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Enzymatic Preparation of an (*S*)-Amino Acid from a Racemic Amino Acid

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Abstract:

The (*S*)-amino acid, (*S*)-2-amino-3-(6-*o*-tolylpyridin-3-yl)propanoic acid (3), is a key intermediate needed for synthesis of an antidiabetic drug candidate. Three enzymatic routes to 3 were explored. (*S*)-Amino acid 3 could be prepared in 73% isolated yield with 99.9% ee from racemic amino acid 1 using (*R*)-amino acid oxidase from *Trigonopsis variabilis* expressed in *Escherichia coli* in combination with an (*S*)-aminotransferase using (*S*)-aspartate as amino donor. The (*S*)-aminotransferase was purified from a soil organism identified as *Burkholderia* sp. and cloned and expressed in *E. coli*. (*S*)-Amino acid 3 with 100% ee was also prepared in 68% solution yield and 54% isolated yield from 1 using recombinant (*R*)-amino acid oxidase from *T. variabilis* and an (*S*)-amino acid dehydrogenase from *Sporosarcina ureae*. The cofactor NADH required for the reductive amination reaction was regenerated using formate and formate dehydrogenase. The chemoenzymatic dynamic resolution of 1 by (*R*)-selective oxidation with Celite-immobilized (*R*)-amino acid oxidase in combination with chemical imine reduction using borane−ammonia complex gave an 81% solution yield and 68% isolated yield of 3 with 100% ee.

Introduction

(*S*)-Amino acids are useful intermediates for the synthesis of many pharmaceuticals.[1,2] Several enzymatic approaches have been applied for their preparation, including (*S*)-hydantoinases combined with (*S*)-carbamoylases or HNO_2,[3,4] (*S*)-acylases,[5,6] (*S*)-amidases,[7,8] (*S*)-transaminases,[9,10] (*S*)-amino acid dehydrogenases,[11,12] and dynamic resolution.[13−16] The (*S*)-amino acid, (*S*)-2-amino-3-(6-*o*-tolylpyridin-3-yl)propanoic acid (**3**), is a key intermediate needed for synthesis of glucagon-like peptide-1 (GLP-1) mimics or GLP-1 receptor modulators potentially useful for the treatment of type II diabetes.[17−22]

We have previously used (*S*)-amino acid dehydrogenases to convert keto acids to the corresponding unnatural (*S*)-amino acids,[23−26] which were used in the synthetic routes to several pharmaceutical candidate compounds. In this report we describe the conversion of the racemic amino acid **1** to (*S*)-amino acid

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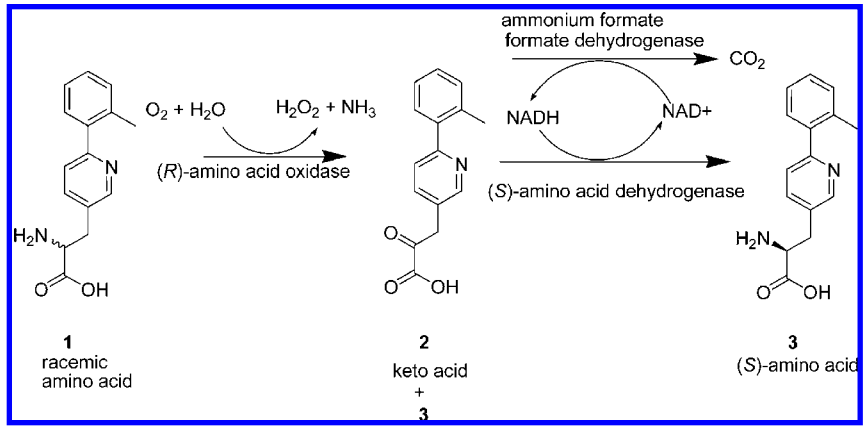
Scheme 1. **Conversion of racemic amino acid 1 to (*S*)-amino acid 3 using (*R*)-amino acid oxidase and (*S*)-amino acid dehydrogenase**



3 using a recombinant (*R*)-amino acid oxidase from *Trigonopsis variabilis* immobilized on Celite followed by an (*S*)-amino acid dehydrogenase from *Sporosarcina ureae*. The larger scale preparation of amino acid **3** from racemic amino acid **1** using the (*R*)-amino acid oxidase cloned from *T. variabilis* and an (*S*)-aminotransferase cloned from *Burkholderia* sp., each separately expressed in *Escherichia coli* is also reported. The chemoenzymatic dynamic resolution of racemic amino acid **1** was also examined using (*R*)-selective oxidation with Celite-immobilized (*R*)-amino acid oxidase in combination with chemical imine reduction.

Results and Discussion

The basic strategy was to carry out deracemization of the racemic amino acid **1** by combining an amino acid oxidase with another enzyme or a chemical reducing agent. First, the amino acid oxidase carries out the enantioselective oxidation of only the (*R*)-amino acid leaving the (*S*)-amino acid **3** intact. The (*R*)-amino acid is oxidized by the amino acid oxidase to form the imine **4** bound to the enzyme which can be hydrolyzed to the keto acid **2**. The keto acid **2** is converted to the desired (*S*)-amino acid **3** by an amino acid dehydrogenase or a transaminase. Alternatively, the imine **4** bound to the enzyme is reduced chemically to the racemic amino acid **1** for recycling.

