Organic Process Research & Development

Article

pubs.acs.org/OPRD

Enzymatic Preparation of an *R*-Amino Acid Intermediate for a γ-Secretase Inhibitor

Ronald L. Hanson,* Robert M. Johnston, Steven L. Goldberg, William L. Parker, and Animesh Goswami

Chemical Development, Bristol-Myers Squibb, One Squibb Drive, New Brunswick, New Jersey 08903, United States

ABSTRACT: (*R*)-5,5,5-Trifluoronorvaline, an intermediate for a γ-secretase inhibitor (BMS-708163) under development, was initially prepared from the corresponding keto acid using a commercially available D-amino acid dehydrogenase for reductive amination and glucose dehydrogenase for cofactor recycling. This amino acid could also be prepared using a D-amino acid transaminase with alanine as the amino donor, but the transamination also requires lactate dehydrogenase, NAD, formate, and formate dehydrogenase to remove pyruvate in order to bring the reaction to completion. An effective proprietary D-amino acid dehydrogenase was constructed by modification of the D-diaminopimelic acid dehydrogenase gene from *Bacillus sphaericus*, and a glucose dehydrogenase gene was cloned from *Gluconobacter oxidans*. Both genes were expressed in the same strain of *Escherichia coli*, and the glutamate dehydrogenase gene was inactivated in the expression strain to eliminate background production of the *S*-amino acid and improve the ee of the product to 100%. The amino acid could be isolated or converted without isolation to a *p*-chlorophenylsulfonamide carboxamide intermediate needed for the synthetic route to the γ-secretase inhibitor development candidate.

INTRODUCTION

Amyloid-β peptides (Aβ) are a major component of the plaques that are found in the brains of Alzheimer's disease patients and have been proposed to play a causative role in the disease.[1] These peptides are produced from amyloid precursor protein by initial cleavage at the β site by β-secretase followed by further cleavage at sites near the C-terminus by γ-secretase to generate Aβ forms, with Aβ42 most closely associated with Alzheimer's disease and Aβ40 most abundant. Inhibition of γ-secretase has been pursued as a pharmaceutical target to decrease the formation of Aβ peptides.[2] γ-Secretase has other substrates and functions in addition to the processing of amyloid precursor protein, with Notch processing an important activity. BMS-708163, a γ-secretase inhibitor (**6**, Figure 1), causes a significant decrease of Aβ40 levels at concentrations having minimal effects on Notch signaling.[2]

The initial synthetic route to **6** used an asymmetric Strecker reaction to prepare (*R*)-5,5,5-trifluoronorvaline amide (**7**, Figure 1), a key intermediate.[2] (*R*)-5,5,5-Trifluoronorvaline (**1**, Figure 1) has been prepared by synthesis of the racemic *N*-acetyl amino acid and resolution with an L-amino acid acylase, followed by hydrolysis of the separated (*R*)-*N*-acetyl-5,5,5-trifluoronorvaline.[3] Other enzymatic resolution approaches have also been used for preparation of *R*-amino acids, including transformations with D-acylases,[4] D-hydantoinases,[5,6] D-amidases,[7] and *S*-amino acid oxidases combined with an excess of reducing agent to recycle the imine product.[8] When the corresponding keto acid can be synthesized, enzymatic reductive amination or transamination are attractive approaches for preparation of *R*- or *S*-amino acids since a resolution is not required. D-Amino acid dehydrogenases catalyzing reductive aminations with broad substrate specificity have become available as a result of directed evolution of *Corynebacterium glutamicum meso*-2,6-diaminopimelic acid D-dehydrogenase,[9] and D-transaminases[10,11] have also been used for preparation of *R*-amino acids. As an alternative approach to **1**, we have explored the use of D-amino acid dehydrogenases and D-transaminases to convert the corresponding keto acid **2** to the *R*-amino acid. This report describes alternative preparations of **1** using D-amino acid transaminase (Scheme 2) or D-amino acid dehydrogenase (D-AADH, Scheme 1) and development of the latter process, initially using a commercially available D-amino acid dehydrogenase and glucose dehydrogenase (GDH). Further development of the process included construction of a D-amino acid dehydrogenase and cloning and expressing a glucose dehydrogenase for NADPH regeneration together with the D-amino acid dehydrogenase, knockout of glutamate dehydrogenase from the *Escherichia coli* expression strain to improve ee, and conversion of the *R*-amino acid to the required *p*-chlorophenylsulfonamide carboxamide intermediate.

RESULTS AND DISCUSSION

Screen for Conversion of 5,5,5-Trifluoro-2-oxopentanoic Acid, 2, to (*R*)-5,5,5-Trifluoronorvaline, 1. Six D-amino acid dehydrogenases (Biocatalytics) were screened for conversion of keto acid **2** to *R*-amino acid **1** (Scheme 1). All of the enzymes carried out the conversion and gave a single enantiomer. Seven L-amino acid dehydrogenases from various sources all gave the opposite enantiomer as a product. When the D-amino acid dehydrogenases were tested using 1% enzyme weight, the most effective D-amino acid dehydrogenase was D-AADH-102 which gave complete conversion in 16 h and was selected for further development.

Biocatalytics D-transaminase and an in-house D-transaminase (from a soil isolate of *Bacillus thuringiensis* cloned and expressed in *E. coli*)[10] also converted keto acid **2** exclusively to *R*-amino acid **1**, using alanine as an amino donor. Whereas complete conversion occurred with the amino acid dehydrogenases,

