Organic Process Research & Development

Article

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Development of an Immobilized Ketoreductase for Enzymatic (*R*)-1-(3,5-Bis(trifluoromethyl)phenyl)ethanol Production

Hongmei Li,* Johannah Moncecchi, and Matthew D. Truppo

Department of Process Chemistry, Merck Research Laboratories, Merck & Co., Inc., P.O. Box 2000, Rahway, New Jersey 07065, United States

ABSTRACT: The development of an immobilized ketoreductase via covalent binding on resin EC-HFA has demonstrated that it is highly active and stable in organic solvents and can be recycled and reused many times in both batch mode and flow reactor mode. (*R*)-1-(3,5-Bis(trifluoromethyl)phenyl)ethanol, a key chiral intermediate in the synthesis of EMEND, was synthesized in 98% yield with >99% ee using the immobilized ketoreductase in a 50% hexanes/40% IPA/10% water solvent system. The immobilized ketoreductase has also been applied to the synthesis of various chiral alcohols.

INTRODUCTION

Enzymes are naturally renewable, biodegradable, and sustainable catalysts, and biocatalysis has emerged as an important technology for meeting the growing demand for green and sustainable chemicals manufacture in the synthesis of flavours, fragrances, and other fine chemicals.[1]Particularly, advances in protein engineering technology have enabled biocatalysis to become a preferred approach for the synthesis of enantiomerically pure compounds in the pharmaceutical industry because of its excellent chemoselectivity, regioselectivity, and enantioselectivity.[2] The reduction of prochiral ketones to chiral alcohols is an excellent way to introduce enantioselectivity into the synthesis of a molecule, as the enantioenriched alcohol functionality can be easily transformed into other useful functional groups without racemization.[3] Many ketoreductases (KREDs) are now commercially available for the synthesis of chiral alcohols in good yields with excellent selectivity (often >99% ee).[4,5]They generally operate under mild reaction conditions and obviate the need for an ee upgrade, which is often required with less selective metal-catalyzed transfer hydrogenation approaches. Over the past few years the application of KREDs to the synthesis of chiral alcohols has been demonstrated on industrial scale.[6,7] The ketoreductase reaction is typically run in an aqueous buffer system and requires the cofactor nicotinamide adenine dinucleotide phosphate (NAD(P)H). The most efficient and cost-effective cofactor recycling approach is the cosubstrate approach, in which isopropanol (IPA) is used as a hydride source to recycle the nicotinamide cofactor by the same KRED catalyst employed for the reduction of the carbonyl substrate.[8,9] Despite many advantages and recent successes in biocatalysis, the use of enzymes is often hampered by a lack of long-term operational stability due to low substrate solubility in the aqueous reaction system coupled with poor enzyme stability in commonly used organic solvents and to difficulties in recovery and reuse of the enzyme. Immobilization of enzymes provides one of the most attractive approaches to overcome these drawbacks.[10−12] We recently reported the successful immobilization of a transaminase that is capable of operating in organic solvent for many reaction cycles.[13] A variety of immobilized hydrolases are also widely commercially available and used.[14] However, immobilization of ketoreductases still remains challenging. To our knowledge, only few examples of immobilization of ketoreductases have been reported.[15−17] One example uses a ketoreductase that was immobilized by physical deposition on a nonporous glass support, which was used in a continuous gas-phase reactor without solvents.[16] Yeast alcohol dehydrogenase was reported to be immobilized covalently on Fe_3O_4 magnetic nanoparticles.[17] The limitation of KRED immobilization work done to date is that the immobilized ketoreductases did not tolerate operation in organic solvents. Here we report the first demonstration of an immobilized ketoreductase via covalent binding that is active and stable in organic solvents and can be recycled and reused.

