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Highly Enantioselective Reduction of a Small Heterocyclic Ketone: Biocatalytic Reduction of Tetrahydrothiophene-3-one to the Corresponding (*R*)-Alcohol

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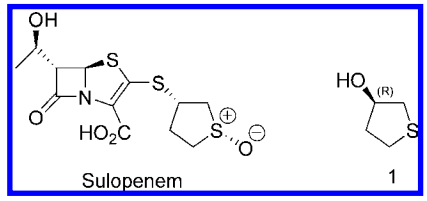
Abstract:

By leveraging enzyme evolution technologies, the enantioselectivity of a KetoREDuctase (KRED) towards the nearly spatially symmetrical ketone tetrahydrothiophene-3-one was increased from 63% ee to 99.3% ee. The biocatalytic process gives (*R*)-tetrahydrothiophene-3-ol in one step from a commodity chemical and supplants the original multistep hazardous processes starting from the chiral pool. The biocatalytic process has been successfully scaled to 100 kg.

Introduction

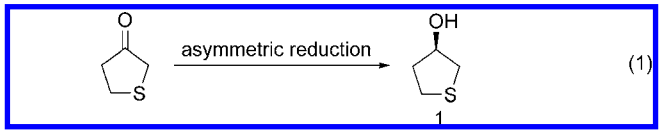
(*R*)-Tetrahydrothiophene-3-ol (**1**) is a key component in sulopenem, a potent antibacterial, a prodrug of which is being developed by Pfizer.



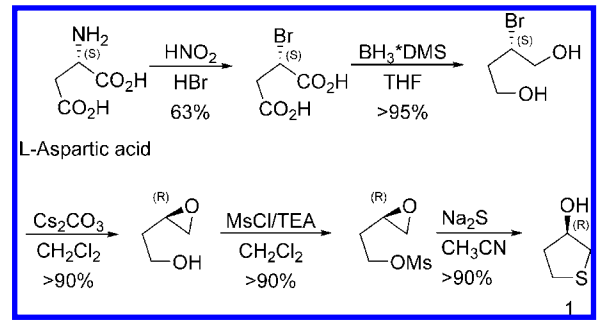
In Pfizer's original synthesis, the chiral alcohol **1** was obtained in five steps from L-aspartic acid (Scheme 1).[1]

Although the raw material L-aspartic acid is inexpensive and readily available and the yields in this five-step process are generally good, the synthesis involves hazardous conditions (diazotization in step 1), a sensitive reagent (BH_3·dimethylsulfide in step 2), high energy intermediates (epoxide in steps 3 and 4) and a noxious reagent (Na_2S in step 5).[2,3] The optical purity of **1** thus produced was 96−98% ee.[1]

At a quick glance, the most straightforward approach is the asymmetric reduction of tetrahydrothiophene-3-one (eq 1).[4]



Scheme 1. **Synthesis of the alcohol 1 starting from the chiral pool**



However, because the ketone is nearly spatially symmetrical (β-CH_2 vs β-S), asymmetric reductions via either CBS-borane[5] or catalytic hydrogenation[6] only resulted in 23−82% ee. Only the use of a stoichiometric and bulky chiral reducing agent, (−)-diisopinocampheylborane for the hydroboration of 2,3-dihydrothiophene at 25 °C gave the (*S*)-alcohol **1** in 100% ee.[7] Furthermore, biocatalysts also failed to produce the alcohol **1** with a synthetically useful optical purity; horse liver alcohol dehydrogenase gave the undesired (*S*)-enantiomer in 33% ee,[8] and whole-cell fungal systems gave the desired (*R*)-alcohol in 81−91% ee.[9,10]

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(1) (a) Volkmann, R. A. U.S. Patent 4,921,792, 1990. (b) Volkmann, R. A.; Kelbaugh, P. R.; Nason, D. M.; Jasys, V. J. *J. Org. Chem.* **1992**, *57*, 4352.

