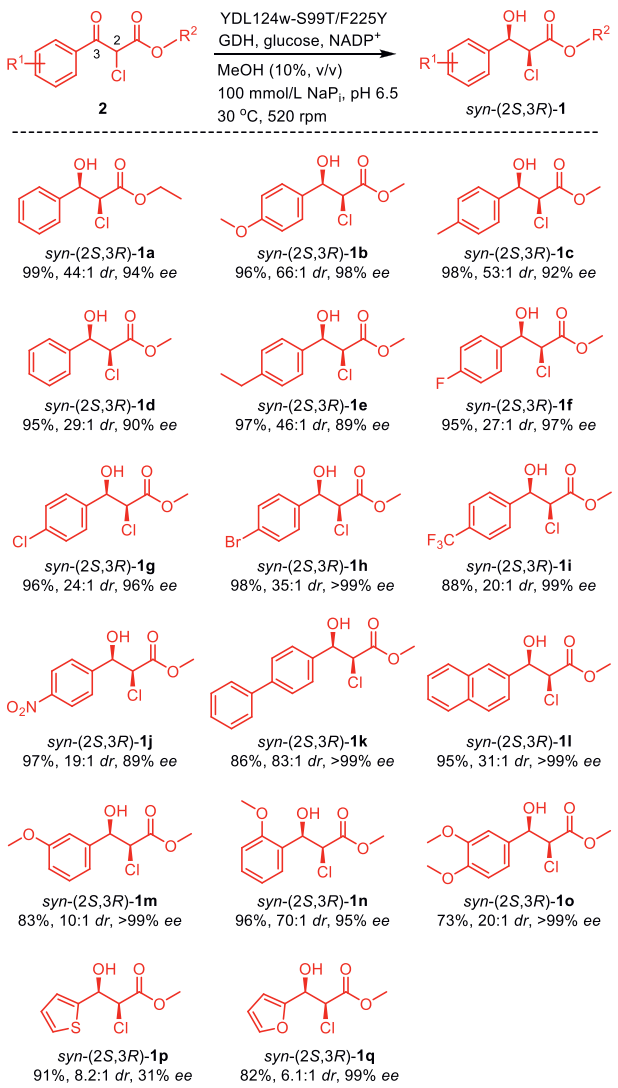
Entry	Enzyme	Conv. (%)[b]	*dr* (*syn:anti*)[b]	*ee* (%)[b] (*syn*)	*ee* (%)[b] (*anti*)
1[c]	KRED-F42	92.0	6.2:1	>99 (2*S*,3*R*)	33.3 (2*R*,3*R*)
2[c]	KdoADH	93.0	1.1:1	75.7 (2*S*,3*R*)	55.8 (2*S*,3*S*)
3[c]	KRED-Bt	18.3	2.5:1	>99 (2*S*,3*R*)	95.3 (2*R*,3*R*)
4	YDL124w	98.4	46:1	98.1 (2*S*,3*R*)	n.d.[d]

[a] A reaction mixture (60 mL) composed of **2b** (10 mmol/L), glucose (20 mmol/L), $NADP^+$ (0.2 mmol/L), 10 mL 15% (w/v) cell-free extract (CFE) of KREDs in NaP_i buffer (100 mmol/L, pH 7.0), 1 mL 15% (w/v) CFE of GDH in NaP_i buffer (100 mmol/L, pH 7.0), 6 mL MeOH, and 43 mL NaP_i buffer (100 mmol/L, pH 7.0) was stirred at 30 °C with 520 rpm for 12 h.
[b] The conversions, *dr* and *ee* values were determined by chiral HPLC analysis, with racemic product standards obtained through $NaBH_4$-mediated reduction of **2b**.
[c] Data reported in Ref. [7].
[d] n.d.: not determined.

Table 2
Specific activity of the purified YDL124w and its evolved variant YDL124w-S99T/F225Y toward substrates **2a, 2b**, and **2c**.[a]

Substrate	Enzyme	Specific activity (U/mg)	Fold enhancement
 **2a**	YDL124w	0.127 ± 0.001	1.4
	YDL124w-S99T/F225Y	0.176 ± 0.002	
 **2b**	YDL124w	0.086 ± 0.003	1.9
	YDL124w-S99T/F225Y	0.160 ± 0.004	
 **2c**	YDL124w	0.072 ± 0.001	1.4
	YDL124w-S99T/F225Y	0.098 ± 0.002	

[a] Determined at 30 °C, pH 6.5.



