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Biocatalytic asymmetric ring-opening of dihydroisoxazoles: a cyanide-free route to complementary enantiomers of β -hydroxy nitriles from olefins†‡

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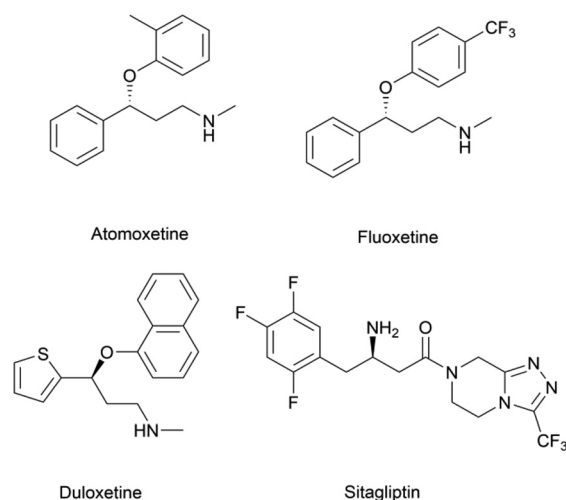
By combination of the cyanide-free synthesis of chiral nitriles and the Kemp elimination reaction catalyzed by aldolase dehydratases, we herein report a new application of aldolase dehydratase in the asymmetric ring-opening of 5-sub-4,5-dihydroisoxazoles to synthesize chiral β -hydroxy nitriles with broad substrate scope, excellent enantioselectivity (up to 99% ee), and good turnover number (up to 11 s⁻¹). Upon simple isolation and treatment with an alkaline reagent, the remaining chiral 5-sub-4,5-dihydroisoxazoles can be easily transformed into their corresponding β -hydroxy nitriles. Using site-directed mutagenesis, a ferrous Heme-containing active site was confirmed and two possible deprotonation pathways were proposed. To the best of our knowledge, this is the first enzymatic reaction used to construct a chiral hydroxyl group and nitrile group in one-step starting from a simple alkene, which provides a novel and useful strategy for the synthesis of complementary enantiomers of β -hydroxy nitriles.

Introduction

Chiral β -hydroxy nitriles are versatile building blocks that can be easily transformed into chiral β -hydroxy acids, γ -hydroxy amines, lactones *etc.*,^{1–4} which are important motifs of pharmaceutical and biologically active molecules (Scheme 1), and can be used as potential monomers for the preparation of biodegradable plastics.⁵

Due to the versatility of this family of compounds, the methods used for their efficient synthesis have been extensively explored over the last few decades. The most common strategy used in organic synthesis involves asymmetric transfer hydrogenation (ATH) to reduce keto-nitrile precursors into their corresponding hydroxyl nitrile compounds using transition-metal complexes such as ruthenium(II)-complex^{6–10} and iridium-complexes.^{11–13} In addition, alternative methods using the β -borylation of organoborane compounds with acceptor of α,β -unsaturated nitriles catalysed by Copper-complex following

with oxidation to synthesize corresponding alcohols have been reported.^{14–16} What's more, the asymmetric cross aldol reaction of aldehydes with different nucleophilic addition donors and catalysts, such as acetonitrile/Cu alkoxide-chiral phosphine complex,¹⁷ TMSCH₂CN/copper fluoride,¹⁸ organolithium reagents,¹⁹ cyanomethylzinc bromide/DPMPM²⁰ have been well developed. Moreover, the region-selective ring-opening of epoxide with cyanide was also a classic synthetic



Scheme 1 Biologically-active molecules containing the motifs derived from β -hydroxy nitriles.

