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Development of a Continuous Enzymatic Process for the Preparation of (*R*)-3-(4-Fluorophenyl)-2-hydroxy Propionic Acid

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Abstract:

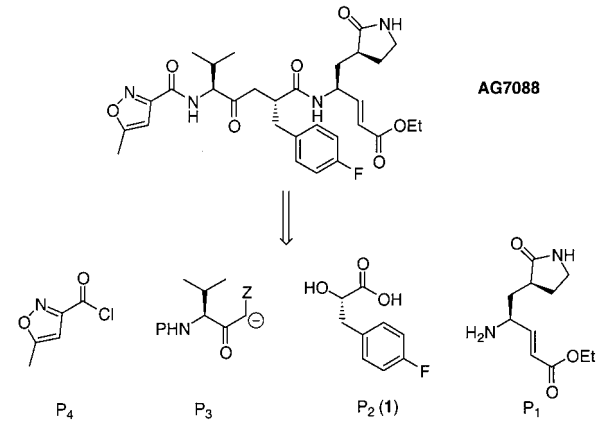
While many methods have been reported for the synthesis of chiral 2-hydroxy acids, few of them have proven to be reliable toward the synthesis of the title compound in terms of overall yield and enantioselectivity. Herein we describe a continuous enzymatic process for an efficient synthesis of (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid at multikilogram scale with a high space−time yield (560 g/(L·d)) using a membrane reactor. The product was generated in excellent enantiomeric excess (ee > 99.9%) and good overall yield (68−72%).

Introduction

(*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid **1** is a building block for the synthesis of **AG7088**, a rhinovirus protease inhibitor currently in human clinical trials to treat the common cold (Scheme 1).[1,2] Retrosynthetically, **AG7088** was prepared from four fragments: the lactam derivative P_1, the chiral 2-hydroxyacid P_2 (compound **1**), the valine derivative P_3, and an isoxazole acid chloride P_4 (Scheme 1). In this study the preparation of **1** using a biocatalytic reduction performed in a membrane reactor will be discussed in detail.

To our knowledge, no methods have been described for the preparation of **1** although many have been reported for the synthesis of related chiral 2-hydroxy acids in the literature.[3] Among them, only a few are concise and practical for preformance at large scale (Scheme 2). For example, optically active α-hydroxy acids could be prepared enzymatically from an aldehyde and a cyanide equivalent upon hydrolysis if $n = 0$, where *n* denotes the number of carbons between the aldehyde and the arene (path a, Scheme 2).[4]

Scheme 1



However, the stereoselectivity is usually quite low for substrates where $n = 1$, which is needed for the preparation of **1**. Extensive studies have been reported to synthesize chiral α-hydroxy acids from ketoesters using asymmetric hydrogenation (path b, Scheme 2). In this strategy excellent enantioselectivity could be obtained if $n = 2$.[5] Unfortunately, in our hands the stereoselectivity is far from ideal if $n = 1$. Borane-mediated asymmetric reductions have also been investigated for the same purpose.[6] However, most of them require the use of a stoichiometric amount of an expensive reducing agents or substrates which are not readily available. In addition, enzymatic reduction methods have been reported to prepare compounds where $n = 2$.[7] Only a few were targeted to substrates where $n = 1$, and all of them were performed only on small scales. Alternatively, hydroxy acids could be obtained from an enol acetate using asymmetric hydrogenation[8] (path c, Scheme 2) or ring-opening of glycidic acid derivatives by a metalated nucleophilic species[9] (path d, Scheme 2). In our hands, both approaches suffer