(2) The (*S*)-alcohol has been prepared in five steps from (*S*)-dimethyl malate in ∼11% overall yield and involves LAH in Et_2O, Li_2S, protection/deprotection and chromagraphic purification. (a) Ghosh, A. K.; Thompson, W. J.; Lee, H. Y.; McKee, S. P.; Munson, P. M.; Duong, T. T.; Darke, P. L.; Zugay, J. A.; Emini, E. A.; Schleif, W. A.; Huff, J. R.; Anderson, P. S. *J. Med. Chem.* **1993**, *36*, 924. (b) Ghosh, A. K.; Lee, H. Y.; Thompson, W. J.; Culberson, C.; Holloway, M. K.; Mckee, S. P.; Munson, P. M.; Duong, T. T.; Smith, A. M.; Darke, P. L.; Zugay, J. A.; Emini, E. A.; Schleif, W. A.; Huff, J. R.; Anderson, P. S. *J. Med. Chem.* **1994**, *37*, 1177.

(3) A recent patent revealed a three-step synthesis starting from ethyl (*R*)-4-chloro-3-hydroxybutanoate (41% overall yield). The sulfur atom was introduced by 1.5 equiv of Na_2S. Hinoue, K.; Mikami, M. U.S. Patent 7,262,310, 2007.

(4) Tetrahydrothiophene-3-one is used as a food additive and can be synthesized via the condensation between thioglycolic ester and acrylic ester followed by decarboxylation. (a) von Karrer, P.; Schmid, H. *Helv. Chim. Acta* **1944**, *27*, 124. (b) Buchman, E. R.; Cohen, H. *J. Am. Chem. Soc.* **1944**, *66*, 847. (c) Woodward, R. B.; Eastman, R. H. *J. Am. Chem. Soc.* **1946**, *68*, 2229. (d) Schuster, K. H.; Kuhlein, K.; Mix, K.; Muller, R. German patent DE 3318775, 1984.

(5) *n*-Bu-CBS gave 23% ee. Quallich, G. J.; Woodall, T. R. *Tetrahedron Lett.* **1993**, *34*, 785.

(6) (a) Ir-BINAP catalysts gave 66−82% ee. Taketomi, T.; Atukagawa, S.; Kumobayashi, H.; Takaya, H.; Mashima, K. U.S. Patent 5,159,093, 1992. (b) Zhang, X.; Taketomi, T.; Yoshizumi, T.; Kumobayashi, H.; Akutagawa, S.; Mashima, K.; Takaya, H. *J. Am. Chem. Soc.* **1993**, *115*, 3318.

(7) Brown, H. C.; Prasad, J. V. N. V. *J. Am. Chem. Soc.* **1986**, *108*, 2049.

(8) Jones, J. B.; Schwartz, H. M. *Can. J. Chem.* **1981**, *59*, 1574.

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Table 1. **KRED process target**

substrate loading	≥100 g/L (≥1 M)
KRED loading	≤1 g/L[a,b]
conversion	≥98%
reaction time	≤24 h
optical purity of the product	≥99.0% ee

[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") will henceforth be based on this preparation. [b] On a *molar* basis, the catalyst loading is on the order of 10^{-4} to 10^{-3} mol %.

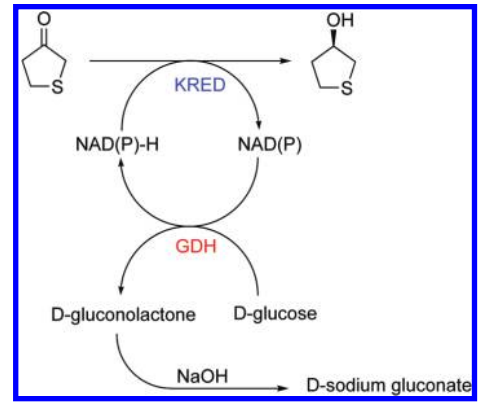
Despite these discouraging precedents, it was believed that biocatalysis,[11] especially in light of the recent advances in enzyme engineering via *in vitro* evolution techniques,[12] offered the best chance to effect the desired asymmetric transformation (eq 1).[13]

Results and Discussions

Critical to the success of a directed evolution program is the definition of the end-point criteria and process environment *before* the program starts. Within a well-defined environment (i.e., reaction conditions and process goals), the activity, selectivity, and stability of the enzyme can be evolved such that the only variable is the amino acid sequence of the catalyst. The targets we set for the evolved ketoreductase (KRED) are shown in Table 1. The substrate loading is based on the "rule of thumb" process typical concentration of ∼10 wt %/vol and the substrate/catalyst ratio of ≥100:1 wt/wt as well as an optical purity of ≥99% ee.