Conversion of Racemic Amino Acid 1 to (*S*)-Amino Acid 3 Using (*R*)-Amino Acid Oxidase and (*S*)-Amino Acid Dehydrogenase. Our initial approach for preparation of (*S*)-amino acid **3** is shown in Scheme 1. Starting from racemic amino acid **1**, the aim was to oxidize the (*R*)-amino acid to the corresponding keto acid **2**, and then carry out reductive amination of **2** to **3** by addition of ammonium formate, NAD^+, formate dehydrogenase and a suitable (*S*)-amino acid dehydrogenase. (*R*)-Amino acid oxidase from *T. variabilis*, cloned and overexpressed in *E. coli* [*E. coli* BL21(pBMS1000 DAAO)] and then immobilized on Celite, was effective for the initial oxidation step. After screening of microbial cultures for a suitable amino acid dehydrogenase active with keto acid **2**, *S. ureae* SC16048 was identified as a source of the desired enzyme for the conversion of keto acid **2** to (*S*)-amino acid **3.** Cell extracts of *S. ureae* were used as a source of (*S*)-amino acid dehydrogenase. Racemic amino acid (1.95 g) was oxidized with the (*R*)-amino acid oxidase, and after 5.5 h the ee was 89%. The reductive amination reaction was initiated using (*S*)-amino acid dehydrogenase after 7.5 h and was continued for 19 h until the keto acid peak was not detected by HPLC. Under the conditions of the reductive amination reaction, the oxidase was inhibited, so a two-step procedure was needed. The solution yield of (*S*)-amino acid **3** was 68% with >99% ee and the isolated yield was 1.06 g, 54% overall. A phenylalanine dehydrogenase of known sequence from *S. ureae* was cloned by polymerase chain reaction (PCR) and expressed in *E. coli* but was not effective for this transformation, although it was shown to be active for reductive amination of phenylpyruvate.

Conversion of Racemic Amino Acid 1 to (*S*)-Amino Acid 3 Using (*R*)-Amino Acid Oxidase and (*S*)-Aminotransferase. A similar approach was used for deracemization of amino acid **1** using (*R*)-amino acid oxidase combined with an (*S*)-transaminase instead of an (*S*)-amino acid dehydrogenase (Scheme 2). This procedure had the advantage that both enzymes could be added at the start of reaction. A bacterial strain producing a suitable transaminase was isolated from a soil sample and identified as a *Burkholderia* sp. by comparison of its 16S rRNA gene to publicly available sequences. The (*S*)-transaminase was purified to homogeneity from extracts of this strain by a four-step procedure (Table 1) as described in the Experimental Section.

Although an N-terminal sequence could not be obtained, four internal sequences of the purified protein were obtained following tryptic digestion. A BLAST2 homology search showed regions of homology to adenosylmethionine-8-amino-7-oxononanoate aminotransferases (AAOA) from *Burkholderia cenocepacia* and *Borrelia cepacia*. Oligonucleotide primers were prepared based on the corresponding codons of the amino acids for use in PCR. The direction of the primers (i.e., sense and/or antisense) were determined using the likely location of the amino acid sequence within the protein as compared to similar aminotransferases. The (*S*)-aminotransferase gene was cloned and overexpressed in *E. coli* as described in the Experimental Section.

Fermentation processes for production of (*R*)-amino acid oxidase and (*S*)-aminotransferase in recombinant *E. coli* were developed and scaled up to 250 L. About 11−13 kg of wet cell paste was obtained from *E. coli* expressing (S)-aminotransferase and 7 kg of wet cell paste was obtained from *E. coli*

Scheme 2. **Conversion of racemic amino acid 1 to (*S*)-amino acid 3 using (*R*)-amino acid oxidase and (*S*)-aminotransferase**

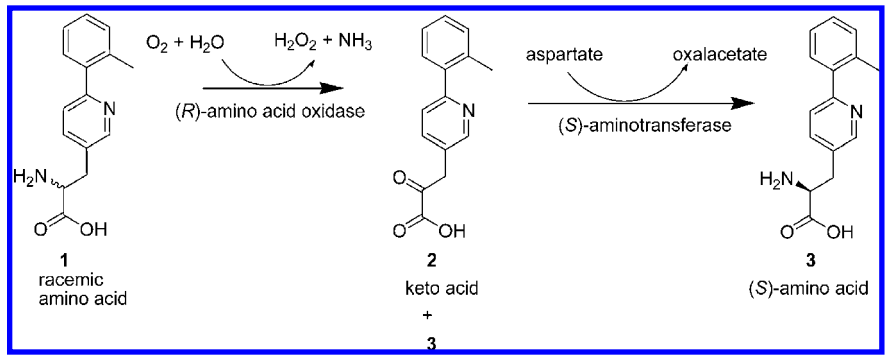


Table 1. **Purification of (*S*)-aminotransferase from *Burkholderia* sp**

step	total protein (mg)	total activity (U)	specific activity (U/mg)	purification (fold)
crude extract	345	8.02	0.021	1
butyl-Sepharose	42.6	6.37	0.150	7
Red-120	3.65	2.25	0.616	29
UnoQ	0.56	1.99	3.18	148
Superdex 200	0.024	0.87	7.22	336

Table 2. **Summary of fermentation runs of recombinant *E. coli* for production of (*S*)-aminotransferase and (*R*)-amino acid oxidase**

strain	enzyme	cell yield (kg)	activity (U/g cells)
E. coli SC16541	(*S*)-aminotransferase	11.2	0.72
		11.2	0.78
		13.3	0.78
E. coli SC16544	(*R*)-amino acid oxidase	7.1	33.4
		6.8	38.8

expressing (*R*)-amino acid oxidase (Table 2). On the basis of the much higher activity of cells containing (*R*)-amino acid oxidase relative to that of cells containing (*S*)-amino acid transaminase, a 5:1 ratio of transaminase:oxidase cells was used to prepare a combined cell extract containing both activities.