Received: January 18, 2013
Published: March 7, 2013

dx.doi.org/10.1021/op400013e | *Org. Process Res. Dev.* 2013, 17, 693−700

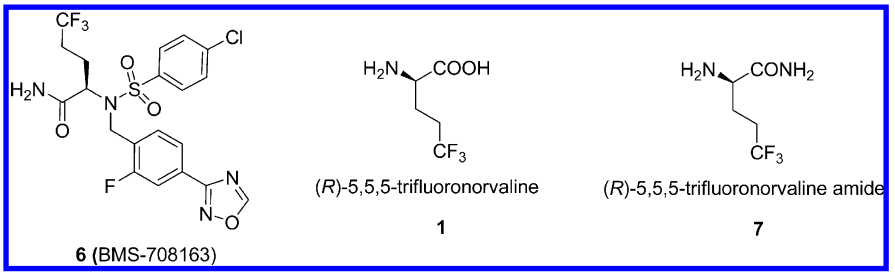
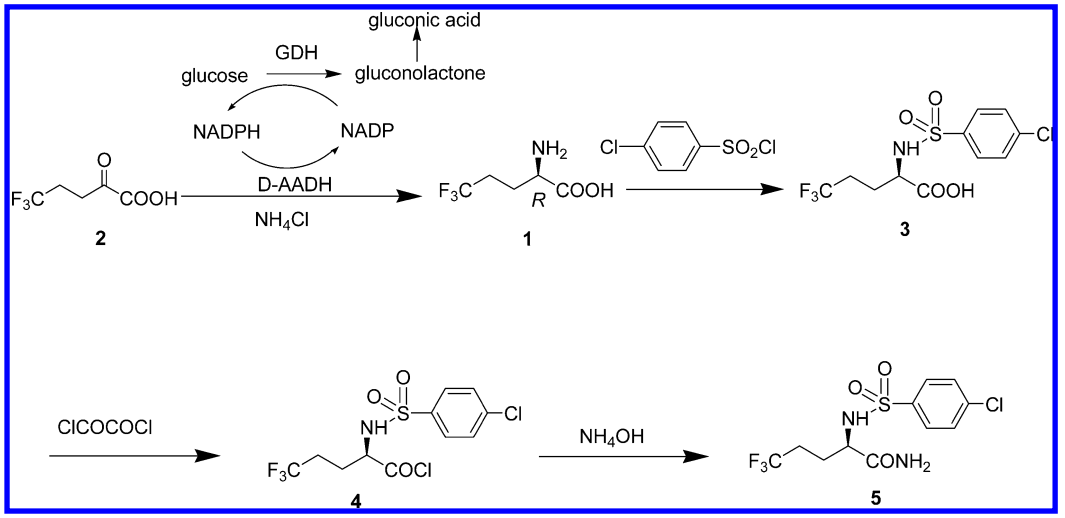


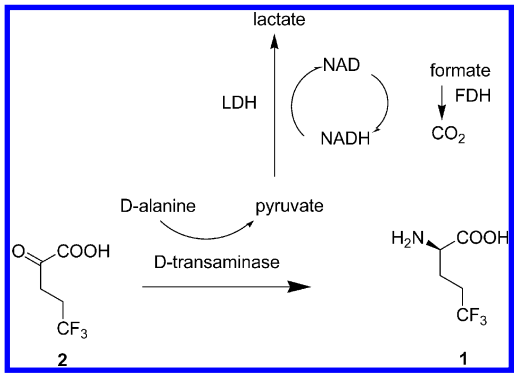
Figure 1. Structure of γ-secretase inhibitor **6** and (*R*)-5,5,5-trifluoronorvaline **1**.

Scheme 1. Reaction scheme for the preparation of *R*-amino acid 1 from keto acid 2 and further conversion to a synthetic intermediate 5 for a γ-secretase inhibitor



~50% of the keto acid remained with the transaminases. These results are expected on the basis of the equilibrium constants for the two types of reaction. The D-transaminases can effectively convert keto acid **2** to *R*-amino acid **1**, provided NAD, formate, lactate dehydrogenase (LDH), and formate dehydrogenase (FDH) are added to remove inhibitory pyruvate (Scheme 2) from the reaction (Table 1). However, because of

Scheme 2. Preparation of *R*-amino acid 1 with D-transaminase



the anticipated difficulty in separating the product **1** from the remaining alanine and the need for adding additional enzymes, the transamination approach was not pursued further.

The intermediate used in the initial synthesis of **6** was the primary amide **7**. The activities of the six D-amino acid dehydrogenases and two D-transaminases were compared with those of 5,5,5-trifluoro-2-oxopentanamide and keto acid **2** as

Table 1. Preparation of *R*-amino acid 1 with transaminase

D-transaminase	time (h)	keto acid **2** (mg/mL)	*R*-amino acid **1** (mg/mL)	yield (%)
Biocatalytics	21	10.66	10.82	54
	44	9.27	9.95	50
in-house	21	9.78	9.76	48
	44	9.47	9.73	48
Biocatalytics + LDH, FDH, NAD, formate	21	0.00	18.98	94
in-house + LDH, FDH, NAD, formate	44	0.00	18.34	94

substrates. Not surprisingly, in all cases enzyme activity with the keto amide was <1% of the activity with the keto acid. The slight activities observed in the spectrophotometric assays may be due to traces of acid in the amide sample. An attempted reaction using the in-house D-transaminase did not give any product. In addition to the lack of enzyme activity, the low water solubility of the ketoamide makes it a problematic substrate for an enzyme reaction.

Process Optimization. Initial development was done with D-amino acid dehydrogenase-D-AADH-102 and a commercial glucose dehydrogenase (Amano) for NADPH regeneration. Reductive aminations gave solution yields by HPLC of 84, 74, and 63% at 5, 7.5, and 10% keto acid concentrations, respectively, with only 1−2% keto acid remaining at the end of the reactions. Keto acid **2** was found to be stable in the reaction buffer for 1 day at 2 mg/mL. When stability of the keto acid was tested at higher concentrations, however, the stability was markedly lower, suggesting occurrence of a bimolecular reaction such as an aldol condensation. Because of the

dx.doi.org/10.1021/op400013e | Org. Process Res. Dev. 2013, 17, 693−700

instability at higher concentrations, subsequent reactions were carried out with 50 mg/mL keto acid **2** and an enzyme concentration sufficient to complete most of the reaction in 2 h.

With keto acid **2** as substrate, the pH optimum for D-AADH-102 is ~9.3 (Figure 2), and the apparent K_m for NADPH is

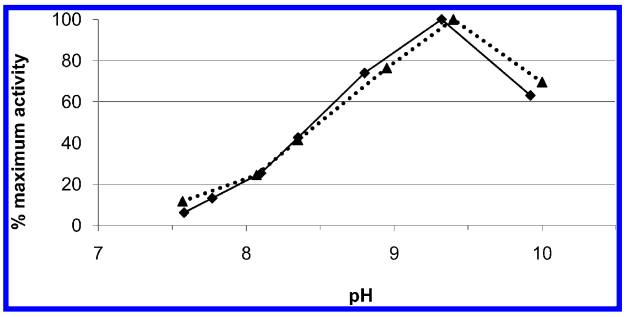


Figure 2. pH optima for D-amino acid dehydrogenases. (▲) In-house D-AADH; (♦) D-AADH-102.

0.49 mM. The pH optimum for glucose dehydrogenase is 8−9, and the K_m for NADP is 0.043 mM (Amano Technical Bulletin). A pH of 9 and 0.5 mM NADP were used for the reaction.