RESULTS AND DISCUSSION

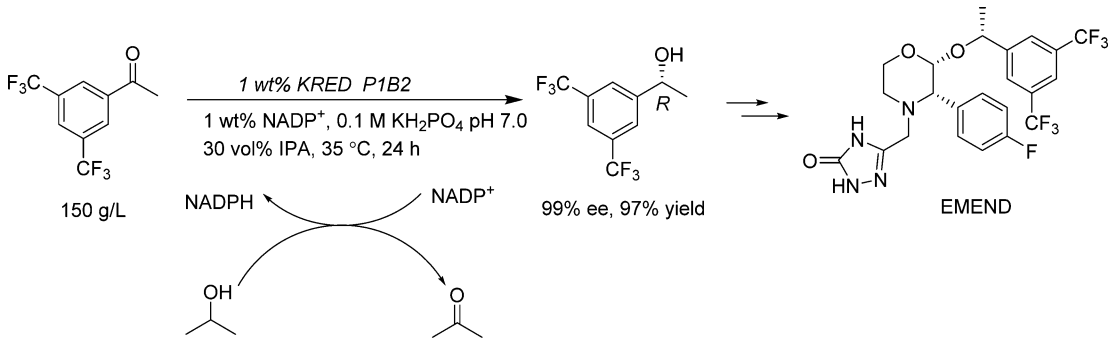
Both enantiomers of 1-(3,5-bis(trifluoromethyl)phenyl)ethanol are pharmaceutically important alcohol intermediates for the synthesis of NK-1 receptor antagonists. The enzymatic synthesis of (*S*)-1-(3,5-bis(trifluoromethyl)phenyl)ethanol was demonstrated with an enzyme isolated from *Rhodococcus erythropolis* with excellent ee (>99%) and good conversion (>98%).[18] (*R*)-1-(3,5-Bis(trifluoromethyl)phenyl)ethanol is currently incorporated into the orally active NK1 receptor antagonist (EMEND) for chemotherapy-induced emesis and has been synthesized by ruthenium-catalyzed transfer hydrogenation or oxazaborolidine-catalyzed borane reduction with 91−93% ee, requiring an ee upgrade to >99% via a recrystallization.[19,20]We were pleased to find that this important chiral intermediate (*R*)-1-(3,5-bis(trifluoromethyl)phenyl)ethanol can be synthesized with the commercially available ketoreductase P1B2 from Codexis (Scheme 1). After thorough reaction optimization, the optimized aqueous reaction conditions for the ketone reduction were found to be 150 g/L 3,5-bis(trifluoromethyl)acetophenone, 1 wt % lyo KRED P1B2, and 1 wt % $NADP^+$ in 30% (v/v) IPA and 70% (v/v) 0.1 M potassium phosphate buffer (pH 7) at 35 °C. The reaction produces the desired (*R*)-alcohol product with 98−99%

Special Issue: Sustainable Chemistry

Received: October 13, 2014

DOI: 10.1021/op5003215
Org. Process Res. Dev. XXXX, XXX, XXX−XXX

Scheme 1. Enzymatic reduction of EMEND ketone with ketoreductase P1B2



conversion and >99% ee. A drawback of the aqueous reaction system, which is common to many aqueous enzymatic reaction systems, is the need for an extractive reaction workup to isolate the product away from the enzyme catalyst. The extractive workup results in denatured enzyme that cannot be reused and that has the possibility to generate an emulsion layer that can complicate the product isolation. The desire to eliminate these issues led us to evaluate the immobilization of ketoreductase P1B2 with several polymer-based resins.[21] The resins selected include three epoxide-functionalized supports for covalent immobilization (EC-EP, EC-HFA, and EXE119) and two adsorption supports for immobilization through hydrophobic interactions (EXE120 and HP2MG).[21]

The lyophilized ketoreductase P1B2 and cofactor $NADP^+$ were dissolved in buffer and incubated with each resin at 5 °C for 48 h. The resins were then recovered, washed with buffer to remove unbound enzyme, and dried under vacuum with a nitrogen sweep at room temperature. Each resin was then evaluated for activity on the 1-(3,5-bis(trifluoromethyl)phenyl)-ethanone substrate in primarily organic solvent systems (90% IPA and 10% buffer). All of the immobilized enzyme preparations showed activity in the 90% IPA system, with several examples reaching >95% conversion within 24 h (Figure 1).