Several batches containing 9.11 g (15 g of the monosulfate monohydrate, corrected for potency) of **1** were run in a Braun Biostat B 2-L reactor for the purpose of developing a procedure for scale-up of the bioconversion. The reactor was controlled at 30 °C, 300 rpm agitation, pH 7.5 and 1 L/min aeration. Samples were taken hourly to analyze ee and conversion. The aeration was stopped after about 6 h when the oxidation was complete as indicated by an ee of (*S*)-amino acid >99%. The reaction was continued for an additional 16 h without aeration to allow for conversion of **2** to **3**. The solution yield was 85% and the ee was 99.5%. The reaction mixture was ultrafiltered to remove cellular material and the permeate mixed with ammonium sulfate and extracted with *n*-butanol. The product was crystallized from the extract as the monosulfate monohydrate in 73% overall yield by adding H_2SO_4.

The reaction was scaled up with 607 g (1 kg of the monosulfate monohydrate corrected for potency) of **1** to give a 66% isolated yield of **3** as the monosulfate monohydrate, ee 99.9%.

Dynamic Resolution of Racemic Amino Acid to (*S*)-Amino Acid. The chemoenzymatic dynamic resolution of racemic amino acid **1** was examined using Celite-immobilized

Scheme 3. **Conversion of racemic amino acid 1 to (*S*)-amino acid 3 by dynamic resolution using (*R*)-amino acid oxidase and chemical reduction of imine**

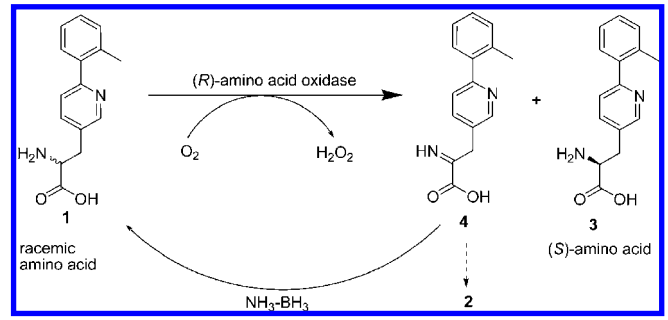


Table 3. **Dynamic resolution of racemic amino acids using (*R*)-amino acid oxidase and 10 equiv of NH_3-BH_3**

pH	yield (%)	ee (%)
5.0	70	51
5.5	68	82
6.0	78	100
6.5	79	100
7.0	76	100
7.5	73	100
8.0	67	100

(*R*)-amino acid oxidase in combination with chemical reduction of the imine (initial product of the oxidase reaction) with borane−ammonia complex (Scheme 3) at various pH values. Before the enzyme bound imine (**4**) hydrolyzes to the keto acid, borane−ammonia reduces **4** to the racemic amino acid **1** in this dynamic resolution process.[15] A suspension of racemic amino acid **1** in phosphate buffer with ammonium formate was added to a mixture of immobilized (*R*)-amino acid oxidase and borane−ammonia complex and the suspension stirred for 20 h. Results using 10 equiv of borane−ammonia complex are shown in Table 3. The maximum yield of **3**, 76−79%, was obtained at pH 6.0−7.0, with ee values reaching >99.9% at pHs 6−8. Using 20 equiv of borane−ammonia complex did not significantly improve the results.

Conclusion

Three methods were evaluated for deracemization of amino acid **1** to (*S*)-amino acid **3.** A recombinant (*R*)-amino acid oxidase from *T. variabilis* expressed in *E. coli* selectively oxidized the (*R*)-enantiomer to keto acid **2,** which was converted to (*S*)-amino acid **3** by an (*S*)-transaminase. An (*S*)-transaminase from a soil organism identified as a strain of *Burkholderia* sp. was purified, cloned, and expressed in *E. coli* and found to be

effective for conversion of **2** to (*S*)-**3.** The (*S*)-amino acid **3** was also prepared from the corresponding racemic amino acid **1** using (*R*)-amino acid oxidase in combination with an (*S*)-amino acid dehydrogenase from *S. ureae*. The cofactor NADH required for reductive amination reaction was regenerated using NAD^+, formate and formate dehydrogenase. The chemoenzymatic dynamic resolution of racemic amino acid **1** was also demonstrated. (*R*)-selective oxidation with Celite-immobilized (*R*)-amino acid oxidase in combination with chemical imine reduction with borane−ammonia afforded (*S*)-amino acid **3**. Ultimately, we selected the very efficient (*R*)-amino acid oxidase/(*S*)-transaminase process for the large-scale preparation of **3**.

Experimental Section

Source of Racemic Amino Acid 1. Racemic amino acid **1** was prepared in the Process Research and Development department of Bristol-Myers Squibb. Both **1** and **3** afford crystalline monohydrate monosulfates. The calculated amino acid content is 68.8%. All assays are in terms of the free amino acid. Amino acid **1** was used as either the free amino acid or the monohydrate monosulfate.

