

Efficient Enzymatic Process for the Production of (2S)-4,4-Difluoro-3,3-dimethyl-N-Boc-proline, a Key Intermediate in the Synthesis of HIV Protease Inhibitors

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Abstract:

(2S)-4,4-Difluoro-3,3-dimethyl-N-Boc-proline (**3**) is a key intermediate for the synthesis of HIV protease inhibitors. Here, several approaches for the preparation of enantiopure **3** and its analogues are disclosed. Among these methods, one strategy relies on resolving the racemic methyl ester of **3** through a protease-catalyzed enantioselective hydrolysis. Despite the fact that this resolution was applied to prepare kilogram quantities of optically pure acid **3** for clinical trials, this process suffered from low efficiency, high cost and difficulties in improvement by medium engineering. An alternative much more efficient and cost-effective enzymatic process was therefore developed by switching the protective group of the proline esters from a Boc to a benzyl moiety. This new process has a much higher throughput (6.3 mmol/h/L vs 0.11 mmol/h/L), and the cost of the process was also dramatically reduced to only 5% of the protease resolution process.

Introduction

Ongoing treatment of HIV-infected patients with commercially available protease inhibitors has led to the development of mutant viruses that possess proteases that are resistant to the inhibitory efficacy of these agents. Thus, there continues to be a need for developing new broad-spectrum HIV protease inhibitors against the newly emerging mutant strains of the viruses.^{1a–c} Compounds **1** and **2** fall into this category of inhibitor and are currently entering human clinical trials. Both of the molecules contain a chiral difluoroproline moiety (bracketed, Figure 1) and 3-amino-2-hydroxy-4-phenylbutanoic acid (AHPBA) that are essential for their inhibitory activity.

4,4-Difluoro-3,3-dimethyl-N-Boc-proline **3** is a key intermediate for the synthesis of protease inhibitors **1** and **2**. Although numerous efficient syntheses of AHPBA are

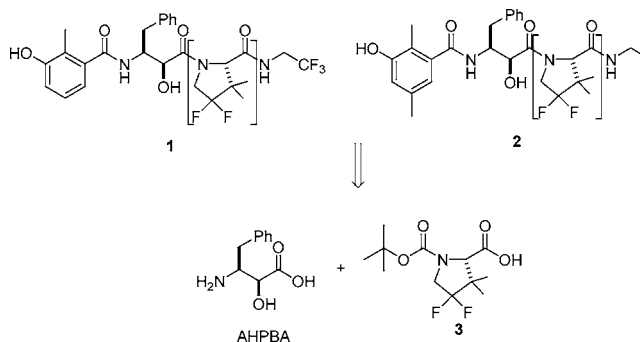


Figure 1. HIV protease inhibitors.

known,^{2a–d} there are no good synthetic methods available for the preparation of difluoroproline **3**. Thus, to prepare compounds **1** and **2** on a large scale for clinical trials, it is essential to develop an efficient, scalable, and cost-effective process for the production of enantiopure **3** or its derivatives with high optical purity. We disclose here several efficient enzymatic preparations of optically pure acid **3**. The strategies for improving the efficiency and the throughput of the enzymatic process, i.e., by switching the protective groups of substrates, will be described in detail.

Results and Discussion

The initial synthetic approach to **3** is composed of nine steps starting from *N*-Boc-glycine, which included a difficult and expensive fluorination step together with one chromatography purification (Scheme 1).^{1c,d} Optically pure **3** is obtained through the resolution of any one of racemic **4** (or **4'**)–**6** (Scheme 1) by enzymatic hydrolysis or diastereomeric resolution.

Here, an automated enzyme screening protocol recently developed in our lab was used to identify the ideal enzymes.³ After screening 94 hydrolases including lipases, proteases, esterases, and acylases, an alkaline protease from *Bacillus licheniformis* (BL), commonly known as subtilisin Carlsberg was found to catalyze the enantioselective hydrolysis of the (*S*)-esters of racemic mixtures **4**–**6**. In all three cases, the