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method accessing to β -hydroxy nitriles.²¹ In general, all these synthetic routes inevitably use costly chiral transition-metal complexes as catalysts to construct the chiral centre. Nowadays, benefitting from the great development of biotechnology and green chemistry, a variety of synthetic strategies to β -hydroxy nitriles have been developed using purified enzymes or whole cell catalysts. Among them, lipases,^{2,22,23} reductases,^{1,24–26} dehydrogenases,²⁷ and nitrilases^{28,29} have been mostly employed toward the synthesis of chiral β -hydroxy nitrile *via* the kinetic resolution of racemic hydroxyl nitriles or the asymmetric reduction of β -oxo-nitriles. In addition, few publications have reported the use of monooxygenase for the asymmetric hydroxylation of 3-phenylpropanenitrile³⁰ or multi-enzymatic biosynthesis system of alcohol dehydrogenase/halohydrin dehalogenase treating on α -halo ketones and cyanide³¹ to form this family of chiral compounds. In part, these biocatalytic methods overcome the use of costly transition-metal catalysts, but are confined to a limited scope of precursors, including keto-nitriles and racemic hydroxyl nitriles, which are prepared using tedious synthetic processes or inevitably use poisonous cyanide as a starting material. By combination of cyanide-free synthesis of chiral nitriles (Scheme 2a)³² and the Kemp elimination reaction (Scheme 2b)³³ catalysed by aldoxime dehydratases (EC 4.99.1.5, EC 4.99.1.6, EC 4.99.1.7), we herein report a novel cyanide-free enzymatic approach to construct a chiral hydroxyl group and nitrile group in one-step toward the efficient synthesis of the complementary enantiomers of β -hydroxy nitriles *via* the asymmetric ring-opening of 5-sub-4,5-dihydroisoxazoles (Scheme 2c).

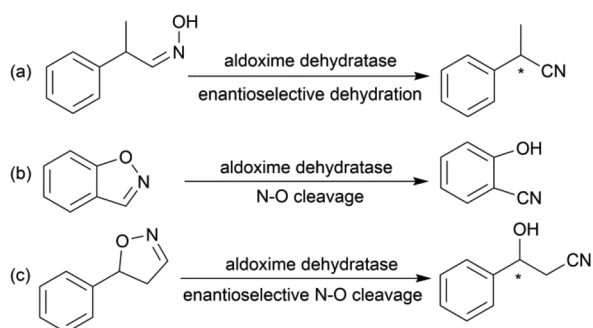
Aldoxime dehydratase is a class of Heme-containing enzyme involved in the cyanogenesis pathway observed in nature, which catalyses the dehydration of aldoximes to their corresponding nitrile derivatives.³⁴ Using the dehydration function of the enzyme, we established a cyanide-free platform for the synthesis of nitriles utilizing aldoxime precursors.^{35–38} Recently, we discovered the enzyme catalyses the Kemp elimination reaction *via* a $-\text{Fe}-\text{N}-$ complex catalytic pathway,³³ which is different from the classic acid–base mechanism.^{39,40} In this

enzymatic elimination reaction, the N–O bond is initially opened with the action of the $-\text{Fe}-\text{N}-$ complex (Heme Fe-proximal histidine) followed by deprotonation by a distal histidine residue to form the nitrile triple-bond. Upon investigation of the catalytic mechanism, it has been reported that the Kemp elimination reaction can also occur based on the redox catalysis of Heme-containing P450-BM3 *via* heterolysis to liberate an intermediate containing a nitrogen radical moiety ($\text{Fe}(\text{III})-\text{N}^{\bullet}$) and phenoxyl anion. The product is formed by bond rotation and proton-transfer to the resulting phenoxyl anion.⁴¹ It was also mentioned that the metabolism of leflunomide, an immunomodulatory therapeutic drug, could be transferred into teriflunomide *via* isoxazole ring-opening catalysed by human cytochrome P450. This provides the idea of synthesizing a chiral β -hydroxy nitrile product using a prochiral substrate containing the dihydroisoxazole motif. To verify this hypothesis, the substrate 5-phenyl-4,5-dihydroisoxazole was designed and tested in the reaction with aldoxime dehydratase Oxd-B obtained from *Bacillus* sp. OxB-1.^{42–44}