Scheme 2. Preparative-scale synthesis of *syn*-(2*S*,3*R*)-**1** catalyzed by YDL124w-S99T/F225Y. Reaction conditions (60 mL): **2** (10 mmol/L), glucose (15 mmol/L), $NADP^+$ (0.2 mmol/L), 10 mL of 15% (w/v) CFE of YDL124w-S99T/F225Y in NaP_i buffer (100 mmol/L, pH 6.5), 0.2 mL of 15% (w/v) CFE of GDH in NaP_i buffer (100 mmol/L, pH 6.5), and 6 mL of MeOH, in 44 mL of NaP_i buffer (100 mmol/L, pH 6.5). Reaction mixtures were incubated at 30 °C with 520 rpm stirring for 12 h. Isolated yield was given. The *dr* values were determined by the ^1H NMR analysis of the crude product. The *ee* values were determined by chiral HPLC analysis. The racemic product standards were obtained through $NaBH_4$-mediated reduction of **2**.

(2*S*,3*R*)-**1** were isolated in 88%–98% yield along with 19:1–66:1 *dr* and 89%∼>99% *ee*. In comparison, *anti*-(2*S*,3*S*)-**1f**, *anti*-(2*S*,3*S*)-**1g**, and *anti*-(2*S*,3*S*)-**1h**, containing electron-withdrawing groups (F, Cl, Br), were synthesized with only moderate diastereoselectivities (5.3:1–9.2:1 *dr*) through ketoreductase LfSDR1-catalyzed DYRKR [7]. Secondly, sterically demanding substrates, including **2k** and **2n** with a *para*-phenyl and an *ortho*–methoxy substituent, respectively, the naphthyl-based **2l**, and the bis–methoxy groups containing **2o** were all reduced smoothly by YDL124w-S99T/F225Y, providing the desired products (*syn*-(2*S*,3*R*)-**1k**, *syn*-(2*S*,3*R*)-**1n**, *syn*-(2*S*,3*R*)-**1l**, and *syn*-(2*S*,3*R*)-**1o**) in good to excellent yields with excellent stereoselectivities (73%–96% yield, 20:1–83:1 *dr*, 95%∼>99% *ee*). Thirdly, not only methyl ester, but ethyl ester **2a** was accepted by YDL124w-S99T/F225Y, furnishing *syn*-(2*S*,3*R*)-**1a** in 99% yield with 44:1 *dr* and 94% *ee*. Finally, although ketone **2m** with a *meta*–methoxy-substituted phenyl ring and heteroaryl-based substrates (**2p** and **2q**) could be transformed to the respective chlorohydrins *syn*-(2*S*,3*R*)-**1m**, *syn*-(2*S*,3*R*)-**1p**, and *syn*-(2*S*,3*R*)-**1q** in 82%–91% yield, the diastereoselectivities were inadequate (6.1:1–10:1 *dr*). In the future, reaction optimization and/or protein engineering might be utilized to boost the stereoselectivity.

The application potential of the currently developed DYRKR method was further showcased by the reduction of **2b** at an industrially useful concentration of 120 g/L. In our previous study of LfSDR1-catalyzed reduction of 100 g/L of **2b**, a continuous fed-batch operation was necessary in order to achieve high reaction conversion, as LfSDR1 was severely inhibited by **2b** at substrate concentration of 40 mmol/L or greater [7]. Fortunately, no obvious substrate inhibition was observed for YDL124w-S99T/F225Y with up to 80 mmol/L of **2b**. Hence, a single-dose of **2b** was employed for the current study. In practice, **2b** (120 g, 120 g/L), glucose (178.5 g, 2.0 equiv.), $NADP^+$ (149 mg, 0.2 mmol/L), methanol (100 mL), cell-free extracts of YDL124w-S99T/F225Y (corresponding to 90 g/L of wet cells), and cell-free extracts of GDH (corresponding to 3.3 g/L of wet cells) in NaP_i buffer (900 mL, 100 mmol/L, pH 6.5) were stirred at 30 °C, while maintaining the reaction pH between 6.0 and 6.5 by titrating a 4 mol/L NaOH solution. After the reaction went to nearly completion (98% conversion) as indicated by the chiral HPLC analysis, celite was added, followed by filtration. The filter cake was washed three times with ethyl acetate, and the two layers of filtrate were separated. The aqueous solution was extracted further with ethyl acetate for 2 times. Finally, the combined organic layer was washed with brine, dried, and concentrated *in vacuo* to give *syn*-(2*S*,3*R*)-**1b** (110.6 g, 91.4% yield) with excellent purity (93.6% chemical purity, 41:1 *dr*, and 98.4% *ee*).