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Scheme 1

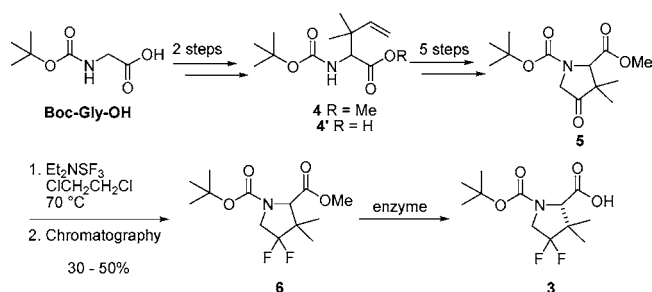
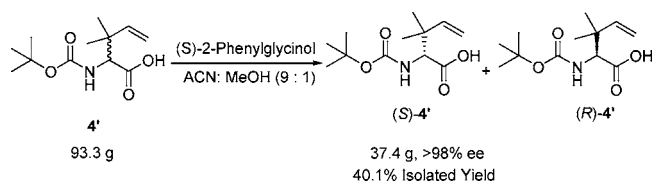


Table 1. Subtilisin Carlsberg-catalyzed enantioselective hydrolysis of esters 4–6^a

substrate	time (h)	yield (%)	ee (%)	<i>E</i>	config
4	51	42	91.4	37	<i>S</i>
5	16	45	98	>200	<i>S</i>
6	260	40–45	96	74	<i>S</i>

^a The yields, ee and configurations are reported for the produced acids.

Scheme 2

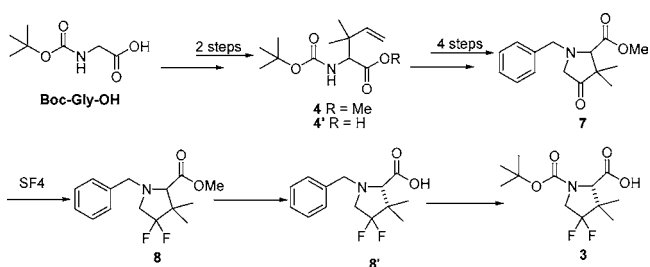


desired (*S*)-acids were obtained in high enantioselectivity and good yields (Table 1).

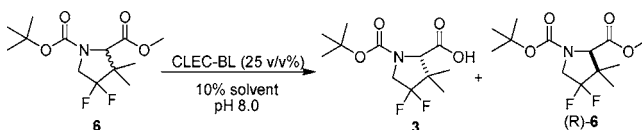
Alternatively, the racemic acid 4' was resolved using (*S*)-(-)-2-phenylglycinol as the diastereomeric resolving agent to afford the desired (*S*)-acid 4' in 40% isolated yield and >98% ee (Scheme 2). This classical resolution process gives a higher optical purity than the enzymatic resolution of 4. Moreover, the classic resolution also cuts off the chemical esterification step from 4' to ester 4.

Although the enzymatic or classical resolution of the early intermediates 4, 4', and 5 is more efficient than the BL protease resolution of 6, concerns regarding the loss of chirality during downstream, harsh chemical steps and the development of other more efficient route(s) prohibited us from further optimization and scale-up of these resolutions. We have thus chosen to scale-up the resolution of the proline intermediate 6 using a cross-linked form of BL commercialized as CLEC-BL by Altus. Compared to the solution form of the enzyme, CLEC-BL showed several advantages for the hydrolysis of 6. These included the recyclability of the enzymes and the increased rate of reaction as compared to the solution form of the same enzyme. In the production of 1 kg of optically pure acid 3 for clinical trials, this enzymatic process worked fairly well, and the relatively high cost of the enzyme was not the major issue compared to the issues of the fluorination step. After 10–12 days of reaction with 25% v/v of CLEC-BL suspension, acid 3 was obtained with 98% optical purity and 35% isolated yield. At this stage, the quick delivery of high-quality material was more important than the allocation of resources to optimize the process and the exploration of other resolution processes.

Scheme 3



Scheme 4



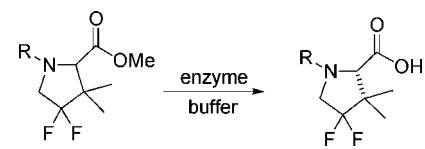
However, when multikilogram quantities of 3 were required for further clinical trials, it became very difficult to provide such large amounts of material using the discovery route due to the low efficiency and extremely high cost of this process (<3% overall yield, Scheme 1). A modified process based on this route was subsequently developed (Scheme 3, 10–15% overall yield),^{1c,d} and one of major improvements was to use a much more efficient fluorination process to produce benzyl-protected proline ester 8. As the issues for chemical steps were resolved, the enzymatic step became the bottleneck for the entire synthesis.

