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Development of a Biocatalytic Process as an Alternative to the (−)-DIP-Cl-Mediated Asymmetric Reduction of a Key Intermediate of Montelukast

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Abstract:

A KetoREDuctase (KRED) engineered via directed evolution technologies catalyzed the asymmetric reduction of (*E*)-methyl 2-(3-(3-(2-(7-chloroquinolin-2-yl)vinyl)phenyl)-3-oxopropyl)benzoate to the corresponding (*S*)-alcohol, a key intermediate in the synthesis of montelukast sodium (Singulair). Through synergistic efforts in process chemistry, molecular biology, bioinformatics and high throughput screening, a KRED with very high enantioselectivity (>99.9% ee) was developed for an economical and simple process that takes advantage of the physical properties of the substrate and product. The evolved KRED is an efficient and robust enzyme for catalyzing the reaction of an essentially water insoluble substrate (*c* log *P* ≈ 7) at a 100 g/L loading in the presence of ~70% organic solvents at 45 °C. The biocatalytic process currently runs at >200 kg scale.

Introduction

Montelukast sodium (Singulair) is a leukotriene receptor antagonist developed by Merck to control the symptoms of asthma and allergies (Figure 1).[1] Although its structure and consequently its synthesis is complex, montelukast sodium has only a single stereocenter, which Merck installed via a (−)-*B*-chlorodiisopinocampheylborane (i.e., (−)-DIP-Cl or (−)-Ipc_2BCl)-mediated asymmetric reduction of the bulky and highly functionalized ketone **1** (Scheme 1).[2,3] Perusing the patent literature revealed that the chiral alcohol (*S*)-**2** is also the intermediate used by many generic companies in their syntheses of montelukast.[4]

The presence of a variety of functional groups (labeled in red, Scheme 1) in the ketone **1** that are labile or sensitive to metal hydrides and/or hydrogenation/hydrogenolysis conditions precluded the deployment of most of the commonly used tools in the chemists' arsenal for enantioselective reduction of ketones.[5,6] In fact, until very recently, (−)-DIP-Cl was the only practical option.[7−9]

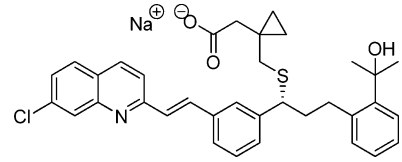

Figure 1. **Structure of montelukast sodium (Singulair).**

Despite the shortcomings associated with (−)-DIP-Cl, such as corrosivity and moisture sensitivity as well as poor atom economy, tedious workup, and heavy burden in the waste stream (at least 1.5−1.8 equiv is needed), (−)-DIP-Cl has been nevertheless successfully used in an industrial setting, primarily because it is a mild and selective *reagent*. It is desirable that a

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(1) (a) Belley, M. L.; Leger, S.; Labelle, M.; Roy, P.; Xiang, Y. B.; Guay, D. U.S. Patent 5,565,473, 1996. (b) Belley, M. L.; Leger, S.; Roy, P.; Xiang; Y. B.; Labelle, M.; Guay, D. European Patent EP 408,717, 1998. (c) King, A. O.; Corley, E. G.; Anderson, R. K.; Larsen, R. D.; Verhoeven, T. R.; Reider, P. J.; Xiang, Y. B.; Belley, M.; Leblanc, Y.; Labelle, M.; Prasit, P.; Zamboni, R. J. *J. Org. Chem.* **1993**, *58*, 3731.

(2) For a recent review of boron reagents in process chemistry, see: Burkhardt, E. R.; Matos, K. *Chem. Rev.* **2006**, *106*, 2617.

(3) Researchers at Merck cleverly took advantage of the asymmetric amplification effect in their preparation of (−)-DIP-Cl such that (−)-DIP-Cl prepared *in situ* from the cheaper 85% ee (+)-(α)-pinine gave the alcohol **2** in 94−97% ee (recrystallized to 99% ee). See ref 1c; also (a) Zhao, M.; King, A. O.; Larsen, R. D.; Verhoeven, T. R.; Reider, P. J. *Tetrahedron Lett.* **1997**, *38*, 2641. (b) King, A. O.-P.; Larsen, R. D.; Verhoeven, T. R.; Zhao, M. U.S. Patent 5,693,816, 1997.