In the ketoreductase system, the hydride is delivered via an enzyme-bound cofactor nicotinamide adenine dinucleotide (phosphate), NAD(P)H. Since the cofactor is expensive, it is used in millimolar equivalent amounts and is regenerated *in situ*. In this case, the cofactor regeneration was carried out by

Scheme 2. **Cofactor re-generation via an irreversible glucose−GDH coupled two-enzyme system**



a glucose−glucose dehydrogenase (GDH) coupled two-enzyme system as depicted in Scheme 2.[14] The ultimate reducing agent in this example is D-glucose.

We have previously reported on the evolution for a robust and active glucose dehydrogenase.[15] The resulting GDH was used in this process without further optimization. Since the immediate product of the glucose−GDH recycle system is gluconic acid (producing a decrease in pH), the pH of the reaction needs to be maintained via a feedback-controlled addition of 4−8 M NaOH. Thus, the progress of the reaction is directly correlated to the amount of NaOH added. It should be noted that the two-enzyme system has the advantage of being irreversible. With certain classes of KREDs, it is possible to use isopropanol (IPA) as the ultimate reducing agent and thereby negate the need for a second enzyme (e.g., GDH). However, this option was rejected from the outset as it would be a reversible system (eq 2).

(2)

We expected the aliphatic ketone substrate to have reduction potential similar to that of acetone (i.e., $K_{eq} \approx 1$). Experimentally, in the presence of 50 vol % of IPA (∼6 M), the 100 g/L (∼1 M) reaction stalled at the thermodynamic limit (equilibrium) of ∼70% conversion (K_{eq} ∼0.3; ∼85% conversion expected for $K_{eq} = 1$). In order to achieve ∼99% conversion, it would be necessary to drive the reaction by removing the acetone byproduct via fractional distillation. We chose the GDH option because we envisioned pH control to be a simpler and more robust unit operation than fractional distillation.

Screening of 43 commercially available KREDs and in-house collection of mutants revealed that the enantioselectivity of these enzymes varied, giving chiral purities that ranged from 81% ee for the undesired (*S*)-**1** to 63% ee for the desired product (*R*)-**1**. Notably, all KREDs screened exhibited some activity on the small substrate tetrahydrothiophene-3-one. Most impor-

(9) (a) Nagai, H.; Konuki, K.; Ito, S.; Sameshima, T. U.S. Patent application U.S. 2008/0050787, 2008. (b) Nagai, H.; Konuki, K.; Ito, S.; Sameshima, T. PCT Patent Application WO/2005/054491, 2005.

(10) Kinetic resolution of the racemic butyrate ester of **1** using lipase from *Pseudomonas cepacia* (CPL) gave a specificity factor (*E*) of ∼1.4. Honig, H.; Shi, N.; Polanz, G. *Biocatalysis* **1994**, *9*, 61.

(11) 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.

(12) 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. Biosci. Bioeng.* **2001**, *91*, 109. (d) Song, J. K.; Rhee, J. S. *Biochim. Biophys. Acta* **2001**, *1547*, 330.

(13) For selected reviews and articles of 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.

(14) Other examples of irreversible two-enzyme coupled co-factor regeneration systems include formate−formate dehydrogenase (FDH) and phosphite−phosphite dehydrogenase (PDH) system. The glucose−GDH system was chosen for this project because Codexis had an already evolved active and robust GDH.

(15) Davis, S. C.; Jenne, S.; Krebber, A.; Newman, L. M. U.S. Patent Application U.S. 2005/0095619, 2005.

tantly, control experiments showed that even KREDs native to the host strain used for production of the enzyme (lyophilized cells in the absence of a recombinant KRED gene) were active on the substrate to give (*R*)-**1** in 15−26% ee. Although the activity of the native KRED(s) was low (∼2−5% of the targeted rate, Table 1), calculations estimated that the optical purity of (*R*)-**1** would have been capped at ∼98% ee. Four options were available to overcome the interfering background reaction from the host organism: thermal/chemical deactivation/inhibition of the host KREDs, selective precipitation of the host KREDs during biocatalyst manufacturing, optimization of a psychrotrophic KRED to run the reaction at ≤0 °C, or evolution of an ultra-active KRED to complete the reaction in less than ∼6 h. All four options were explored, and each led to some success, albeit no combinations were able to completely suppress the background reaction. However, it was discovered that a *purified N*-His-tagged evolved KRED exhibited enantioselectivity the *same* as that of the *crude* (i.e., "contaminated" with background/host KREDs) evolved KRED powder, suggesting that in the presence of an overexpressed, evolved KRED the background activity was muted. Although the reason for this is unclear, two possibilities exist: the overproduction of the exogenous KRED could have suppressed the expression of endogenous reductases, or the evolved KRED could have a much higher binding affinity (lower K_M) than host strain KREDs, effectively outcompeting the native enzyme(s) for the substrate.