To address these issues, initially we attempted to improve the CLEC-BL resolution process through further optimization (Scheme 4), i.e., adding different cosolvents, tuning reaction pH, changing the buffer or the temperature. Unfortunately, after an extensive investigation of a variety of parameters, only a slight improvement was achieved for the process. Under the optimized conditions, the resolution reached 45% conversion with 20 g/L of substrate concentration in 10% DMSO and Tris-buffer mixture at 40 °C for 6 days. This process not only has a low throughput (0.18 mmol/h/L), but it also is still very expensive and not feasible for the production of optically pure acid at multikilogram scale in an efficient and economic manner.

At this stage, the development of an alternative strategy for the efficient production of enantiopure acid 3 was becoming the most urgent and challenging task. Although directed enzyme evolution is a promising approach for the improvement of enzyme activity,⁴ it is time-consuming and difficult to obtain mutated enzymes within the desired time frame. Thus, we had to look for a simple, fast, and efficient solution to meet the delivery timeline for the clinical trials. It is well-known that in many cases the activity and the stereoselectivity of enzymes can be improved through the modification of substrates. Fine-tuning of substrate structures, i.e., by switching the protective groups of the substrates, may improve the enzyme activity and selectivity dramatically.⁵ After a thorough study of the modified route (Scheme 4),

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Table 2. Enzymatic hydrolysis of proline esters with different protective groups^a



substrate	enzyme	rate	<i>E</i>	config
6 R = Boc	BL	1	72	<i>S</i>
8 R = Benzyl	PLE	> 100	> 200	<i>S</i>
9 R = CBz	PLE	45	8	—
10 R = H	BL	> 100	5	<i>R</i>
	PLE	> 100	1	—

^a The experiments were performed under the enzymatic screening conditions.³

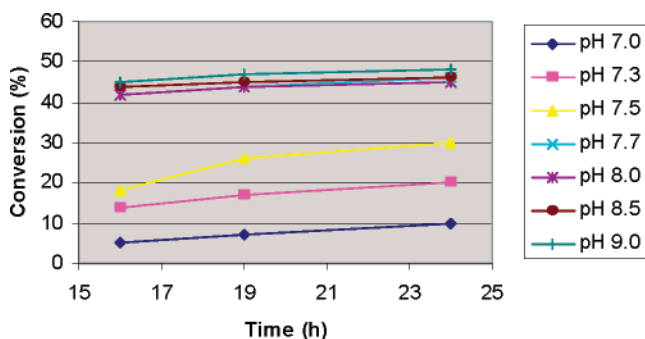


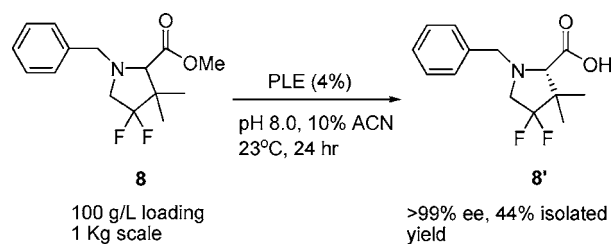
Figure 2. pH effect on PLE hydrolysis of benzyl proline ester **8**.

any of the proline derivatives **8–10** were examined as potential substrates for enzymatic hydrolysis.

After screening our hydrolase library against substrates **8–10**, the benzyl-protected proline **8** was identified as the best substrate, and in this case, pig liver esterase (PLE) was found to be the most active and enantioselective enzyme (Table 2). The rate of the reaction with PLE was dramatically increased compared to that of CLEC-BL. Further experiments indicated that the substrate concentration was readily increased to 100 g/L, and in this case the reaction rate was comparable to that of lower concentration of substrate (45% conversion in 24 h). It was also found that the appropriate pH of the reaction medium was crucial for the enzyme activity (Figure 2). Higher pH values from 7.7 to 9.0 gave a much faster rate than lower pH values from 7.0 to 7.5 (40–45% vs 10–20% in 24 h). It is difficult to explain this unexpected phenomenon from a molecular level since the crystal structure of PLE is not available. However, it is well-known that in many cases the pH change will impact significantly enzyme kinetics, so the optimization of pH will be essential for improving an enzymatic process.⁶

The PLE process showed a much higher efficiency and throughput than the CLEC-BL process. The acid production was 6.3 mmol/h/L for the resolution of **8** vs 0.18 mmol/h/L for the CLEC-BL resolution of **6**. Under the optimal conditions, the ratio of the enzyme to the substrate was significantly reduced to 2.5:1 from 10:1. The cost of the PLE process was thus dramatically reduced to only 5% of that of CLEC-BL resolution of **6**. Moreover, a lower concentration

Scheme 5



of PLE significantly reduced the emulsion issue during the downstream separation of the remaining ester from the produced acid by extraction. The PLE process was successfully used to resolve multikilograms of **8**, and a significant cost savings was achieved via this new process (Scheme 5).