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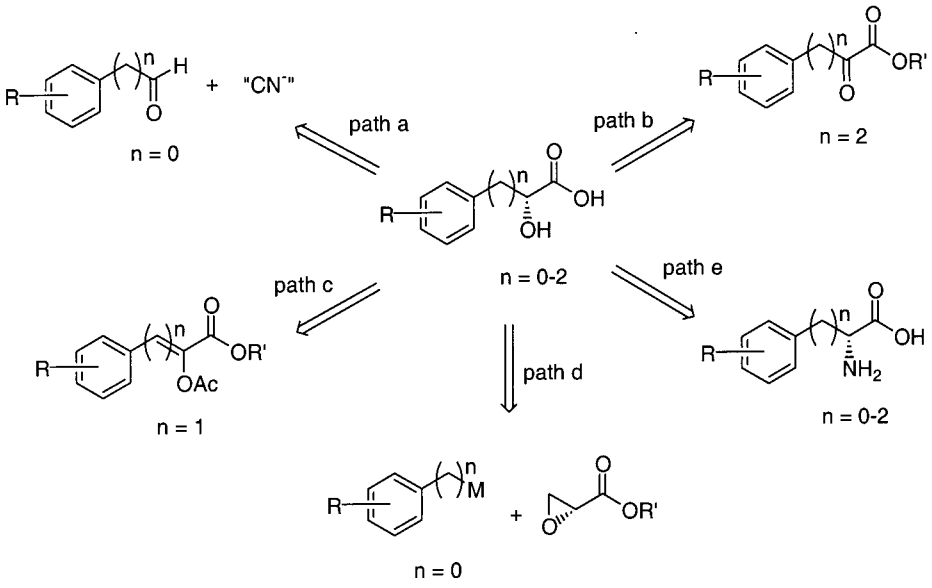
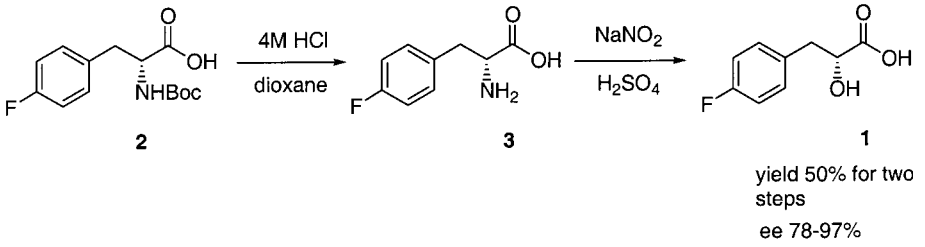
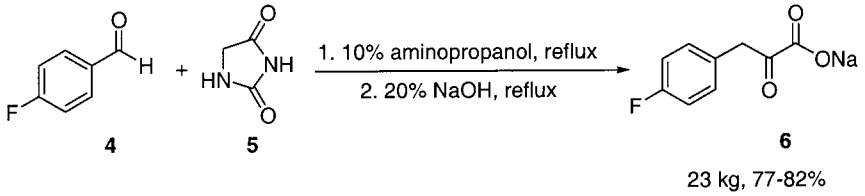
Scheme 2



Scheme 3



Scheme 4



from either low yields or poor stereocontrol. In the first campaign for scaling up the preparation of **1**, the only pathway we could rely on utilizes amino acids as starting materials (path e, Scheme 2).[10]

Using this method, the preparation of **1** could be accomplished starting from a protected (*R*)-4-fluorophenylalanine **2** (Scheme 3). Upon deprotection and diazotization, the desired product could be obtained in a modest yield of 50% for two steps via the isolated intermediate **3**. However, the starting amino acid **2** is quite expensive, and the optical purity of the product **1** varied from a low 78% to a high 97% in scaled-up runs, indicating that the process is not robust. Both problems render this method impractical for production at large scale.

These issues motivated us to seek an alternative way to prepare the desired optically active α-hydroxy acid. Herein we describe an efficient and concise synthesis of **1** at multikilogram scale using a continuous enzyme membrane reactor.

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Results and Discussion

The route began from 4-fluorobenzaldehyde **4** and hydantoin **5**, which are inexpensive and readily available (Scheme 4). Upon condensation and saponification using a modified literature protocol,[11] α-keto acid salt **6** was obtained as a white solid in one step in good yields (77−82%). By this method, an overall quantity of 23 kg of **6** was prepared.

