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Enzymatic Preparation of a D-Amino Acid from a Racemic Amino Acid or Keto Acid

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Abstract:

The D-amino acid (*R*)-2-amino-3-(7-methyl-1 *H*-indazol-5-yl)propanoic acid (3) is a key intermediate needed for synthesis of a drug candidate compound. Enzymatic routes to 3 were explored. D-Amino acid 3 was prepared in 68% isolated yield with >99% ee from racemic amino acid 1 using L-amino acid deaminase from *Proteus mirabilis* expressed in *Escherichia coli* in combination with a commercially available D-transaminase using D-alanine as amino donor. The D-enantiomer was also prepared in 79% isolated yield with >99% ee from the corresponding keto acid 2 using the D-transaminase with racemic alanine as the amino donor. The rate and yield of this reaction could be accelerated by addition of lactate dehydrogenase (with NAD, formate and formate dehydrogenase to regenerate NADH) to remove the inhibitory pyruvate produced during the reaction. A D-transaminase was purified from a soil organism identified as *Bacillus thuringiensis* and cloned and expressed in *E. coli*. The D-transaminase was very effective for the preparation of 3 and gave a nearly complete conversion of 2 to 3 without the need for additional enzymes for pyruvate removal.

Introduction

D-Amino acids are useful intermediates for the synthesis of β-lactam antibiotics and other pharmaceuticals.[1,2] Many enzymatic approaches have been applied for their preparation including D-hydantoinases combined with D-carbamoylases or HNO_2,[3,4] D-acylases,[5] D-amidases,[6] D-transaminases[2] and recently a D-amino acid dehydrogenase has been developed.[7] Racemic amino acids have also been deracemized to give D-amino acids using an L-amino acid oxidase to selectively deplete the L-amino acid combined with an excess of reducing agent to recycle the imine product to the racemic amino acid.[8] The D-amino acid (*R*)-2-amino-3-(7-methyl-1 *H*-indazol-5-yl)propanoic acid (**3**) is a key intermediate needed for synthesis of antagonists of calcitonin gene-related peptide receptors.[9] Such antagonists are potentially useful for the treatment of migraine and other maladies.[9,10] We have previously used the combination of a D-amino acid oxidase and an L-amino acid dehydrogenase to convert a racemic amino acid to an L-amino acid.[11] In this report we describe an analogous procedure for the conversion of the racemic amino acid **1** to D-amino acid **3** using an L-amino acid deaminase cloned from *Proteus mirabilis* and expressed in *Escherichia coli* together with a commercially available D-transaminase.

The low solubility of **1** at neutral pH combined with the localization of the deaminase enzyme in an insoluble fraction of the cell makes the oxidation of the L-amino acid a slow process. With the availability of the more soluble keto acid **2**, D-amino acid **3** was more conveniently prepared using the commercially available D-transaminase without the necessity for an oxidation step. Starting the process from keto acid **2** instead of racemic amino acid **1** was an improvement, but the reaction was still slow to reach completion, apparently due to inhibition by pyruvate. This report also describes the isolation, cloning, and overexpression of a D-transaminase, from a soil isolate identified as *B. thuringiensis*, which proved to be more effective for the conversion of **2** to **3**.

Results and Discussion

Cloning of the *Proteus mirabilis aad* Gene. Our initial approach for preparation of *R*-amino acid **3** is shown in Scheme 1. Starting from racemic amino acid **1**, the aim was to oxidize the L-amino acid to keto acid **2**, then transaminate **2** to **3** with a D-transaminase. Screening of seven strains reported to have L-amino acid deaminase (also called L-amino acid oxidase) for conversion of **1** to give **3** identified *Providencia alcalifaciens* SC9036 (92.3% ee) and *Proteus mitajiri* SC13814 (100% ee) as effective strains for the selective oxidation of the undesired *S*-isomer. After initial oxidation and transamination experiments with these strains further work was performed with a recombinant L-amino acid deaminase to achieve better productivity. The gene sequence of the related *P. mirabilis* amino acid deaminase gene has been published.[12] Oligonucleotide sequences for amplification of this gene and subsequent cloning into our proprietary expression vector pBMS2004 by the

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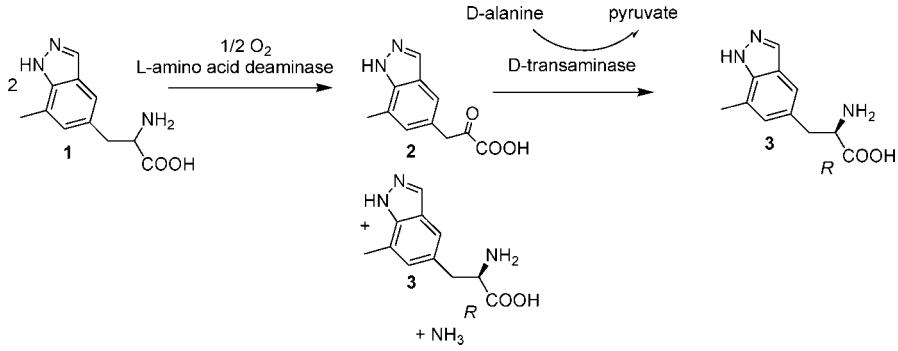
Scheme 1. **Enzymatic reactions used for the conversion of racemic amino acid 1 to *R*-amino acid 3**



Polymerase Chain Reaction (PCR) are given in the Experimental Section. Proprietary *E. coli* expression vector pBMS2004 is a variant of the previously described expression vector pBMS2000[13] differing only in the number of restriction sites contained within the multiple cloning sequence.

The primer sequences were tried with the FailSafe series of PCR buffer and *P. mirabilis* chromosomal DNA as template. A prominent fragment of the expected size (~1450 base pairs) was seen upon electrophoresis on a 1.0% TAE agarose gel in buffers "D" through "L". This fragment was excised from the gel and purified. The PCR fragment was ligated to cloning vector pTOPO4 Blunt and subsequently transformed into TOP10 cells. Six kanamycin (Km)-resistant colonies were tested for the presence of the PCR fragment by colony PCR using the primers and thermocycling conditions described in the Experimental Section; 3/6 supported amplification. Plasmid DNA was isolated and cleaved with restriction endonuclease *Eco*RI. All plasmids gave the expected fragments (based on restriction maps of the vector and insert) of 3900, 1031, 242, and 150 bp.

Plasmid DNA from one of these samples was digested with *Nde*I and *Bam*HI. The 1450-bp fragment was isolated and ligated to *Nde*I−*Bam*HI-digested pBMS2004. Thirty-six Km^R colonies were tested for the presence of the *aad* gene by colony PCR (see above) using FailSafe buffer "H". Following electrophoresis, 34/36 colonies supported amplification of the 1450-bp fragment. After plasmid isolation from four of the colonies and digestion with *Nde*I + *Bam*HI, all four plasmids gave the expected 4500-bp vector and 1450-bp insert fragments. One such plasmid was selected and denoted as pBMS2004-PMAAD (for *P. mirabilis* amino acid deaminase).

Heterologous Expression of PMAAD. pBMS2004-PMAAD was transformed into chemically competent *E. coli* BL21 cells and streaked for single colonies onto an LB Km agar plate. Growth and induction of expression as well as assay for deamination were performed as described in the Experimental Section. A positive reaction indicated by the formation of a dark green color, was seen in all *E. coli* samples containing pBMS2004-PAAAD, including a sample where the inducer β-isopropyl-thio-D-galactoside (IPTG) was not added. These results suggested that some transcription/translation of the cloned *aad* gene was occurring even without addition of inducer, although the reaction was significantly more intense using cells that had been exposed to IPTG. However, *E. coli* possessing pBMS2004 alone did not change color, showing that activity is not due to a wild-type *E. coli* enzyme. In addition, SDS-polyacrylamide gel electrophoresis of the sonicates of induced cells revealed a highly overexpressed protein of the expected molecular weight of the PMAAD (~51,000 Da).