HPLC Methods. Quantitation with C18 column: Samples were analyzed with a YMC ODS A C18 15 × 0.46 cm column. The mobile phase was a gradient of 10 to 80% acetonitrile/water (0.05% trifluoroacetic acid in both) from 0 to 12 min at a flow rate of 1 mL/min, with detection at 282 nm, temperature 40 °C, and an injection volume of 10 μL. Retention times were: amino acid **1** or **3,** 5.4 min; keto acid **2,** broad peak centered at 6.6 min.

Direct determination of enantiomeric excess with chiral column: The same samples were analyzed with a Chirobiotic T 25 × 0.46 cm 5 μ column. The mobile phase was: A. water: methanol 90: 10:05% acetic acid; B. methanol: water 90:10: 0.05% acetic acid with a gradient: 0−3 min, 10% B; 3−15 min, 10−100% B; 15−20 min, 100%B. Flow rate was 1.2 mL/min, detection was at 215 nm, temperature was 25 °C (ambient), and injection volume was 5 μL. Retention times were: (*S*)-enantiomer **3**, 14.1 min; (*R*)-enantiomer 14.9 min.

Determination of ee via derivatization with Marfey's reagent:[27] Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide from Pierce) was used following the procedure of the manufacturer to give diasteromeric derivatives that could be separated with a C18 column. Samples were analyzed with a YMC ODS A C18 15 × 0.46 cm column. The mobile phase was a 25% acetonitrile/75% water (both containing 0.05% trifluoroacetic acid), flow rate was 1.5 mL/min, detection was at 340 nm, temperature was 40 °C, and injection volume was 10 μL. Retention times were: (*S*)-enantiomer, 10.5 min; (*R*)-enantiomer 15.3 min.

Enzyme Activity Assays. The (*R*)-amino acid oxidase assay mixture (0.5 mL) contained 50 μL of racemic amino acid **1** (10 mg/mL), 0.5 μL catalase, 0.44 mL 50 mM potassium phosphate pH 8.0, and 10 μL cell extract. The reaction was initiated by addition of the cell extract and incubated at 30 °C for one hour. For amino acid quantitation, the reaction mixture was mixed with 0.5 mL methanol. After centrifugation, the supernatant was assayed by HPLC. The conversion of (*R*)-amino acid to keto acid was expressed in μmol/min/mL.

The (*S*)-aminotransferase assay mixture (0.5 mL) contained 0.1 mL of keto acid **2** (10 mg/mL, prepared by treating racemic amino acid **1** with (*S*)-amino acid oxidase from *Proteus mirabilis* cloned and expressed in *E. coli* and (*R*)-amino acid oxidase), 0.1 mL sodium aspartate (20 mg/mL), 0.2 mL 50 mM potassium phosphate pH 7, and 0.1 mL cell extract. The reaction was initiated by addition of the extract and incubated at 30 °C for 60 min. The reaction was quenched with 0.5 mL ethanol and centrifuged to remove precipitated proteins. The supernatant was used to determine amino acid **3** by HPLC. The formation of (*S*)-amino acid **3** was expressed in μmol/min/mL.

Purification of (*S*)-Aminotransferase. A bacterial strain with a suitable (S)-aminotransferase activity was isolated from a soil sample by selection for growth on 0.1% (S)-1-(4-(2-(4-methyl-2-phenyloxazol-5-yl)ethoxy)phenyl)ethanamine as the sole nitrogen source. The strain was identified as a *Burkholderia* species by comparison of its 16S rRNA gene sequence to publicly available data banks. For preparation of crude cell-free extracts, 25 g (wet weight) of cells was suspended in 300 mL of 50 mM potassium phosphate buffer pH 7.0 containing 1 mM dithiothreitol (DTT) and 25 μM pyridoxal 5′-phosphate (PLP) (buffer A), then passed three times through a Microfluidizer at 12,000 psi. The disintegrated cells were centrifuged at 45,000 × g for 90 min at 4 °C to remove the cell debris. The supernatant was collected and stored at −80 °C for subsequent enzyme purification.

(*S*)-Aminotransferase was purified to homogeneity by a four-step procedure as follows. Crude extract (65 mL, 345 mg of protein) containing 0.5 M ammonium sulfate was loaded on a 20-mL Butyl-Sepharose 4 fast flow (Sigma) column equilibrated with buffer A containing 0.5 M ammonium sulfate. The enzyme was eluted with 100 mL of ammonium sulfate with a linear gradient (0.5 to 0 M) at a flow rate of 1 mL/min collecting 3-mL fractions. The active fractions (21 mL) were combined and concentrated with a PM-30 membrane (Amicon) to approximately 3 mL, followed by dilution to 20 mL with 10 mM potassium phosphate pH 7.0 buffer containing 1 mM DTT and 5 mM magnesium sulfate (buffer B). The diluted solution was applied to a Red-120 dye (Sigma) affinity column (18 mL) equilibrated with buffer B, and the column was then washed with 25 mL of buffer B. The flow-through and wash fractions (45 mL) were combined and concentrated with a PM-30 membrane to approximately 2 mL. The concentrated enzyme solution was applied to a UnoQ column (1 × 3.8 cm, Bio-Rad) equilibrated with 25 mM potassium phosphate pH 7.0 containing 1 mM DTT and 0.1 M NaCl at flow rate of 1 mL/min. After washing with 12 mL of the buffer, the column was eluted with 16 mL of a linear gradient (0.1 to 0.2 M NaCl) collecting 0.5-mL fractions. The enzyme activity was in the unbound fractions, and the active fractions (8 mL) were combined and concentrated to 0.5 mL. The enzyme solution was injected at flow rate of 0.5 mL/min to a Superdex column (1.2 × 30 cm, Pharmacia) equilibrated with 25 mM potassium phosphate pH 7.0 buffer containing 1 mM DTT and 100 mM NaCl. The enzyme was eluted with 23 mL of the column buffer

(27) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591.

collecting 0.5-mL fractions. The active fractions (1.5 mL) were combined and concentrated to 0.2 mL. Analysis by sodium dodecyl sulfate acrylamide gel electrophoresis confirmed purity of a 54 kDa protein.