Results and discussion

In the initial screening process, the reaction was carried out in the absence of a reductant, which showed excellent enantioselectivity on the cleavage of the N–O bond as expected, but the activity was barely satisfactory. In view of the important role of a reductant in the aldoxime dehydration⁴⁵ and Kemp elimination reactions,³³ which can significantly enhance the enzyme activity, different reductants were tested for the asymmetric ring-opening of 5-phenyl-4,5-dihydroisoxazole (Fig. S1†). The enhanced effect on the enzymatic activity upon the addition of a reductant was observed, which showed the activity can be improved 1.9–6.0 fold using a variety of reductants. In addition, the enantioselectivity of the enzyme was not affected by the additives with the ee of the product being maintained at $\geq 99\%$ in each case. The reductant effect implies a ferrous-Heme active state was involved in the reaction, which is consistent with the sensitivity of Oxd B to oxygen.⁴²

The classic Kemp elimination reaction can take place under alkaline conditions *via* an acid–base mechanism.⁴⁰ Accordingly, this reaction was investigated in a range of different pH buffers (Fig. 1a). Although the yield varied significantly at different pH in a various buffer types, the enzyme could work at pH 5.0–10.0 with the desired product formed in 2–36% yield, and the optical purity of the product was maintained at $\geq 99\%$ at pH 5.0–8.0. From the control results, spontaneous reactions were detected in pH 9.0–10.0 (Fig. 1b), which caused a sharp decrease in the product ee. Enlightened by these results, an useful method to prepare racemic β -hydroxy nitriles is described from alkenes *via* a 5-sub-4,5-dihydroisoxazole intermediate^{46,47} upon treatment with a methanolic NaOH solution at room temperature (Scheme 3, and detailed data are shown in the ESI, section 5†). The result is consistent with the previous reports that β -hydroxy nitriles were formed by treating dihydroisoxazoles



Scheme 2 (a) Enantioselective dehydration catalysed by aldoxime dehydratase. (b) The Kemp elimination reaction catalysed by aldoxime dehydratase. (c) Asymmetric ring-opening of dihydroisoxazole catalysed by aldoxime dehydratase.



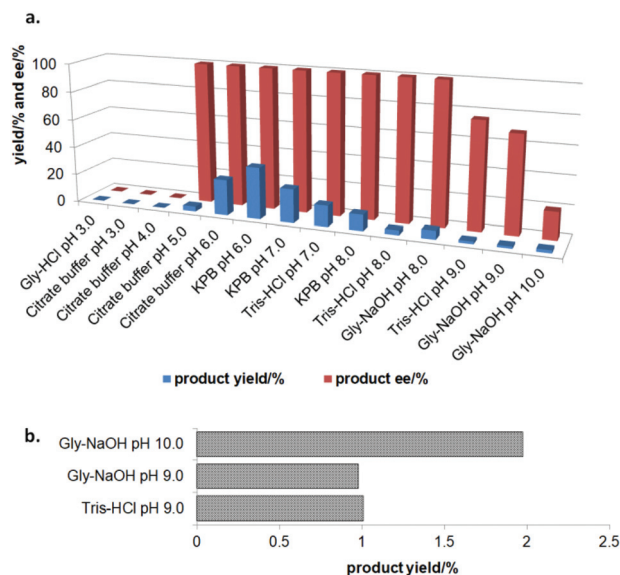
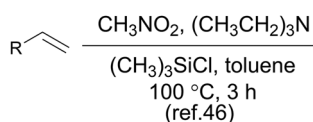
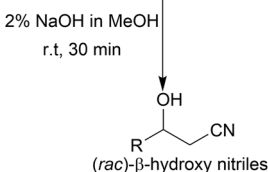
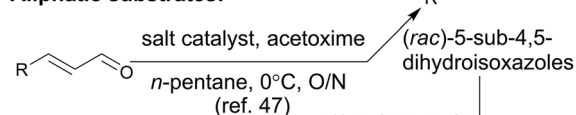


Fig. 1 (a) The pH effect on the activity of Oxd B in the catalytic asymmetric ring-opening of 5-phenyl-4,5-dihydroisoxazole. The reactions were performed on a 500 μ L scale using 100 mM buffer (pH 3.0–10.0), 1 U Oxd-B, 20 mM 5-phenyl-4,5-dihydroisoxazole, 0.05 mM FMN, and 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ at 30 $^\circ\text{C}$ for 30 min. The buffer solutions used were Gly-HCl (pH 3.0), Citrate-buffer (pH 3.0, 4.0, 5.0, and 6.0), KPB (pH 6.0, 7.0, and 8.0), Tris-HCl (pH 7.0, 8.0, and 9.0), and Gly-NaOH (pH 8.0, 9.0, and 10.0). (b) The control experiments were carried out without Oxd B at pH 9.0 and pH 10.0. The yield and ee were determined using chiral HPLC ($n = 2$).