An enzyme assay was established for L-amino acid deaminase measuring conversion of **1** to keto acid **2** during a 1-h reaction by HPLC. Whole cells were as active as sonicated extracts. Two fermentation batches produced 12 kg of cell paste with an activity of 27 U/g and 13 kg of cell paste with an activity of 19.9 U/g. All of the deaminase activity could be pelleted from a cell extract by centrifuging at 108,860*g* for 2 h. In *P. mirabilis* the deaminase is localized in the cell envelope and uses a respiratory chain to transfer electrons to O_2.[14]

Conversion of Racemic Amino Acid 1 to *R*-Amino Acid 3. A D-transaminase (Biocatalytics) was used for conversion of **1** to **3** in combination with *P. mirabilis* L-amino acid deaminase expressed in *E. coli*. The initial batch contained D-aspartic acid (2 g, 15.03 mmol), racemic amino acid **1** (1 g, 4.56 mmol), and 20 g of *E. coli* wet cells expressing L-amino acid deaminase from *P. mirabilis* in 0.1 M potassium phosphate buffer pH 7.5 containing 0.1 mM pyridoxal phosphate and D-transaminase (10 mg, 55 units from Biocatalytics). After 18 h, ee was 100%, and only a trace of keto acid was seen by HPLC. Incubation was continued for 42 h; then the pH was adjusted from 7.97 to 1.5 with a few milliliters of concentrated HCl and the cells were removed and washed by centrifugation. The combined supernatants contained 853 mg (3.89 mmoles) **3**, 100% ee, in a volume of 224 mL.

Other amino donors, higher concentrations of **1**, and various additives were evaluated. Propylene glycol or toluene at 5% concentration did not improve the yield of **3** or significantly increase the solubility of **1**. The best amino donor (comparing D-aspartate, D-glutamate, D-alanine, L-alanine, L-aspartate, and β-alanine) was D-alanine. Although the recombinant *E. coli* catalyzed racemization of D- and L-alanine, racemic alanine was not an effective amino donor (65% yield, 88% ee after 88 h). The additional L-alanine may compete with **1** for L-amino acid deaminase, although the *P. mirabilis* enzyme was reported to have only slight activity with L-alanine.[14]

Several 1-L batches were run in a Braun Biostat B for the purpose of developing a procedure for scale-up. All batches contained **1** (20 g, 0.0912 mol), D-alanine (40 g, 0.448 mol),

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pyridoxal phosphate monohydrate (2.65 mg, 0.01 mmol), 100 g of *E. coli* frozen cell paste containing cloned L-amino acid deaminase (27 U/g cells wet weight) from *P. mirabilis*, Biocatalytics D-transaminase (200 mg, 4.4 U/mg), K_2HPO_4 (6.97 g, 0.04 mol), and KH_2PO_4 (1.36 g, 0.01 mol). The pH was maintained at 7.5, 8.0, or 8.5 by addition of 25% H_2SO_4 and 25% NaOH. SAG 5693 antifoam was used to control foaming. Best yields and ee were obtained at pH 7.5 with 1 L/min aeration. Three batches run under these conditions gave an average 85% solution yield with 96.5% ee.

Downstream Processing. At the end of the initial batch, the pH was adjusted to 1.0 with H_2SO_4 to dissolve the amino acid **3**. Ultrafiltration with a 30K filter led to failure of the filter due to the low pH. Later batches were adjusted to pH 12 to dissolve the amino acid **3** and still be compatible with the downstream processing materials and equipment. At pH 12 the mixture became quite viscous, presumably from release of DNA from the cells. Batches were homogenized with an ultraturrax to shear the DNA and reduce viscosity, centrifuged 2 min at 15000*g* to remove material that slowed the ultrafiltration, and passed through a 30K UF membrane to prepare for crystallization.

For isolation the ultrafiltrate (pH 12) was warmed to about 95 °C, neutralized, cooled to 5 °C, and the resultant crystalline product was isolated by filtration, washed with water, and dried *in vacuo* to give **3** as a monohydrate. The isolated yield was 68% with ee >99%. The excess alanine remains soluble during the isolation procedure. To determine the effectiveness of the isolation procedure for enhancement of the ee, a batch was stopped after 3 days (88.1% ee) instead of 4. After isolation, the ee of the crystalline solid was >99%, and the ee of the mother liquor was 33.2%.

Stability samples for **3** (>99% ee) kept for 5 days at pH 12 showed no change at 5 or 21 °C, but some racemization occurred at 101 °C (97% ee after 16 h, 77% ee after 120 h). There was no change in the concentration or ee of **3** in a crude reaction mixture adjusted to pH 12 and kept for 10 days at room temperature.

Scale-Up. The conversion of 2.9 kg **1** to **3** was carried out in two batches in a pilot plant. The ee rose as rapidly initially as during laboratory batches but became slower at later times and did not reach the 96% ee target. Foaming was a much bigger problem than in the laboratory batches. The average yield before isolation was 79% with 91% ee. Isolation of the product gave **3** with 98.6% ee. A total of 1.79 kg of **3** (62% isolated yield) was obtained.

Transamination of Keto Acid 2 to *R*-Amino Acid 3. L-Amino acid deaminase from *P. mirabilis* has good enantioselectivity, but improvement of ee in the deracemization process was hampered by the poor solubility of **1** near neutral pH. Keto acid **2** is quite soluble at neutral pH and was therefore a better starting material for the preparation of **3**. The product amino acid **3** has low solubility and its ee can be substantially improved by crystallization. D-transaminase (Biocatalytics) afforded the direct conversion of **2** to **3** with excellent ee. The reaction conditions were optimized on a 1-mL scale for the conversion. Glycine and isopropylamine did not serve as amino donors. Racemic alanine could be substituted for expensive D-alanine. Addition of alanine racemase to the reaction gave

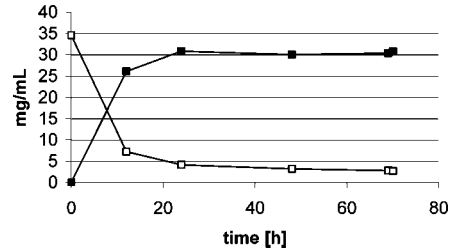


Figure 1. **Time course of reaction for conversion of 2 to 3 using D-transaminase.The reaction was carried out as described in the Experimental Section except scaled up from 30 to 300 g. ■, 3; □, 2.**

no improvement in yield or ee. Although D-aspartate can serve as an amino donor, the keto acid **2** was unstable when aspartate was the amino donor. Racemic glutamate, lysine, valine, and serine were also tried as amino donors. Only glutamate gave conversions similar to alanine. Since racemic alanine is considerably cheaper than racemic glutamate, alanine was used for the transamination. Variation of pH, temperature, concentration of **2**, alanine concentration, pyridoxal phosphate concentration, and addition of dithiothreitol, EDTA, or glycerol were evaluated to maximize the yield of the transamination reactrion. Concentrations of 0, 10, 20, 50, and 100 μM pyridoxal phosphate were equally effective for the conversion of keto acid to amino acid.

Three 1-L batches that contained **2** (30 g, 0.137 moles) D,L-alanine (120 g, 1.35 mol), 0.1 mM pyridoxal phosphate, 1 mM dithiothreitol and 0.3 g D-transaminase in 50 mM potassium phosphate buffer pH 7.5 at 30 °C. were completed. The average solution yield after 70 h was 89%, and the average isolated yield was 76% with ee >99%. For isolation, the mixture was acidified to pH 1 to dissolve the product and to precipitate proteins. The mixture was filtered and the filtrate passed through a column of reversed phase resin to remove nonpolar impurities. Then **3** was crystallized by neutralization at elevated temperature as described above.

Two 10-L batches that contained 300 g (1.37 mol) of **2** were completed with similar results. The average isolated yield was 79% (244 g) with ee >99%. The time course for the first batch is shown in Figure 1.