The key step was an aqueous enzymatic reduction using D-lactate dehydrogenase (D-LDH) and formate dehydrogenase (FDH) (Scheme 5).[7e,12] Mechanistically, the keto acid salt **6** is stereoselectively reduced to the corresponding α-hydroxy acid in the presence of D-LDH by NADH. The cofactor itself is oxidized to NAD in the process. Subsequently, in the presence of FDH, NAD is reduced back to NADH by ammonium formate, which was oxidized to CO_2 and NH_3. In this fashion the expensive cofactor NAD is regenerated by FDH, and only a catalytic amount of NAD

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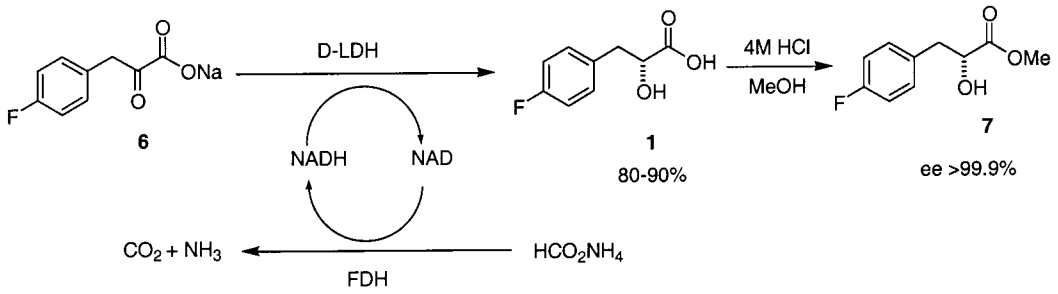
Scheme 5



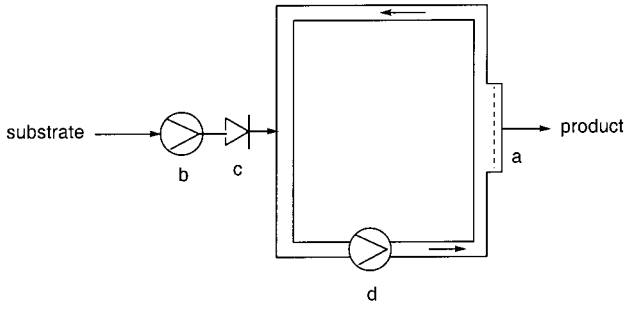


Figure 1. **Schematic drawing of the enzyme membrane reactor.**

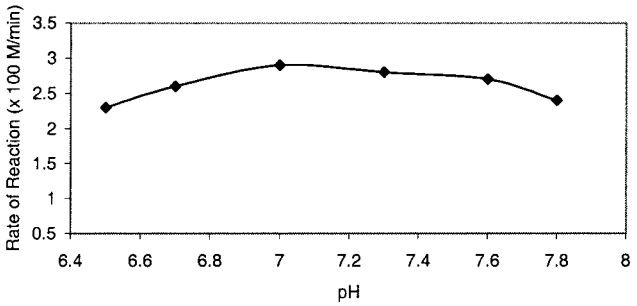


Figure 2. **Effect of pH on reaction rate.**

is required. Overall the ketone group in **6** is reduced stereoselectively to the hydroxy group in the presence of two enzymes with byproducts being CO_2 and NH_3. The optical purity of the product was determined by converting **1** to the methyl ester **7** (Scheme 5). At small scale using a batch process, the desired 2-hydroxy acid could be obtained in good yields (80−90%) and excellent enantiomeric excess (>99.9%).

To scale-up the method, however, both D-LDH and FDH have to be recycled to make the process economically feasible. While the starting material **6** could be prepared readily in large scale and only a catalytic amount of NAD is needed for the reaction, neither of the commercially available enzymes is inexpensive. Initial recycling efforts were directed to a batch process using either membrane-enclosed enzyme catalysis or enzyme immobilization methods. In our hands, these reactor systems were not convenient for enzyme recycling and not ideal for scaling-up due to the volume issue. The substrate has only modest solubility in water. After a period of trial and error, we found that a continuous membrane reactor, shown schematically in Figure 1, would allow recycling of both D-LDH and FDH and could fit both the critical path time line of product delivery and the cost requirement.[13]

The key part of the reactor is a ultrafiltration membrane unit (a), which allows the permeation of small molecules but not macromolecules such as enzymes. In operation, the reactor is initially charged with D-LDH and FDH before the start of the reaction. An aqueous mixture, which consists of **6**, ammonium formate and a catalytic amount of NAD, is then continuously fed into the reactor by a peristaltic pump (b). After passing a check valve (c), the substrate solution is mixed with enzymes inside the reactor by a circulation pump (d). The product is collected continuously as an effluent out of the filtration membrane unit. In this fashion, both enzymes are retained inside the reactor by the membrane leading to high turnover.