Initial activity of the most (*R*)-selective natural enzyme, *Lactobacillus kefir* KRED (Genebank accession number AAP94029 GI:33112056; overexpressed in *Escherichia coli*), was reasonable: complete conversion of 40 g/L of substrate was achieved in 2 h with 4 g/L of KRED. The focus of the enzyme evolution efforts was therefore placed on improving the enantioselectivity of the *L. kefir* KRED.[16] We applied a combination of evolution technologies, including random mutagenesis to identify "hot spots", gene shuffling and semisynthetic shuffling, and ProSAR analysis to deconvolute the contributions of individual mutations.[17] In this particular example, the reaction was miniaturized to a 96-well plate format for tiered high-throughput screening (HTS). A spectrophotometric assay that monitors the disappearance of NADPH in the presence of the substrate was used in the first tier. Variants that were poorly active or inactive (i.e., little or no disappearance of NADPH) were eliminated. The second-tier assay was a micropreparative scale reaction that generated sufficient amount of product for chiral HPLC assay to determine the enantioselectivity of the variants. Thermal challenge (incubation at elevated temperatures) was installed either before or after either of the two tiers to select for thermal stability. The sequences of genes encoding variants that exhibited improvement (and occasionally those that showed strong negative responses) in any of the assays were determined. After ProSAR analysis,[17d] the beneficial mutations were taken forward to the next iteration of screening ("next generation"). The process was repeated until the target was met.[18] After eight generations of biocatalysts evolution ("rounds" of iterative HTS), a KRED that met all criteria in Table 1 was obtained (Table 2).

Table 2. **Improvement in enantioselectivity[19,20]**

round	*T* [°C]	% conv.[a]	% ee	er[b]	number of mutation(s) from parent	number of mutation(s) from previous round
parent	rt	>99[c]	63.0	4:1	NA	NA
1	rt	>99	80.0	9:1	1	1
2	rt	>99	95.2	41:1	1	2
3	rt	>99	97.3	73:1	2	1
4	rt	>99[d]	97.4	76:1	6	4
5[e]	rt	95	98.2	110:1	7	1
6	rt	98	98.6	142:1	8	1
7	rt	94	99.1	221:1	9	2
8	25[f]	99	99.2	249:1	10	1
8	20	97	99.3	285:1		
8	15	96	99.4	332:1		

[a] Per cent conversion of 100 g/L of substrate using 1 g/L of KRED, 0.25 g/L of GDH, and 0.4 g/L of NADP-Na in 100 mM pH 7.0 triethanolamine·HCl with 2 mM $MgSO_4$ in 24 h. [b] er = enantiomeric ratio *R*/*S*. [c] The activity of the parent was determined under the initial screening conditions: >99% conversion of 40 g/L of substrate in 2 h with 4 g/L of KRED. [d] Reaction completed in ∼16−20 h under conditions prescribed in footnote a. [e] A stringent thermal challenge was installed between rounds 4 and 5: all active variants were incubated at up to 60 °C for 24 h prior to retest. [f] A total of 0.5 g/L of GDH is needed at 25 °C (0.25 g/L initially; additional 0.25 g/L added at t = 16 h).

A detailed discussion regarding the "structure−activity relationship" between mutations and selectivity is beyond the scope of this paper. Briefly, with the exception of a "quantum leap" (>4×) in selectivity between round 1 (er = 9) and round 2 (er = 41) caused by a single active site mutation, all subsequent increases in selectivity were painfully incremental (<1.5×). This is not unexpected, given the nearly symmetrical nature of the substrate. The rigorous thermal challenge introduced between rounds 4 and 5 ensured the innate stability of all subsequent improved enzyme variants (it was done for the "wrong reason" of trying to knock out the phantom background reaction). The final variant, designated as CDX-033, is completely stable as the lyophilized powder after one month under air at room temperature and after at least two years under air at −20 °C (the standard storage condition).