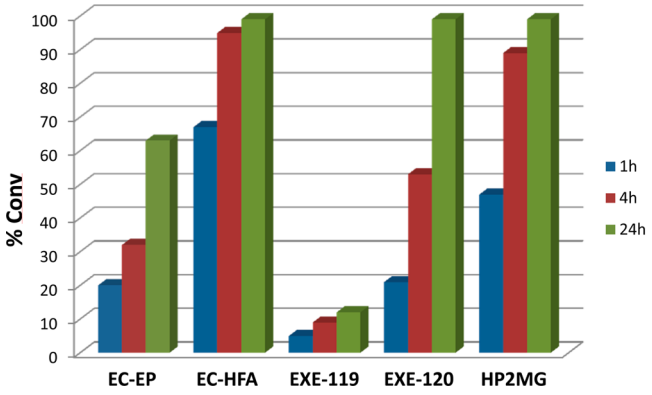


Figure 1. Conversion vs time for ketoreductase P1B2 immobilized on each resin. Reaction conditions: 50 g/L ketone and 200 g/L immobilized ketoreductase in 90% IPA and 10% buffer at 30 °C.

Immobilization on EC-HFA resin provided the highest activity, with over 60% conversion in the first hour. EC-HFA is an amino−epoxy-functionalized polymethacrylate resin, and the enzyme is immobilized on it through covalent binding to surface protein lysine residues (Figure 2). While the soluble lyophilized KRED P1B2 can tolerate up to 60% IPA cosolvent, the enzyme stability drops dramatically with increasing levels of

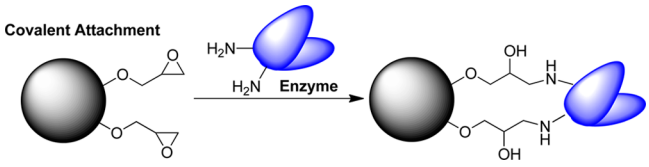


Figure 2. Illustration of enzyme immobilization through covalent binding.[22]

organic solvent, and the reaction fails in 90% IPA. We further optimized the immobilization conditions to provide for maximum activity and stability. We found that increasing the incubation temperature to room temperature provided for a shortened incubation time of 6 h. Washing the resin with buffer solution containing the $NADP^+$ cofactor helped increase the activity of the immobilized enzyme. The active enzyme loading on the EC-HFA resin was estimated to be ∼5 wt % on the basis of an activity comparison with the lyophilized enzyme. The ketoreductase P1B2 immobilized on EC-HFA resin via covalent binding was chosen and investigated further for activity and stability.

To study the long-term operational stability of the immobilized enzyme, the activity and stability of immobilized ketoreductase P1B2 in various solvents were investigated, using IPA as a cosubstrate for cofactor regeneration. The use of buffer was found to be unnecessary, with water providing similar or better performance than buffer under the same reaction conditions. This is an exciting result for immobilized ketoreductase since aqueous buffer conditions are essential and required to retain the activity and stability for general enzymatic reactions. It was found that the level of water content in the organic solvent system is crucial for the activity of the immobilized ketoreductase. As shown in Figure 3, when no water was added, the immobilized ketoreductase showed minimal reactivity, with <10% conversion after 2 h. However, with the addition of only 2% water, the conversion jumped to 40% in 2 h. Good activity (70% conversion in 2 h) was observed in IPA with a water concentration of 10%. In addition to IPA used for cofactor regeneration, additional cosolvents were studied. The immobilized KRED was found to be active in a wide variety of organic solvents. Hexanes, toluene, methyl *tert*-butyl ether (MTBE), methyltetrahydrofuran (Me-THF), and cyclopentylmethyl ether (CPME) as cosolvents (with a cosolvent/IPA/water ratio of 50/40/10) all gave activities and conversions comparable to those under 90/10 IPA/water conditions (Figure 3). A study of the effect of reaction temperature showed that 40 °C is the optimal reaction temperature with the highest conversion. The conversion slightly decreased when the temperature was increased to 60 °C (Figure 4). Reduction of 1-(3,5-bis(trifluoromethyl)phenyl)ethanone using P1B2 immobilized