(4) Many of the generic companies go through the alcohol **2** and then employ different strategies for installing/assembling the side chain. For example (U.S. or PCT Patent/Application that either explicitly uses **2** or cites the Merck process): (a) Ray, U. K.; Boju, S.; Pathuri, S. R.; Meenakshisunderam, S. (Aurobindo Pharma Limited, India). PCT Patent Application WO/2008/001213, 2008. (b) Wang, Y.; Wang, Y.; Brand, M.; Kaspi, J. (Chemagis Ltd., Israel). PCT Patent Application WO/2007/088545, 2007. (c) Turchetta, S.; Tuozzi, A.; Ullucci, E.; de Ferra, L. (Chemi S.P.A.; Italy). European Patent Application EA 1,693,368, 2008. (d) Srinivas, P. L.; Rao, D. R.; Kankan, R. N.; Relekar, J. P. (Cipla Limited, India). PCT Patent Application WO/2006/064269, 2006. (e) Reguri, B. R.; Bollikonda, S.; Bulusu, V. V. N. C. S.; Kasturi, R. K.; Aavula, S. K. (Dr. Reddy's Laboratory, India). U.S. Patent Application U.S.2005/0107612, 2005. (f) Coppi, L.; Bartra Sanmarti, M.; Gasanz Guillen, Y.; Monsalvatje Llagostera, M.; Talavera Escasany, P. (Esteve Quimica, S.A., Spain). PCT Patent Application WO/2007/051828, 2007. (g) Hung, J. T.; Wei, C. P. (Formosa Laboratories, Inc., Taiwan). U.S. Patent Application U.S.2008/0097104, 2008. (h) McGarrity, J.; Bappert, E.; Belser, E. (Lonza A.G., Switzerland). PCT Patent Application WO/2008/131932, 2008. (i) Suri, S.; Sarin, G. S.; Mahendru, M. (Morepen Laboratories Limited, India). PCT Patent Application WO/2006/021974, 2006. (j) Avdagic, A.; Mohar, B.; Sterk, D.; Stephan, M. (Pliva-Istrazivanje Razvoj D.O.O., Croatia). PCT Patent Application WO/2006/000856, 2006. (k) Overman, A.; Gieling, R. G.; Zhu, J.; Thijs, L. (Synthon B.V., Holland). PCT Patent Application WO/2005/105479, 2005. (l) Shapiro, E.; Yahomoli, R.; Niddam-Hildesheim, V.; Sterimbaum, G.; Chen, K. (Teva Pharmaceuticals Industries Ltd., Israel). PCT Patent Application WO/2005/105751, 2005. (m) Achmatowicz, O.; Wisniewski, K.; Ramza, J.; Szelejewski, W.; Szechner, B. (Zaklady Farmaceutyczne Polpharma, S.A., Poland). PCT Patent Application WO/2006/043846, 2006.

(5) For an example, in the presence of 20−25 mol % (*R*)-Me-CBS, 3−10% of the over-reduced by-product (from a CH=CH bridge to a CH_2CH_2 bridge) was observed. Shinkai, I.; King, A. O.; Larsen, R. D. *Pure Appl. Chem.* **1994**, *66*, 1551.

(6) Researchers at Lonza (ref 4h) showed that under certain ruthenium- or iridium-catalyzed asymmetric hydrogenation conditions, the olefin-reduced compound is the major product.

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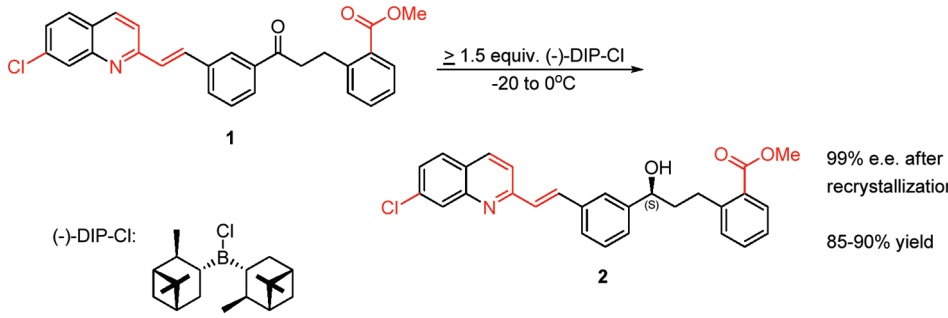
Scheme 1. **Installation of the lone stereocenter of montelukast**



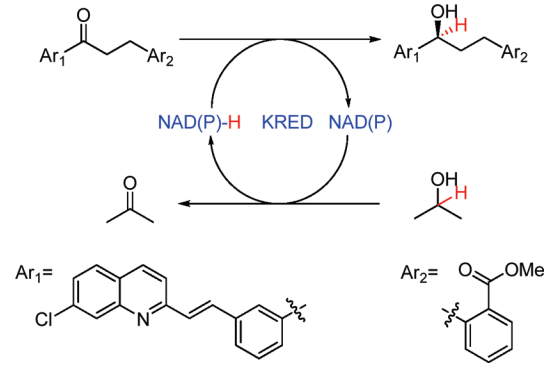
mild and selective *catalyst* be developed to replace (−)-DIP-Cl in this process.