Development of an In-House D-Amino Acid Dehydrogenase. *meso*-Diaminopimelic acid D-dehydrogenase (DAPD) from *Corynebacterium glutamicum* was the starting point for evolution of D-amino acid dehydrogenases with broad substrate specificity reported by Vedha-Peters et al.[9] DAPD has also been reported from *Bacillus sphaericus*. Using the GenBank amino acid sequence submission for a *B. sphaericus* DAPD to design primers, the gene from *B. sphaericus* ATCC 4525 was cloned and expressed in *E. coli* BL21(DE3)-Gold. From the nucleotide sequence, the encoded protein displayed 95% identity (311 amino acids out of 327) with the GenBank submission for *B. sphaericus* DAPD and was shown to catalyze the oxidative deamination of *meso*-diaminopimelic acid to (*S*)-α-amino-ε-ketopimelic acid but had no detectable activity for the reductive amination of keto acid **2**. Oligonucleotide mutagenesis was used to introduce nucleotide substitutions into the *B. sphaericus* DAPD gene, changing five amino acids to those previously found to be important for broad substrate specificity in the evolved *C. glutamicum* DAPD.[9] After the resulting modified gene was ligated into plasmid pBMS2004 and expressed in *E. coli* BL21(DE3)-Gold, the resulting D-amino acid dehydrogenase was highly efficient for the reductive amination of keto acid **2** despite the fact that the sequence identity of *B. sphaericus* DAPD compared to that of *C. glutamicum* DAPD is only 51% (Figure 3). The amino acid sequence of the evolved *B. sphaericus* D-amino acid dehydrogenase is shown in Figure 4.

Preparation of *R*-Amino Acid 1 with In-House D-Amino Acid Dehydrogenase and Procedure for *p*-Chlorobenzenesulfonylation of 1 and Conversion of the Resulting Acid, 3, to the Carboxamide, 5. For the in-house D-amino acid dehydrogenase, the K_m for NADPH was determined to be 0.63 mM, and the pH optimum was ~9.5 (Figure 2), similar to those for D-amino acid dehydrogenase-102, and the same reaction conditions were used for the in-house enzyme as had been used for the commercial enzyme.

The enzymatic transformation mixture containing *R*-amino acid **1** was the starting material for preparing acid **3** (Scheme 1) by arylsulfonylation with *p*-chlorobenzenesulfonylchloride. Acid **3** can be isolated as a crystalline solid, but a satisfactory way to do this in good yield was not developed. The crude acid was converted to the acid chloride **4** with oxalyl chloride and then to carboxamide **5** with aqueous ammonia.

Enzymatic synthesis of **1** from **2** using the in-house D-AADH followed by *N*-sulfonylation to **3** and conversion to carboxamide **5** was done at a 3.5 mol scale (600 g input of the keto acid). The sequence was done without isolation of intermediates, giving a 65% overall yield of **5** with a purity of 98.4 wt % and an ee of >99.9%. The ee of the intermediate amino acid **1** was 98.9%, and thus, the final isolation by recrystallization from *n*-butanol gave good purification with respect to chiral homogeneity.

Cloning of Glucose Dehydrogenase and Development of a Dual-Expression Strain. A putative NADP-dependent glucose 1-dehydrogenase gene was identified from the sequenced genome of *Gluconobacter oxidans* (National Center for Biotechnology Information (NCBI) accession number NC 006677). Primers based on this sequence were used for PCR amplification of the gene from *G. oxidans* chromosomal DNA; the gene was ligated into plasmid pBMS2004 and expressed in *E. coli* strain BL21. Maximum activity was obtained at pH 9.0 and 40 °C. Near maximal activity was seen between pH 8.0 to 10.0 and 35 to 50 °C. The resulting glucose dehydrogenase activity was demonstrated to be effective for NADPH regeneration during the reductive

```
B. sphaericus   MSAIRVGIVGYGNLGRGVEFAISQNPDMELVAVFTRRDPSTVSVASNASVYLVDDAEKFQDDIDVMILCGGSATDLPEQG
C. glutamicum   MTNIRVAIVGYGNLGRSVEKLIAKQPDMDLVGIFSRR----ATLDTKTPVFDVADVDKHADDVDVLFLCMGSATDIPEQA
                         10        20        30        40        50        60        70        80

B. sphaericus   PHFAQWFNTIDSFDTHAKIPEFFDAVDAAAQKSGKVSVISVGWDPGLFSLNRVLGEAVLPVGTTYTFWGDGLSQGHSDAV
C. glutamicum   PKFAQFACTVDTYDNHRDIPRHRQVMNEAATAAGNVALVSTGWDPGMFSINRVYAAAVLAEHQQHTFWGPGLSQGHSDAL
                         90        100       110       120       130       140       150       160

B. sphaericus   RRIEGVKNAVQYTLPIKDAVERVRNGENPELTTREKHARECWVVLEEGADAPKVEQEIVTMPNYFDEYNTTVNFISEDEF
C. glutamicum   RRIPGVQKAVQYTLPSEDALEKARRGEAGDLTGKQTHKRQCFVVADA-ADHERIENDIRTMPDYFVGYEVEVNFIDEATF
                         170       180       190       200       210       220       230       240

B. sphaericus   NANHTGMPHGGFVIRSGESGANDKQILEFSLKLESNPNFTSSVLVAYARAAHRLSQAGEKGAKTVFDIPFGLLSPKSAAQ
C. glutamicum   DSEHTGMPHGGHVITTGDTGGFN-HTVEYILKLDRNPDFTASSQIAFGRAAHRMKQQGQSGAFTVLEVAPYLLSPENLDD
                         250       260       270       280       290       300       310       320

B. sphaericus   LRKELL
C. glutamicum   LIARDV
```

Figure 3. Amino acid alignment of DAPD from *B. sphaericus* and *C. glutamicum*. Those residues that are changed during the subsequent mutation of the original sequence are underlined and bolded.

dx.doi.org/10.1021/op400013e | Org. Process Res. Dev. 2013, 17, 693−700

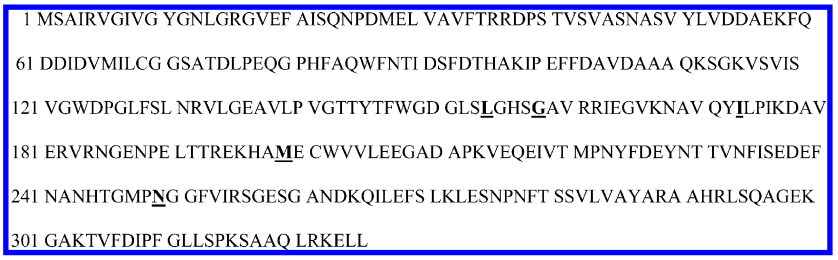


Figure 4. Amino acid sequence of evolved D-AADH. Those residues that were changed by mutation of the original sequence are underlined and bolded.

amination of **2** with either D-amino acid dehydrogenase-102 or our in-house D-amino acid dehydrogenase under the same conditions used for the commercial glucose dehydrogenase.