Additionally, the resolution of other intermediates, i.e., 4-oxo-substituted proline **7** through enzymatic hydrolysis, and racemic acid **3** by diastereomeric resolution, may provide alternative efficient pathways for the preparation of optically pure acid **3**. Clearly, the process efficiency can also be improved significantly through the development of an efficient racemization process for the wrong enantiomers of **3**, **6**, or **8**.

PLE represents the most useful esterase for the preparation of optically pure fine chemicals.⁶ One of the major reasons for cautious use of enzymes from mammalian sources is the concern that they might be contaminated with infectious pathogens of BSE. As a result, mammal enzymes are generally used to prepare early pharmaceutical intermediates rather than late and final active pharmaceutical ingredients. Recently, recombinant PLE was successfully cloned and functionally expressed in the methylotropic yeast *Pichia pastoris*.⁷ It is possible that commercialized PLE free from mammal sources will be available in the coming years. This will clearly broaden the application of PLEs in pharmaceutical manufacturing processes.

In conclusion, an efficient and cost-effective enzymatic process was developed for the production of (2*S*)-4,4-difluoro-3,3-dimethyl-*N*-Boc-proline and its analogues in high yield and excellent optical purity. The issues of low throughput and high enzyme cost for the protease resolution process were overcome by switching the protective groups of the substrates. This simple strategy led to an efficient, cost-effective, and scalable enzyme process for the production of (2*S*)-4,4-difluoro-3,3-dimethyl-*N*-Boc-proline at a multikilogram scale.

Experimental Section

Enzyme Screening. A screening kit was thawed for 5 min, and 80 μ L of potassium phosphate buffer (0.1 M, pH 7.0) was then dispensed into each well via a multichannel pipet. A 10 μ L amount of ACN solution of the substrate

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(10–20 mg of substrate/mL) was then added to each well, and the plate was incubated at 23 °C on a thermomixer (900 rpm). The reactions were quenched with 100 μ L of acetonitrile after 5 h. The 96-well plate was then centrifuged, and the supernatant was transferred from each well into another 96-well plate and analyzed with automated HPLC using a short C₁₈ column (30 \times 4.6 mm, 3 μ m) and ACN/H₂O containing 0.1% TFA as the solvent system.

Method A: Preparation of (2S)-4,4-Difluoro-3,3-dimethyl-N-Boc-proline (3) by Enzymatic Hydrolysis of 6. To a 50-L reactor equipped with a pH electrode, an overhead stirrer, a heating coil, and a base addition line², was added the CLEC-BL solution (7 L of fresh CLEC + 5 L of recycled CLEC (80% of the initial activity) and 24 L of di-water). The pH of the suspension was adjusted to 8.0 by addition of 20 mL of 2 N NaOH. Then, the racemic ester I solution (400 g, 1.36 mol, 1.00 equiv, in acetonitrile, 3.6 L) was added. The suspension was then stirred at 30 °C for 262 h. The pH of the solution was maintained at 8.0 by adding 2 N NaOH. Reaction was followed by RP-HPLC for both conversion and % ee of the product, and stopped after 45–50% starting material had been consumed. The % ee of the acid was measured as 95.5% (after 262 h under these conditions, 246 mL of base were added).