Enzymes are renowned as mild and selective catalysts. The potential advantages of an enzymatic or biocatalytic transformation of **1** to **2** were recognized early on by researchers at Merck. However, only two microorganisms were identified as having activity on the bulky and hydrophobic substrate **1**.[10,11] The extremely low volumetric productivity (0.5 g/L in 280 h)[11b] combined with the lack of technologies at the time for improving the biocatalysts inevitably prevented the initial efforts from becoming practical.[12,13]

Table 1. **Solubility of the ketone 1 at room temperature**

solvent	solubility [g/L]	solvent system [v/v]	solubility [g/L]
DMSO	>50	1:1 DMSO/H_2O	<0.05
DMF	>50	1:1 DMF/H_2O	<0.05
THF	>20	1:1 THF/H_2O	<0.05
IPA	<0.2	1:1 IPA/H_2O	<0.05
H_2O	≪0.01		
		1:5:3 THF/IPA/H_2O	∼1

Scheme 2. **Codexis' biocatalytic alternative to DIP-Cl**



Results and Discussion

We recognized the highly hydrophobic nature of the substrate **1** (c log $P \approx 7$)[14] as the major challenge in developing a commercially viable biocatalyst. It has been known for many years that enzymes could be highly active in hydrophobic organic solvents, and it has been proposed by Klibanov[15] that maintenance of the active structure is possible by maintaining several layers of hydration in nonpolar solvents. Until now, it was unclear whether enzymes, designed by nature to be active in aqueous systems, could be evolved to have commercially relevant activity and stability in the high concentrations of water-miscible organic solvents required to dissolve appreciable amounts of the substrate **1**.[16] The ketone **1** is virtually insoluble in water. Even in 50:50 water-miscible organic solvent/water systems, the solubility of **1** is <0.05 g/L. The highest solubility of the ketone **1** that we found is ∼1 g/L at room temperature in a ternary system of 1:5:3 THF/IPA/water (Table 1).

Armed with this knowledge and the physical properties of the product **2** (*vide infra*), we envisioned a biocatalytic conversion of the ketone **1** to the (*S*)-alcohol **2** (Scheme 2, NAD(P) = nicotinamide adenine dinucleotide (phosphate)).

Fundamentally, the KRED-NAD(P) complex transfers a hydride from isopropanol (IPA) to the ketone **1** to generate the

(7) The first report of asymmetric transfer hydrogenation of **1**: Fujii, A.; Hashiguchi, S.; Uematsu, N.; Ikariya, T.; Noyori, R. *J. Am. Chem. Soc.* **1996**, *118*, 2521. However, both the optical purity and the yield were modest (92% ee and 68% yield).

(8) Researchers at Lonza (ref 4h) used the [(*R*)-BINAP-$RuCl_2$−(*R*)-DAIPEN] system to achieve the desired transformation in 99% conversion (84% yield) and up to 96.9% ee (crude). However, chlorobenzene was used the cosolvent (5:1 chlorobenzene/IPA; ∼5:1 wt/wt chlorobenzne/product).

(9) The best result to date is by researchers at Pliva (ref 4m): 1 mol % [$RuCl_2$(mesitylene)]$_2$ and 2 mol % (1*S*,2*S*)-piperidyl-*N*-sulfonyl-1,2-diphenylethylenediamine (∼3 wt % catalyst) under the Noyori transfer hydrogenation conditions (ref 7) to obtain the desired product in 95−99.5% ee; up to 2% styrene-reduced byproduct. See also: Avdagic, A.; Jerkovic, J.; Pavlicic, D. PCT Patent Application WO 2008/009970, 2008; 99.8% ee in 83% yield and 96% chemical purity.

(10) *Mucor hiemalis* IFO 5834. Chen, S.-S. T.; Shafiee, A. U.S. Patent 5,427,933, 1995.

(11) *Microbacterium campoquemadoensis* (MB5614) ATCC 55557 (isolated from a soil sample collected in a field at Santa Rosa Memorial Park, Guanacaste Pr., Costa Rica. The field had been subjected to burning 48 hours prior to sampling). (a) Chartrain, M. M.; Chen, S.-S. T.; Garrity, G. M.; Heimbuch, B.; Roberge, C.; Shafiee, A. U.S. Patent 5,491,077, 1996. (b) Roberge, C.; King, A.; Pecore, V.; Greasham, R.; Chartrain, M. *J. Ferment. Bioeng.* **1996**, *81*, 530. (c) Shafiee, A.; Motamedi, H.; King, A. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 709.

(12) For selected reviews and articles on biocatalysis in a process chemistry environment, see: (a) Ran, N.; Zhao, L.; Chen, Z.; Tao, J. *Green Chem.* **2008**, *10*, 361. (b) Tao, J.; Zhao, L.; Ran, N. *Org. Process Res. Dev.* **2007**, *11*, 259. (c) Sylvestre, J.; Chautard, H.; Cedrone, F.; Delcourt, M. *Org. Process Res. Dev.* **2006**, *10*, 562. (d) Leresche, J. E.; Meyer, H.-P. *Org. Process Res. Dev.* **2006**, *10*, 572. (e) Wells, A. *Org. Process Res. Dev.* **2006**, *10*, 678. (f) Chikusa, Y.; Hirayama, Y.; Ikunaka, M.; Inoue, T.; Kamiyama, M.; Moriwaki, M.; Nishimoto, Y.; Nomoto, F.; Ogawa, K.; Ohno, T.; Otsuka, K.; Sakota, A.; Shirasaka, N.; Uzura, A.; Uzura, K. *Org. Process Res. Dev.* **2003**, *3*, 289. (g) Gotor, V. *Org. Process Res. Dev.* **2002**, *6*, 420. (i) Lye, G. J.; Dalby, P. A.; Woodley, J. M. *Org. Process Res. Dev.* **2002**, *6*, 434. (h) Wandrey, C.; Liese, A.; Kihumbu, D. *Org. Process Res. Dev.* **2000**, *4*, 286.