The two 10-L batches were carried out for 70 and 75 h, respectively. Most of the reaction took place during the first 24 h, and then the reaction became very slow. Pyruvate was shown to be a strong product inhibitor of the reaction. Addition of pyruvate decarboxylase and the cofactor thiamine pyrophosphate to remove inhibitory pyruvate did not accelerate the reaction. Either L-alanine dehydrogenase from *Bacillus* or L-lactate dehydrogenase from rabbit muscle was added in 1-mL reactions to try to remove the pyruvate. In both cases formate dehydrogenase, NAD, and either ammonium formate for alanine dehydrogenase or sodium formate for lactate dehydrogenase were also added for cofactor regeneration. Alanine dehydrogenase was not effective, but lactate dehydrogenase (Scheme 2) brought the reaction to near completion in as little as 4 h as shown in Figure 2. With the transamination coupled to the lactate dehydrogenase and formate dehydrogenase reactions, the overall equilibrium constant is much improved compared to that of the transamination reaction alone, and the reaction would be expected to need only a small excess of D-alanine to go to

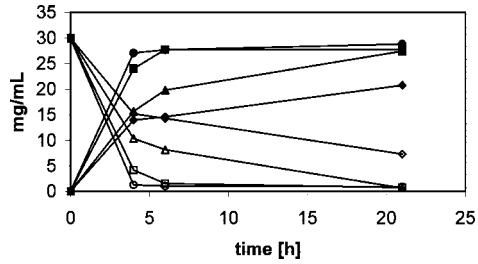
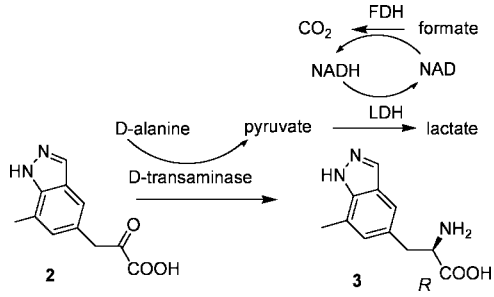


Figure 2. **Reaction rate increase from addition of lactate dehydrogenase. Reactions contained in 1 mL at 30 °C: 30 mg (0.137 mmol) keto acid 2, 120 mg (1.347 mmol) D,L-alanine, 13.6 mg (0.2 mmoles) sodium formate, 0.1 mM pyridoxal phosphate, 1 mM dithiothreitol, 1 mM NAD, 1.77 U formate dehydrogenase from *Candida boidinii* (Boehringer), 0.3 mg D-transaminase (Biocatalytics) and varying amounts of rabbit muscle lactate dehydrogenase in 0.1 M potassium phosphate buffer pH 7.5. Amino acid 3 concentration with 0 (♦), 0.91 (▲), 2.3 (■), and 4.5 (●) U/mL lactate dehydrogenase added. Keto acid 2 concentration with 0 (◇), 0.91 (Δ), 2.3 (□), and 4.5 (○) U/mL lactate dehydrogenase added.**

Scheme 2. **Enzymatic reactions used for removal of pyruvate**



completion instead of the 4.9 equiv used in the previous procedure. A large excess of lactate dehydrogenase gave a new impurity peak which LC/MS indicated was the hydroxyacid (mass 220.1), but this peak was small for the reactions shown in Figure 2. Cloned lactate dehydrogenases (Biocatalytics) from chicken heart and rabbit muscle were compared at 2 U/mL. Both gave a complete reaction after 15 h, and the hydroxyacid impurity was 3.09% (% HPLC peak area relative to amino acid peak area) for rabbit muscle and 7.66% for the chicken heart enzyme.

One-milliliter reactions were tried using 30 or 60 mg/mL (137 or 275 mM) **2** and 30 or 60 mg (336 or 673 mM) racemic alanine (1.225 equiv of D-alanine), with or without sodium formate, NAD, formate dehydrogenase, and lactate dehydrogenase. Complete conversion of 60 mg/mL **2** to **3** in good yield was possible only when using the auxiliary enzymes (Table 1).

Purification of D-Transaminase from a Strain of *B. thuringiensis* Isolated from a Soil Sample. D-Amino acid transaminase has been found in some species of bacteria where it functions to provide D-glutamate for cell wall biosynthesis and may also provide D-amino acids for synthesis of antibiotics that are produced by some species.[15–19] D-Transaminase has been particularly studied in several species of Bacillus, and the gene has been cloned and sequenced from some of these strains.[17–19]

Screening of strains isolated from soil samples was undertaken to identify a more effective transaminase for conversion of **2** to **3**. Whole cells of 10 soil isolates were tested for transamination of keto acid **2** to D-amino acid **3**. Five isolates produced **3** when D-phenylalanine or D-ala was the amino donor. One of the isolates was selected as the enzyme source due to the relatively high activity of its extract and the fact that extract activity was stable during six days of storage at 4°. This isolate was identified as *B. thuringiensis* by the sequence of its 16S rRNA gene, and it was added to the culture collection as SC16569. Whole cells and extracts were found to possess the desired D-amino acid aminotransferase activity. The bacterium was grown in two 15-L fermentors to provide enough cells for purification of the enzyme.

The D-amino acid aminotransferase was purified 773-fold by sequential passage through four columns (Table 2). The N-terminal sequence of the purified protein was determined to be MKATHKDWILFNGRM, and an internal sequence was determined to be RHYVITLAK. The sequencing results indicated strong homology with known Bacillus D-amino acid aminotransferases and enabled the design of PCR primers to allow cloning and expression of the D-transaminase as described in the Experimental Section.

Cloning and Expression of the D-Transaminase Gene from *B. thuringiensis* SC16569. Amino acid sequences derived from the purified amino acid transferase protein were used to design synthetic oligonucleotides to serve as primers for PCR amplification of the corresponding gene from the chromosomal DNA. The DNA sequence of the PCR product contained a single open reading frame of 665 bp, encoding a sequence of 221 amino acids. Based on the molecular weight of the purified *B. thuringiensis* SC16569 aminotransferase protein (34,000 Da) and the molecular weight of the amino acid sequence encoded by the PCR fragment (26,000 Da), the PCR fragment encompassed approximately 75% of the intact aminotransferase gene.

To isolate the remaining portion of the gene, a standard Southern blot hydbridization was performed using various restriction digests of *B. thuringiensis* SC16569 chromosomal DNA as the target and DIG-labeled sample of the 665 bpPCR fragment as the probe. A *Hind*III restriction fragment of approximately 8500 bp hybridized strongly to the probe. DNA sequence analysis of the 8500 bp *Hind*III fragment revealed that it contained the entire open reading frame for the intact gene (now called BtDAAT) encoding a protein with a molecular weight of 33,766 Da, consistent with the purified *B. thuringiensis* SC16569 aminotransferase protein. The nucleotide and amino acid sequences of BtDAAT are shown in Figure 3.

The C-terminus of the BtDAAT gene had to be modified by the addition of a *Bam*HI restriction site to facilitate ligation into the expression vector pBMS2004. Plasmid pBMS2004 was digested with *Nde*I/*Bam*HI and the (*Nde*I/*Bam*HI) BtDAAT insert fragment was ligated downstream of the plasmid's IPTG-

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Table 1. **Effect of lactate dehydrogenase on conversion of 2 to 3[a]**

input keto acid **2** (mg/mL)	LDH, FDH	time (h)	keto acid **2** (mg/mL)	*R*-amino acid **3** (mg/mL)
30	−	15	8.19	13.6
	−	87	5.47	19.8
	+	15	0.00	23.2
	+	87	0.00	25.5
60	−	15	25.3	22.0
	−	87	31.0	26.0
	+	15	16.2	32.1
	+	87	0.00	54.2

[a] Reactions contained in 1 mL: 30 or 60 mg (137 or 275 mM). **2**, 30 or 60 mg racemic alanine (336 or 673 mM), 0.3 or 0.6 mg/mL D-transaminase, 1 mM dithiothreitol, 0.1 mM pyridoxal phosphate, 1 mM NAD, and where indicated, 0.2 or 0.4 M sodium formate, 2 or 4 units formate dehydrogenase (FDH), and 2 or 4 units rabbit muscle lactate dehydrogenase (LDH).

