Available online at www.sciencedirect.com
SCIENCE DIRECT
Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 17 (2006) 554–559

Effective synthesis of (*S*)-3,5-bistrifluoromethylphenyl ethanol by asymmetric enzymatic reduction

David Pollard,[a,*] Matthew Truppo,[a] Jennifer Pollard,[a] Cheng-yi Chen[b] and Jeffrey Moore[a]

[a]*Bioprocess R&D, Merck Research Laboratories, Merck & Co. Inc. Rahway, NJ 07065, USA*
[b]*Process R&D, Merck Research Laboratories, Merck & Co. Inc. Rahway, NJ 07065, USA*

Received 3 November 2005; accepted 7 January 2006
Available online 10 March 2006

Abstract—The synthesis of (*S*)-3,5-bistrifluoromethylphenyl ethanol, a pharmaceutically important alcohol intermediate for the synthesis of NK-1 receptor antagonists, was demonstrated from a ketone via asymmetric enzymatic reduction. The isolated enzyme alcohol dehydrogenase from *Rhodococcus erythropolis* reduced the poorly water soluble substrate with excellent ee (>99.9%) and good conversion (>98%). The optimized process was demonstrated up to pilot scale using high substrate concentration (390 mM) using a straightforward isolation process achieving excellent isolation yields (>90%) and effective space time yield (100–110 g/L d). Process improvements, demonstrated at preparative scale, increased the substrate concentration to 580 mM achieving a space time yield of 260 g/L d.

1. Introduction

Efficient routes to enantiomerically pure compounds are required to meet the increasing complexity of pharmaceutical drugs with multiple chiral centers. Optically active phenylethanol and its derivatives are useful building blocks for the synthesis of complex molecules as the alcohol functionality can be easily transformed, without racemization, into other useful functional groups. The trifluoromethyl substituents are important to a number of active research compounds as a useful tool to prevent degradation of the active compound by human metabolism. For example, both enantiomers of 3,5-bistrifluoromethylphenyl ethanol are intermediates for the synthesis of NK-1 receptor antagonists. (*R*)-3,5-Bistrifluoromethylphenyl ethanol is incorporated into the orally active NK1 receptor antagonist (EMEND®) for chemotherapy induced emesis,[1] while (*S*)-3,5-bistrifluoromethylphenyl ethanol is incorporated into a number of antagonists currently under clinical evaluation.

The use of commercially available isolated enzymes for the reduction of ketones can provide efficient and economic processes with good yields and excellent enantioselectivity (>99%). They generally use mild reaction conditions without the need for ee upgrade, which is often required for routes using metal catalyzed transfer hydrogenation. Biocatalytic reductions can be completed using whole cell systems but are frequently hampered by low productivity, and complicated by the presence of multiple ketoreductases, which can lower the selectivity and produce additional impurities.[2] The isolated enzymes provide simple reaction systems with few reaction components, making them amenable to the rapid process development required to meet the aggressive development timelines for clinical phase drug supply. The use of recombinant technology with high expression systems along with protein engineering has made isolated ketoreductases readily available with high activity.[3] Cofactor regeneration is no longer an issue due to the availability of both glucose dehydrogenase and formate dehydrogenase to recycle NADPH and NADH, respectively. The cofactor can be provided by in situ regeneration using formate dehydrogenase (FDH), which irreversibly oxidizes the formate to carbon dioxide that is easily removed as a gaseous product.[4] This has successfully been demonstrated on a tonne scale for the production of *tert*-leucine.[5]

This contribution demonstrates the first example of the effective synthesis of (*S*)-3,5-bistrifluoromethylphenyl ethanol by enzymatic reduction. Process development is presented from the initial ketoreductase screening success, which identified the alcohol dehydrogenase

* Corresponding author. E-mail: david_pollard@merck.com

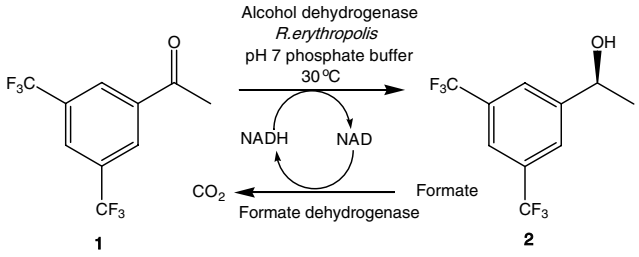
0957-4166/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetasy.2006.01.039

Rhodococcus erythropolis, through to the effective pilot plant demonstration. Particular challenges were overcome including the poor solubility of ketone substrate in water, which often limits enzymatic reactions to low substrate concentrations (5–10 mM) and product crystallization, which was hampered by the high solubility of the alcohol product in most organic solvents.