DOI: 10.1021/op5003215
Org. Process Res. Dev. XXXX, XXX, XXX−XXX

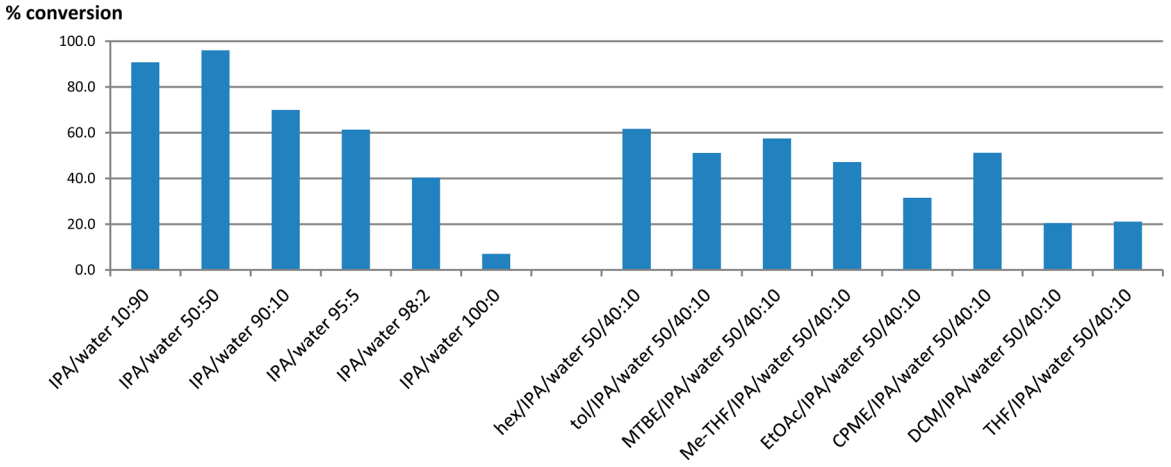


Figure 3. Immobilized ketoreductase performance in different organic solvents and water concentrations. Reaction conditions: 50 g/L ketone and 100 g/L KRED immobilized on EC-HFA resin at 30 °C for 2 h.

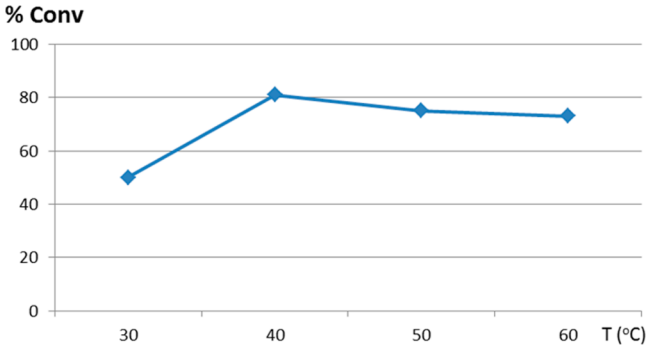


Figure 4. Conversion as a function of reaction temperature. Reaction conditions: 50 g/L ketone and 100 g/L KRED immobilized on EC-HFA resin in 90% IPA and 10% water for 2 h.

on EC-HFA resin was demonstrated on a 50 g scale in batch mode under the following reaction conditions: 50 g/L ketone and 100 g/L immobilized P1B2 in 50/40/10 hexanes/IPA/water at 40 °C. The reaction gave the desired (*R*)-1-(3,5-bis(trifluoromethyl)phenyl)ethanol with 99% conversion, 98% yield and >99% ee.

With the optimized reaction conditions in hand, the stability of the immobilized ketoreductase was evaluated through recycling for multiple rounds. The enzyme was found to be quite stable in the 90% IPA and 10% water system. Although the initial rate of the reaction (1 h) decreased slightly after the first three rounds of recycling, the reaction rate over the next 7 rounds remained consistent. In total, we demonstrated 10 consecutive recycles of the immobilized ketoreductase over a 200 h time period (Figure 5). Tight binding of the $NADP^+$ cofactor to the immobilized KRED obviated the need for cofactor addition at each round, further demonstrating the efficiency of the reaction system.