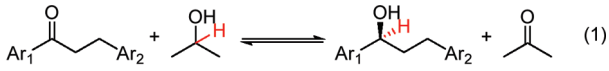
(13) For recent reviews on asymmetric reduction of ketones using biocatalysis, see: (a) Matsuda, A.; Yamanaka, R.; Nakamura, K. *Tetrahedron: Asymmetry* **2009**, *20*, 513. (b) Moore, J. C.; Pollard, D. J.; Kosjek, B.; Devine, P. N. *Acc. Chem. Res.* **2007**, *40*, 1412. (c) Nakamura, K.; Yamanaka, R.; Matsuda, T.; Harada, T. *Tetrahedron: Asymmetry* **2003**, *14*, 2659.

(14) ChemDraw 10.0. For a comparison, *n*-heptane has a c log P of ∼4.

(15) Kilbanov, A. M. *Nature* **2001**, *409*, 241–246.

(*S*)-alcohol **2** and the acetone coproduct in a mechanism that is akin to transfer hydrogenolysis/ionic hydrogenation. The overall reaction is eq 1:

Equation 1: The overall/net reaction of a ketone reduction using IPA as the hydride donor.



(1)

Ar_1 and Ar_2 as shown in Scheme 2.

The equilibrium dictates that in order to achieve >99% conversion at an industrially viable substrate loading (e.g., 100 g/L), it is imperative that the process includes a driving force. Typically, this type of reaction is driven by concomitant removal of the acetone coproduct via distillation. Fortuitously, the *monohydrate* of (*S*)-alcohol **2** readily crystallizes from all of the organic−aqueous media studied.[17] The reaction is therefore "self-driven" to >99% conversion by the precipitation of the product from the reaction mixture. Had a crystalline form of enantiopure **2** not existed, the oiling out of **2** would have led to the entrapment of the substrate, necessitating the presence of a water-immiscible cosolvent to dissolve/disperse the product. The resulting process would have been significantly more complicated as mass transfer (partition of the highly hydrophobic substrate from a water-immiscible solvent into the reactive aqueous phase) would be the limiting factor.

For the biocatalysis approach to be a practical alternative to the (−)-DIP-Cl process, the enzyme must exhibit the following characteristics:

(1) Capable of using IPA for cofactor (NAD or NADP) regeneration.[18]
(2) Capable of accepting both the bulky substrate **1** and the small cosubstrate IPA.
(3) Tolerate up to 70% polar/water miscible organic solvent(s), preferably at elevated temperatures.
(4) Efficient at low substrate concentration (aqueous saturation) of ∼0.2 mM (high k_{cat} at low K_M).
(5) Not inhibited or deactivated by the accumulation of the acetone coproduct, a potential competitive inhibitor.
(6) High enantioselectivity (>99% ee).

Upon initiation of the program, screening revealed that that no commercially available enzymes had any measurable activity on the ketone **1**. However, we found a number of NADP-dependent KREDs in Codexis' panel collection were active on the ketone **1**. More importantly, the Codexis variants were all (*S*)-selective and exhibited an enantioselectivity of >99.9% ee.

Table 2. **Initial activity versus target activity**

	enzyme loading[a]	product titer[b]
initial	∼10 g/L	∼0.1−0.2 g/L after 24 h
target	≤5 g/L	≥100 g/L in ≤24 h
improvement needed	**≥2-fold**	**≥500−1000-fold**

[a] Lyophilized cell lysate (semipurification to remove cell debris and polynucleotides). Approximately 20−30 wt % of the lyophilized powder is the catalyst of interest. All references to enzyme loading ("g/L") henceforth will be based on this preparation. [b] Merck's biocatalyst (whole cell of MB-6416) has a product titer of ∼0.5 g/L after 280 h.[11b]

Although the activity of these initial variants was very low with respect to the target activity (Table 2), these variants nevertheless provided a valuable starting point for our enzyme evolution program. It was estimated that the initial activity needed to be improved by 1000−2000 fold for the biocatalyst to be commercially viable (Table 2). In addition to low activity, even at room temperature, all of the initial variants were unstable at the high level of organic solvents mandated by the process design and were subject to acetone inhibition.

Because the initial variants exhibited essentially absolute enantioselectivity on a rather bulky substrate, we hypothesized that the active site of the enzyme can only accept the substrate in the pro-(*S*) conformation. Therefore, the evolution efforts were focused on improving the activity and stability of the biocatalyst alone.[19] Subsequent events proved the hypothesis to be correct: the undesired enantiomer was never detected.