2. Results and discussion

2.1. Ketoreductase library screen

A library of commercially available ketoreductases were screened for their ability to reduce ketone **1** (Scheme 1). An example of some of the screening results is shown in Table 1 where the alcohol dehydrogenase (ADH) from *R. erythropolis* (ADH RE) gave an ee >99% for the (*S*)-alcohol **2** followed by the ADH's of *Candida boidinii* and *Candida parapsilosis* with ee of 94%. The screen also showed that the (*R*)-enantiomer can be synthesized by a number of enzymes including KRED 101 with ee of 99%.



Scheme 1.

Table 1. A sample of the screening data from the ketoreductase library for the reduction of 3,5-bistrifluoromethyl acetophenone

Enzyme catalyst	ee (%)	Cofactor dependency
ADH *Rhodococcus erythropolis*	>99 (*S*)	NADH
ADH *Candida parapsilosis*	96 (*S*)	NADH
ADH *Candida boidinii*	94 (*S*)	NADH
KRED 101	>99 (*R*)	NADPH
KRED 110	98 (*R*)	NADPH
KRED 112	97 (*R*)	NADPH
KRED 113	96 (*R*)	NADPH
ADH *Lactobacillus brevis*	87 (*R*)	NADPH
ADH *Lactobacillus kefir*	83 (*R*)	NADPH

The NADH cofactor dependent alcohol dehydrogenase isolated from *R. erythropolis* (ADH RE) is known to catalyze the conversion of ketones to chiral alcohols, particularly medium chain alkanones and aromatic ketones, including acetophenone with halogenated substituents.[6] Preparative scale examples of the acetophenone reductions with 4-chloro and 4-bromo acetophenone have been shown with good yields and enantiomeric excesses (>95%).[7] The relative activity of ADH RE for ketone **1** was found to be similar to that of 4-chloroacetophenone indicating that the reduction of ketone **1** was amenable for development into an effective process. Process development for the pilot plant demonstration was pursued using formate dehydrogenase (FDH) to regenerate NADH.

2.2. Process development using formate dehydrogenase for cofactor regeneration

2.2.1. Substrate delivery and mass transfer. The poor solubility of ketone substrates in aqueous buffer systems can lead to mass transfer issues and limit the reaction to slow rates and low substrate concentrations. Despite the poor solubility of ketone **1** in aqueous buffer (<0.05 g/L (<2 mM)), reactions proceeded to completion provided that adequate mixing was applied to disperse the hydrophobic liquid ketone as a second phase. For this biphasic system, the mixing intensity affected the rate of reaction by impacting the ketone droplet size. This dependency of the initial reaction rate to mixing is shown in Figure 1. A minimum speed was required, in the case of the lab reactor >600 rpm, before the initial rate of reaction was independent of agitation. Reactions, which were run below the minimum agitation, were slow and often led to incomplete conversion. Adequate mixing was especially important as the alcohol product was a solid at 30 °C and so precipitated out of solution during the reaction. Once conversions reached >60% completion, the residual liquid ketone **1**, which formed a second phase in water, became encapsulated by the precipitated alcohol. This limited the availability of the ketone to the enzyme causing reduced rates and incomplete conversion. The rate of reaction was maintained by increasing agitation, in the case of the lab reactor this meant $\geqslant$700 rpm (Fig. 1), which minimized the alcohol solid particle size and the amount of encapsulated ketone.