The immobilized KRED P1B2 was also tested in a continuous reaction process. Immobilized ketoreductase (1 g) was packed in a plug flow reactor (PFR) (6 cm length and 5 mL volume). A 50 g/L ketone solution in 90/10 IPA/water was flowed through the reactor via syringe pump, and the alcohol product was collected in a flask from the other end. A plot of reaction conversion versus residence time is shown in Figure 6. A residence time of 4 h gave 93% conversion, and a residence time of 10 h gave 98% conversion. After 7 days of holding the PFR at 40 °C, we were pleased to find that the immobilized ketoreductase P1B2 still retained 94% of its initial activity.[23]

Finally, we demonstrated the generality of this immobilized ketoreductase in the synthesis of several chiral alcohols starting from their prochiral ketones in batch mode, as shown in Table 1.[24] The ability to perform immobilized ketoreductase reactions in organic solvent (90% organic solvent and 10% water) has significant advantages compared with running reactions with soluble lyophilized enzyme in aqueous systems. Aqueous reactions typically require the use of buffer to stabilize the reaction pH in order to keep the enzymes active. Aqueous soluble

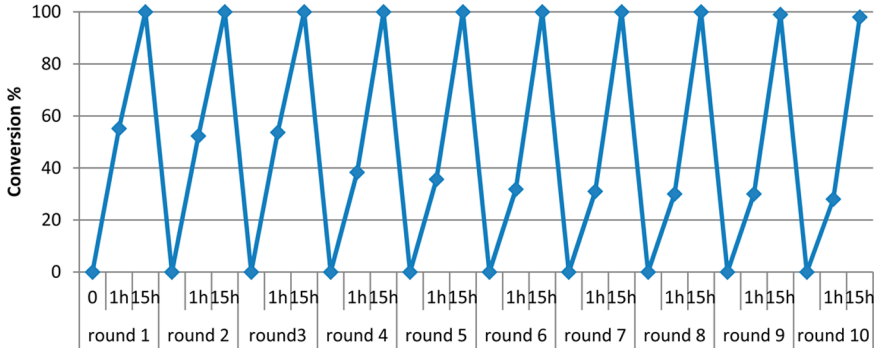


Figure 5. Demonstration of reuse of immobilized ketoreductase for 10 rounds. Reaction conditions: 50 g/L ketone and 100 g/L KRED immobilized on EC-HFA resin in 90% IPA and 10% water at 30 °C.

DOI: 10.1021/op5003215
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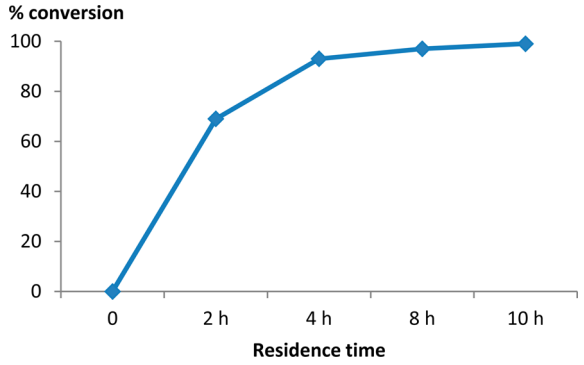
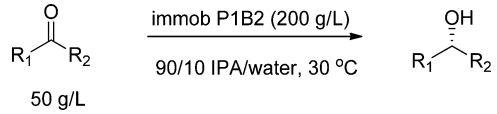
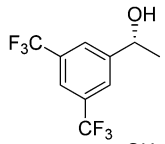
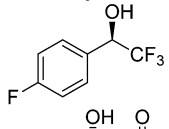
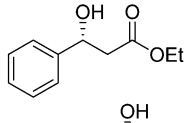
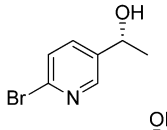
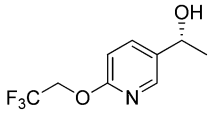
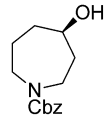
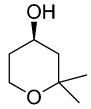


Figure 6. Test of immobilized KRED P1B2 in a continuous reaction process. Conditions: plug flow reactor, 50 g/L ketone, 90% IPA and 10% water, 40 °C.