The mixture was extracted 3 \times with 16 L of MTBE each, and the combined organic layers were dried with Na₂SO₄ and concentrated under vacuum to afford 220 g of crude (*R*)-enriched ester 4. The remaining aqueous slurry was filtered (to remove the CLEC-BL) through Whatman paper 1. CLEC paste was removed from the paper and stored at 4 °C.³ The remaining aqueous solution was acidified to pH 5.5 and extracted twice with 16 L of MTBE each (pH was set up to 5.5 after first extraction). Two more extractions were performed, one at pH 5.0 and subsequently one at pH 4.0 (It was determined by HPLC that three extractions were sufficient to remove most of the acid). The acid fractions were pooled and concentrated under vacuum. The solid residue was then suspended in hot tap water (1000 mL) and allowed to cool overnight. The slurry was filtered and the crystals dried in a vacuum oven at 40 °C overnight. A white solid was obtained (133 g, 98% ee, 69.8% yield based on (*S*)-enantiomer, >98% HPLC pure).⁴ ¹H NMR (300 MHz, CDCl₃): δ 7.9 (bs, 1H), 4.10 (d, 1H), 3.89 (dd, 2H), 1.5 (s) +1.45 (s) (9H), 1.3 (s, 3H), 1.15 (s, 3H). HPLC Methods: (A) Nonchiral HPLC conditions: detector wavelength 200 nm; Luna C-18, 4.6 mm \times 30 mm; flow rate 1.5 mL/min; injection volume 10 μ L; mobile phases: (a) 25 mM KH₂PO₄ pH 2.5, (b) acetonitrile; gradient 35–70% ACN in 5 min. (B) Chiral HPLC for acid 3: detector wavelength 195 nm; Chiralcel OJ-R, 3 μ m, C-18, 4.6 mm \times 150 mm; flow rate 0.5 mL/min; injection volume 10 μ L; mobile phases: (a) 25 mM KH₂PO₄ pH 2.0, (b) acetonitrile, (c) HPLC grade H₂O. Isocratic 75% A and 25% B for 17 min, then 75% B and 25% C for 3 min, and finally 75% A and 25% B for 15 min. Retention times: acid 3 14.85 (*R*) and 15.84 (*S*).

Method B: Enzymatic Resolution of Racemic 4,4-Difluoro-3,3-dimethyl-N-benzyl-proline Methyl Ester (8). To 7.85 L of phosphate buffer (pH 8.0, 100 mM) was added

0.4 L of PLE ammonium sulfate solution (Biocatalytics, Inc, CA, USA; 2 \times 10⁶ units/L of solution), and the mixture was stirred at 700 rpm. Then 1.0 kg of benzyl ester 8 was added. The pH of the reaction was controlled at 8.0 with a titrator by continuously adding 1 N NaOH. The reaction was monitored by HPLC (achiral and chiral methods). After the conversion reached ~50% in 24 h, 15% of NaCl (1.6 kg, w/w) was added, and the mixture was stirred for 5–10 min. After the addition of toluene (0.5 v, 5.0 L) the mixture was stirred for 15–30 min. After allowing the mixture to settle for 30 min, the toluene layer was removed. The aggregated enzyme formed was quickly filtered by vacuum filtration and was washed with distilled water (0.5 L). The above extraction procedure was repeated once to remove the residual (*R*)-ester. The pH of the aqueous layer was adjusted to 3.5 by the addition of 36% HCl slowly. MTBE (0.5 v, 5.0 L) was added, and the mixture was gently stirred for 30 min. After settling for 30 min, the organic layer was collected, and the aqueous layer was extracted twice with MTBE. The combined organic layer was dried over Na₂SO₄, and after the removal of the organic solvent the pure acid 8' was obtained in high yield and excellent optical purity (398 g, 44%, >99% ee). Compound 8': ESI [M – H][–] 268.1. ¹H NMR (300 MHz, CDCl₃): δ 7.26–7.40 (m, 5H), 3.94 (d, *J* = 12.9 Hz, 1H), 3.66 (d, *J* = 12.9 Hz, 1H), 3.32–3.57 (m, 2H), 3.05 (m, 1H), 1.26 (s, 3H), 1.11 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 171.10, 136.19, 129.16, 128.68, 127.73, 74.83, 60.67, 56.49, 53.75, 46.48, 20.40, 18.77. Chiral HPLC methods: compound 8', Chiralcel AD-RH (4.6 mm \times 100 mm, 3 μ m); flow rate 0.6 mL/min; injection volume 5 μ L; mobile phases ACN/H₂O (20:80), detection at 254 nm. Compound 8: Chiralcel OD-RH (4.6 mm \times 100 mm, 3 μ m); flow rate 0.6 mL/min; injection volume 5 μ L; mobile phases ACN/H₂O (60:40), detection at 254 nm.

Method C: Preparation of (S)-3,3-Dimethyl-N-Boc-vinylglycine by Enzymatic Hydrolysis of 4. To a 5-L three neck flask equipped with a pH electrode, an overhead stirrer, a heating mantle, and a titrator was added the racemic ester 4 (78 g, 0.3 mol, 1.00 equiv) in acetonitrile (280 mL). A mixture of Alcalase (350 mL from a 5 \times concentrated crude solution from a commercial solution) and distilled water (2.8 L) was then prepared, and the pH of the solution was set to 7.0. The enzyme solution was charged into the reaction flask. The suspension was then stirred at 30 °C for 51 h. The pH of the solution was maintained at 7.0 by adding 1 N NaOH. Reaction was followed by RP-HPLC for both conversion and % ee of the product and was stopped after 45% starting material had been consumed (after 51 h under these conditions, 95.8 mL of base was added). The mixture was extracted 3 \times with 1.75 L of MTBE each, and the combined organic layers were dried with MgSO₄ and concentrated under vacuum to afford 50.81 g of crude (*R*)-enriched ester 4 (>55% yield, approximately 56% ee). This crude mixture contained some carboxylic acid <7%, which was recovered later by acid–base extraction. The remaining aqueous solution was passed through a Pellicon 2 tangential flow filtration equipped with an Ultracel cellulose membrane. During this step, most of the enzyme is removed from the