On the basis of the initial data, we envisioned the reaction to be carried out in a slurry-to-slurry mode in a ternary solvent system of 1:5:3 THF/IPA/buffer. The reactions were miniaturized to a 96-well plate format for the purpose of HTS and enzyme evolution. Because we had developed a very efficient HPLC method (∼1000 samples per day per HPLC)[19] for determining the conversion (i.e., enzyme activity), no prescreening was deemed necessary. That is, every live cell (i.e., those with a recombinant KRED gene) was subjected to the miniaturized reaction directly, and no attempts were made to identify and eliminate the inactive variants prior to the reaction.[20] The variants that exhibited equivalent or superior activity to the positive control(s) were further subjected to a thermal challenge to select for thermal stability. The mutations found in the superior variants were collected, analyzed, and combined for the next generation of evolution, and the process was repeated until a commercially viable variant was found.[21,22]

After three "rounds" of evolution, the activity of the biocatalyst was improved from an initial product/catalyst mass ratio of ∼1:50 to ∼8:1. The enhancement in catalyst productiv-

(16) For leading references in the area of directed evolution towards solvent tolerant biocatalysts, see: (a) Ogino, H.; Uchiho, T.; Doukyu, N.; Yasuda, M.; Ishimi, K.; Ishikawa, H. *Biochem. Biophys. Res. Commun.* **2007**, *358*, 1028. (b) Zumárraga, M.; Bulter, T.; Shleev, S.; Polaina, J.; Martínez-Arias, A.; Plou, F. J.; Ballesteros, A.; Alcalde, M. *Chem. Biol.* **2007**, *14*, 1052. (c) Orgino, H.; Ishikawa, H. J. *Bioscience Bioeng.* **2001**, *91*, 109. (d) Song, J. K.; Rhee, J. S. *Biochim. Biophys. Acta* **2001**, *1547*, 370.

(17) During the synthesis of reference compounds, we realized that racemic **2** and the *monohydrate* of the enantiopure **2** are crystalline solids; however, the *anhydrous* enantiopure **2** is a viscous honey-like liquid. For reports of (*S*)-**2** monohydrate as a solid, see: (a) Bhupathy, M.; McNamara, J. M.; Sidler, D. R.; Volante, R. P.; Bergan, J. J. PCT Patent Application WO/1995/18107 (Example 5, step 1) and ref 1b (Example 146, step 2).

(18) Theoretically, the cofactor can also be regenerated via a two-enzyme coupled system (e.g., glucose/glucose dehydrogenase). However, due to the rather rigorous process demands (high level of cosolvents at elevated temperature), we believed that further evolution of the cofactor recycling enzyme would be necessary as well (i.e. we would need *two* enzyme evolution programs).

(19) By assuming that only the (*S*)-product can be formed, we used a fast *achiral* HPLC method (1.1 minute per sample, see Experimental Section) for assaying the conversion (i.e., activity) only. The chiral method (see Experimental Section) would have taken ∼8 minutes per sample.

(20) For example, prior to the miniaturized reaction, we could have carried out a spectrophotometric assay with a throughput of $>10^3$ variants per day to reject variants that are incapable of regenerating the cofactor in the presence of IPA.

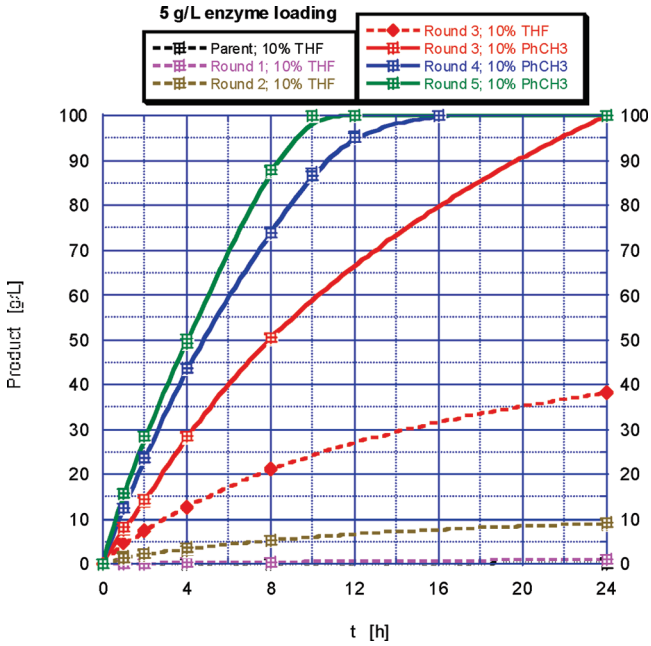


Figure 2. **Progress in activity of evolved KREDs.**

ity was attributed to an increase in the intrinsic activity of the catalyst in conjunction with the elimination of competitive inhibition by acetone and the improvement in thermal stability of the biocatalyst—we were able raise the reaction temperature from room temperature to 40 °C. At this point, we conducted another cosolvent screen. Rather unexpectedly, the activity increased by ~2.5 fold and reached our target outlined in Table 1 when the cosolvent was changed from 10% THF to 10% toluene even thought the enzymes had been evolved in 10% THF up to this point.[23] From Figure 2, it can be seen that the beneficial effect of toluene is due to the rate enhancement alone—the rate difference between 10% toluene (solid red line) and 10% THF (dashed red line) is ~2.5-fold throughout, implying that the catalyst has similar stability in either solvent system.