Table 1. Synthesis of chiral alcohols from ketones using immobilized ketoreductase[24]



Entry	Alcohol products	% Conv.	% ee	Absolute Conf.
1		100	99	(R)
2		100	93	(R)
3		100	>99	(R)
4		100	>99	(R)
5		90	>99	(R)
6		100	96	(R)
7		92	86	(R)

Reaction conditions: 50 g/L ketone and 200 g/L immobilized ketoreductase P1B2 in 90% IPA and 10% water at 30 °C for 20 h. Reaction scale: 500 mg of ketone, reaction volume of 10 mL.

enzyme reaction systems typically require an extractive workup in order to recover and isolate the desired product. Extractive workups denature the enzyme, rendering it inactive, and can potentially complicate the isolation because of the formation of an emulsion layer. The immobilized enzyme reaction in organic solvent greatly simplifies the workup to a simple filtration and, most importantly, enables the enzyme catalyst to be reused over multiple rounds, maximizing the utility of enzymes.

CONCLUSION

The first example of an immobilized ketoreductase capable of operating in organic solvent has been developed and demonstrated. Ketoreductase P1B2 was immobilized on a polymer-based resin via covalent binding, and this catalytic system was applied to the synthesis of (*R*)-1-(3,5-bis(trifluoromethyl)-phenyl)ethanol, a key chiral intermediate in the synthesis of EMEND. The immobilized enzyme exhibited excellent selectivity and conversion and demonstrated that it could be reused multiple times both in batch mode and in a continuous plug flow reactor. Furthermore, this immobilized ketoreductase was shown to be a generally useful tool for the rapid synthesis of various chiral alcohols from ketones.

EXPERIMENTAL SECTION

Reagents and Enzymes. The ketoreductase enzyme P1B2 was purchased from Codexis. The resins used for immobilization were purchased from Resindion or Chiralvision.

Analysis of the extent of conversion was performed by reversed-phase HPLC using an Agilent HPLC system with an Ascentis Express C18 column (2.7 μm, 100 mm × 4.6 mm) with mobile phase A (water with 0.1 vol % H_3PO_4) and mobile phase B (acetonitrile) in a gradient from 90/10 A/B to 10/90 A/B over 5 min and then holding at 10/90 A/B for 2 min. UV absorbance was monitored at 210 nm. Both ketone substrates and alcohol products were readily visible with this method, and the ee was determined by chiral HPLC or supercritical fluid chromatography (SFC) using the conditions listed below. Optical rotation was measured on a PerkinElmer model 341 polarimeter.

Preparation of (*R*)-1-(3,5-Bis(trifluoromethyl)phenyl)-ethanol from Lyo Ketoreductase P1B2. A mixture of 0.1 M KH_2PO_4 (pH 7.0) buffer solution (140 mL), enzyme P1B2 (300 mg), and cofactor NADP (300 mg) was gently dissolved to form a solution at rt, and the solution was heated to 30 °C. 1-(3,5-Bis(trifluoromethyl)phenyl)ethanone (30 g) in IPA (60 mL) was added via an addition funnel over ∼60 min while the temperature was kept at <35 °C. After the addition was finished, the reaction mixture was aged at 35 °C for 24 h until 99% conversion by HPLC was reached. The reaction mixture was cooled to rt, and MTBE (150 mL, 5 vol) was added. The mixture was allowed to settle for 30 min for layer separation. The layers were cut, and the emulsion between the layers was cut to the aqueous phase, after which the aqueous layer was extracted with additional MTBE (90 mL, 3 vol). The combined organic layers were washed with brine (150 mL, 5 vol). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure (40 °C, 100 Torr), and the alcohol product was obtained as an oil (29.2 g, 97% yield, >99% ee).

General Enzyme Immobilization Procedure for Resin Screening. A 25 g/L solution of ketoreductase P1B2 from Codexis was made in 100 mM potassium phosphate buffer (pH 7.0) containing 2 g/L NADP. Immobilization resin (20 g) was added to 10 mL of the enzyme solution, and the mixture was placed on a shaking incubator at room temperature for 24 h. The resin was then filtered away from the enzyme solution and rinsed four times with 10 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 2 g/L NADP cofactor. The immobilized enzyme resin was then dried under a nitrogen sweep for 2 h.