Aromatic substrates:



Aliphatic substrates:



1a: R = Ph; **1b:** R = 2-Me-Ph; **1c:** R = 3-Me-Ph; **1d:** R = 4-Me-Ph; **1e:** R = 2-Cl-Ph; **1f:** R = 3-Cl-Ph; **1g:** R = 4-Cl-Ph; **1h:** R = 4-MeO-Ph; **1i:** R = 1-naphthalene; **1j:** R = 2-thiophene; **1k:** R = *n*-propyl; **1l:** R = *n*-butyl

Scheme 3 Synthesis of dihydroisoxazoles and (*rac*)- β -hydroxy nitriles from alkenes.

with bases (such as trimethylamine,⁴⁸ 1,8-diazabicyclo[5.4.0]undec-7-ene,⁴⁹ NaOMe,⁵⁰ ring-opening of 3-bromo-2-isoxazolines upon treatment with an alkanethiolate.⁵¹

To further improve the product yield, the reaction was monitored for a prolonged time of 3 h (Fig. 2). During the enantioselective transformation of the substrate, the ee of the substrate 5-phenyl-4,5-dihydroisoxazole (**1a**) increased to 99% over 2 h and the product (**2a**) ee was maintained at 99%, indicating the excellent catalytic enantioselectivity of Oxd B on this substrate. Additionally, it is often beneficial to agitate a reaction to improve the mass transfer process and as a result, enhance the reaction rate. However, a \sim 5-fold decrease in the yield of the β -hydroxy nitrile product was observed upon shaking the reaction when compared to the static-state reaction over 1 h. As reported in the dehydration of aldoximes, the activity of Oxd B can be increased by 5-fold under anaerobic conditions.⁴² Therefore, the oxygen sensitivity of Oxd B seems to be the reason of the decrease in the enzymatic activity in a shaking mode. By shaking, the enzyme is much more susceptible to exposure to oxygen in the air, which leads to the oxidation of ferrous-Heme to ferric-Heme, the resting-state of Heme in the ring-opening reaction of dihydroisoxazole.^{52,53}

With the optimized reaction conditions, a series of substrates were investigated using Oxd B (Table 1). Oxd B exhibited good selectivity toward most aromatic substrates with desired product *S/R*-2 formed in 48–99% ee. Among them, substrates bearing an electron-donating group on the aryl ring showed relatively higher ee values than those bearing an electron-withdrawing group. In addition, the enzyme preferred substrates bearing a substituent at the *para*-position (Table 1, **1d**, **1g**, and **1h**), rather than the *ortho*- (Table 1, **1b** and **1e**) or *meta*-position (Table 1, **1c** and **1f**). The selectivity toward heterocyclic aromatic substrates (Table 1, **1j**) was also very excellent, but a slight decrease in activity was observed. However, increasing the size of the aromatic ring to a naphthalene group leads to the reaction conversion becoming negligible (Table 1, **1i**),