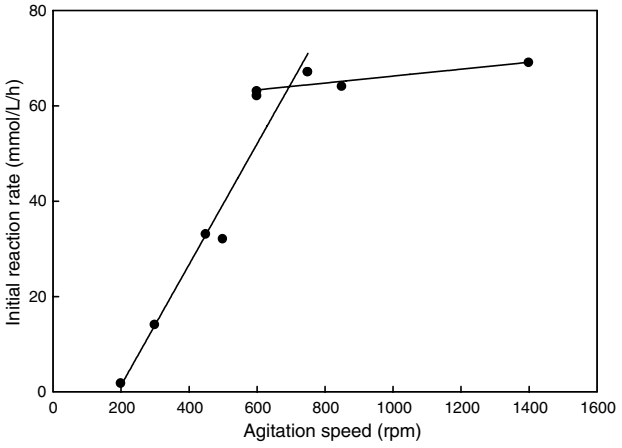


Figure 1. Effect of agitation on the initial rate of reaction for 3′,5′-bistrifluoromethyl acetophenone (ADH RE 5 KU/L, FDH 4 KU/L, NAD 1 g/L, 30 °C, pH 7.0, substrate 4 g, 40 mL Multimax reactor).

2.2.2. Optimization of the activity and stability of two enzyme process. A good understanding of the factors affecting the activity and stability of both enzymes is essential for process optimization of isolated enzyme systems. The pH for the optimum activity of ADH RE

was 6.5, whereas the optimum for FDH was closer to pH 8.0 (Fig. 2). This meant that a compromise is required for the two enzyme systems to work efficiently. An operating region (Fig. 2: shaded area) around pH 7 was chosen as the optimum region for both enzymes. Both ADH RE and FDH showed improved activity at elevated temperatures (Fig. 2) but the stability of both enzymes must also be considered. Studies of the enzyme stability in the buffer showed the deactivation profiles, at a range of pH and temperatures, followed the first order kinetic model ($r^2 > 0.98$) enabling half-life times to be compared. Figure 3 shows the inferior thermostability of FDH compared to ADH RE. This limits the reaction operating temperature to around 30 °C when using FDH for cofactor regeneration. The buffer pH was also shown to affect enzyme stability as ADH RE was most stable at pH <7.0 while FDH was most stable at pH values >6.5. Combining the activity and stability data showed that the optimum conditions for activity and stability for the two enzyme system (ADH RE with FDH) was to be at the temperature of 30–32 °C and pH between 6.8 and 7.2.

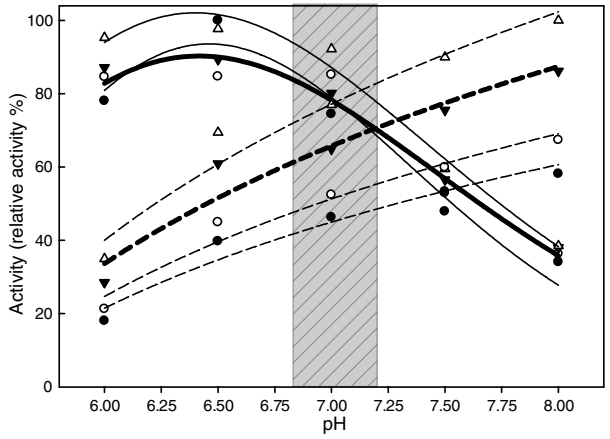


Figure 2. Effect of temperature and pH on the activity of ADH RE&FDH. (25 °C: closed circle, 30 °C: open circle, 34 °C: closed triangle, 38 °C open triangle). One hundred percent relative activity refers to maximum activity for each enzyme across all temperature and pH ranges.

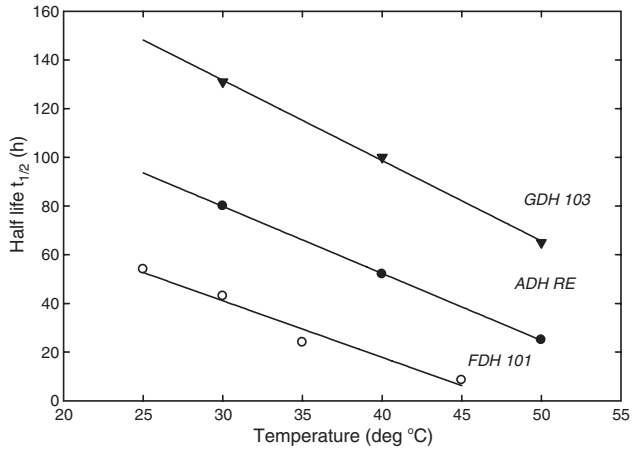


Figure 3. Stability comparison of alcohol dehydrogenase (*R. erythropolis*), formate dehydrogenase (101), and glucose dehydrogenase (103): the effect of temperature on the half life $t_{1/2}$ of the enzyme in 100 mM phosphate buffer (pH 7).