DOI: 10.1021/op5003215
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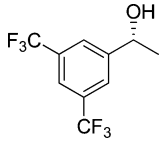
Optimized Enzyme Immobilization Procedure for EC-HFA Resin. A 100 g/L solution of ketoreductase P1B2 from Codexis in 100 mM potassium phosphate buffer (pH 7.0) containing 2 g/L NADP was made. Then 20 g of EC-HFA resin (Chiralvision) was added to 10 mL of the enzyme solution, and the mixture was placed on a shaking incubator at room temperature for 6 h. The resin was then filtered away from the enzyme solution and rinsed four times with 10 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 2 g/L NADP cofactor. The immobilized enzyme resin was then dried under a nitrogen sweep for 2 h. The typical water content of the immobilized resin was 2−5 wt %. The immobilization yield was ∼98% (immobilization yield = 100% × [enzyme added (units/g of carrier) − unbound enzyme (units/g of carrier)]/[enzyme added (units/g of carrier)]). The active enzyme loading on the EC-HFA resin was estimated to be ∼5 wt % on the basis of an activity comparison with the lyophilized enzyme.

General Immobilized Ketoreductase Reaction Protocol. The ketone substrate (50 g/L) was dissolved in 90% 2-propanol and 10% water, and 100 g/L immobilized ketoreductase P1B2 was added to the substrate solution. The reaction mixture was heated to 30−40 °C and stirred with an orbital shaker for 20 h. For immobilized ketoreductase reuse testing, the immobilized enzyme was filtered away from the reaction solution and rinsed with 3 volumes of 90/10 IPA/water to ensure removal of the starting material and product. The recovered KRED was then used directly in the subsequent reaction with no further manipulation.

Preparation of (*R*)-1-(3,5-Bis(trifluoromethyl)phenyl)-ethanol from Immobilized KRED P1B2. A mixture of P1B2 immobilized on EC-HFA resin (100 g), water (100 mL), IPA (400 mL), hexanes (500 mL), and 1-(3,5-bis(trifluoromethyl)-phenyl)ethanone (50 g) was stirred and heated to 40 °C and aged for 15 h. The reaction gave >99% conversion by HPLC. The immobilized enzyme was filtered off and rinsed with 95/5 IPA/water (200 mL). The combined fitrate was concentrated and flashed with IPA to remove water under reduced pressure (40 °C, 100 Torr), and finally the clean alcohol product was obtained as an oil (32 g, 92 wt % by NMR, 98% yield, >99% ee). The recovered immobilized enzyme could be reused several times.

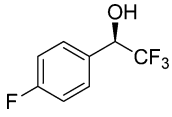
NMR Data and Chiral Methods for Table 1.

Entry 1.



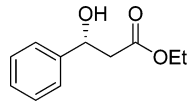
Normal-phase HPLC with a Chiralcel OD-H column, 98% hexanes/2% 2-propanol, 1 mL/min, 10 min run.[18] ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.53 (d; J = 6.51 Hz; 3H); 2.19 (s; 1H); 5.02 (q; J = 6.50 Hz; 1H); 7.78 (s; 1H); 7.83 (s; 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.5, 69.2, 121.2 (d), 125.6 (d), 119.3−127.4 (q), 131.2−132.2 (q), 148.2 ppm. $[\alpha]_{589}^{25}$ +23.6 (c 1.0, DMF).

Entry 2.



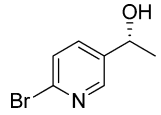
Normal-phase HPLC with a Chiralcel OD-H column, 95% heptane/5% EtOH, 40 °C, 1 mL/min, 12 min run. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.78 (br s; 1H); 5.01 (q; J = 6.64 Hz; 1H); 7.12−7.08 (m; 2H); 7.47−7.44 (m; 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 71.7−72.6 (q), 115.5−115.8 (d), 122.7−129.3 (q), 129.3 (d), 129.8, 162.2−164.6 (d) ppm. $[\alpha]_{589}^{25}$ +51.1 (c 1.0, DMF).

Entry 3.



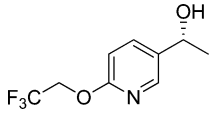
SFC with an AD-H column, iso 5% EtOH/25 mM isobutylamine/CO_2, 8 min run, 2 mL/min, 35 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.28 (t; J = 7.15 Hz; 3H); 2.81−2.69 (m; 2H); 3.31 (s; 1H); 4.20 (q; J = 7.14 Hz; 2H); 5.15 (dd; J = 8.70, 4.07 Hz; 1H); 7.32−7.28 (m; J = 7.70 Hz; 1H); 7.40−7.36 (m; 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 43.3, 60.9, 70.3, 125.7, 127.8, 128.5, 142.5, 172.4 ppm. $[\alpha]_{589}^{25}$ −11.6 (c 1.0, DMF).