A bicistronic expression plasmid, pBMS2004-GDH+D-AADH was subsequently created to produce both D-amino acid dehydrogenase and glucose dehydrogenase from the same mRNA transcript. In this construct the D-AADH gene is downstream from the termination codon of the GDH gene and possesses its own ribosome binding site before the ATG initiation codon. The plasmid was transformed into *E. coli*, forming dual-expression strain BL21-Gold/pBMS2004-GDH+(D)-AADH.

Preparation of *R*-Amino Acid with the Dual-Expression Strain. The dual-expression strain was grown in 250-L fermentations. An extract containing both enzyme activities was prepared by microfluidization of a 15% w/v cell (11.75 kg cell paste) suspension in potassium phosphate buffer, then clarified by treatment with 0.25% polyethylene imine and centrifugation. Two preparations of clarified extract contained 38 and 33 U/mL of D-amino acid dehydrogenase and 2174 and 2304 U/mL of glucose dehydrogenase and gave 87% and 88% yields of **1**, with 98.8% and 98.6% ee in 1-g use tests. The extracts were stored at −80 °C until used for preparation of *R*-amino acid **1** and conversion to **5** on a 50-kg scale.[12]

Glutamate Dehydrogenase *gdhA* Knockout Strain. The ee for **1** prepared using Biocatalytics D-amino acid dehydrogenase-102 was close to 100% (*S*-enantiomer was not detectable), whereas the extract containing the in-house D-amino acid dehydrogenase from the dual-expression strain gave 98−99% ee. Although crystallization of **5** increased the ee to 99.9%, analysis[13] of the ternary phase diagram of the enantiomers and solvent indicated that 98.2% ee of **5** was required to purge all *S*-enantiomer by crystallization under equilibrium conditions.[12] During development of the fermentation process, some batches yielded enzyme preparations that produced the *R*-amino acid with considerably lower ee. Although in practice excellent upgrade of **5** was obtained by crystallization (ee 75% improved to 99.6%) apparently because of failure of the racemic compound to nucleate, achieving a higher ee for **1** in the enzymatic process is desirable to ensure the ee of **5** is >99.5. *E. coli* contains L-glutamate dehydrogenase, and we reasoned that this endogenous enzyme was responsible for converting some of the keto acid **2** to (*S*)-5,5,5-trifluoronorvaline, resulting in a lower ee. In support of this possibility, a commercial preparation of L-glutamate dehydrogenase from beef liver was found to completely convert keto acid **2** to the *S*-amino acid.

To further test the idea, an extract from cells that produced *R*-amino acid **1** with only 89.8% ee was fractionated on a 1-mL column of Q-Sepharose. Fractions were assayed for the ee of *R*-amino acid produced, activity of amino acid dehydrogenase with keto acid **2** as substrate, and activity of amino acid dehydrogenase with α-ketoglutarate as substrate (glutamate dehydrogenase activity). The results in Table 2 show that glutamate dehydrogenase activity eluted mainly at 0.4 M NaCl which correlated with a low ee of *R*-amino acid **1** produced. Amino acid dehydrogenase activity with keto acid **2** as substrate peaked at 0.3 M NaCl, which correlated with 99.6% ee of *R*-amino acid produced. The fraction giving a product with the lowest ee had the highest amount of glutamate dehydrogenase activity, and the elevated activity in this fraction is believed to result from endogenous L-glutamate dehydrogenase found in *E. coli*. Low levels of glutamate dehydrogenase activity measured in the other fractions can result from D-AADH activity with α-ketoglutarate as a substrate. These results provided an impetus for eliminating the L-glutamate dehydrogenase activity from the expression strain to obtain consistently higher ee for the *R*-amino acid preparations.

Table 2. Fractionation of cell extract enzyme activities by chromatography on Q-Sepharose

fraction	ee of amino acid **1** produced (%)	amino acid **1** dehydrogenase (U/mL)	glutamate dehydrogenase (U/mL)
sonicate	89.8	21.2	1.72
flow through	98.8	0.31	0.12
0 M NaCl	98.8	0.05	0.08
0.1 M NaCl	98.9	0.11	0.12
0.2 M NaCl	99.8	0.48	0.12
0.3 M NaCl	99.6	13.9	0.13
0.4 M NaCl	83.6	4.39	1.77

The TargeTron Gene Knockout System (Sigma-Aldrich) was used to disrupt the chromosomal *gdhA* gene in BL21-Gold by directed insertion of a genetic element. Colonies containing the knockout were identified by PCR of the *gdhA* gene showing an ~1000 bp size increase due to the presence of the intron and confirmed by glutamate dehydrogenase enzyme assays showing the depletion of the enzyme activity. The *gdhA* knockout strain, BL21-Gold($gdhA^{minus}$), was used as an expression strain for the bicistronic GDH+D-AADH plasmid construct. Growth of the BL2Gold($gdhA^{minus}$)/pBMS2004GDH+D-AADH strain in a 15-L fermentation produced cells used to prepare a cell extract that was utilized to prepare *R*-amino acid **1** on a 1-g scale with 89% yield and 100% ee (undetectable *S*-enantiomer).

dx.doi.org/10.1021/op400013e | *Org. Process Res. Dev.* 2013, 17, 693−700

CONCLUSION

(*R*)-5,5,5-Trifluoronorvaline was prepared from the corresponding keto acid using a D-amino acid dehydrogenase and converted without isolation to a *p*-chlorophenylsulfonamide carboxamide intermediate needed for the synthesis of a γ-secretase inhibitor development candidate. A proprietary strain was constructed to produce enzymes for the reductive amination reaction. The strain expressed a D-amino acid dehydrogenase developed by modification of *B. sphaericus* D-diaminopimelic acid dehydrogenase and also expressed a glucose dehydrogenase from *G. oxidans* for NADPH regeneration. Extracts from this strain were used for production of intermediate **5** on a 50-kg scale. The endogenous glutamate dehydrogenase gene *gdhA* was subsequently inactivated in the expression strain to improve the ee of *R*-amino acid **1**, resulting in amino acid yields of 89% with no detectable *S*-enantiomer.

EXPERIMENTAL SECTION

HPLC Analysis. Samples of 0.02 mL containing *R*-amino acid **1** were diluted with 0. 98 mL water and placed in a boiling water bath for 2 min. After cooling, samples were filtered into HPLC vials. Samples were analyzed with a Regis Davankov Ligand Exchange 15 cm × 0.46 cm column. The mobile phase was 20% methanol/80% water/1 mM $CuSO_4$, flow rate was 1 mL/min, detection was at 235 nm, temperature was 40 °C, and injection volume was 10 μL. Retention times were: *S*-enantiomer of **1**, 4.7 min; *R*-amino acid **1**, 12.7 min; keto acid **2**, 5.6 min.