A sample of the purified protein was submitted for partial amino acid sequencing. No N-terminal sequence could be obtained, but following tryptic digestion, four internal sequences were acquired:

1) APHATQA
2) KADGVYLWDSDGN
3) KELADAAYR
4) DEGIVER

Cloning and Expression of the *Burkholderia* sp. (*S*)-Aminotransferase Gene. For use in polymerase chain reaction (PCR), degenerate oligonucleotide primers were prepared based on the corresponding codons of the amino acids. The direction of the primers (i.e., sense and/or antisense) were determined using the likely location of the amino acid sequence within the protein as compared to similar aminotransferases.

Two sets of oligonucleotide pairs were used with the FailSafe series of PCR buffer (Epicentre Technologies) and *Burkholderia* sp. chromosomal DNA as template. The PCR (10 μL final volume) was carried out in a Hybaid PCR Express thermocycler with the following parameters:

stage 1	stage 2	stage 3	stage 4	stage 5
94° 1 min	94° 30 s	94° 30 s	94° 30 s	72° 5 min
	55° 30 s	55°→40° 30 s	40° 30 s	
	72° 30 s	72° 30 s	72° 30 s	
1 cycle	4 cycles	20 cycles	5 cycles	

A fragment of the expected size (~1056 bp based on comparison to known AAOA genes) was obtained using oligos based on partial amino acid sequences 1 and 4. The reaction was scaled up 40-fold and the entire reaction mix electrophoresed on a 1.0% agarose gel for 2 h at 100 v in TAE buffer (0.04 M Trizma base, 0.02 M acetic acid, and 0.001 M EDTA, pH 8.3) containing 0.5 μg/mL ethidium bromide. The fragment was excised from the gel and purified using a Qiagen Gel Purification Kit.

To isolate the entire gene, *Burkholderia* sp. DNA was first cleaved with a series of 9 restriction endonucleases and transferred to Hybond N+ nylon filters under alkaline conditions using the VacuGene vacuum blotting unit (Amersham-Pharmacia). A digoxygenin-labeled probe was prepared using specific primers based on the known DNA sequence of the adenosylmethionine-8-amino-7-oxononanoate aminotransferase (AAOA) gene from *B. cenocepacia* (GenBank accession no. CP000958.1) since there was near-identity of the partial amino acid sequences obtained from *Burkholderia* sp. and the *B. cenocepacia* AAOA protein. Hybridization to the blotted DNA digests was performed in EasyHyb solution (Roche) under stringent wash conditions. A single hybridizing *Bgl*II fragment of ~3800 base pairs was seen. On the basis of this result, *Bgl*II-digested *Burkholderia* sp. chromosomal DNA from ~3200−4200 base pairs was isolated and ligated to pZero2 vector DNA digested with *Bam*HI (the overhanging nucleotides are compatible with those of *Bgl*II) using the FastLink kit (Epicentre). DNA was transformed by electroporation into *E. coli* DH10B competent cells (Invitrogen). Colonies containing recombinant plasmids were selected on LB agar plates containing 50 μg/mL kanamycin sulfate (Sigma Chemicals, St. Louis, MO). Approximately 15,000 colonies on a Hybond N+ membrane were hybridized with the labeled PCR probe described above. Approximately 100 strongly hybridizing colonies were obtained; six were removed from the master plate, inoculated into LB-kanamycin liquid medium, and grown at 37 °C for 24 h, 250 rpm. Plasmid DNA was isolated using the Fast Plasmid DNA kit from Eppendorf. PCR with AAOA-specific primers gave strong amplification of the expected 600-bp fragments. One plasmid was chosen for further study and named "pZerO2-AAOA."

For rapid DNA sequencing of the insert in pZerO-AAOA, primer sites were introduced at random using the New England Biolabs Genome Priming System kit. The AAOA gene was identified within the 4000-base pair insert by the presence of sequences corresponding to those expected for the partial amino acid sequences obtained.

Expression of the (*S*)-Aminotransferase (AAOA) Gene. Primers were prepared for amplification of the AAOA for ligation to expression vector pBMS2000:

primer name	sequence	corresponds to (direction)
Oligo 569	GACATATTTAAAT-CATATGACTTAC-CGCAACGAATCT-GCC	*Swa*I/*Nde*I/5′ end *Burkholderia* sp. AAOA (sense)
Oligo 570	GATTTAAATC-CCGGGTTAC-GAAATACCCAGT-TGCTGGGCGG	*Swa*I/*Sma*I/stop/3′ end *Burkholderia* sp. AAOA gene (anti-sense)

The FailSafe series of buffers were tested using oligos 569 + 570 with pZerO2-AAOA as template for PCR. Buffer "D" gave strong amplification of the expected ~1450-bp fragment and was used to scale up the reaction 20-fold. A MinElute column (Qiagen) was used to purify the fragment after addition of 5 vol buffer PB before loading onto the column.