Molecular docking was adopted to shed light on the molecular basis for the increased catalytic activity of YDL124w-S99T/F225Y. First of all, 3-D structure models of YDL124w and YDL124w-

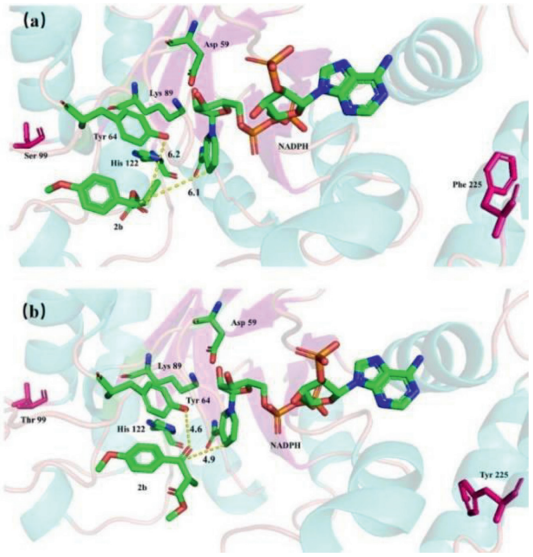


Fig. 2. Docking of substrate 2b into the active sites of wild-type of YDL124w (a) and variant YDL124w-S99T/F225Y (b).

S99T/F225Y were built with SWISS-MODEL server [22], by using the crystal structure of the conjugated polyketone reductase C2 (CPR-C2) from *Candida parapsilosis* IFO 0708 as the template (PBD ID: 4H8N) [23]. The amino acid sequence identity between the wild-type YDL124w and CPR-C2 is 47%. Both models thus generated were considered as reasonable, based on the respective VERIFY [24] values of 88.8% and 88.5%, ERRAT [25] values of 92.3 and 90.9, as well as both 100% of the residues being found in the allowed region of the Ramachandran plots [26]. Then, substrate **2b** was docked into the modeled structures of wild-type of YDL124w and variant YDL124w-S99T/F225Y, respectively (Fig. 2). The distance between the hydride of C4 from the nicotinamide of NADPH and the carbon atom of the carbonyl group of **2b**, as well as the distance between the oxygen atom of the carbonyl group of **2b** and the phenoxyl hydrogen of the catalytic residue Tyr64, were both shorter for YDL124w-S99T/F225Y than those for the wild-type enzyme (4.9 Å and 4.6 Å *versus* 6.1 Å and 6.2 Å). These results indicated that compared with WT, YDL124w-S99T/F225Y had higher chances to conduct both events of the hydride and the proton transfer from NADPH and Tyr64, respectively, to the carbonyl group of **2b**, hence, in accordance with the improved catalytic activity of this variant determined experimentally. Finally, the two mutated residues, Ser99 and Phe225 were found to be located distal from the active site. Although at this moment it is unclear how these mutations exactly affect the catalytic activity, one possibility is that nonlocal structural perturbations occurred after mutation, thereby influencing the catalytic activity indirectly through changing the enzyme's conformational motions as well as the probability of sampling conformations conducive to catalysis [27].

In summary, epPCR-based directed evolution of ketoreductase YDL124w enabled the development of an enzyme variant, YDL124w-S99T/F225Y, exhibiting 1.4–1.9 fold enhancement of specific activity relative to the wild-type enzyme towards α–chloro β-keto esters **2a**, **2b**, and **2c**. Compared with YDL124w, the soluble expression level of YDL124w-S99T/F225Y was much improved as well. Seventeen synthetically useful *syn*-aryl-(2*S*,3*R*)-2–chloro-3–hydroxy esters (*syn*-(2*S*,3*R*)-**1**) were synthesized through YDL124w-S99T/F225Y-catalyzed dynamic reductive kinetic resolution of racemic aryl α–chloro β-keto esters (**2**) in 73%–99% isolated yields, along with 6.1:1–83:1 *dr* and 31%∼>99% *ee*. The practical synthesis potential of the method developed in this study was demonstrated by a nearly complete reduction of 120 g/L of **2b** at a hectogram scale, furnishing *syn*-(2*S*,3*R*)-**1b**, a synthetic intermediate to the cyclic nucleotide gated channel blocker L-*cis*-diltiazem (**5**), in 91.4% isolated yield with 41:1 *dr* and 98.4% *ee*. Molecular docking was performed to help provide insights for the improvement of catalytic activity of the evolved enzyme variant.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.108178.

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