In later experiments, samples were analyzed with a Phenomenex Chirex 3126 (D-Penicillamine Ligand Exchange) 50 mm × 4.6 mm column. The mobile phase was 2 mM $CuSO_4$ in 5% isopropanol/95% water, flow rate 1 mL/min, detection at 235 nm, temperature 40 °C, and injection volume 10 μL. Retention times were: *S*-enantiomer of **1**, 3.75 min; *R*-amino acid **1**, 5.86 min; keto acid **2**, 26.3 min.

Solutions of **3** and **5** in 40% MeCN were analyzed by HPLC on a Phenomenex Luna C18(2), 5 μm, 4.6 mm × 50 mm column, eluting at 2 mL/min with MeCN/water/TFA, 300:700:1 and detected at 235 nm. Retention times were 7.2−7.7 min for compound **3** and 3.3 min for compound **5**.

Enzyme Assays. Enzyme assays were done using 1 cm path-length cuvettes in a spectrophotometer at 30 °C. One unit (U) of enzyme activity gives 1 μmol of product/min. The D-AADH assay solution contained 5 mg/mL (29.4 mM) keto acid **2**, 1 M NH_4Cl, 0.1 M Na_2CO_3, 0.2 mM NADPH at pH 9.0 in a volume of 1 mL. After addition of a suitably diluted enzyme solution, the absorbance decrease/min at 340 nm was used to calculate enzyme activity. A blank was run with no keto acid.

The glucose dehydrogenase assay solution contained 0.1 M potassium phosphate buffer pH 8, 0.5 mM NADP, and 0.1 M glucose in a volume of 1 mL. After addition of diluted enzyme solution, the absorbance increase/min at 340 nm was used to calculate enzyme activity. A blank was run with no enzyme.

The glutamate dehydrogenase assay contained 5 mg/mL (29.7 mM) α-ketoglutarate monosodium salt, 1 M NH_4Cl, 0.1 M Na_2CO_3, and 0.2 mM NADPH at pH 9.0 in a volume of 1 mL. After addition of a diluted enzyme solution, the absorbance decrease/min at 340 nm was used to calculate enzyme activity. A blank was run with no keto acid.

The *meso*-2,6-diaminopimelic acid dehydrogenase assay contained 100 mM glycine-KCl buffer (pH 10.5), 25 mM *meso*-2,6-diaminopimelic acid, 10 μL of cell lysate, and water to a final volume of 990 μL. The reactions were initiated by the addition of 10 μL of 100 mM NADP. The absorbance increase/min at 340 nm was used to calculate enzyme activity. A blank was run with no enzyme.

Keto Acid 2. Keto acid **2** is known[14] and was prepared at Bristol-Myers Squibb.

Transaminase Reactions. A solution, containing keto acid **2** (100 mg, 0.588 mmol), D,L-alanine (200 mg, 2.24 mmol), and 0.02 mM pyridoxal phosphate, in 0.1 M potassium phosphate buffer, pH 7.5, was incubated with D-transaminase AT-103 from Biocatalytics (5 mg, 44 U) or with cloned D-transaminase from *B. thuringiensis*[10] (0.5 mL, 10 U) at 30 °C in a total volume of 5 mL. For reactions with auxiliary enzymes to reduce pyruvate to lactate, NAD (3.31 mg, 5 μmol), L-lactate dehydrogenase cloned from rabbit muscle (Biocatalytics LDH-103, 0.107 mg, 15 U), and formate dehydrogenase (0.5 mL, 15 U cloned from *Pichia pastoris* and expressed in *Escherichia coli*) were also added. Samples were taken at 21 and 44 h for HPLC analysis.

Conversion of Keto Acid 2 to *R*-Amino Acid 1 with Commercial D-Amino Acid Dehydrogenase and Glucose Dehydrogenase. Keto acid **2** (60 g, 0.353 mol), NH_4Cl (64.2 g, 1.2 mol), glucose (86.4 g, 0.48 mol), Na_2CO_3 (12.72 g, 0.12 mol), NADP (458 mg, 0.60 mmol), glucose dehydrogenase (Amano, 67 mg, 10490 U), D-AADH-102 (Biocatalytics, 600 mg, 2129 U) in 975 mL of water were incubated for 19 h in a 2-L reactor at 30 °C, pH 9 (maintained by addition of 5 N NaOH from a pH stat). The solution yield was 50.5 g (84%), and the ee was 100%.

Production of *E. coli* Cells and Cell Extract Containing D-Amino Acid Dehydrogenase and Glucose Dehydrogenase. *E. coli* BL21-Gold/pBMS2004-GDH+ D-AADH was used to inoculate 1 L of MT5mod2/kan medium (40 g/L glycerol, 20 g/L pea hydrolysate, 18.5 g/L Tastone, 6 g/L Na_2HPO_4, 1.25 g/L $(NH_4)_2SO_4$, 50 mg/L kanamycin sulfate) in a 4-L shake flask. After overnight incubation at 37 °C and 225 rpm, the 1-L culture was used to inoculate 250 L of MT5mod2/kan in a 275-L Braun fermentor, yielding an initial OD_{600} of ∼0.15. The cells were grown at 37 °C, 150 L per min air input, 10 psig pressure, 320 rpm, and pH 7.2. The pH level was not controlled and drifted downward during the course of the fermentation. When the OD_{600} of the culture reached ∼5, sterile isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1.0 mM for induction. The fermentation was continued until the CO_2 off-gas dropped precipitously, indicating depletion of the growth medium. The cells were harvested by centrifugation, and the cell paste, 11.75 kg from two pooled tanks, was analyzed for both GDH and D-AADH activity.

For small-scale use, 180 g of frozen *E. coli* cell paste was added to 60 mL of 1 M potassium phosphate buffer pH 7 in 960 mL of water to give 15% w/v cells in 50 mM potassium phosphate buffer, pH 7. The cells were suspended in the buffer with an Ultraturrax homogenizer, and the cell suspension was passed through a microfluidizer one time at 12000 psi to give 1200 mL of extract. To 1100 mL of magnetically stirred extract was added 16.5 mL of 20% w/v poly(ethyleneimine) to give 0.3% final concentration. Stirring was continued for 10 min, and then the extract was kept on ice for another 30 min. The extract was centrifuged for 2 min at 18000*g*, and the clarified supernatant (960 mL containing 21.9 U/mL D-AADH and 1886 U/mL GDH) was stored at −20 °C until use. The procedure was scaled up to process 11.75 kg of cells to give

dx.doi.org/10.1021/op400013e | Org. Process Res. Dev. 2013, 17, 693−700

76.2 kg of enzyme solution which was stored at −80 °C until use.