The PCR fragment obtained with oligos 569 + 570 (~2 μg) was cleaved with 10 U each *Nde*I and *Sma*I in a 40 μL volume (37 °C, 2 h). Samples were electrophoresed and purified after excision from the agarose gel using the MinElute kit. The digested PCR fragment (100 ng) was ligated to 30 ng *Nde*I−*Sma*I-cut pBMS2000 using the Fast Link kit. One plasmid that was verified to contain the correct insert was named pBMS2000-AAOA and transformed into *E. coli* BL21, forming strain SC 16541.

Growth of Recombinant *E. coli* Expressing (*S*)-Aminotransferase. For shake flask expression work, SC 16541 was initially grown in MT5-M2 for 20−24 h, 30 °C, 250 rpm. MT5-M2 contained 2.0% Quest Hy-Pea, 1.85% Tastone-154, 0.6% Na_2HPO_4, 0.125% $(NH_4)_2SO_4$, and 4.0% glycerol, pH 7.2 presterilization. Kanamycin (Km) sulfate to 50 μg/mL (from a 50 mg/mL filter-sterilized stock in dH_2O) was added after autoclaving. The optical density at 600 nm (OD_{600}) was recorded and fresh medium inoculated with the culture to a starting OD_{600} of 0.30. The flask was incubated as described above until the OD_{600} reached ~0.8−1.0. Isopropyl-thio-β-D-galactoside (IPTG)

was added from a 1 M filter-sterilized stock in dH_2O to the desired final concentration (50 μM or 1 mM), and the culture was allowed to grow for varying lengths of time before harvesting by centrifugation.

Fermentation Process for Production of (*S*)-Aminotransferase. Two frozen vials of *E. coli* SC16541 were thawed, and the contents (1.5 mL) were transferred to two 500-mL flasks containing 100 mL of MT5 medium (2.0% Yeastamin, 4.0% glycerol, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.125% ammonium sulfate, 0.0246% magnesium sulfate heptahydrate and 0.005% Km sulfate [added postautoclaving from a filter-sterilized solution]). The flasks were incubated at 30 °C and 250 rpm for ~24 h; then 2 mL of the broth was used to inoculate 1 L of the same medium in each of four 4-L flasks. The second-stage flasks were incubated at 30 °C and 230 rpm for 18−20 h (average OD_{600} 5.5 AU/cm). The contents of each flask were then pooled and used to inoculate a fermentor containing 250 L of MT5-M2 medium with 0.04% UCON LB625 antifoam (Dow Chemical) and 0.005% Km sulfate (prepared separately and filter-sterilized) resulting in a starting OD_{600} of 0.08 to 0.1 AU/cm. The fermentation process conditions for the tank were as follows: 30 °C; no pH control; agitation, 300 rpm; aeration, 250 Lpm; back pressure, 10 psi; foam controlled by the addition of UCON LB625 on demand.

At an OD_{600} of ~0.8 to 1.0 U/cm (about 5.5 h after inoculation), an IPTG solution (2.98 g in 500 mL of deionized water, filter-sterilized) was added to yield a final concentration of 50 μM. About 24−25 h postinoculation, the whole broth was harvested by centrifugation. During centrifugation, the cells were washed with 50 mM pH 7.0 potassium phosphate buffer. The cell paste (12.5 kg) was then stored at −70C until used for the next processing step. (*S*)-Aminotransferase activity of 0.72 to 0.78 U/g of wet cells was obtained.

Fermentation Process for Production of (*R*)-Amino Acid Oxidase. (*R*)-Amino acid oxidase from *T. variabilis* was cloned and overexpressed in *E. coli* (*E. coli* BL21[pBMS1000-DAAO], designated SC 16544) by our Technical Operations group in Syracuse, New York. Cloning and expression of this gene has been described previously by other groups.[28,29] Two frozen vials of SC16544 were thawed and the entire contents (1.5 mL) were transferred to each of two 500-mL flasks containing 100 mL of MT5 medium. The F1 stage flasks were incubated at 28 °C and 250 rpm for 24 h. From each F1 flask, 5 mL was transferred to two 4-L flasks containing 1 L of the same MT5 medium (total: 4 flasks). The four F2 flasks were incubated at 28 °C and 230 rpm for an additional 22 −24 h. The 4 L of pooled inoculum from the F2 flasks was then transferred to a 250 L working volume tank containing MT5-M2 medium. The fermentation process was carried out at 30 °C with 300 rpm agitation, 250 Lpm aeration, at 10 psi pressure. At an OD_{600} of ~2.0 AU/cm, IPTG (8.93 g dissolved in 500 mL deionized water and filter-sterilized) was added to yield a final concentration of 150 μM. This addition time corresponded to a CO_2 off-gas value of ~0.11−0.15. After 24−25 h postinoculation, the whole broth was harvested by centrifugation. During centrifugation, the cells were washed with 50 mM pH 7.0 potassium phosphate buffer. The cell paste (7.2 kg) was then stored at −70 °C until used for the next processing step. (*R*)-Amino acid oxidase activity of 36 U/g of wet cells was obtained.