Further fine-tuning of the enzyme evolution efforts in 10% toluene gave the final variant with an activity that exceeded the initial target by ~2 fold (Table 3). Lastly, the reaction may be carried out via either higher enzyme loading (5 g/L) over a shorter period of time (10 h) or lower enzyme loading (3 g/L) over a longer period of time (24 h).

Boundary condition scanning indicated that the final variant is insensitive to pH in the range between pH 7.5−9.8,[24] tolerates a variability in the toluene cosolvent loading between 8 and 12% (standard loading is 10%), and can withstand a temperature excursion up to 55 °C for 1 h in the reaction mixture. Stability studies further revealed that the final variant CDX-026 retains 85−90% of its activity after incubation as the dry powder under air at 40 °C for 4 days and that it is completely stable at −20 °C under air, the standard storage condition, for at least 2 years.

The final process conditions are as follows: 100 g/L of the substrate ketone **1** in 1:5:3 v/v/v $PhCH_3$/IPA/100 mM pH 8.0 triethanolamine·HCl (with 2 mM $MgSO_4$) at 45 °C using a biocatalyst system of 3 g/L KRED and 0.1 g/L NADP to give a slurry-to-slurry reaction mixture. Typical reaction time is 22−24 h. The process was successfully scaled up from a 20-mL vial with stir bar to a 2-L jacketed flask with overhead stirring to a 20-L in-house kilo-lab reactor without any difficulty. Subsequently, this process was transferred to Arch PharmaLabs Limited (India) and was initially scaled up to 125 kg batches (Table 4) and is currently running at a 230-kg scale (see Experimental Section).

A comparison between the (−)-DIP-Cl protocol and the biocatalytic alternative is shown in Table 5.[25] Furthermore, recent focus on green chemistry prompted us to analyze our process against the standard DIP-Cl process in terms of process mass intensity (PMI, defined as the ratio of the quantity of all material input to the quantity of the product output),[26] and the result is shown in Figure 3.[25] The KRED process reduces the PMI by ~30% (and the organic solvent usage by ~25%). The numbers for the KRED process are based on the 230-kg scale reactions carried out by Arch (see Experimental Section) where the product was isolated via recrystallization. On a smaller scale (100 g), we have shown that product of similar quality can be obtained by direct filtration and water rinse (see Experimental Section). The PMI of this protocol is ~18 (water~13.0, IPA~3.9, and toluene~0.9), underscoring the impact of workup/isolation conditions on PMI (and "greenness" in general).

Conclusion

Thus far, the lone stereocenter in montelukast sodium (Singular) had been set via the (−)-DIP-Cl-mediated asymmetric reduction. As Merck indicated, "despite the availability of this method, alternative economical and environmentally acceptable procedures are desired".[11c]

This challenge has been met by developing an engineered ketoreductase that is highly active and selective in a mixed aqueous organic media previously thought to be inactivating for such catalysts. After envisioning the optimal process conditions, the activity of the biocatalyst was improved by 3000-fold by subjecting variants to

(21) For references on Codexis' Enzyme Evolution technology, see: (a) Huisman, G. W.; Sligar, S. G. *Curr. Opin. Biotechnol.* **2003**, *14*, 357. (b) Fox, R. *J. Theor. Biol.* **2005**, *234*, 187. (c) Huisman, G. W.; Lalonde, J. J. *Biocatal. Pharm. Biotechnol. Ind.* **2007**, 717. (d) Fox, R. J.; Davis, S. C.; Mundorff, E. C.; Newman, L. M.; Gavrilovic, V.; Ma, S. K.; Chung, L. M.; Ching, C.; Tam, S.; Muley, S.; Grate, J.; Gruber, J.; Whitman, J. C.; Sheldon, R. A.; Huisman, G. W. *Nat. Biotechnol.* **2007**, *25*, 338. (e) Fox, R.; Huisman, G. W. *Trends Biotechnol.* **2008**, *26*, 132.

(22) For experimental details on the molecular biology and HTS aspects as well as the amino acid sequence of the final variant, see: Liang, J.; Borup, B.; Mitchell, V.; Mundorff, E.; Lalonde, J.; Huisman, G. PCT Patent Application WO/2009/042984, 2009, pp 115−125.

(23) Interestingly, THF turned out to be one of the worst cosolvents. Other co-solvents that exhibited beneficial effects were CH_2Cl_2, EtOAc, 2-Me-THF, and IPA (i.e. *without* THF).

(24) Triethanolamine, 100 mM, has a pH of 9.8.

(25) The numbers for the DIP-Cl process are based on ref 1c: a 3-kg scale (largest scale published by Merck) reaction for the (*R*)-enantiomer of **2** (*J. Org. Chem* **1993**, *58*, 3731).