Table 2. **Purification of the D-transaminase from *B. thuringiensis* SC 16569**

stage	volume (mL)	activity (U)	protein (mg)	specific activity (U/mg)
crude extract	520	17.2	2673	0.006
Q Sepharose	45	13.6	273	0.05
Phenyl Sepharose	32	7.7	194	0.04
Sephacryl S-200	12	2.5	0.60	4.64
UNO Q1	1	0.24	0.05	5.24

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        M  K  A  T  H  K  D  W  I  L  F  N  G  R  M  V  N  T  K  E
   1    ATGAAAGCTACACATAAAGATTGGATTTTATTTAACGGAAGAATGGTAAATACGAAGGAA
        E  Q  P  M  V  A  L  E  E  R  G  F  Q  F  G  D  G  I  Y  E
  61    GAACAGCCGATGGTTGCATTAGAAGAGCGAGGCTTCCAATTTGGTGATGGTATATATGAG
        V  F  R  L  Y  G  G  K  P  H  L  L  D  L  H  L  D  R  F  F
 121    GTATTCAGACTATACGGTGGTAAGCCTCATTTATTAGATTTACATTTGGACCGTTTTTTT
        K  S  M  E  E  I  K  L  I  P  P  F  T  K  E  E  L  V  E  E
 181    AAATCCATGGAAGAAATTAAATTAATTCCACCATTTACAAAGGAAGAATTAGTGGAAGAG
        L  H  Q  M  I  E  K  N  Q  F  Q  E  D  G  N  V  Y  L  Q  I
 241    TTGCATCAAATGATTGAAAAAAATCAATTTCAAGAAGATGGAAATGTATATTTGCAAATA
        S  R  G  A  Q  P  R  N  H  V  Y  E  S  D  L  Q  P  T  Y  F
 301    TCAAGAGGCGCCCAACCACGTAATCATGTATATGAGTCAGATCTACAACCTACATATTTT
        A  N  I  V  S  F  L  R  P  I  A  T  M  E  A  G  I  K  V  T
 361    GCAAATATTGTTTCGTTCCTAAGACCAATCGCTACGATGGAAGCAGGTATAAAGGTAACG
        V  E  E  D  I  R  W  K  F  C  H  I  K  S  L  N  L  L  P  N
 421    GTAGAAGAGGATATAAGATGGAAATTTTGTCATATAAAATCTTTAAATCTTCTTCCTAAT
        I  M  I  K  N  K  I  N  E  Q  G  Y  Q  E  A  I  L  V  R  D
 481    ATCATGATTAAAAATAAAATAAATGAACAAGGGTATCAAGAAGCGATTTTAGTACGAGAT
        G  V  V  T  E  G  C  H  S  N  F  F  I  V  K  N  D  K  L  I
 541    GGAGTTGTAACAGAGGGATGTCATTCCAATTTCTTTATAGTTAAGAATGATAAACTGATT
        T  H  P  A  D  H  F  I  L  H  G  I  T  R  H  Y  V  I  T  L
 601    ACACATCCAGCTGATCACTTCATTTTGCATGGTATTACTCGTCATTATGTTATAACATTA
        A  K  E  L  H  I  E  V  E  E  R  E  F  S  V  Q  E  V  Y  E
 661    GCGAAAGAGTTACATATTGAAGTAGAAGAACGAGAATTTTCAGTACAAGAGGTATATGAG
        A  D  E  C  F  F  T  A  T  P  L  E  I  F  P  V  V  Q  I  G
 721    GCTGATGAGTGCTTCTTTACGGCGACACCACTTGAAATATTCCCGGTTGTTCAAATTGGT
        D  E  Q  F  G  S  G  E  R  G  A  I  T  K  K  L  Q  A  A  Y
 781    GATGAACAGTTTGGAAGCGGCGAAAGAGGGGCAATTACGAAAAAGCTACAAGCTGCATAT
        E  E  T  I  S  L  F  K  V  T  N  *
 841    GAAGAAACTATTAGTCTTTTTAAAGTTACTAATTAA
```

Figure 3. **DNA and amino acid sequences for D-transaminase from *B. thuringiensis* SC16569.**

inducible promoter. This recombinant plasmid was used to transform electrocompetent *E.coli* expression strain BL21. SDS/PAGE analysis revealed a highly overexpressed novel protein following IPTG induction of the expression culture, with the majority of the heterologous BtDAAT aminotransferase present in the soluble fraction. This protein increased in concentration with increased induction time and was not present before introduction of IPTG to the culture. The molecular weight of this heterologously expressed protein was approximately 34,000 Da, in agreement with the anticipated size of the BtDAAT protein. Growth of *E. coli* BL21(pBMS2004-BtDAAT) SC16577 cells expressing D-aminotransferase in a 100-L fermentation and preparation of cell extracts containing D-transaminase activity are described in the Experimental Section.

Conversion of 2 to 3 with D-Transaminase from *B. thuringiensis* Expressed in *E. coli* SC16577. In initial experiments with crude extract, the cloned D-transaminase was more effective than the commercially available D-transaminase by going nearly to completion with 60 mg/mL of **2** without the additional enzymes used for removal of pyruvate. Although the equilibrium constant for most amino acid transaminase reactions is close to one,[2] the precipitation of **3** during the reaction should allow the reaction to go nearly to completion provided that the product inhibition by pyruvate is not severe. In addition to being highly active with keto acid **2** as a substrate, Figure 4 shows that the transaminase expressed in *E. coli* SC16557 is less sensitive to inhibition by pyruvate than the commercial transaminase.

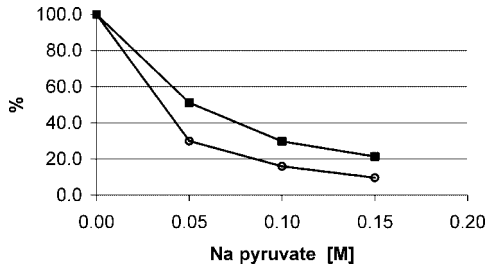

Figure 4. **Inhibition of transamination by pyruvate. Reactions contained in 1 mL at 30 °C: 15 mg (0.0687 mmol) keto acid 2, 60 mg (0.6735 mmol) D,L-alanine, 0.1 mM pyridoxal phosphate, 1 mM dithiothreitol, 0.3 mg of D-transaminase (Biocatalytics) or 0.03 mL of extract containing D-transaminase from *B. thuringiensis* and varying amounts of sodium pyruvate in 0.1 M potassium phosphate buffer, pH 7.5. (■) D-transaminase from *B. thuringiensis*. (○) Biocatalytics D-transaminase. The concentration of amino acid 3 was measured after 1 h of incubation time. In the absence of added pyruvate the concentration was 5.91 mg/mL for D-transaminase from *B. thuringiensis* and 4.90 mg/mL for Biocatalytics transaminase. Percent of uninhibited reaction rate is plotted versus pyruvate concentration.**

Using an extract (derived from 2 g wet weight *E. coli* SC16577) prepared by microfluidization and clarified with 0.2% polyethyleneimine (PEI), **2** (6 g, 0.0275 mol) was converted to 5.33 g **3** (92% solution yield, 79% isolated yield after correction for purity, >99% ee). The batch also contained racemic alanine (12 g, 0.135 moles), 0.1 mM pyridoxal phosphate, 1 mM dithiothreitol, and 80 mM potassium phosphate buffer, pH 8, in a volume of 100 mL. This is twice the keto acid concentration and half the equivalents of racemic alanine used for batches with the commercial enzyme. Some variations in the reaction conditions were tried. Although the transaminase has three cysteines, omission of dithiothreitol did not affect the conversion. Decreasing pyridoxal phosphate from 0.1 mM to 0.01 mM only slightly decreased the reaction rate. The reaction rate was slower at pH 7 and about the same at pH 7.5 or 8. Whole cells of the *E. coli* SC16577 were equally as active as the extract in carrying out the conversion. Transaminase activity in the extract (20% w/v) clarified with 0.2% PEI was stable for 1 year frozen at −20 °C.

Other Approaches to *R*-Amino Acid 3. D-Amino acid dehydrogenases have recently been developed by Biocatalytics.[7] This allows another approach in addition to our original transaminase approach. All six Biocatalytics dehydrogenases gave **3** with 100% ee under the screening conditions with 2 mg/mL **2** input, 10 mg/mL NADPH, 1 M NH_4Cl, and 0.1 M $NaHCO_3$, pH 9. The most active enzyme, D-AADH-102, was tested with 50 and 100 mg/mL keto acid input, (amino acid dehydrogenase and glucose dehydrogenase concentrations 1% of **2**, 1 M glucose, 1 M NH_4Cl, and 1 mM NADP), using a pH stat set to pH 8 or pH 9. Very little conversion of **2** to **3** occurred under these conditions.

A dynamic resolution to produce **3** by treating the *N*-acetyl derivative of racemic amino acid **1** with D-acylase (Amano) and *N*-acetylamino acid racemase (Biocatalytics) gave 50% conversion with 100% ee after 15 h, and 71% conversion with 44.7% ee after 88 h. Thus, the D-acylase was active with this substrate, but the racemase was not.

Conclusion