Preparation of *S. ureae* Cells Containing (*S*)-Amino Acid Dehydrogenase. Growth/fermentation medium was as follows: 1.0% L-phenylalanine, 1.0% peptone, 0.5% yeast extract, 0.2% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4$ adjusted to pH 7 with K_2HPO_4 or KH_2PO_4. Broth (0.5 mL) from frozen vials containing *S. ureae* SC16048 was used to inoculate 100 mL medium in each of four 500-mL flasks. After incubation at 28 °C, 200 rpm for two days 200 mL of the culture was used to inoculate 15 L medium in each of two fermentors. Fermentation was carried out at 15 L/min airflow, 500 rpm agitation and 28 °C. No pH control was used during the fermentation. After 48 h, cells were collected with a Sharples centrifuge, washed with 10 mM potassium phosphate buffer pH 7, then stored at −70 °C until used. 412 g cell paste was recovered from the two tanks.

Conversion of Racemic Amino Acid 1 to (*S*)-Amino Acid 3 by (*R*)-Amino Acid Oxidase and (*S*)-Amino Acid Dehydrogenase. *S. ureae* SC16048 cells (20 g, stored frozen at −70 °C) were suspended in 50 mM ammonium formate, pH 8, containing 1 mM DTT, to a volume of 100 mL using an Ultraturrax homogenizer, and then disrupted by sonication for 3 min. The sonicated suspension was centrifuged at 27,500xg for 20 min, and the supernatant was used as a source of (*S*)-amino acid dehydrogenase. Racemic amino acid 1 (1.95 g, 7.61 mmol) was dissolved in 95 mL water, 5 mL 1 M potassium phosphate buffer pH 7 added and the solution adjusted to pH 8. Immobilized (*R*)-amino acid oxidase (*T. variabilis* enzyme expressed in *E. coli* and immobilized on Celite, 4 g, 280 units from Bristol-Myers Squibb Industrial Division) and catalase (0.2 mL, 7840 units from Biocatalytics) were added and the mixture was shaken at 28 °C, 250 rpm in a 250-mL flask. After 5.5 h the ee was 89%. After 7.5 h, ammonium formate (2.52 g, 40.0 mmol), dithiothreitol (31 mg, 0.2 mmol), NAD (66 mg, 0.1 mmol), formate dehydrogenase (20 mg, 10 units from Boehringer) and extract from *S. ureae* SC16048 (60 mL) were added, and the pH was adjusted to 8.0 with 1N NaOH. The flask was incubated at 28 °C, 40 rpm, for 19 h at which time keto acid **2** was not detected by HPLC analysis. The flask was placed in a boiling water bath for 5 min to precipitate proteins, cooled to room temperature, then centrifuged at 27,000 × *g* for 20 min. The pellet was washed with 20 mL water and centrifuged again. The combined supernatants contained 1.33 g (68% solution yield) (*S*)-amino acid **3** with 100% ee in 176 mL.

The reaction mixture was adjusted to pH 7.0 (H_2SO_4), mixed with 35 g of Na_2SO_4 and **3** extracted into *n*-butanol (176- and 44-mL portions). The amino acid was extracted from the combined rich organic phase with one 49-mL and two 10-mL portions of 0.1 M H_2SO_4. The combined aqueous phase was concentrated to 12 g (crystallization was abundant by 25 g), mixed with 12 mL of EtOH, and left at room temperature to equilibrate. Filtration and drying *in vacuo* gave 1.70 g of **3** as the monohydrate monosulfate, potency 62.1 w% (Na_2SO_4

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contaminant), ee −99.9%. The isolated product thus contains 1.06 g (4.12 mmol) of **3** for an overall yield of 54% from **1**.

Conversion of Racemic Amino Acid 1 to (*S*)-Amino Acid 3 with (*R*)-Amino Acid Oxidase and (*S*)-Aminotransferase. Cell extracts were prepared from 200 g (wet weight) of recombinant *E. coli* SC16541 cells containing (*S*)-aminotransferase and 40 g (wet weight) of recombinant *E. coli* SC16544 containing (*R*)-amino acid oxidase. Combined cells were suspended in 760 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 5 μM pyridoxal 5′-phosphate (PLP), and then disintegrated with a Microfluidizer at 10,000 psi in three successive passes. To the cell extract, 5 g of 50% polyethyleneimine (PEI) was added to a final concentration of 0.2% and mixed. The PEI treated mixture was centrifuged at 15,000 × *g* for 2 min at 4 °C to remove the cell debris. The supernatant was collected and used for the bioconversion process.

Bioconversion of racemic amino acid **1** to (*S*)-amino acid **3** was conducted in a 2-L reactor controlled by a Braun Biostat B for optimization of the process. Racemic amino acid **1** (15 g of the monohydrate monosulfate, potency 60.72%, 0.0355 mol), sodium aspartate monohydrate (25 g, 0.144 mol) and ascorbic acid (5 g, 0.028 mol) were dissolved in 50 mL of propylene glycol and 430 mL of water. The solution was adjusted to pH 7.5 with 10 N NaOH, then 2 mL of catalase (392 KU), 500 mL of cell extract containing 165 units of (*R*)-amino acid oxidase and 72 units of (*S*)-aminotransferase and 2 mL of antifoam SAG-5693 were added. The reaction was carried out at 30 °C, pH 7.5, with stirring at 300 rpm, and aeration at 1 L/min. Samples were taken every hour to analyze ee and conversion yield. The aeration was stopped at 6 h when the oxidation was complete (ee of >99% by HPLC analysis). The transamination reaction was continued for an additional 16 h without aeration, and the reaction mixture was harvested.