2.2.3. Effect of substrate and enzyme concentration. The relationship of substrate and enzyme concentrations must be understood for any enzymatic system. Ketone reductions with >95% completion were achieved for substrate concentrations up to 390 mM (100 g/L) (Fig. 4) with a satisfactory space time yield (STY) of 107 g/L d. Comparison of initial rates showed a linear increase with substrate concentration, so no substrate inhibition was observed up to 200 g/L. The 200 g/L reaction could reach completion if the initial concentrations of ADH RE and FDH were increased by 50%. The effect of enzyme concentration was also studied and as expected, the initial rates of reaction were proportional to the enzyme concentration.

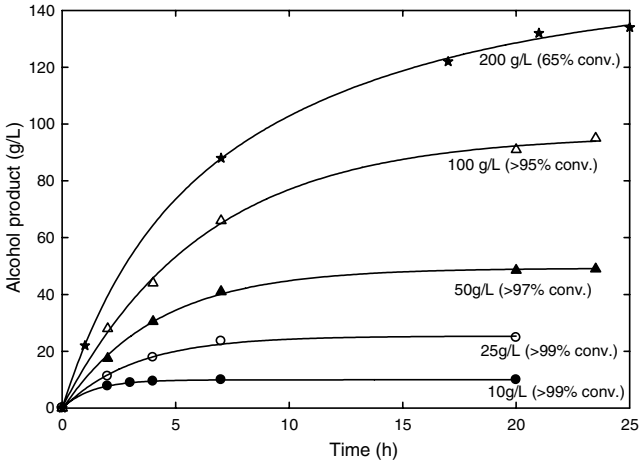


Figure 4. Effect of substrate concentration on the reduction of 3′,5′-bistrifluoromethyl acetophenone (ADH RE 5 KU/L, FDH 4 KU/L, NAD 1 g/L, 30 °C, pH 7.2).

2.2.4. Isolation process development. Crystallization of alcohol **2** proved challenging as an antisolvent could not be found due to the high solubility of the alcohol in most organic solvents. Heptane was chosen as the crystallization solvent due to a steep solubility curve with temperature. Above 35 °C, the solubility of alcohol in heptane was >200 g/L, which fell to <5 g/L at 0 °C. Therefore a simple isolation was developed, which enabled heptane to be used as a single organic solvent for the complete isolation process (extraction and crystallization). Hot heptane was used for the extraction of the alcohol from the aqueous buffer reaction, followed by a water wash to remove residual enzyme and then concentration by vacuum distillation. The heptane extract was cooled to −10 °C with crystal seeding at 35 °C. This crystallization was found to reject up to 20% residual ketone in the unlikely event that a reaction did not reach completion. This ketone could then be recycled back for enzymatic reduction. For the preparative scale demonstration this isolation procedure gave an overall yield of 95% with high purity (>99.9% purity).

2.2.5. Scale up of the 3,5-bistrifluoromethyl acetophenone reduction. The optimized process of pH 7 and 30 °C, was demonstrated with ketone **1** at 100 g/L (390 mM) and scaled to 1.1 kg with conversion >99%, ee >99.5% and overall yield after crystallization of 95% with purity >99%. Similar results were achieved at 25 kg scale with space time yield (STY) of 100–110 g/L/d (Fig. 5). The process was robust and able to withstand operating ranges for the pilot plant of 28–32 °C and pH 6.8–7.2. The process ran in a standard glass lined pilot scale reactor with the addition of a recycle loop for pH monitoring and subsurface acid addition for pH control. The initial rate of reaction was similar at all scales indicating that mixing was sufficiently maintained (Fig. 5).