Conversion of Keto Acid 2 to Amino Acid 1 Using Extract Containing D-AADH and GDH. Water (800 mL) was added to a 2-L reactor and stirred magnetically. NH_4Cl (26.75 g, 500 mmol), glucose (62.5 g, 347 mmol), and keto acid **2** (50 g, 294 mmol) were added, followed by 29.5 mL of 10 N NaOH. Additional NaOH was added dropwise with continued stirring to bring the pH to 9. NADP (382 mg, 0.5 mmol) and a solution containing D-AADH (1250 U) and GDH (75800 U) were added to start the reaction. The pH was maintained at 9.00 with 5 N NaOH from a pH stat, and the reaction temperature was kept at 30 °C. After 22 h the pH was adjusted to 2.0 with 64 mL of concentrated HCl. The final reaction mixture contained 44.5 g (260 mmol, 88.5% solution yield, 98.9% ee) of *R*-amino acid **1** in 1100 mL.

Conversion of Keto Acid 2 to *R*-Amino Acid 1 Using Extract Containing D-AADH and GDH from *E. coli-gdhA*^{minus} Cells. Keto acid **2** (1 g, 5.88 mmol), NH_4Cl (0.535 g, 10 mmol), glucose (1.25 g, 6.94 mmol), and water (16 mL) were charged to a 20-mL reactor, and the mixture was stirred with a magnet at 30 °C to dissolve the solids. NaOH (10 N, 0.59 mL) was added and then more dropwise to bring the pH to 9. NADP (7.65 mg, 0.01 mmol) and extract from *gdhA*^{minus} cells (0.919 mL containing 25 U of D-AADH and 1319 U of GDH) were then added in that order. The reaction mixture was stirred at 30 °C and maintained at pH 9.00 by addition of 1 N NaOH from a pH stat. After 20 h the solution yield of *R*-amino acid **1** was 0.889 g, 89% yield, 100% ee.

Isolation of *R*-Amino Acid 1. A reaction mixture obtained from the keto acid to amino acid conversion using the in-house D-AADH, 1200 g, pH 2.0, containing 44.5 g of *R*-amino acid **1**, was filtered to remove precipitated protein. The filtrate was adjusted to pH 7.0 with NaOH, diluted with 1-butanol (to prevent foaming and bumping), and concentrated in vacuo to 286 g of wet solid. This was mixed with 1430 mL of MeOH and the mixture refluxed briefly. The hot mixture was filtered, and the solids were washed with a little MeOH. The solids, 123 g after drying, contained 22 g of the amino acid **1**. The solids were mixed with 500 mL of MeOH and refluxed, and the hot mixture was filtered and washed with 100 mL of MeOH. The remaining solids were extracted with another 500-mL portion of methanol in the same way. The resulting methanol-insoluble solids, 106 g, contained 7 g of residual amino acid **1**.

The combined methanol filtrates were concentrated to dryness in vacuo and the residue, 92 g, was dissolved in 370 mL of water at the boiling point. To remove a small quantity of precipitated protein, the hot solution was filtered, rinsing with 50 mL of hot water. Crystallization proceeded as the filtrate cooled. The mixture was cooled to 4 °C and filtered, washing with 40 mL of ice-cold water. Drying in vacuo at room temperature gave 21.4 g of *R*-amino acid **1** as nacreous platelets, ee >99.8%, yield 42.5%. Recrystallization from water gave **1**, mp 289 °C (dec). ^{1}H NMR (500 MHz, D_2O, δ relative to internal 3-(trimethylsilyl)propionic acid-d_4 sodium salt): δ 2.14 (m, 2H), 2.37 (m, 2H), 3.81 (t, 1H, J = 6.3 Hz). Anal. Calcd for $C_5H_8F_3NO_2$: C, 35.09; H, 4.71; N, 8.19; F, 33.31. Found: C, 34.88; H, 4.76; N, 8.04; F, 33.00.

***p*-Chlorobenzenesulfonylation of *R*-Amino Acid 1 and Conversion of the Resulting Acid 3 to the Acid Chloride 4 and Then to Carboxamide 5.** (*R*)-2-(4-Chlorophenylsulfonamido)-5,5,5-trifluoropentanamide, **5**, was prepared from 15 kg of an enzymatic reaction solution containing *R*-amino acid acid **1** derived from 600 g (3.53 mol) of keto acid **2**. The solution from the conversion of keto acid to amino acid was acidified to pH 2.10 (HCl), and after the solution was held at 5 °C for several days, the precipitated protein was filtered out. The filtrate was concentrated in vacuo to 8.5 L, adjusted to pH 12.30 (NaOH), and further concentrated in vacuo with addition of water to remove ammonia. The resulting solution, 6.5 L, contained 556 g (3.25 mol, 92% in process yield) of *R*-amino acid **1**, ee 98.9%.

p-Chlorobenzenesulfonyl chloride, 1099 g (5.20 mol), and 2.2 L of water were cooled to 5 °C, combined and blended with a homogenizer to give a fine suspension of the solid (warmed to 14 °C during the homogenization). The pH of the amino acid solution was adjusted to 10.5 (HCl) and the *p*-chlorobenzenesulfonyl chloride−water mixture added in one portion. The reaction was stirred, keeping the pH between 10.3 and 10.7 for 8 h by addition of 10 M NaOH. Water (a total of 8 L) was also added as needed to maintain effective stirring. Stirring was continued overnight, and the pH, 4.7, was adjusted to 10.5 (NaOH). After an additional 5 h the mixture, 20 L, was stirred with 8 L of MTBE and the pH adjusted to 2.5 (HCl). The lower phase was separated and discarded. The organic phase contained 910 g (2.63 mol, 80.9% in process step yield) of (*R*)-2-(4-chlorophenylsulfonamido)-5,5,5-trifluoropentanoic acid, **3**, by HPLC analysis. A sample of **3** isolated by crystallization from heptane−dichloromethane melted at 115−116.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ relative to internal TMS): δ 1.89 (m, 1H), 2.15 (m, 1 H), 2.25 (m, 2H), 4.00 (dt, 1H, J = 8.6, 8.6, 4.6 Hz), 5.28 (d, 1H, J = 8.6 Hz), 7.50 (dt, 2H, J = 8.8, 2.2, 2.2 Hz), 7.79 (dt, 2H, J = 8.7, 2.3, 2.3 Hz). Anal. Calcd for $C_{11}H_{11}ClF_3NO_4S$: C, 38.12; H, 3.21; Cl, 10.25; F 16.47; N, 4.05; S, 9.27. Found: C, 38.16; H, 3.04; Cl, 10.42; F, 16.20; N, 3.96; S, 9.27.