(26) There are many ways to benchmark "greenness", none of which is perfect. The Green Chemistry Institute Pharmaceutical Round Table uses PMI, see: (a) Henderson, R. K.; Kindervater, J.; Manley, J. B. Lessons learned through measuring green chemistry performance - The pharmaceutical experience (http://portal.acs.org:80/portal/fileFetch/C/CTP_005585/pdf/CTP_005585.pdf). (b) Curzons, A. D.; Constable, D. J. C.; Mortimer, D. N.; Cunningham, V. L. *Green Chem.* **2001**, *3*, 1. (c) For some of the debates over the merits and shortcomings of the various "greenness" metrics, see: Constable, D. J. C.; Curzons, A. D.; Cunningham, V. L. *Green Chem.* **2002**, *4*, 521. and. (d) Sheldon, R. A. *Green Chem.* **2007**, *9*, 1273.

Table 3. **Progress in activity of evolved KREDs[a]**

round	*cosolvent*	*T* [°C]	KRED [g/L]	product [g/L]	time [h]	number of mutation(s) from parent	number of mutation(s) from previous round
parent	THF	rt	~10	0.1−0.2	24	NA	NA
1	THF	rt	5	1	24	1	1
2	THF	35	5	10	24	5	4
3	THF	40	5	40	24	10	5
3[b]	*PhCH$_3$*	40	5	100	24	10	5
4	*PhCH$_3$*	45	5	100	16	17	7
5[c]	*PhCH$_3$*	45	5	100	10	19	2
5[c]	***PhCH$_3$***	**45**	**3**	**100**	**24**	**19**	**2**

[a] Reaction medium = 1:5:3 *cosolvent*/IPA/100 mM pH 8.0 triethanolamine·HCl with 2 mM $MgSO_4$. [b] Targeted activity. [c] Final variant, designated as CDX-026.

Table 4. **Batches (125 kg) of the alcohol 2 produced by Codexis' evolved biocatalyst[a]**

run	% yield[b]	% ee	chemical purity [LC area %][b]
1	93	>99.9	99.38
2	86	>99.9	98.17
3	92	>99.9	98.41
4	92	>99.9	98.23
5	92	>99.9	99.14
6	90	>99.9	98.59
7	90	>99.9	98.99
8	88	>99.9	99.35
9	**98**	**>99.9**	**99.36**

[a] Reaction conditions: 100 g/L of the substrate **1** in 1:5:3 v/v/v $PhCH_3$/IPA/100 mM pH 8.0 triethanolamine*HCl (with 2 mM $MgSO_4$) at 40−45 °C for 22−34 h. [b] Reaction time, yield, and chemical purity of the product depend on the purity of the substrate **1**.

Table 5. **Process conditions and performances of the DIP-Cl process vs the KRED process**

	(−)-DIP-Cl	KRED
substrate loading	~100 g/L	100 g/L
reaction conditions	moisture sensitive	aqueous
reagent or catalyst loading	at least 1.8 equiv (at least 150 wt %)	3−5 wt %
optical purity	crystallization needed to upgrade >99% ee	crude product is >99.9% ee (upgrade not needed)
yield	85−90%	90−98%

directed evolution technologies under the prescribed process parameters.

The resulting biocatalytic process is economical and robust. The product is obtained in >95% yield in >99.9% ee and >98.5% chemical purity in 200+ kg scale.

Experimental Section

General. The substrate ketone **1** was provided by Arch PharmaLabs, India, and used without further purification. The KRED (CDX-026) was produced by Codexis and can be purchased from Codexis. NADP-Na was purchased from Oriental Yeast. All other solvents and reagents were used "as is" from commercial vendors.

The optical and chemical purities of the product were determined via an Agilent 1100 HPLC (sample concentration: 0.5 mg/mL in MeOH). Optical purity (20 μL injection): 2.1 × 150 mm ChiralPak AS-H column (with 10 mm AS-H guard cartridge) with a mobile phase of 10:90 IPA/heptane at 0.5 mL/min, 40 °C (detection at 287 nm). The elution order (retention time) is as follows: Ketone **1** (4.2 min), the undesired (*R*)-alcohol (5.3 min), the desired (*S*)-alcohol (6.5 min). Chemical purity (20 μL injection): 4.6 × 250 mm SS Wakosil II column with a mobile phase of 85:15 MeOH/pH 3.0 phosphate buffer at 1.5 mL/min, 15 °C (detection at 220 nm). The elution order (retention time) is as follows: product (12.1 min) and substrate (21.4 min). Purity reported as uncorrected LC area %. The HTS HPLC method: 2.1 mm × 50 mm Eclipse XDB column with a mobile phase of 3600:400:1 ACN/H_2O/TFA at 1.0 mL/min; 40 °C; 287 nm detection (product at 0.4 min; substrate at 0.7 min).

KRED Reduction of 1 to 2 (Three Consecutive Runs at 100 g Scale; Direct Filtration). To a jacketed 2-L, three-neck round-bottom flask under nitrogen and equipped with internal thermometer and mechanical stirrer at 300 rpm was added 100 mM pH 8.0 triethanolamine·HCl with 2 mM $MgSO_4$ buffer (300 mL), KRED CDX-026 (3.0 g), and NADP-Na (0.1 g) to give a pale-yellow solution in less than 2 min with no detectable temperature change. To the biocatalyst solution was added the substrate **1** (100 g) in portions over ~5 min to give a thick tan slurry. To the thick tan slurry was added IPA (500 mL) and toluene (100 mL) to give a thick tan slurry with no detectable temperature change. The internal temperature was raised to 45 °C over ~15 min.