Racemic amino acid **1** can be deracemized to *R*-amino acid **3** using a recombinant L-amino acid deaminase from *P. mirabilis* expressed in *E. coli* to selectively oxidize the *S*-enantiomer to the keto acid **2**, which can be converted to *R*-amino acid **3** by a D-transaminase. The process is slow because of the low solubility of the amino acid at neutral pH. A better procedure starts from keto acid **2** which is much more soluble at neutral pH. *R*-Amino acid **3** was prepared in 79% isolated yield with >99% ee from keto acid **2** using a commercially available D-transaminase. Addition of lactate dehydrogenase with NAD and formate plus formate dehydrogenase for NADH regeneration greatly increased the rate of reaction by reduction of inhibitory pyruvate. A D-transaminase from a soil organism identified as a strain of *B. thuringiensis* was purified, cloned, and expressed in *E. coli* and found to be very effective for conversion of **2** to **3.** This transaminase gave nearly a complete conversion of **2** to **3** without a requirement for additional enzymes for pyruvate removal.

Experimental Section

Source of Keto Acid 2 and Racemic Amino Acid 1. Racemic amino acid **1** and keto acid **2** were purchased from DSM Pharma Chemicals, Regensburg, Germany. The compounds were prepared by scaling up procedures developed by chemists from Process Research and Development, Bristol-Myers Squibb.

Preparation of *R*-Amino Acid 3 from Racemic Amino Acid 1. A solution of K_2HPO_4 (6.97 g, 0.04 mol), KH_2PO_4 (1.36 g, 0.01 mol), and pyridoxal phosphate monohydrate (2.65 mg, 0.01 mmol) in 800 mL of water was added to 100 g of *E. coli* frozen cell paste containing cloned L-amino acid deaminase (27 U/g cells wet weight) from *P. mirabilis*. The cells were suspended with a homogenizer. Racemic amino acid **1** (20 g, 0.0912 mol) and D-alanine (40 g, 0.448 mol) were added to a 2-L vessel of a Braun Biostat B followed by the cell suspension with additional water to bring the volume to 1 L. The mixture was stirred at 400 rpm, adjusted to pH 8 (NaOH), and brought to 30 °C. D-transaminase (200 mg, 4.4 U/mg from Biocatalytics) dissolved in 10 mL of 50 mM potassium phosphate buffer, pH 7.5, was added, and aeration was begun at 1 L/min to start the reaction. The pH was maintained at 8.0 by addition of H_2SO_4 and NaOH. SAG 5693 antifoam was used to control foaming. After 4 days when ee was >96%, the pH was adjusted to 12 (NaOH) and the reaction mixture stirred to dissolve the amino acid **3** that had precipitated. The viscous suspension was homogenized for 10 min to decrease the viscosity and centrifuged for 2 min at 15000*g*. The supernatant was ultrafiltered with a Millipore Pellicon 2 filter equipped with a Biomax 30K polyethersulfone 0.1 m^2 ultrafiltration membrane, diafiltering with 0.01 M NaOH until the concentration of **3** in the effluent was <0.6 mg/mL. The ultrafiltrate (pH ≈ 12, ∼1980 mL) contained 16.5 g of **3**.

The solution was heated to 90−95 °C and the pH (10.8) adjusted to 6.0 with sulfuric acid. As the mixture cooled, crystallization of **3** commenced at 65 °C. The mixture was further cooled to 5 °C, and stirring continued for 1 h. The mixture was filtered, washing the cake with four 50-mL portions

of ice-cold water, monitoring the conductivity of the filtrate (17 μS for the final wash). The product was dried *in vacuo* at room temperature, giving 14.5 g of **3** as a monohydrate with a purity of 96.8% and an ee of >99%. The yield (correcting for purity of the product and starting material) was 67.6%. The ee of the amino acid in the mother liquor was 65.2%.

Preparation of *R*-Amino Acid 3 from Keto Acid 2. Water (445 mL), keto acid **2** (30 g, 0.137 mol), D,L-alanine (120 g, 1.35 mol), K_2HPO_4 (6.97 g, 0.04 mol), and KH_2PO_4 (1.36 g, 0.01 mol) were added to a 2-L vessel of a Braun Biostat B. NaOH (25%, 7.96 M, 17.3 mL, 1 equiv) was then added to the vessel, and the suspension was stirred at 200 rpm for about 10 min to dissolve most of the solids. An additional 445 mLof water was added to bring the volume to 1 L, and stirring was continued for about 20 min to completely dissolve the solids. The pH was adjusted to 7.5 with a few drops of 25% NaOH. Dithiothreitol (154 mg, 1 mmol) dissolved in 2 mL of water and pyridoxal phosphate monohydrate (26.5 mg, 0.1 mmol) dissolved in 2 mL of 0.1 M potassium phosphate buffer pH 7.5 were then added to the stirred solution. The stirrer was set to 50 rpm and the temperature to 30 °C. A pH electrode was used to monitor the pH during the reaction, but no addition of acid or base was needed during the reaction because the pH changes were small. D-Transaminase (300 mg, 4.4 U/mg from Biocatalytics) dissolved in 25 mL of 0.1 M potassium phosphate buffer, pH 7.5, was then added to start the reaction, and stirring was continued at 30 °C and 50 rpm. Once the product amino acid started to precipitate (about 3 h), the stirring speed was temporarily increased to 150 rpm before taking samples for analysis. The reaction was continued until HPLC analysis showed that the keto acid peak had <10% of the area counts of the amino acid peak, which took about 72 h. Sulfuric acid was added to adjust the pH to 1 and bring the precipitated amino acid into solution. The solution was filtered (Whatman no. 4 paper), and the filtrate was passed through a 2.5 cm i.d. × 9.1 cm column of Amberlite XAD-16 resin, washing with 0.1 M H_2SO_4 to remove nonpolar impurities. (The resin had been prewashed sequentially with water, methanol, and water.) The column effluent was heated to 100 °C and then slowly adjusted to pH 7.0 with NaOH. The amino acid began to crystallize at pH 1.3−1.5. The mixture was cooled to room temperature, stirred for 15 h, and filtered, washing the cake with deionized water. The cake was dried *in vacuo* at room temperature, giving 24.6 g of **3** as the monohydrate, ee >99%. The yield from the ketoacid was 77.1%, correcting for purity of the input keto acid (94.2%) and isolated material (96.5%).

^{1}H NMR (400 MHz, acetic acid-d_4) 2.54 (s, 3H), 3.27 (dd, J = 14.5, 8.2 Hz, 1H), 3.45 (dd, J = 14.7, 4.6 Hz, 1H), 4.39 (dd, J = 7.9, 4.8 Hz, 1H), 7.16 (s, 1H), 7.54 (s, 1H), 8.11 (s, 1H).

Calculated for $C_{11}H_{13}N_3O_2 \cdot H_2O$: C 55.68, H 6.37, N 17.71; found: C 55.62, H 6.45, N 17.63. Water, calculated for the monohydrate 7.59; found: 7.50 (Karl Fisher).

$[\alpha]^{29}_D$ +17.3 (*c* 0.8, HOAc).

Cloning of the L-Amino Acid Deaminase Gene. Chromosomal DNA was prepared from *P. mirabilis* cells using the procedure described in Ausubel et al.[20] Plasmid DNA was isolated from 1 mL of *E. coli* culture grown in TB medium + 50 μg/mL Km sulfate (Sigma) using the Fast Plasmid kit from Eppendorf. A typical preparative restriction digest contained 2 μg of plasmid DNA and 10 U of restriction endonuclease in a 40 μL reaction volume. Digests were carried out in the recommended buffer and temperatures for 2−4 h.