In the final process the KRED reduction was run at 15 °C for 16 h to reach ∼90% conversion, followed by 7 h at 25 °C to reach >99% conversion. The temperature change was implemented to ensure >99% conversion and to obviate the need for a second charge of GDH (Table 2, footnote[f]). As a

(16) Throughout the evolution process, the activity of the successively more enantioselective variants typically varied within ±10−20% of the round 1 variant. Interestingly, the activity of a number of less (*R*)-selective (more (*S*)-selective) variants increased by 2−3×.

(17) 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.

(18) For the detailed experimental procedures (tiered HTS screening and catalyst preparations) and the amino acid sequence of the final variant, CDX-033, see: Liang, J.; Jenne, S.; Mundorff, E.; Voladri, R.; Lalonde, J.; Huisman, G. PCT Patent Application WO/2009/029554 (Experimental Section in pp 115−121), 2009.

(19) Extensive medium engineering at rounds 2 and 3 showed that the presence of 20 vol % of 30 co-solvents individually had little or no effect on the enantioselectivity.

(20) The reactions are carried out at pH 7.0. pH excursions of ±0.5 is well tolerated. The reaction rate approximately halved at pH 6.0, and GDH is unstable at pH 8.0 (deactivation within 2 h).

preventative mechanism, at the end of the reaction, 5 mol % of $NaHSO_3$ was added to sequester any unreacted ketone as the water-soluble (i.e., organic unextractable) bisulfite adduct.[21] After extraction with EtOAc and filtration through Celite 545 and solvent strip, the product **1** was obtained in 85−88% yield in >99% chemical purity in 99.3% ee.

Conclusions

The successful application of directed evolution technology enabled the unprecedented highly enantioselective reduction of tetrahydrothiophene-3-one to the corresponding (*R*)-alcohol in >99% ee. This process allows for the production of a key component to a family of potent antibiotics starting from a commodity chemical and obviates the need for hazardous reactions, reagents, and intermediates used in the current chiral pool route. Codexis has produced a total of ∼1 MT of the chiral alcohol **1** in 100+ kg batches using the evolved biocatalyst.

Experimental Section

On the R&D scale, tetrahydrothiophene-3-one was obtained from Alfa Aesar and was redistilled prior to use.[22] On the manufacturing scale, tetrahydrothiophene-3-one was procured from bulk suppliers in China and was used without further purification. KRED (CDX-033) and GDH (CDX-901) were manufactured by Codexis and are available from Codexis. NADP-Na was obtained from Oriental Yeast (Andover, MA).

Conversion and optical purity was determined via Chiralcel AD-H column on either an Agilent 1100 or an Agilent 1200 HPLC: Chiralpak AD-H column (4.6 mm × 150 mm, plus 10 mm guard cartridge) with a mobile phase of 2:98 IPA/hexanes at 1.5 mL/min (40 °C) and detection at 210 nm. Order of elution (and retention time) as follows: ketone (7.4 min), undesired (*S*)-alcohol (12.0 min), desired (*R*)-alcohol (12.7 min). For the purpose of determining conversion, the response factor is 2.1× LC area of the ketone.

Biocatalytic Reaction Procedure. To a 3-L three-neck jacketed flask at room temperature and fitted with a mechanical stirrer at ∼300 rpm, a thermometer, a pH probe, and an 8 N NaOH dosing probe from a Schott Titronic universal titrator (VWR catalogue number 13502-814) was added 100 mM pH 7.0 triethanolamine·HCl with 2 mM $MgSO_4$ buffer ("the buffer" henceforth, 850 mL) and glucose (338 g, 1.88 mol, 1.25 equiv.) in portions over ∼5 min under nitrogen. The temperature was lowered to 15 °C. To the reaction mixture was added tetrahydrothiophene-3-one (150 g, 1.47 mol) in one portion to give a biphasic reaction mixture[23] followed by KRED CDX-033 (1.5 g)[24] in 50 mL of the buffer, GDH CDX-901 (0.37 g)[24] in 50 mL of the buffer, and NADP-Na (0.60 g) in 50 mL of the buffer.