The solution was concentrated in vacuo, adding portions of 2-methyltetrahydrofuran (MeTHF) until the water content in the distillate was <0.1 weight%. The solution was diluted to 4 L with MeTHF, and oxalyl chloride (335 mL, 3.96 mol) was added in 30-mL portions at 1 min intervals. After 30 min, four 26-mL portions of a 5 vol % solution of DMF in MeTHF were added at 10-min intervals. By 75 min gas evolution (CO and CO_2) had stopped. The solution was concentrated in vacuo with addition of MeTHF until the distillate gave negligible gas evolution when mixed with water. Compound **4** was not isolated.

A mixture of 7.8 L of MeTHF and 7.8 L of 5 M ammonium hydroxide was cooled to 10 °C and the acid chloride solution (2.8 kg) added rapidly with stirring, which was continued for 10 min. The lower phase was discarded. The organic phase was stirred and adjusted to pH 2.8 with 0.5 M sulfuric acid. The resulting aqueous phase was discarded and the organic phase washed with 500-mL portions of water until the extract was neutral.

The organic phase was concentrated in vacuo with addition of 1-butanol to replace the MeTHF, and the resulting mixture was diluted with 1-butanol to bring the volume to 5.4 L. Heating to 100 °C dissolved all but a small quantity of the solid. The mixture was stirred while cooling to 0 °C over 6 h and then at 0 °C for an additional 10 h. The mixture was filtered and the solid washed with cold 1-butanol and dried on the funnel with suction, giving 800 g of (*R*)-2-(4-chlorophenylsulfonamido)-5,5,5-trifluoropentanamide, **5**, as an off-white free-flowing granular solid, mp 211−213.5 °C, ee >99.9, purity 98.4 wt %, corrected weight 787 g, 2.28 mol, 86.9% step yield, 65%

dx.doi.org/10.1021/op400013e | Org. Process Res. Dev. 2013, 17, 693−700

overall yield from **2**. Characterization of **5** is presented in reference 2.

The mother liquor/wash contained 68 g of 2-(4-chlorophenylsulfonamido)-5,5,5-trifluoropentanamide, **5**. A small portion, purified by preparative TLC (not recrystallized), had ee 74.5%. The mother liquor therefore contained 59 g of the(*R*-enantiomer. A second crop was obtained by crystallization from 1-butanol, giving 30 g of (*R*)-2-(4-chlorophenylsulfonamido)-5,5,5-trifluoropentanamide, **5**, ee 99.6%, purity 98.9 wt %.

Cloning and Expression of *meso*-Diaminopimelic Dehydrogenase from *B. sphaericus*. *B. sphaericus* (ATCC 4525) was grown at 30 °C in LB medium, and chromosomal DNA was prepared from the harvested cell paste using a standard proteinase K/SDS/NaCl/CTAB bacterial DNA purification protocol.[15] This chromosomal DNA was used for a target for PCR reactions to amplify the *B. sphaericus* DAPD gene. The GenBank amino acid sequence submission for a comparable *B. sphaericus* DAPD protein (GenBank accession number BAB07799) was used to design synthetic oligonucleotides to prime amplification of the DAPD gene from chromosomal DNA. The completed PCR reaction was analyzed by ethidium bromide-stained agarose gel electrophoresis. A single DNA band of about 1000 base pairs was strongly amplified, consistent with the expected size of a *B. sphaericus* DAPD gene.

The DNA from the band was purified and ligated into cloning vector pCR4-TOPO (Invitrogen), using the manufacturer's protocol, and then transformed into *E. coli* strain TOP10 by electroporation. Plasmid DNA purified from transformed cells was used for DNA sequence analysis using plasmid based primers that flanked the fragment insert site in PCR4-TOPO.

The DAPD gene was cloned into expression vector pET30a and transformed into electrocompetent *E. coli* strain BL21-(DE3)-Gold (Agilent, Santa Clara, CA). A single positive colony of the transformed strain was grown overnight in MT5mod2/kan broth at 37 °C, 250 rpm and then subcultured in the same medium at 30 °C at a starting optical density at 600 nm (OD_{600}) of 0.15. At OD_{600} of 1.0, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 200 μM to induce transcription of the DAPD gene from the T7 promoter. Growth was continued overnight at 30 °C. A cell extract derived from the DAPD expression culture displayed a novel, highly overexpressed protein with an apparent molecular weight of ~36 kD on an SDS gel. The extract catalyzed the oxidative deamination of *meso*-2,6-diaminopimelic acid to (*S*)-α-amino-ε-ketopimelate. The oxidative deamination activity level (10.3 U/mg protein) in the recombinant DAPD *E. coli* expression strain was about 1000-fold higher than the level observed in the native strain used to isolate the DAPD gene, clearly demonstrating that the cloned gene encoded a DAPD.

Construction of the Evolved D-AADH Enzyme. A QuikChange Multisite Mutagenesis kit (Stratagene) was used to introduce nucleotide substitutions into the *B. sphaericus* DAPD gene, altering the amino acid sequence of the encoded protein to include five amino acid mutations. Four specific mutagenic oligonucletide primers were used to initiate PCR reactions that amplified a modified version of the native DAPD gene. Each primer replicated the sense strand of the DAPD gene in the region flanking the site of the desired mutation but substituted an alternate codon at the point of the amino acid change.

This mutagenized plasmid preparation was used to transform competent *E. coli* strain XL10-Gold, selecting transformants by growth on LB/kan agar plates. Plasmid was prepared from 20 isolates, and DNA sequencing identified six colonies that contained a plasmid containing an evolved version of the DAPD with all five of the desired mutations (Q155L/D159G/T174I/R200M/H250N). One such plasmid was selected and used for subsequent experiments.

Presence of Glutamate Dehydrogenase and Its Effect on Reaction Yield and Ee. Extract from cells expressing D-AADH and GDH was prepared by sonication of 835 mg of cells in 5 mL of 50 mM potassium phosphate buffer, pH 7. All further steps were carried out at 4 °C. The extract was centrifuged for 10 min at 43000*g*, and 2 mL of the supernatant was added to a 1-mL column of Q-Sepharose equilibrated with 20 mM tris-chloride, pH 7.4. The column was eluted with 2-mL portions of 20 mM tris-chloride (pH 7.4) containing 0, 0.1, 0.2, 0.3, and 0.4 M NaCl, and 2-mL fractions were collected. Each fraction was assayed for glutamate dehydrogenase activity, D-AADH activity, and ee of **1** produced by the fraction.