Z-Taq DNA polymerase (Takara Corp.) was used for PCR with the FailSafe series of 2X buffers (Epicentre Technologies, Madison, WI) which contain 400 μM dNTPs. Reactions also included 25 nM of each primer [GACATATTTAAATCATATGAACATTTCAAGGAGAAAGCTA (*Swa*I/*Nde*I/5′ end of *P. mirabilis* deaminase gene (sense) and GACATTTAAATGGATCCTTACTTCTTAAAACGATCCAAACT (*Swa*I/*Bam*HI/3′ end of *P. mirabilis* deaminase gene (antisense)] and 10−100 ng of chromosomal DNA. A Hybrid PCR Express thermocycler (Thermo Hybaid, Franklin, MA) was used under the following conditions: 94 °C, 1 min for one cycle, followed by 94 °C, 30 s; 50 °C, 30 s; 72 °C, 1 min for 30 cycles. DNA samples were electrophoresed at 100 v for the appropriate length of time on a 1.0% agarose gel with TEA running 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. Where required, DNA was isolated from gel slices using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

Ligation of PCR fragments to pTOPO4 (Invitrogen) were carried out as recommended by the manufacturer. Transformation into chemically competent cells was performed by incubating the ligation mix with the cells for 30 min on ice in a Falcon 2054 tube before heat shock (42 °C, 30 s). SOC medium was immediately added (0.25 mL; SOC = 0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose per liter), and the cells were incubated in a shaker for 1 h at 37 °C and 225 rpm. Colonies containing recombinant plasmids were selected on Luria−Bertani (LB) agar plates containing 50 μg/mL Km sulfate. Transformation of plasmid DNA into *E. coli* expression strain BL21 (Stratagene) were performed similarly.

Ligations using pBMS2004 as vector included 400 ng (2.0 μL) of isolated fragment, 20 ng (1 μL) of vector, 1.5 μL of 10x FastLink ligation buffer (Epicentre), 1.5 μL of 10x (10 mM) ATP (Epicentre), 1.0 μL of FastLink T4 DNA ligase (Epicentre) plus water to 15.0 μL. The reaction was carried out at room temperature for 15 min. DNA was precipitated by addition of 22.5 μL of water and 375 μL of 1-butanol, and pelleted at 13,000*g* in a microcentrifuge for 5 min. Liquid was removed by aspiration, the pellet was washed with 200 μL of 70% EtOH, and the DNA was dried in a SpeedVac (Savant Instruments, Farmingdale, NY) for 3 min under low heat. The pellet was resuspended in 4 μL of water. The resuspended DNA was transformed by electroporation into 40 μL of *E. coli* DH10B competent cells (Invitrogen) at 25 μF and 250 Ω. SOC medium (0.96 mL) was immediately added, and the cells were incubated in a shaker for 1 h at 37 °C and 225 rpm. Colonies containing

(20) Ausubel, F. M., Brent, R, Kingston, R. E., Moore, D. D., Seidman, J. D., Smith, J. A., Struhl, K., Eds. *Current Protocols in Molecular Biology*; John Wiley and Sons: New York, NY, 1990; Vol. 1, section 2.4.3−2.4.4.

recombinant plasmids were selected on LB agar plates containing 50 μg/mL Km sulfate.

Heterologous Expression. MT5-M2 contained 4% glycerol, 1.85% Tastone 154 (Sensient Bionutrients), 2% Hy-Pea (Quest International), 0.125% $(NH_4)_2SO_4$, and 0.6% Na_2HPO_4. The pH of the medium was adjusted to 7.2 with NaOH. Km sulfate (from a 50 mg/mL filter-sterilized stock in water) was added after autoclaving to a concentration of 50 μg/mL. For shake flask expression work, cells were initially grown in MT5-M2 for 20−24 h, 30 °C, 250 rpm. 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 AU/cm. The flask was incubated as described above until the OD_{600} reached ∼0.8−1.0 AU/cm. IPTG was added from a 1 M filter-sterilized stock in water to the desired final concentration (50 μM or 1 mM) and the culture allowed to grow for varying lengths of time before harvesting by centrifugation.

Detection of L-Amino Acid Deaminase. Cells grown in liquid medium were pelleted by centrifugation at 5000*g* for 7 min and washed by resuspension in 100 mM potassium phosphate buffer, pH 7.0. After recentrifugation, the pellet was stored at −20 °C until required or used immediately. Cells were resuspended in 100 mM potassium phosphate buffer, pH 7.0 (10 mL/g wet cell weight). For preparation of cell extract, sonication was conducted on 0.8 mL of suspension in a 1.5 mL microfuge tube. Cell debris was pelleted by centrifugation for 5 min at 13,500*g* in a microcentrifuge, and the supernatant retained for further use. The assay consisted of incubating 20 μL of extract with 2 mL of 0.1 M L-phenylalanine in 20 mM potassium phosphate buffer, pH 7.0, at room temperature. Five minutes later, 0.5 mL of the sample was added to 0.5 mL of water containing 50 μL of 10% $FeCl_3$. A positive reaction (deamination) is indicated by the formation of a dark green color.[12]

Growth of *E. coli* BL21(pBMS2004-PMAAD) SC16554 Cells Expressing L-Amino Acid Deaminase. Two frozen vials of *E. coli* SC16554 were thawed, and the contents (1.5 mL) were transferred to two 500-mL flasks containing 100 mL of MT5-M0 medium (2.0% Yeastamin, 4.0% glycerol, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.125% ammonium sulfate, 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 ∼20 h (OD_{600} 5.0−6.1 AU/cm). The contents of each flask were then pooled and used to inoculate a 380-L Braun 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 0.08−0.1 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 ∼0.9 to 1.8 AU/cm, (4.5 h into the run), a filter-sterilized IPTG solution was added to a final concentration of 50 μM. The runs were completed by 20 h, at which time OD_{600} had leveled off at 35 AU/cm and the off-gas CO_2 level was 3%. The whole broth was cooled to 4−10 °C, and cells were recovered by centrifugation, followed by washing with 10 mM pH 7 phosphate buffer. A total of 25 kg of cell paste was recovered for two comparable batches and stored at −75 °C. L-Amino acid deaminase activity was 19.9−27.4 U/g.

Selection and Growth of a Bacillus Strain with D-Transaminase. Medium BD contained hydrolyzed soy (Quest) 1.5%, yeast extract 0.2%, HyPea (Quest) 0.2%, glycerol 0.2%, K_2HPO_4 0.2%, and KH_2PO_4 0.2%. Five 50-mL flasks each containing 10 mL of medium BD were inoculated with 0.25 g of each of two soil samples (10 soil samples in total). Flasks were shaken at 225 rpm, at 28 °C. Following the appearance of good growth, broths were streaked onto plates of the same medium solidified with agar. Plates were incubated at 28 °C. Isolated colonies were restreaked onto plates of the same medium and ultimately grown on slants of the same medium.

Whole-cell suspensions of the isolates (0.2 mL) were screened for D-transaminase activity with a reaction mixture consisting of 0.03 M pH 7.2 sodium phosphate buffer, 10 mg of either D-alanine or D-phenylalanine, and 2.5 mg of keto acid **2** in a final volume of 1 mL. Incubation was at 30 °C with slow shaking, usually overnight. An isolate with high activity and good stability of D-transaminase was identified as *B. thuringiensis* by the sequence of its 16S RNA gene, and it was added to the culture collection as strain SC16569.

Four vials of *B. thuringiensis* SC 16569 were used to inoculate individual 500-mL flasks each containing 100 mL of medium BD. The flasks were incubated 24 h at 28 °C. Each of two 15-L fermentors was inoculated with 150 mL of the 24-h broth. The tanks each contained 15 L of medium BD; the temperature was 28°, aeration was 1 vvm, agitation was 400 rpm, and pH was not controlled. Harvesting of the tanks began at 27 h into the run. Cells (0.5 kg) were recovered by centrifugation and stored at −80° until use.