Once a prototype reactor had been designed, optimum conditions were obtained by studying reaction kinetics. D-LDHs from *Leuconostoc mesenteroides* and *Staphylococcus epidermidis* showed the same activity toward the substrate **6**, and the former was chosen for subsequent studies solely for economic reasons. FDH from *Candida boidinii* was preferred over the one from yeast since this preparation is not only more reactive but also significantly cheaper. To maximize the throughput and reduce the working volume of the process, a saturated aqueous substrate solution was used in all studies. The concentration was calculated to be at 44 g L^{-1} or 0.2 M. As for ammonium formate, 4 equiv were used to achieve a high reaction rate. Below that level, the reactivity of the system goes down proportionally. However, no significant advantage was achieved at a higher concentration. The optimum concentration of the cofactor NAD was found to be at 0.167 M or 1% with respect to the substrate. Above that level, no significant benefits were obtained. Below that level, however, the rate of the reaction is almost proportional to the concentration of NAD. In general, the enzymatic reduction attains the highest reactivity at a pH range between 7.0 and 7.3, which was chosen for subsequent investigations (Figure 2). To reach this pH range inside the reactor and obtain a conversion above 90%, the substrate solution was maintained at pH 6.3 before being fed into the reactor.

The concentration ratio of D-LDH to FDH has an important effect on the reactivity as well and the optimum ratio was found to be around 20 (Figure 3). Below that level,

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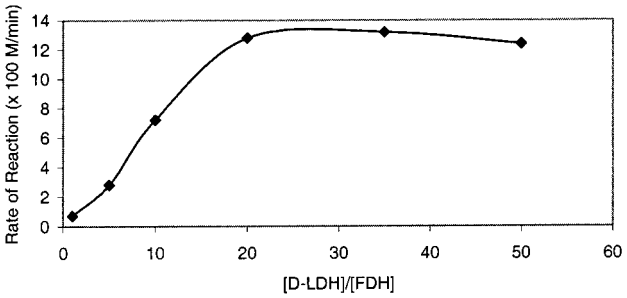

Figure 3. **Effect of concentration ratio of enzymes on reaction rate.**

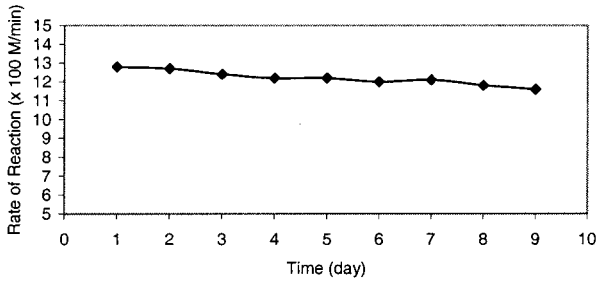

Figure 4. **Enzyme deactivation in the membrane reactor.**

the reaction slows down significantly. Above that level, a slight gain in the rate of reaction occurs at the expense of a large excess of D-LDH. Moreover, the reactivity starts to level off when the ratio is above 30. In the final material production, a concentration of 400 units mL^{-1} was used for D-LDH and 20 units mL^{-1} for FDH.

Enzyme deactivation was a key factor in determining the overall cost of the process. In a period of 9 days without adding fresh D-LDH and FDH, the rate of reaction decreases slowly, and the enzymes lose their activity at a rate of only about 1% per day (Figure 4). Both EDTA and mecaptoethanol were added to enhance enzyme stability. During actual production, the reactor was charged with new enzymes periodically to get a high conversion of above 90%.[14]

In the full-scale production of the chiral hydroxy acid, a continuous membrane reactor with a volume of 2.2 L was used to meet the time line for product delivery. Under the optimum conditions, a substrate solution with pH 6.3 was fed into the reactor at a rate of 12.0 mL min^{-1}, resulting in a residence time of about 3 h. The average conversion was maintained above 90%. Upon workup by extraction, the desired product was obtained in a yield of up to 88% with ee's >99.9%. The NAD is water soluble and left in the aqueous phase. The cost of NAD is insignificant at this scale in comparison to that of the enzymes, and no efforts have been devoted to its recycling. Using this process, a total of 14.5 kg of the desired chiral 2-hydroxy acid **1** was prepared within a period of 4 weeks with a productivity of ca. 560 g/(L·d) (gram per liter per day). The overall cost of goods of the current process is significantly less expensive than the diazotization route.