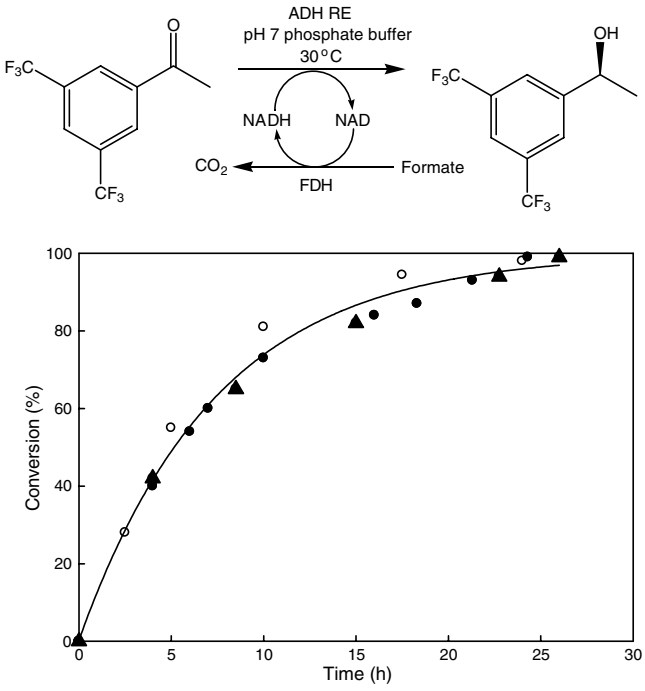
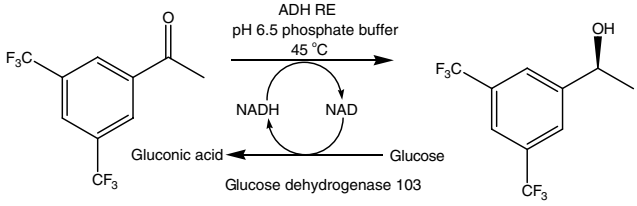


Figure 5. Scale up of the enzymatic reduction of 3′,5′-bistrifluoromethyl acetophenone to 25 kg (3 g: closed circles, 1.1 kg: triangles, 25 kg: open circles) (ADH RE 3KU/L, FDH 4 KU/L, NAD 1 g/L, 30 °C, pH 7.2).

This enzymatic process with the bioconversion stage followed by simple isolation using organic solvent extraction and crystallization was certainly more convenient and practical to operate than existing technologies. Similar space time yields (STY) (60–104 g/L h) to this work have been shown for continuous acetophenone reduction.[8] The system required a specialized enzyme membrane reactor and solvent extractor connected by external loop that was far more complicated than the procedure with isolated enzymes described in this paper. The reduction of 4-chloroacetophenone by ADH RE, using cofactor regeneration from FDH, co-expressed in the same *E. coli* cell has also been demonstrated.[7] However, this intensive process required several steps: fermentation of the cells, cell separation/concentration, bioconversion then product isolation. The ability to use commercially available isolated enzymes conveniently separates the issues of enzyme manufacture by recombinant fermentation from the bioreduction. The biocatalytic routes certainly provide superior enantiomeric excess (>99%) over the conventional chemo catalysis routes, which often require a difficult upgrade procedure from ee values of 90–92%.[1,9] The isolated enzyme route provides an ease of operation with the aqueous process compared to the handling issues often faced for chemo catalysis, which include air and moisture sensitive catalysts and hazardous reagents such as in the case of borane reduction.

2.3. Further process improvements using glucose dehydrogenase for cofactor regeneration

After implementation of the ADH RE/FDH process into the pilot plant, a glucose dehydrogenase became available (GDH 103, Biocatalytics Inc.) that had enhanced temperature tolerance and was capable of regenerating either NADH or NADPH with similar activity. The thermostability of GDH 103 was significantly improved to that of FDH, as shown in Figure 3. This allowed the reaction temperature to be raised to 45–50 °C above the 30 °C limit set by the process using FDH. The pH activity optimum for GDH is 6.5, which matched the optimum for ADH RE. This allowed both enzymes to work at their optimum pH for activity compared to the previous process using FDH where both enzymes had to work at the suboptimal pH of 7.0 (between the two activity optimums of pH 6.5 for ADH RE and pH 8.0 for FDH). The GDH was also 10-fold more active than FDH, which meant the protein levels in the reaction were reduced by 10-fold. This made a cleaner interface during the heptane extraction and simplified the phase separation between aqueous and organic solvent for pilot plant operation. These combined effects enabled reactions to be operated at 45 °C and pH 6.5 (Scheme 2). Preparative scale demonstrations at 100 g/L, 390 mM showed the increased rate of reaction at 45 °C allowed space time yield improvements from 100 to 170 g/L d. with similar 99% ee, 96% conversion and overall yield of 93%. A twofold increase of the concentration of ADH RE allowed the substrate concentration to be increased to 580 mM with a space time yield of 260 g/L d.



Scheme 2.