Purification of D-Transaminase from *B. thuringiensis* SC16569. One hundred grams of thawed cells was suspended in 500 mL of 0.1 M pH 7.2 sodium phosphate buffer. The suspension was passed through a Microfluidizer three times. Solids were removed by centrifugation. Purification of the transaminase was conducted at 4°. Crude extract (520 mL) was applied to a 7 cm × 1.5 cm column of Q Sepharose (GE Healthcare). The enzyme was eluted with a gradient of NaCl, from 0 to 1 M in 20 mM pH 7.6 phosphate buffer, at a flow rate of 2 mL/min.

Four active fractions were pooled to give about 45 mL. A 3-mL sample was removed, and ammonium sulfate was slowly added to the remaining pool to a concentration of 1 M. Forty milliliters of this solution was applied to a 10 cm × 1.5 cm column of Phenyl Sepharose (GE Healthcare), and enzyme was eluted with a gradient of 1 to 0 M ammonium sulfate in 50 mM phosphate buffer at a flow rate of 2 mL/min.

Three active fractions were pooled and concentrated about 6-fold with an Amicon ultrafiltration stirred cell. A 90 cm × 1.5 cm column of Sephacryl S-200 (GE Healthcare) was equilibrated with 20 mM pH 7.2 phosphate buffer containing 0.1 M NaCl, 10 μM pyridoxal phosphate, and 1 mM dithiothreitol (DTT). Five milliliters of concentrate was applied to

the column at a flow rate of 1 mL/min, followed by elution with 1 mL/min of column buffer.

Two milliliters from an active fraction were applied to a 1.3 mL UNO Q1 (Bio-Rad) column equilibrated with the buffer described for the Sephacryl column. Elution was with a gradient of 0.1 to 0.5 M NaCl. The activity peak fractions were concentrated with an Amicon Microcon-10 and subjected to SDS gel electrophoresis and then blotted onto a PVDF membrane. Sequencing of the N-terminus from the blot and two internal peptides from a gel slice was done by the Keck Sequencing facility, Yale University.

Cloning of the *B. thuringiensis* D-Aminoacid Transferase Gene and Expression of the Gene in Recombinant *E. coli.* *B. thuringiensis* SC16569 was grown in 250 mL of medium BD until late log-phase, and the cells were harvested by centrifugation (5000*g*/10 min). The cell pellet was resuspended in 25 mL of water and recentrifuged, and the pellet was used for a chromosomal DNA isolation using a standard proteinase K/SDS/NaCl/CTAB bacterial DNA purification protocol.[21] The resulting crude preparation was extracted three times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was removed to a fresh tube, and 0.6 vol of isopropanol was added. The DNA was precipitated by centrifugation at 6500*g*/20 min. The DNA pellet was washed with 70% ethanol (in water) and recentrifuged. The chromosomal DNA pellet was air-dried overnight. The dried nucleic acid pellet was resuspended in 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0) containing 50 μg/mL RNase, incubated at 37° for 15 min to digest contaminating RNA, reprecipitated by the addition of 0.1 vol of 3 M sodium acetate (pH 4.8) and 2 vols of 100% ethanol, centrifuged, washed with 70% ethanol, and reprecipitated as previously described. The final chromosomal DNA pellet was resuspended in 500 μL of TE buffer. The DNA concentration was determined by absorbance at 260 nM (1 unit = 50 μg/mL DNA) and adjusted to a final concentration of 1 μg/mL. This chromosomal DNA was used for a target for PCR reactions to amplify portions of the SC16569 D-amino transferase gene as well as a source to construct gene libraries.

The N-terminal primer representing the DNA sequence encoding the N-terminal amino acid sequence, with the addition of an *Nde*I restriction cut site (underlined) upstream of the initiator ATG codon was 5′-CCGCATATGAAAGCWACW-CATAAAGATTGGAT-3′.

The internal primer representing the complement of the DNA sequence encoding the internal amino acid sequence was: 5′-TTWGCWARWGTWATWACATAATG-3′. Both of these primers were resuspended in TE buffer at 100 pM/mL.

A PCR reaction was prepared using standard Z-Taq reagents and Z-Taq polymerase (Takara Bio Inc., Japan). A 25 μL reaction contained 1 μL of chromosomal DNA as the target, 0.5 μL of N-terminal primer, 0.5 μL of internal primer, and 0.2 μL of Z-Taq polymerase. The "touchdown" cycling conditions were 94 °C/1 min/1X, 94 °C/30 s/55 °C/30 s/72 °C/30 s/5X, 94 °C/30 s/65 °C (minus 1 deg/successive cycle)/30 s/72 °C/30 s/16X (ending at a final annealing temperature of 50 °C/16X, 94 °C/30 s/50 °C/30 s/72 °C/30 s/5X, 94 °C/30 s/50 °C/30 s/72 °C/5 min. The amplified PCR product was isolated by agarose gel electrophoresis and found to have a size of ~650 base pairs (bp). This PCR product was excised from the gel, and the DNA was purified into 10 μL of TE buffer using a Qiaquik gel purification kit (Qiagen Inc.) according to the manufacturer's protocol. Two microliters of the purified PCR product was ligated into the *E. coli* cloning plasmid pCR4-TOPO (Invitrogen) according to the manufacturer's protocol and was used to transform electrocompetent *E. coli* (strain TOP10). Transformants were selected by plating on LB agar containing 50 μg/mL Km sulfate, and the presence of the desired insert was verified by PCR (as previously described) using the colony cells as the target. An acceptable recombinant transformant was used to prepare plasmid DNA for DNA sequence analysis.

The DNA sequence of the PCR product contained a single open reading frame of 665 bp which encompassed approximately 75% of the intact aminotransferase gene. To isolate the remaining portion of the gene, a standard Southern blot hydbridization was performed using various restriction digests of *B. thuringiensis* SC16569 chromosomal DNA as the target and a DIG-labeled sample of the 665 bpPCR fragment as the probe. (DIG High Prime DNA Labeling and Detection Kit, Roche Diagnostics). A *Hind*III restriction fragment of approximately 8500 bp hybridized strongly to the probe. *Hind*III fragments of *B. thuringiensis* SCI6569 chromosomal DNA of 8000−9000 bp were isolated, purified, and ligated into *Hind*III digested pZERO-2 vector (Invitrogen) using a Fast-Link DNA Ligation Kit (Epicentre Biotechnologies) following the manufacturers' protocols. This ligation reaction was used to transform an electrocompetent *E. coli* strain, selecting by plating on LB/Km agar plates. Transformants that had incorporated the desired *Hind*III fragment were detected by evaluating hybridization to the DIG-labeled 665 bp PCR fragment. Plasmid DNA was prepared from one of the strongly hybridizing recombinant transformants, and DNA sequence analysis of the 8500 bp *Hind*III fragment revealed that it contained the entire open reading frame for the intact gene.

The C-terminal of BtDAAT gene had to be modified by the addition of a *Bam*HI restriction to facilitate ligation into the intended expression vector. A antisense PCR primer was designed to add the *Bam*HI site following the stop codon as well as change the nucleotide at position 837 from an "A" to a "G" to eliminate an internal *Nde*I cut site without changing the amino acid sequence. The primer sequence (*Bam*HI site underlined, altered nucleotide underlined in italics) was 5′GGGG-GATCCTTAATTAGTAACTTTAAAAAGAC-TAATAGTTTCTTCATA*C*GCAGCTTGTAGC3′.