The reaction was maintained at pH 7.00 ± 0.1 via pH-stat controlled dosing of 8 N NaOH in 20 μL intervals. After stirring at 15 °C for 16 h (163 mL of 8 N NaOH was dosed), the circulator temperature was raised to 25 °C (internal temperature reached 25 °C in ∼1/2 h). After a total of 23 h (16 h at 15 °C and 7 h at 25 °C; a total of 171 mL of 8 N NaOH was dosed),[25] an aliquot was taken, and HPLC analysis revealed that >99% conversion had been achieved. The pH control was stopped, and 5.0 g of $Na_2S_2O_5$ was added.[26] After stirring for an additional 1 h at 25 °C (24 h total), EtOAc (1200 mL) was added. After 15 min, the aqueous and organic phases were separated. and the bottom, yellow aqueous phase was returned to the reaction vessel. The top milky organic phase (containing some emulsion) was passed through 30 g (∼3 in. diameter × ∼1/2 in. height) of Celite 545 over ∼2 min. A clean phase split occurred with both the top organic and the bottom aqueous phases being homogeneous. The bottom aqueous phase was returned to the reaction vessel (combined with the original aqueous phase). The Celite was rinsed with EtOAc (1200 mL). The EtOAc used in the Celite rinse was added to the pooled aqueous phase and stirred at 300 rpm. After 15 min, the phases were separated (clean split). The top organic phase was combined with the organic phase from the first extraction, and to the pooled organic phases was added heptane (600 mL) to phase out and split off additional water. All aqueous waste streams were treated with bleach and disposed of as alkaline aqueous waste. The combined organic phases were concentrated under reduced pressure at 40 °C to yield 136 g (88% yield, 99.3% ee) of the product as a pale-yellow oil that contained ∼1 wt % EtOAc (by ^{1}H NMR) and <0.2% of the ketone substrate.[27] ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.56 (m, 1H), 2.74−2.97 (m, 4H), 2.09 (m, 1H), 2.00 (br, 1H), 1.82 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 74.7, 39.9, 38.0, 28.1.

130 kg Runs. The above process was transferred to CMOs for manufacturing campaigns. The following is taken from the campaign summary from one of the CMO. The reaction was carried out according to the Codexis process description in a 4000-L glass-lined reaction vessel in two 130-kg batches. The

(21) When a reaction was purposely stopped at ∼66% conversion, we have shown that that it was possible to "rescue" the product in >99% chemical purity by adding the appropriate amount of $NaHSO_3$ or $Na_2S_2O_5$.

(22) Substrates from Alfa Aesar, Sigma Aldrich, Fluka, and Lancaster behaved essentially identically. However, some were darker in appearance than others. There was no difference between the redistilled and "as is" material. Redistillation was carried out to remove colored impurities that interfered with the spectroscopic high-throughput assays (see the Experimental Section of reference 18).

(23) The solubility of the substrate in the initial aqueous phase (buffer plus ∼1.3 M of glucose) is ∼5 g/L. The solubility decreases with increasing conversion due to the increase in ionic strength caused by the sodium gluconate byproduct. Since the catalyst was evolved for stability, activity, and selectivity under the biphasic conditions, the effect of the biphasic reaction mixture (e.g., interfacial deactivation) on the catalyst activity and stability was not determined as it was not of interest.

(24) Lyophilized cell lysate (semi-purification to remove cell debris and polynucleotides). Approximately 20−30 wt % of the lyophilized powder is the catalyst of interest.

(25) 171 mL of 8 N NaOH = 1.37 mol of NaOH (93% of theoretical). Because of the residual buffer capacity of 100 mM pH 7.0 triethanolamine·HCl (and sodium gluconate), the amount of the base required is always less than the theoretical amount by ∼5%.

(26) ∼4 mol % of sodium bisulfite. The residual ketone substrate was sequestered as the water soluble (i.e., organic unextractable) bisulfite adduct.

(27) Since the product **2** is >10 steps away from the API, the level of residual protein was not determined at this point. When the residual protein assay was carried out on a more advanced intermediate, the amount was below the detection level.

pH 7 controlled enzymatic reaction (1.56 kg of the KRED CDX-033 and 0.65 kg of the GDH CDX-901) in a buffer solution with glucose was carried out at 15 °C, later at 22−25 °C. The product was extracted with ethylacetate. Water removal was done with heptane. The product was isolated as crude after flash distillation of the solvents. Production time was 3.5 days. Yields of the crude product were 107 kg (81%) and 115 kg (87%).

Acknowledgment

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