3. Conclusion

This work successfully demonstrated the first example of the synthesis of (*S*)-3,5-bistrifluoromethylphenyl ethanol up to pilot scale by enzymatic reduction using the isolated alcohol dehydrogenase from *R. erythropolis*. The

enzymatic reduction using in situ cofactor regeneration was shown to be an efficient and practical alternative to existing methods achieving superior selectivity of >99.9%. The reduction using high substrate concentration, up to 580 mM, was demonstrated without the need for additional organic co-solvent. The substrate to catalyst molar ratio for substrate **1** was high at 1,000,000:1 and total turnover numbers for NAD estimated to be >1000. The ability to use the improved glucose dehydrogenase 103 for cofactor recycling of either NADH or NADPH allows the same reaction method to be used to synthesize either enantiomers of alcohol **2** by simply switching between the ketoreductase enzyme such as ADH RE or KRED 101. This biocatalytic method provides general applicability of chiral alcohol production for robust manufacturing with effective space time yields using standard chemical processing equipment.

4. Experimental

All solvents and reagents including enzymes were purchased commercially. The ADH RE, formate dehydrogenase 101 and glucose dehydrogenase 103 were from Biocatalytics Inc. Enzyme activity was measured spectrophotometrically using a Biotek powerwave HT plate reader. Quantification of ketone to alcohol conversion was completed using reverse phase HPLC with a Zorbax extend C18 column. The mobile phase was 40% acetonitrile/60% water with 0.1% TFA. The isocratic flow rate was 0.75 mL/min at 25 °C with absorbance monitoring at 265 nm. Chiral analysis for ee determination was by normal phase HPLC with Chiralcel OD-H column using 98% hexanes/2% 2-propanol at 1 mL/min, 25 °C and monitoring at 265 nm.

4.1. Screening protocol

A total of 40 commercially available ketoreductases (KRED 101–131) from Biocatalytics Inc. and alcohol dehydrogenases from Julich Fine Chemicals were used at 2 g/L in 100 mM phosphate buffer (pH 7) containing 1.2 M equiv of NADPH or NADH for the dependency of the enzyme. Substrate (20 mM) was delivered to the reaction in 5% v/v toluene. After overnight incubation at 30 °C, reactions were extracted with three volumes of acetonitrile for reverse phase HPLC, then dried and resuspended in methanol for chiral HPLC analysis.

4.2. Enzyme activity and stability

The activity of the NADH dependent ADH RE was determined spectrophotometrically by measuring the oxidation of NADH at 340 nm ($E340 = 6.22\ mM^{-1}\ cm^{-1}$) in the presence of 2.5 mM 4-chloroacetophenone. Activity was measured at 25 °C in 96 well plate format (200 μL) containing 200 mM potassium phosphate buffer at pH 7.0 with 5 mM NADH. The reaction was started by adding the assay mixture to the enzyme sample. The reaction kinetics were monitored over a 2 min period and one unit of ADH activity was defined as the amount of enzyme that converted 1 μmol of NADH per minute. The activity of the reaction samples was expressed as relative activity % compared to a standard ADH RE solution 2 U/mL (for 4′-chloroacetophenone). The activity of the NAD dependent FDH and GDH 103 was determined spectrophotometrically by measuring the reduction of NAD at 340 nm ($E340 = 6.22\ mM^{-1}\ cm^{-1}$) in the presence of 150 mM sodium formate for FDH or 150 mM glucose for GDH. Activity of the reaction samples was expressed as relative activity % compared to a standard FDH solution or GDH solution 2 U/mol (for sodium formate and glucose, respectively). Studies of ADH RE, FDH, and GDH stability were carried out at a range of pH and temperature conditions at 1 mL scale. Samples (10 μL) were removed during a 48 h period and activity measured as described above. The main activity loss was described by the first order deactivation where the half life ($t_{1/2}$) for each pH and temperature condition with both enzymes was measured from the Ln plot of relative activity against time.