aqueous solution. The remaining solution was acidified to pH 4.0 and extracted three times with 1.75 L of MTBE each. The acid fractions were pooled, dried with sodium sulfate, and concentrated under vacuum. Pale-yellow oil of **4'** was obtained (31 g, 91.4% ee, 42% yield, >98% HPLC pure). ¹H NMR (300 MHz, CDCl₃): δ 10.69 (s, 1H), 5.78 (dd, 2H), 5.02 (m, 2H), 4.96 (s, 1H), 4.09 (d, 1H), 1.36 (s, 9H), 1.06 (s, 6H). Chiral HPLC method for acid **4'**: detector wavelength 200 nm; Chiralcel OJ-R, 3 μm, C-18, 4.6 mm × 100 mm; flow rate 0.5 mL/min; injection volume 10 μL; mobile phases (A) 25 mM NaH₂PO₄ pH 2.0; (B) acetonitrile; isocratic 25% B for 55 min, 3 min post-run; retention times: acid 16.33 (*R*) and 17.97 (*S*); ester 50.40 (*S*), 51.30 (*R*). Chiral HPLC method for ester **4**: Chiralcel OD-RH, 150 mm × 4.6 mm; flow rate 0.8 mL/min; mobile phase 30% ACN and 70% H₂O; wavelength 205 nm.

Method D: Preparation of (*S*)-4'** by Chemical Resolution with (*S*)-Phenylglycinol.** To a 2-L jacketed flask equipped with an overhead stirrer was added the racemic acid **4'** (93.3 g, 386.2 mmol), (*S*)-phenylglycinol (52.6 g, 386.2 mmol), methanol (200 mL) and acetonitrile (1800 mL). The resulting slurry was stirred and heated to 70–80 °C or until fully dissolved. The solution was allowed to crystallize by allowing the solution to cool to room temperature slowly (cooling rate: 10 °C/h) with continuous stirring. The solution was then filtered, and the crystalline salt (containing the desired (*S*)-enantiomer) was washed with 100 mL of cold acetonitrile to wash any residual filtrate from the white crystals. The crystals were then collected and analyzed by HPLC. The acid was then isolated from the salt. The salt was dissolved in 250 mL of ethyl acetate (or MTBE). Water (250 mL) was then added, and the pH of the solution was adjusted to 3 with 5% HCl. The organic layer containing the acid was separated from the remaining aqueous solution,

which was extracted once again with 200 mL of ethyl acetate (or MTBE) to recover any remaining acid. The extract was then dried with sodium sulfate and concentrated under vacuum. The acid was isolated as a clear oil, and following vacuum-drying overnight, (*S*)-**4'** was obtained as a white solid (37.4 g, >98% ee, 40.1% isolated overall yield, >98% pure).

Recycling the (*S*)-Phenylglycinol. The aqueous layer from the above step was adjusted to pH 8.0 with 2.0 N NaOH, and the solution was extracted with 300 mL of ethyl acetate (or MTBE). The extract was then dried with sodium sulfate and concentrated under vacuum. The product was isolated as white solid crystals (12.3 g, >98% pure). Note that recovery was only from 40% of material; the remaining resolving agent can be recovered from the filtrate.

Chiral HPLC methods: detector wavelength 205 nm; column: Chiralcel OJ-RH, 3 μ, C-18, 4.6 mm × 100 mm; flow rate 0.6 mL/min; injection volume 10 μL; mobile phases (A) acetonitrile (0.1% TFA); (B) 75% H₂O (0.1% TFA); isocratic 25% A: 75% B 18 min; retention times: acid: 11.69 (*R*) and 12.9 (*S*)/5.3 *R*-phenylglycinol. Note that in cases where only 90% ee is obtained after the first crystallization, a second crystallization can easily be carried to improve the ee to >98% by adding a fresh batch of acetonitrile and methanol (only 7 volumes).

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