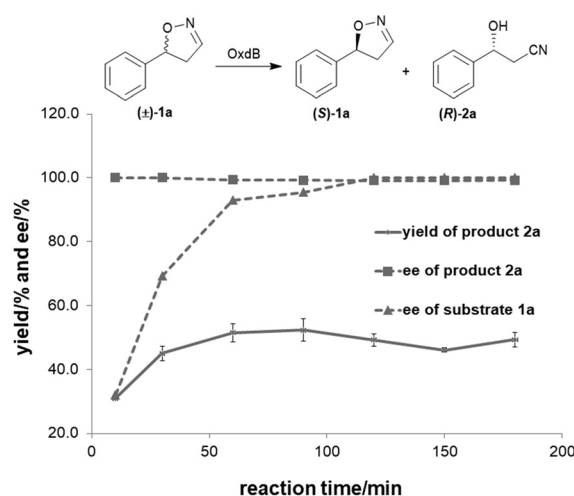


Fig. 2 Time course of the asymmetric ring-opening of 5-phenyl-4,5-dihydroisoxazole using Oxd B. The reactions were performed on a 500 μ L scale using 100 mM KPB buffer (pH 6.0), 1 U Oxd B, 20 mM 5-phenyl-4,5-dihydroisoxazole, 0.05 mM FMN, and 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ at 30 $^\circ\text{C}$. The yield and ee were determined using chiral HPLC ($n = 2$).

Table 1 Investigation of the substrate scope of Oxd A, B, and RE^a

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^a For Oxd A, the reactions were performed on a 500 μ L scale using 100 mM KPB buffer (pH 7.0), 10 U Oxd A, 20 mM substrate, and 4 mM $\text{Na}_2\text{S}_2\text{O}_4$ at 30 $^\circ\text{C}$ for 3 h ($n = 2$); for Oxd B, the reactions were performed on a 500 μ L scale using 100 mM KPB buffer (pH 6.0), 20 U Oxd-B, 20 mM substrate, 0.05 mM FMN, and 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ at 30 $^\circ\text{C}$ for 2 h ($n = 2$); and for Oxd RE, the reactions were performed on a 500 μ L scale using 100 mM KPB buffer (pH 7.0), 1 U Oxd RE, 6 mM substrate, 1 mM Na_2S at 30 $^\circ\text{C}$ for 2 h ($n = 2$). ^b The conversion was calculated using $\text{conv} = \text{ee}_s/(\text{ee}_s + \text{ee}_p)$. ^c The ee was determined using chiral HPLC and chiral GC. ^d The activity for substrate **1a** was taken to be 100%. The relative activity for Oxd B was determined on a 500 μ L scale using 100 mM KPB buffer (pH 6.0), 1 U Oxd B, 20 mM substrate, 0.05 mM FMN, and 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ at 30 $^\circ\text{C}$ for 2 h ($n = 2$).

which may be caused by the lower solubility of the substrate in water. For aliphatic substrates (Table 1, **1k** and **1l**), Oxd B exhibited a deterioration in the reaction selectivity.

To establish an efficient catalytic system for aliphatic substrates, another two aldoxime dehydratases obtained from

Pseudomonas chlororaphis B23 (Oxd A)^{54,55} and *Rhodococcus* sp. N-771 (Oxd RE),^{45,56} were selected and investigated using a range of different substrates. They were found to display higher activity toward aliphatic substrates when compared with aromatic substrates in the aldoxime dehydration reaction.

Prior to investigating the substrate scope, optimization of the reductant was also carried out. Oxd A showed the best activity with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), which was ~ 79 -fold higher than that observed in the control experiment performed in the absence of a reductant (Fig. S7†). Oxd RE preferred sodium sulfide (Na_2S), which exhibited a ~ 8.2 -fold higher activity than the control (Fig. S3†). The optimal reductant concentration for Oxd A and Oxd RE were 4 mM (Fig. S8†) and 1 mM (Fig. S4†), respectively. Upon monitoring the asymmetric ring-opening reaction of 5-phenyl-4,5-dihydroisoxazole with time, Oxd A could catalyse the reaction in 83% yield over 3 h, but gave a nearly racemic product (Fig. S9†). The same result was also obtained using Oxd RE, the product was formed in 28% yield and 53% ee at pH 6.0, and 48% yield and 55% ee at pH 7.0 over 2 h, respectively (Fig. S5†). Our substrate specificity investigations show that Oxd A exhibited very poor enantioselectivity toward the substrates studied, though the conversion is high (Table 1). Oxd RE showed relatively better results toward aliphatic substrates when compared to aromatic substrates. In addition, it also exhibited slightly better enantioselectivity than Oxd B in the asymmetric ring-opening of aliphatic substrates (Table 1; **1k** and **1l**). Interestingly, Oxd A and Oxd RE exhibited different selectivity toward *ortho*-substituted aromatic substrates when compared with Oxd B (Table 1; **1b** and **1e**). What causes this difference in selectivity compared with other substrates and Oxd B is still not clear.