4.3. Enzymatic reduction of ketone 1 using formate dehydrogenase for cofactor regeneration

Development work was completed at the 4 g scale: phosphate monobasic buffer (50 mM, pH 7.2) was added to a Multimax reactor (Mettler Toledo) at a volume of 40 mL with overhead agitation. NAD (1 g/L) at 160 mg was added followed with sodium formate 6 g and the two enzymes ADH RE (3 KU/L) and FDH (2.88 KU/L). The specific activity for ADH RE was 35 U/mg and FDH activity was 4.7 U/mg, calculated using the activity assays outlined in Section 4.2. Reactions were run at 30 °C with automatic pH control at 7.0 using 2 N sulfuric acid. Ketone **1** was added as a liquid. The same procedure was used for the 1.1 kg process using a 20 L glass stirred tank reactor. The reactions were extracted with two half volumes of hot heptane when the substrate was shown to be >95% converted. The combined heptane extracts were washed with a 1/4 volume of water to remove remaining enzyme protein residues, then concentrated by distillation to a 200 g/L concentration. For crystallization the solution was slowly cooled from 45 to 35 °C. Seeding with alcohol product with 1 g (0.1% gram/gram of product) was completed at 35 °C, followed by 1 h of aging and cooled down to −10 °C. The crystallization procedure rejected impurities such as residual ketone and final purity of >99% was obtained. ^{1}H NMR: δ 7.85 (s, 2H), 7.80 (s, 1H), 5.05 (qd, $J = 6.5$, 3.3, 1H), 2.04 (d, $J = 3.3$, 1H), 1.56 (d, $J = 6.5$, 3H). ^{13}C NMR: δ 148.44, 131.99 (q, $J = 33.2$), 125.87 (br q, $J = 2.8$), 123.58 (q, $J = 272.6$), 121.53 (septet, $J = 3.9$), 69.31, 25.79.

The same procedure was followed for the 25 kg pilot plant batch at the 250 L scale with the inclusion of a recycle loop in order to monitor pH.

4.4. Enzymatic reduction of ketone 1 using glucose dehydrogenase 103 for cofactor regeneration

Development work was completed at the 8 g scale following the same protocol as Section 4.3, except that GDH 103 replaced FDH at 5 KU/L. The specific activ-

ity for GDH 103 was 65 U/mg calculated using the activity assay outlined in Section 4.2. The pH was controlled at 6.5 using 2 M sodium hydroxide with temperature at 45 °C.

References

1. Brands, K. M. J.; Payack, J. F.; Rosen, J. D.; Nelson, T. D.; Candelario, A.; Huffman, M. A.; Zhao, M. M.; Li, J.; Craig, B.; Song, Z. J.; Tschaen, D.; Hansen, K.; Devine, P. N.; Pye, P. J.; Rossen, K.; Dormer, P. G.; Reamer, R. A.; Welch, C. J.; Mathre, D. J.; Tsou, N. N.; McNamera, J. M.; Reider, P. J. *J. Am. Chem. Soc.* **2003**, *125*, 2129–2135.
2. Pollard, D. J.; Telari, K.; Lane, J.; Humphrey, G.; McWilliams, C.; Nidositko, S.; Salmon, P.; Moore, J. *Biotechnol. Bioengng.* **2006**, *93*, 674–686.
3. (a) Zhu, D.; Rios, B. E.; Rozzell, D.; Hua, L. *Tetrahedron: Asymmetry* **2005**, *16*, 1541–1546; (b) Kaluzna, I.; Rozzell, D.; Kambourakis, S. *Tetrahedron: Asymmetry* **2005**, *16*, 3682–3689.
4. Hummel, W. *TIBTECH* **1999**, *17*, 487–492.
5. Bommarius, A. S.; Drauz, K.; Hummel, W.; Kula, M.-R.; Wandrey, C. *Biocatalysis* **1994**, *10*, 37–47.
6. Hummel, W.; Abokitse, K.; Drauz, K.; Rollman, C.; Groger, H. *Adv. Synth. Catal.* **2003**, *345*, 153–159.
7. Groger, H.; Hummel, W.; Rollman, C.; Chamouleau, F.; Husken, H.; Werner, H.; Wunderlich, C.; Abokitse, K.; Drauz, K.; Buchholz, S. *Tetrahedron* **2004**, *60*, 633–640.
8. Kruse, W.; Hummel, W.; Kragl, U. *Recl. Trav. Chim. Pays-Bas* **1996**, *115*, 239–243.
9. Hansen, K. B.; Chilenski, J. R.; Desmond, R.; Devine, P. N.; Grabowski, E. J. J.; Heid, R.; Kubryk, M.; Mathre, D. J.; Varsolona, R. *Tetrahedron: Asymmetry* **2003**, *14*, 3581–3587.