As the reaction progressed, the consistency of the slurry gradually turned thicker due to precipitation of fine solid product particles. After stirring at 300 rpm at 45 °C for 24 h, the reaction was judged to be complete according to the HPLC analysis of an aliquot.

The reaction mixture was filtered through three layers of 150 mm diameter Whatman #2 filter paper to give a tan paste that was washed with water (2 × 500 mL). The tan paste was air-dried overnight to give a tan solid in 90−100% yield. The optical purity as determined by chiral HPLC analysis was >99.9% ee. The chemical purity was at least 98.5 LC area %, the major (largest single) impurity being 0.3−0.4 LC area % of the starting material.

KRED Reduction of 1 to 2 (230 kg scale; Product Isolated via Extraction−Crystallization). To a slurry of 2-[3-2[7-chloro-2-quinolinyl]ethenyl]phenyl]-3-oxopropyl]-1-methoxycarbonylbenzene **1** (230 kg, 504 mol) in a mixture of isopropyl alcohol (5 vol), toluene (1 vol), and triethanolamine buffer of pH 8.0 (3 vol) were added ketoreductase enzyme CDX-026 (9.2 kg) and cofactor NADP-Na (0.23 kg) at 40−45 °C under stirring. Reaction was continued at this temperature until

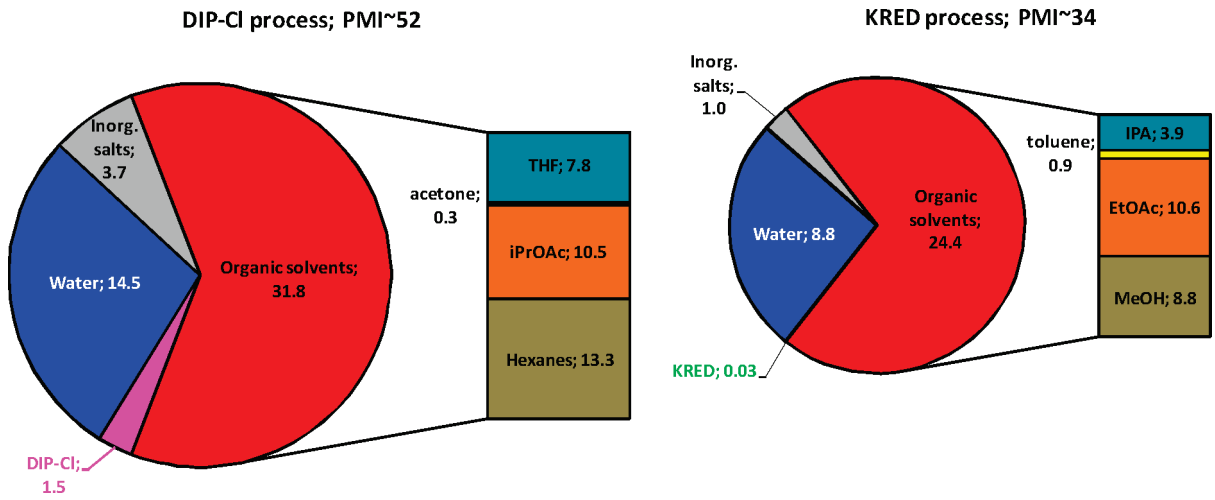


Figure 3. **PMI of the DIP-Cl process versus the KRED process.**

the starting material was completely consumed as shown by TLC (2:1 hexanes/EtOAc on silica gel; R_f of the substrate ≈ 0.6; R_f of the product ≈ 0.4). The total batch time is typically 40−45 h.

To the reaction mass were added saturated NaCl solution (4 vol) and ethyl acetate (10 vol).

The enzyme was filtered at 60−70 °C using a pressure nutsche. The organic phase containing the product was separated. The aqueous layer was extracted with ethyl acetate (2 vol), and the organic phase was combined with the product layer. The combined organic phase was dried and concentrated to give the crude (*S*)-2-[3-2[7-chloro-2-quinolinyl]ethenyl]phenyl-3-hydroxypropyl]benzoic acid methyl ester **2**.

The crude product was purified by recrystallisation from 70% aqueous methanol (14 vol) to get pure product as monohydrate **2** (233 kg; 490 mol), 97.2% yield, >99.9% ee. Melting point 84 °C (DSC); UV (MeOH) λ_{max} (ε) 244.5 nm; ^{1}H NMR (DMSO-d_6) δ 7.26−8.10 (m, 15H, ArH + Styryl CH═CH), 4.75 (s, 1H, OH), 3.89 (s, 3H, OCH_3), 3.0−3.2 (m, 3H, CH_2+CH−OH), 2.12 (m, 2H, CH_2); IR (cm^{-1}) C═O: absent, C−OH: 3149 cm.

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