This C-terminal primer was used for a PCR reaction performed as described above except the new C-terminal primer was substituted for the "internal" primer that generated the initial 665 bp BtDAAT PCR product. All other conditions were kept the same. Agarose gel electrophoresis of the completed PCR reaction revealed a single well-amplified band containing approximately 885 bp. This band was excised from the gel and purified (as previously described), ligated into pCR4-TOP, and

(21) Ausubel, F. M., Brent, R, Kingston, R. E., Moore, D. D., Seidman, J. D., Smith, J. A., Struhl, K., Eds. *Current Protocols in Molecular Biology*; John Wiley and Sons: New York, NY, 1990; Vol. 1, Supplement 27, section 2.4.1.

used to transform electrocompetent TOP10. The presence of the desired insert fragment was verified by colony PCR using the N-terminal and C-terminal BtDAAT primers. An acceptable recombinant transformant was verified by DNA sequencing the pCR4+BtDAAT plasmid. Plasmid pCR4+BtDAAT was digested with both *Nde*I and *Bam*HI, and the band containing the BtDAAT insert was gel separated/excised/purified as previously described.

Plasmid pBMS2004 was digested with *Nde*I/*Bam*HI, and the (*Nde*I/*Bam*HI) BtDAAT insert fragment was ligated downstream of the plasmids IPTG-inducible promoter. This recombinant plasmid was used to transform electrocompetent *E. coli* expression strain BL21 (Stratagene Corporation) according to the manufacturer's instructions, yielding expression strain BL21/pBMS2004+BtDAAT. The expression transformant was used to initiate broth culture in medium MT5-M2 and grown on a rotary shaker (250 RPM) at 30 °C. Cell density was monitored by tracking light absorbance at 600 nm. When the optical density (OD_{600}) of the culture reached 0.8−1.0 AU/cm, IPTG was added to a final concentration 200 μM to induce transcription of the BtDAAT gene. Samples of the culture were collected before induction and at various time points following induction, and the cells were harvested by centrifugation for SDS/PAGE analysis of proteins.

Growth of *E. coli* BL21(pBMS2004-BtDAAT) SC16577 Cells Expressing D-Aminotransferase. Two frozen vials of *E. coli* SC16577 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, and 0.005% Km sulfate [the latter two components added postautoclaving from filter-sterilized solutions]). The flasks were incubated at 30 °C and 250 rpm for 24 h; then 5 mL of the broth was used to inoculate 1 L of the same medium in each of four 4-L flasks. These second-stage flasks were incubated at 30 °C and 230 rpm for 22 h, and two of the flasks (OD_{600} of ∼8.3−8.7 AU/cm) were pooled. A sufficient quantity (∼1.2 L) was then used to inoculate a 147-L Braun fermentor containing 100 L of MT5-M2 medium (see above), resulting in a starting OD_{600} of ∼0.1 AU/cm. The fermentation process conditions for the tank were as follows: 30 °C; no pH control; agitation, 400 rpm; aeration, 100 Lpm; back pressure, 10 psi; foam controlled by the addition of UCON LB625 on demand.

At an OD_{600} of ∼0.9 AU/cm, (3.5 h into the run), a filter-sterilized IPTG solution was added to a final level of 100 μM. The run was completed after 23 h, at which time OD_{600} had leveled off at ∼44 AU/cm, and off-gas CO_2 was ∼3%. The whole broth was cooled to 4−10 °C, and cells were recovered by centrifugation, followed by washing with 50 mM pH 7 phosphate buffer. A total of 5 kg of cell paste was recovered and stored at −75 °C.

Conversion of 2 to 3 with D-Transaminase from *B. thuringiensis* Expressed in *E. coli* SC16577. *E. coli* SC16577 (40 g wet cells/200 mL suspension), suspended in buffer containing 50 mM potassium phosphate pH 7, 1 mM dithiothreitol, and 10 μM pyridoxal phosphate, was passed three times through a microfluidizer at 12000 psi. Polyethyleneimine (0.2%) was added to the extract, and after 30 min on ice the suspension was clarified by centrifugation for 2 min at 15000*g*. Keto acid **2** (6.00 g, 27.5 mmol), 10 N NaOH (2.75 mL), D,L-alanine (12.00 g, 135 mmol), pyridoxal phosphate (2.65 mg, 0.01 mmol), dithiothreitol (15.4 mg, 0.1 mmol), 0.1 M potassium phosphate buffer pH 7.5 (80 mL), and clarified extract (10 mL) were adjusted to pH 7.5 with NaOH and incubated at 30 °C with gentle shaking at 50 rpm. The concentration of **2** remaining after 21 h was 2.2 mg/mL, and after 39 h the concentration was 2.1 mg/mL. After 39 h the pH was adjusted to 1 with H_2SO_4, and **3** was isolated as described previously. The solution yield of **3** was 5.57 g (92.4%) with ee >99%, and the isolated yield was 5.33 g (79%).

Enzyme Assays. The L-amino acid deaminase assay solution contained 5 mg racemic amino acid **1**, 0.975 mL 0.1 M potassium phosphate buffer, pH 7.5, and 0.025 mL of a 10% w/v suspension of *E. coli* SC 16554 in 0.1 M potassium phosphate buffer, pH 7.5. The suspension was incubated in loosely capped 15-mL tubes at 30 °C and 300 rpm for 1 h. Samples of 0.25 mL were quenched with 0.75 mL of methanol and assayed for keto acid **2** by HPLC. Activity units are in μmol/min.

The D-transaminase assay contained in 1.0 mL: 2.5 mg keto acid **2**, 0.04 M pH 7.2 phosphate buffer, 10 mg D-alanine, 0.1 mM pyridoxal phosphate, and enzyme. Incubation time was between 15 and 60 min. Samples of 0.25 mL were quenched with 0.75 mL acetonitrile and analyzed by HPLC for amino acid **3**. Protein was measured with the Bio-Rad Protein Assay.

HPLC Methods. Quantitation with C18 column: Samples (25 μL) from biotransformations were diluted with 0.975 mL 50% 0.1 N HCl/50% methanol to stop the reaction and precipitate proteins; the samples were then filtered through 0.2 μm nylon filters into HPLC vials. Samples were analyzed with a YMC Pak Pro C18 15 cm × 0.46 cm 3 μ column. The mobile phase was a gradient of 10 to 90% acetonitrile/water (0.05% trifluoroacetic acid in both) from 0 to 12 min and 10% acetonitrile/90% water (0.05% trifluoroacetic acid in both) from 12.01 to 15 min at a flow rate of 1 mL/min, with detection at 290 nm, temperature 40 °C, and an injection volume of 5 μL. Retention times were: amino acid **3**, 3.5 min; keto acid **2**, broad peak centered at 5.4 min

Ee with chiral column: The same samples were analyzed with a Chirobiotic T 25 cm × 0.46 cm 5 μ column. The mobile phase was 60% methanol/40% water, flow rate was 1.2 mL/min, detection was at 290 nm, temperature was 25 °C (ambient), and injection volume was 5 μL. Retention times were: *S*-enantiomer, 5.2 min; *R*-enantiomer (**3**) 7.5 min

Ee with Marfey's reagent: Marfey's reagent (Pierce) was used to give diasteromeric derivatives that could be separated with a C18 column. A sample of 10 μL containing about 0.1 mg amino acid, 8 μL 1 M $NaHCO_3$, and 40 μL 1% w/v Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide from Pierce) in acetone were combined in a 1.5 mL microfuge tube and heated for 1 h at 40 °C. The samples were cooled to room temperature, then 8 μL 1 N HCl and 934 μL 40% acetonitrile/60% water were added, and the solutions were vortexed and filtered into HPLC vials. Samples were

analyzed with a YMC Pak Pro C18 15 cm× 0.46 cm 3 μ column. The mobile phase was 28% acetonitrile/72% water (both containing 0.05% trifluoroacetic acid), flow rate was 1 mL/min, detection was at 340 nm, temperature was 40 °C, and injection volume was 20 μL. Retention times were: *S*-enantiomer, 13.6 min; *R*-enantiomer (**3**) 18.5 min, L-ala, 8.5 min; D-ala, 12.4 min.

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