The kinetic parameters for both Oxd B and Oxd RE were determined under the optimized reaction conditions (Table S2†). The turnover number of Oxd B reached 11 s^{-1} with a specific activity of 18 U mg^{-1} , which is about one third of the activity observed for (*Z*)-phenylacetaldoxime dehydration (53 U mg^{-1}) using the assay method containing $\text{Na}_2\text{S}_2\text{O}_5$ (ESI, section 3†). However, the Oxd RE activity was lower than Oxd B at 4 U mg^{-1} with a turnover number of 3 s^{-1} , $\sim 15\%$ of the activity observed for the (*Z*)-phenylacetaldoxime dehydration reaction (30 U mg^{-1}). In addition, the catalytic efficiency of Oxd B ($K_{\text{cat}}/K_{\text{m}} = 3.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) is ~ 9 -fold higher than Oxd RE ($K_{\text{cat}}/K_{\text{m}} = 4.3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$). An obvious substrate inhibition effect was observed using 1 U of the enzyme upon increasing the substrate concentration to 30 mM for Oxd B and 6 mM for Oxd RE, but no any effect on the optical purity of the product (Fig. S2 and S6†). Therefore, a scale-up reaction was performed based on a substrate concentration of 20 mM and 1 U Oxd B. Under the optimal conditions, the reaction concentration could reach 150 mM with the product formed with 50% conversion and 99% ee (Table S1†).

To elucidate the reaction mechanism, a homology model of Oxd B was established based on the crystal structure of Oxd-RE.⁵⁸ In addition, the corresponding residues located in the catalytic pocket of the Heme were selected for an alanine-scanning study (Fig. 3a). In this scanning study, L128A, T202A,



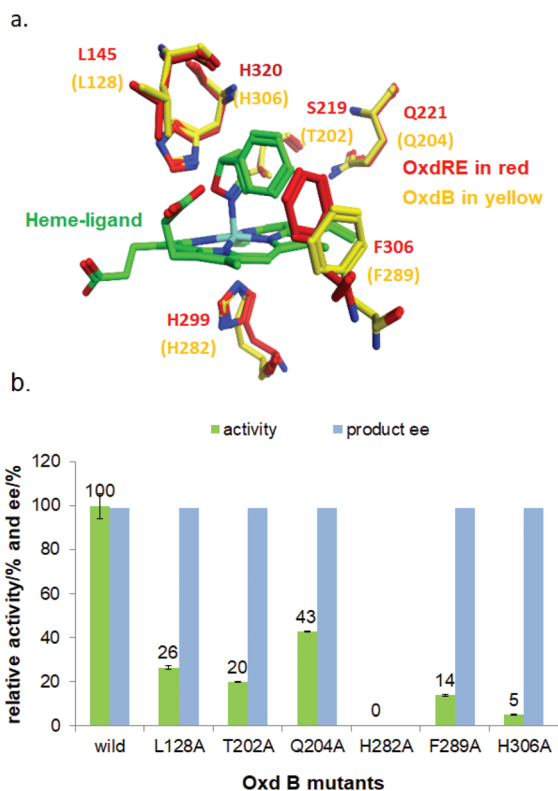
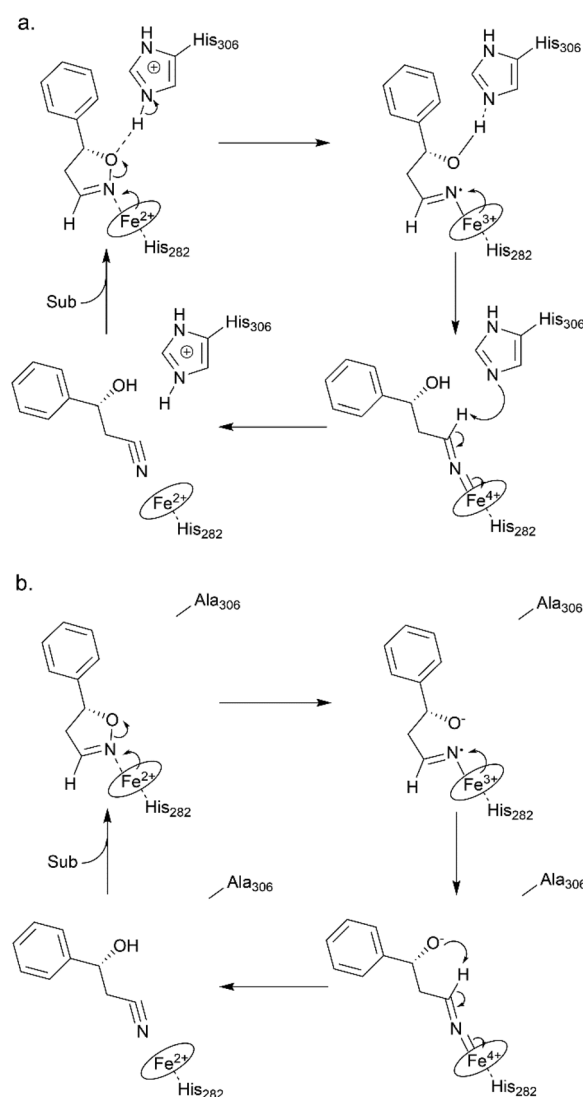