(14) To reach a short residence time and good productivity, about 10% of unreacted starting material was removed upon workup. The cost of the starting material is insignificant compared to that of the two enzymes in this process.

Conclusions

An efficient and practical process has been described for the synthesis of (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid at multikilogram scale with good overall yields (68−72% for two steps), excellent stereoselectivity (>99.9% ee), and significant cost savings. The key to the process is the use of a continuous membrane reactor which was simple in concept, low-cost in design, and provided high space−time yields.

Experimental Section

General Remarks. Commercially available solvents and reagents were used without further purification. D-LDHs from *L. mesenteroides* and *S. epidermidis* were available from Roche Diagnostics and Sigma-Aldrich, respectively. FDH from *C. boidinii* was purchased from Jülich Fine Chemicals, and the yeast preparation, from Sigma-Aldrich. NAD was a product of Roche Diagnostics. The enzyme reactor was constructed as a thermostatic loop using Viton tubing with an ultrafiltration membrane (cutoff: 10 000 D) as a separation unit (Millipore Pellicon 2 Mini Filter Module PLCGC 0.1 m^2). ^1H NMR spectra were recorded at 300 MHz in d_6-DMSO, D_2O, or $CDCl_3$. Reversed-phase HPLC was performed on a Phenomenex Prodigy 3 μm ODS (4.6 mm × 100 mm) column, and chiral HPLC was performed on a Chiralpak AS column. The elemental analyses were carried out by Atlantic Microlab, Inc.

Sodium 3-(4-Fluorophenyl)-2-oxo-propionate (6). 4-Fluorobenzaldehyde **(4)** (3.72 kg, 30 mol), hydantoin **(5)** (3.00 kg, 30 mol), 1-amino-2-propanol (225 g, 3.0 mol), and water (7.5 L) were added to a 50-L reactor equipped with a temperature probe, reflux condenser, agitator, and cooling coils. The resulting mixture was heated and refluxed for approximately 10 h. The reaction was monitored by ^1H NMR and was deemed complete upon disappearance of the hydantoin (**5**) proton signal at δ 3.9 (s, 2H, CH_2) and the appearance of condensed intermediate olefin proton δ 6.4 (s, 2H, CH). Aqueous sodium hydroxide (6.00 kg in 30.0 L) was then added to the bright yellow slurry, and reflux continued until completion as shown by HPLC, leading to a transparent orange solution. The mixture was cooled to 20 ± 5 °C, and sodium chloride (3.51 kg, 60.0 mol) was added with agitation. The pH of the mixture was adjusted to 8.0 using concentrated HCl and the suspension was stirred for 4 h until a pale yellow slurry was obtained. The solids were filtered off and purified via slurrying in methanol (30.0 L) followed by filtration. Upon drying under house vacuum at ambient temperature for 4 days, 5.47 kg of solids (**6**) was obtained with a yield of 82% and HPLC purity above 80%. ^1H NMR (D_2O): δ 4.72 (s, 2H), 7.02−7.19 (m, 4H). Anal. Calcd for $C_9H_6O_3FNa\cdot H_2O$: C, 48.66; H, 3.63; Found: C, 48.64, H 3.74.

(*R*)-3-(4-fluorophenyl)-2-hydroxy Propionic Acid (1). To a 22-L reactor equipped with agitator and gas diffuser was added EDTA (3.35 g, 9.0 mmol), mercaptoethanol (1.41 g, 18 mmol), ammonium formate (908 g, 14.4 mol) and sterile water (18.0 L), which was degassed prior to addition of keto acid salt (**6**) (800 g, 3.6 mol). The suspension was stirred until all solids were dissolved. The resulting solution