Entry 4.



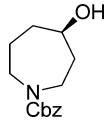
Normal-phase HPLC with a Chiralpak OD-H column, 95% Hex/5% EtOH, 40 °C, 1 mL/min, 210 nm, 15 min run. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.48 (d; J = 6.50 Hz; 3H); 4.90 (q; J = 6.51 Hz; 1H); 7.44 (d; J = 8.24 Hz; 1H); 7.59 (dd; J = 8.21, 2.45 Hz; 1H); 8.32 (s; 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.3, 67.3, 127.9, 136.0, 140.5, 140.8, 147.8 ppm. $[\alpha]_{589}^{25}$ +47.7 (c 1.0, DMF).

Entry 5.



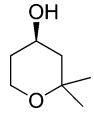
SFC with an AD-H column, isocratic 5% EtOH with 25 μM isobutylamine, 2 mL/min, CO_2 200 psi, 40 °C, total 8 min run. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.41 (q; J = 7.99 Hz; 2H); 4.88 (q; J = 6.52 Hz; 1H); 7.31−7.26 (m; 2H); 8.28 (d; J = 2.58 Hz; 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.2, 66.2−66.6 (q), 68.8, 120.3, 123.5, 136.1, 152.8, 157.5 ppm. $[\alpha]_{589}^{25}$ +40.5 (c 1.0, DMF).

Entry 6.



SFC with an AD-H column, gradient 4−40% EtOH with 25 μM isobutylamine in 6 min, hold 2 min, 2 mL/min, CO_2 200 psi, 40 °C, total 8 min run. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.93 (1.60−2.05; m; 6H); 3.44−3.30 (m; 2H); 3.58−3.47 (m; 2H); 3.88−3.86 (m; 1H); 5.13 (d; J = 1.87 Hz; 2H); 7.34 (t; J = 4.42 Hz; 5H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.9−22.3, 35.1−35.3, 37.1−37.3, 41.0−41.4, 46.2−46.4, 67.0, 70.4−70.6, 127.8, 127.9, 128.5, 136.9, 156.2 ppm. $[\alpha]_{589}^{25}$ −10.1 (c 1.0, DMF).

Entry 7.



Chiral GC with a Beta-Dex-SA column (30 m, 0.25 mm ID, 0.25 um), heater 220 °C, H_2 flow 40 mL/min, air flow

DOI: 10.1021/op5003215
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400 mL/min, makeup flow (He) 40 mL/min, constant pressure 12 psi, split 50:1, oven 170 °C hold 8 min, total 8 min run. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.18 (s; 3H); 1.25 (s; 3H); 1.28−1.31 (m; 1H); 1.40−1.52 (m; 1H); 1.88−1.82 (m; 2H); 3.61 (t; J = 12.11 Hz; 1H); 3.80 (dd; J = 12.29, 4.40 Hz; 1H); 4.00−3.88 (m; 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 23.1, 31.1, 35.6, 45.8, 60.0, 65.3, 72.8 ppm. $[\alpha]_{589}^{25}$ −20.1 (c 1.0, DMF).

AUTHOR INFORMATION

Corresponding Author

*E-mail: hongmei06@gmail.com.

Notes

The authors declare no competing financial interest.

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(23) The remaining activity of 94% for the immobilized enzyme (stability study) was determined from the conversion in ketone reduction with the immobilized enzyme after it was packed for 7 days at 40 °C in the PFR versus the conversion with the immobilized enzyme packed fresh in the PFR under the same reaction conditions and residence time.

(24) In Table 1, the ee values of the alcohol products with immobilized P1B2 are the same as or similar to those with the lyo enzyme P1B2 reaction system. The ee values in entries 6 and 7 could be lower or higher depending on the enzyme loading for both the immobilized and lyo enzyme systems.

DOI: 10.1021/op5003215
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