Fig. 3 (a) Oxd B homology model and superposition to Oxd RE structure docking with substrate **1a**; (b) Comparison of the Oxd B mutants' activities for substrate **1a**. The reactions were performed on a 500 μ L scale using 100 mM KPB buffer (pH 6.0), 2 μ g of Oxd-B mutants, 20 mM 5-phenyl-4,5-dihydroisoxazole, 0.05 mM FMN, and 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ at 30 $^\circ\text{C}$ for 10 min. The yield and ee were determined using chiral HPLC ($n = 2$).

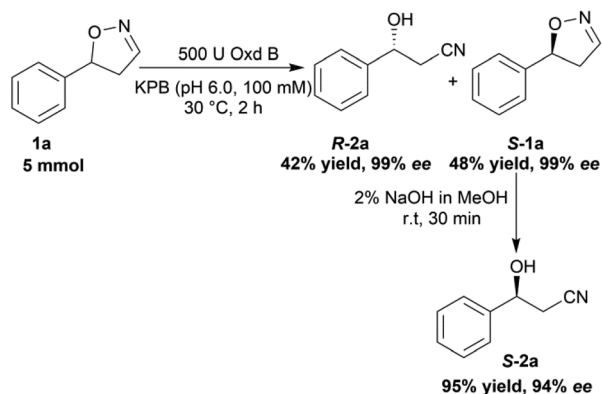
Q204A, H282A, F289A, and H306A mutants were constructed *via* site-mutagenesis (ESI, section 7 \ddagger), which were purified as the wild type of Oxd B to obtain the pure enzyme (ESI, section 2.2 \ddagger) and used for activity comparison. To begin with, the activity of the mutants toward (Z)-phenylacetaldoxime was assayed. As reported in previous publication, no activity was detected for the T202A, H282A, and H306A mutants, which are corresponding to the S219, H299, and H320 residues in Oxd RE, the crucial residues in the dehydration of butyraldehyde oxime.³³ These results reveal that the homology model of Oxd B is workable to some extent, although the protein sequence homology between Oxd B and Oxd RE is only 31%. After verifying the reliability of the homology model, the mutants were used in the 5-phenyl-4,5-dihydroisoxazole ring-opening reaction. As expected, the H282A mutant showed no activity toward the substrate because the proximal axial residue ligating to iron is thought to affect the redox potential of the Heme, thus leading to a great difference in the catalytic activity for bond cleavage.^{59–61} Surprisingly, the activity of the mutants was determined to be 20% for T202A and 5% for H306A when compared with wild-Oxd B (Fig. 3b), which is similar to that reported in the Kemp elimination reaction.³³ To confirm the