The reaction mixture was ultrafiltered with a Millipore 30 kDa NMWL Biomax polyethersulfone membrane, diafiltering with water to recover the product. The combined permeate, 2.8 L, was concentrated *in vacuo* to 0.73 L, washed with ethyl acetate (to remove antifoam agent), mixed with 439 g of ammonium sulfate and extracted with two 183-mL portions of *n*-butanol, The rich extract was washed with two small portions of water to remove residual ammonium sulfate. Assay at this point indicated the extract contained 29.7 mmol of **3**. The extract was mixed with 59.5 mL of methanol and 59.5 mL of 1 M sulfuric acid (100% excess). The resulting mixture was stirred with cooling in an ice bath for 4 h and filtered, washing the solid with 60 mL of ice-cold methanol−water, 9:1. The solid was dried *in vacuo* at room temperature, giving 9.68 g of **3** as the monohydrate monosulfate, potency 68.1%, ee 99.85.

Scale-Up of Bioconversion Process. Frozen SC16541 cells (12 kg) and SC16544 cells (2.4 kg) were thawed and suspended in 46 L of 0.1 M phosphate buffer (pH 8.0) containing 5 μM PLP with agitation at 200 rpm. The 24% cell suspension was passed twice through a microfluidizer. PEI was added to 0.2% concentration, mixed for 30 min, followed by centrifugation with a Sharples centrifuge at 0.4 gal/min to clarify the extract.

To a 100-L vessel containing 29 L of water, stirring at 100 rpm, were added sodium aspartate monohydrate (1.67 kg, 9.65 mol), ascorbic acid (0.33 kg, 1.87 mol), racemic amino acid **1** monosulfate monohydrate (1 kg, potency 60.7%, 2.37 mol), and propylene glycol (3.33 kg). The solution was adjusted to pH 7.0 with 6.25 M NaOH. Subsequently, catalase (0.133 kg, 25500 KU), pyridoxal phosphate (167 mg, 0.63 mmol), clarified cell extract (40 L), and SAG-5693 antifoam (133 g) were added to the reactor. The reaction was carried out at 30 °C, 250 rpm, pH 7.5 and 80 L/min aeration. Samples were taken every 2 h to assay ee of the product and conversion. The oxidation was complete within 2 h, and the bioconversion was complete after 25 h to give a 75% solution yield and ee >99.9% of **3**.

The reaction mixture, 78 L, was ultrafiltered on a 20 ft^2 tangential flow Millipore Biomax polyethersulfone membrane, diafiltering with water. The combined permeate, 105.2 kg, containing 412 g (1.61 mol) of **3**, was concentrated *in vacuo* to 41 L and stirred with 21 kg of ammonium sulfate and 10.3 L of *n*-butanol. The aqueous phase, including some interfacial precipitate, was separated, filtered, and extracted with 4 L of *n*-butanol. The combined rich organic extract was stirred with 3.2 L of methanol and 3.2 L of 1 M sulfuric acid. A small sample of the solution was withdrawn, rubbed with a glass rod to induce crystallization, and the resulting slurry was returned to the reactor. The mixture was stirred at room temperature for one hour and then at 0 °C for 15 h. The product was filtered out and washed with 3.4 L of cold methanol−water, 9:1, and dried *in vacuo* at 40 °C for 27 h, giving 580 g of **3** monosulfate monohydrate, potency 69.5%, ee 99.9%, overall yield 66%.

Dynamic Resolution of Racemic Amino Acid 1 to (*S*)-Amino Acid 3: Effect of pH. Portions of a suspension of **1**, 13.5 mg of the sulfate monohydrate (0.036 mmol) per mL in water containing 0.2 M ammonium dihydrogen phosphate and 0.1 M ammonium formate were adjusted to pH 5.0−8.0 at 0.5 pH unit intervals (NH_4OH). One-mL portions of each was added to a mixture of 50 mg of immobilized (*R*)-amino acid oxidase on Celite and 11 mg of borane−ammonia complex (0.36 mmol) and the suspension stirred at room temperature for 20 h. The mixture was diluted with 1 mL of 0.1 M NaOH followed by 34 mL of 50 mM HCl. HPLC analysis gave the results shown in Table 3.

Preparative Dynamic Resolution of Racemic Amino Acid 1 to (*S*)-Amino Acid 3. A suspension of 1.08 g of **1** monosulfate monohydrate (2.90 mmol) in 80 mL of 0.2 M ammonium phosphate buffer, pH 6.0, containing 50 mM ammonium formate, was adjusted to pH 6.25 (NH_4OH). The mixture was stirred and 0.92 g (29.8 mmol) of borane−ammonia complex added followed by 4.0 g of (*R*)-amino acid oxidase immobilized on Celite. The mixture was stirred at room temperature. At 20 h the pH, 6.84, was adjusted to 6.25 with 1 M H_2SO_4, and this was repeated at 72 h (pH 6.81). HPLC at this point indicated a solution yield of 81% and an ee of 100%. To catalytically decompose the excess borane reagent, 0.1 g of 10% Pd/C was added and the mixture stirred for another 20 h. The pH was adjusted to 4.0 with 2 M H_2SO_4, 5 g of activated charcoal was added, and the mixture was filtered (Celite). The

filtrate was concentrated *in vacuo* to 11 mL, cooled on ice, and filtered to give 0.73 g of **3** as the monosulfate monohydrate, 68% yield, ee 100%.

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