was filtered through a 0.2 μm filter and transferred to a clean 22-L reactor equipped with an argon purge and overhead stirrer, with argon degassing maintained throughout the remaining operations. NAD (23.88 g, 36 mmol) was added and the pH adjusted to 6.3 by addition of 1 N HCl. This substrate solution was then fed into a membrane reactor with ultrafiltration membrane for enzymatic reduction. The reactor was previously filled with an aqueous mixture of enzymes (D-LDH, 400 units mL^{-1} with activity 20 units mg^{-1} and FDH, 20 units mL^{-1} with activity 76 units mL^{-1}). An appropriate feed rate was used to maintain a conversion of 90% or greater when sampled by HPLC. The circulation rate was kept between 15 and 30 times that of the feed rate. Internal pressure was regulated by a permeate control valve. The aqueous effluent solution thus obtained was adjusted to pH 3.0 with 2 N HCl and extracted with MTBE (3 × 5.0 L). The organic layer was evaporated to obtain 972 g of acid (**1**) as an off-white solid in a yield of 88% and UV purity >90% (reversed phase HPLC). ^{1}H NMR ($CDCl_3$): δ 2.95 (dd, 1H, J = 8.0 Hz, 14.0 Hz), 3.15 (dd, 1H, J = 8.0 Hz, 14.0 Hz), 4.50 (dd, 1H, J = 8.0 Hz, 4.0 Hz), 6.97 (t, 2H, J = 8.0 Hz), 7.00−7.25 (m, 4H). To check optical purity, **1** was converted to **7** by a standard esterification procedure, and the enantiomeric excess of **7** was found to be >99.9% by chiral HPLC. In this fashion a total of 14.5 kg of **1** was produced over a period of 2 weeks.

Reactor Set-Up and Preparation. All pumps, temperature probes, and pH meters were calibrated prior to use. Filter membranes were tested under pressure and fully saturated prior to use. All water used was sterile and filtered (0.2 μm). With the effluent valve closed, the reactor was filled with a known amount of water. This volume was used to calculate the enzyme amounts required and was ca. 2.2 L. A solution of 12 L of 0.02% (v/v) peracetic acid in water was prepared. With the circulation pump running, the effluent valve was opened, and the peracetic solution was fed in at a rate of ~ 25 mL min^{-1} until all was consumed. This was to sterilize the assembled system, and the final pH reading was 3.7. The system was then flushed with water at a rate of ~25 mL min^{-1} until 25 L of water was consumed and the internal pH reading rose to ~6.0. Water (12.0 L), EDTA as disodium salt (2.23 g, 6.9 mmol), mercaptoethanol (938 mg, 12 mmol), and ammonium formate (606 g, 9.61 mol) were added to a 22-L reactor equipped with agitator and argon sparge tube. The mixture was stirred until these compounds were dissolved. After the solution was thoroughly degassed, it was filtered through a 0.2 μm filter into a similarly fitted clean 22-L reactor. The solution was adjusted to pH 6.3 before use with either 1 N H_2SO_4 or 1 N NaOH. and fed into the reactor at a rate of ~25 mL min^{-1} until consumed. At this point the reactor was ready for enzyme loading with the internal pH reading 6.6.

Enzyme Loading and Replenishment. Water (1.0 L), EDTA as disodium salt (186 mg, 0. 5 mmol), mercaptoethanol (78 mg, 1.0 mmol), ammonium formate (50.45 g, 800 mmol) were added to a 2-L reactor fitted with agitator. The mixture was stirred to effect solution and the pH was adjusted to 7.0 ± 0.1 with either 1 N H_2SO_4 or 1 N NaOH. This was then filtered through a 0.2 μm filter and transferred to a clean flask. Formate dehydrogenase (44 000 units, 20 units mL^{-1}) and D-lactic dehydrogenase (880 000 units, 400 units mL^{-1}) were dissolved in 500 mL of the solution. The mixture was again filtered through a 0.2 μm filter and then fed into the constructed membrane reactor. The remaining portion of solution without enzymes was used to rinse the mixing flask and feed lines. The reactor was now ready for conversion of the sodium 3-(4-fluorophenyl)-2-oxo-propionate (**6**) to (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid (**1**). Conversion was monitored by HPLC and when deemed to have dropped below 90%, D-LDH was replenished by dissolving it into a solution and feeding it into the reactor as described above.

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