transformation in mutant of H306A, the amount of Oxd B-H306A was increased 10-fold, and the product could be obtained in 3.8% yield and 99% ee over 10 min, 21% yield and 95% ee in 1 h, respectively. This implied a discriminating catalytic mechanism between the ring-opening of dihydroisoxazole and aldoxime dehydration reactions. It seems that the ring-strain in dihydroisoxazole could facilitate the cleavage of the N–O bond *via* redox of ferrous-Heme without the assistance of the other residues (T202 and H306 in the (Z)-phenylacetaldoxime dehydration reaction), even though its catalytic efficiency was slower than that observed under the assistance of the other residues. In particular, weak activity was detected for H306A, which is thought to play an important role in the next deprotonation step to form the nitrile triple-bond (Scheme 4a). This suggests that in addition to the efficient deprotonation step by histidine (H306), it may have another route for deprotonation, which we refer to as the oxygen anion deprotonation



Scheme 4 Proposed reaction mechanism for Oxd B-wild (a) and Oxd B-H306A (b).





Scheme 5 Preparative scale reaction using substrate **1a** with Oxd B. The reaction was performed on a 50 mL scale using 100 mM KPB buffer (pH 6.0), 500 U Oxd B, 100 mM 5-phenyl-4,5-dihydroisoxazole, 0.25 mM FMN, and 5 mM Na₂S₂O₅ at 30 °C for 2 h. The isolated yield was calculated after purification by silica gel column chromatography and the ee was determined by chiral HPLC.

pathway.⁴¹ After ring-opening upon attack of the ferrous-Heme, the resulting oxygen anion will approach the hydrogen atom of H–C=N– (via single bond rotation) followed by deprotonation and electron transfer back to ferric-Heme to produce the active state of ferrous-Heme, which undergoes the next catalytic cycle (Scheme 4b). We also noticed that the product ee did not change significantly in the presence of any mutants. This reveals the cooperative control of the enantioselectivity with the surrounding residues of the Heme, not only from certain residues in the catalytic pocket.

To verify the practicability of this novel method in organic synthesis, a preparative scale reaction of 5-phenyl-4,5-dihydroisoxazole (**1a**) was performed at 30 °C using 5 mmol of substrate, 0.25 mM FMN, 500 U Oxd B (see the activity assay method in ESI, section 3[†]), and 5 mM Na₂S₂O₅ in 50 mL KPB (pH 6.0, 100 mM) stirred slowly for 2 h. After purification by silica gel column chromatography, (*R*)-3-hydroxy-3-phenylpropanenitrile (**R-2a**) was isolated in 42% yield and 99% ee, and (*S*)-5-phenyl-4,5-dihydroisoxazole (**S-1a**) was isolated in 48% yield and 99% ee. The obtained (*S*)-5-phenyl-4,5-dihydroisoxazole (**S-1a**) was transformed into (*S*)-3-hydroxy-3-phenylpropanenitrile (**S-2a**) in 95% yield and 94% ee upon treatment with 2% NaOH methanol solution (10 mL) for 30 min (Scheme 5).

Conclusion

In summary, by combination of cyanide-free synthesis of chiral nitriles and the Kemp elimination reaction, we have achieved the first enzymatic reaction to synthesize chiral β-hydroxy nitriles from alkenes via the key step of asymmetric ring-opening of dihydroisoxazoles catalysed by aldoxime dehydratase with broad substrate scope and excellent enantioselectivity (up to 99% ee). The reaction can be carried out with a good turnover number (k_{cat} up to 11 s^{−1}) and high catalytic efficiency ($k_{\text{cat}}/k_{\text{m}}$ up to 3.7 × 10⁴ M^{−1} s^{−1}). Using site-directed

mutagenesis analysis, the reaction was found to mostly occur in the Heme-containing catalytic pocket involving residues H282 and H306. The proximal histidine ligated to the Heme plays a very important role in improving the redox potential of ferrous-Heme, which ensures its reductive ability to open the N–O bond. Without the assistance of any other residues, the N–O bond can be also slowly opened via its redox reaction with ferrous-Heme. Two kinds of deprotonation pathways (histidine deprotonation and oxygen anion deprotonation) seem to exist in the catalytic process, which both contribute to the formation of the nitrile triple-bond.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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