The ee of **1** produced by the fraction was determined with a reaction mixture containing in a total volume of 1 mL at pH 9.0: 5 mg/mL (29.4 mM) of keto acid **2**, 0.5 M NH_4Cl, 0.347 M glucose, 0.5 mM NADP, and 0.1 mL of the fraction being assayed. After 15 h incubation at 30 °C, the ee of **1** was analyzed by HPLC.

Knockout of *gdhA* Gene and Production of *E. coli* *gdhA*^{minus} Strain. The nucleotide sequence of the *gdhA* gene from *E. coli* strain B, the parental strain of the BL21-Gold expression strain, was obtained from the GenBank sequence database. On the basis of this sequence, oligonucleotide primers were prepared for amplification of the *gdhA* gene by PCR. The amplified fragment was cloned into pCR4-TOPO and confirmed to be the *gdhA* gene by DNA sequence analysis.

The nucleotide sequence from the *gdhA* gene was submitted to Sigma and analyzed using their proprietary algorithm to determine the optimal region for insertion of an intron intended to disrupt the *gdhA* coding sequence. The intron-containing plasmid pACD4 was modified using oligonucleotide primers in order to direct disruption of the *gdhA* locus. All subsequent gene knockout experiments (i.e., amplification of a *gdhA*-modified intron fragment, cloning of the modified intron into the pACD4 vector, and transformation of BL21-Gold to disrupt the native *gdhA* gene) were conducted according to the TargeTron manufacturer's protocol. Since the pACD4 has no selective marker to identify potential intron-disrupted colonies, colony PCR using the original *gdhA* terminal primers was used to detect which colonies contained disrupted *gdhA* genes. Approximately 20% of the colonies amplified a PCR product ~1000 bp larger than the control (the purified pCR4-TOPO +*gdhA* vector) indicating they had incorporated the intron within the *gdhA* coding region. One of these colonies, named BL21-Gold(*gdhA*^{minus}), was selected for further analysis.

Both BL21-Gold and BL21-Gold(*gdhA*^{minus}) were grown in shake flasks containing MT5(mod2) medium at 37 °C to late-log phase. Cell pellets of two wild-type (parental strain of the BL21-Gold expression strain) and two knockout samples were suspended in 5 mL of 50 mM potassium phosphate buffer pH 7, sonicated for 3 min, and then centrifuged for 15 min at 48000*g*. The supernatants were assayed for glutamate dehydrogenase activity. Glutamate dehydrogenase activity in knockouts measured in this assay was <10% of wild-type control activity. The residual activity (ΔA 340/min) found in

dx.doi.org/10.1021/op400013e | *Org. Process Res. Dev.* 2013, 17, 693−700

the knockouts may be due to the presence of a ketoreductase rather than glutamate dehydrogenase.

Expression of D-AADH and GDH in the *E. coli-gdhA*^{minus} Strain. *E. coli* BL21-Gold(*gdhA*^{minus}) was transformed with plasmid pBMS2004-GDH+D-AADH. Transformants were identified by growth on LB/kan agar plates and verified by colony PCR specific for amplification of the GDH +D-AADH gene cassette. A transformant was grown in MT5(mod2)/kan medium with induction at OD_{600} ~1.5 with 0.2 mM IPTG and continued overnight growth. SDS-PAGE and enzyme activity analyses of an extract of the induced cells revealed that both the GDH and D-AADH proteins were highly overexpressed in the *gdhA*^{minus} background.

Production of *E. coli-gdhA*^{minus} Producing Both D-AADH and GDH. *E. coli* BL21-Gold(*gdhA*^{minus})/pBMS2004GDH+D-AADH was used to inoculate 500 mL of MT5mod2/kan broth in a 2-L shake flask. The culture was grown overnight at 37 °C and 225 rpm, and then used to inoculate 15 L of MT5mod2/kan in a 21-L Braun fermentor, yielding an initial OD_{600} of ~0.25. The cells were grown at 37 °C, 150 LPM air input, 10 psig pressure, 320 rpm, at pH 7.2. The pH level was not controlled and drifted downward during the course of the fermentation. When the OD_{600} of the culture reached ~5, sterile IPTG was added to a final concentration of 1.0 mM. The fermentation was continued until the CO_2 off-gas dropped precipitously, indicating depletion of the growth medium. The cells were harvested by centrifugation, and the cell paste was stored at −80 °C.

Preparation of D-AADH and GDH Enzyme Mixture from *E. coli gdhA*^{minus} Cells. A 15% w/v suspension of the harvested culture was prepared in 50 mM potassium phosphate buffer, pH 7.0 (6.0 g frozen cells +34 mL buffer). The cells were suspended in the buffer with an Ultraturrax T25 homogenizer and then sonicated for 3 min. The extract was centrifuged for 15 min at 48000*g*, 4 °C. The supernatant contained 27.2 U/mL D-AADH and 1436 U/mL GDH.

Other Methods. Expression of evolved D-AADH in *E. coli*, cloning and expression of the *G. oxidans* glucose dehydrogenase gene, and construction of the *E. coli* strain expressing both evolved D-AADH and glucose dehydrogenase have been described.[16]

AUTHOR INFORMATION

Corresponding Author

*Telephone: (732)-227-6222. E-mail: ronald.hanson@bms.com.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Hyei-Jha Chung, Thomas Razler, and Michael Cassidy for development of procedures to synthesize keto acid **2**. We thank Emily Reiff and Stephen Guzikowski for optimization of the conversion of amino acid **1** to intermediate **5**, and we thank Michael Randazzo and Ramesh Patel for helpful advice on all of the work.

REFERENCES

(1) Roberts, S. B. *Adv. Drug Delivery Rev.* **2002**, *54*, 1579.
(2) Gillman, K. W.; Starrett, J. E., Jr.; Parker, M. F.; Xie, K.; Bronson, J. J.; Marcin, L. R.; McElhone, K. E.; Bergstrom, C. P.; Mate, R. A.; Williams, R.; Meredith, J. E.; Burton, C. R.; Barten, D. M.; Toyn, J. H.; Roberts, S. B.; Lentz, K. A.; Houston, J. G.; Zaczek, R.; Albright, C. F.; Decicco, C. P.; Macor, J. E.; Olson, R. E. *ACS Med. Chem. Lett.* **2010**, *1*, 120.
(3) Ojima, I.; Koji, K.; Nakahashi, K. *J. Org. Chem.* **1989**, *54*, 4511.
(4) Moriguchi, M.; Ideta, K. *Appl. Environ. Microbiol.* **1988**, *54*, 2767.
(5) Drauz, K. *Chimia* **1997**, *51*, 310.
(6) Pietzsch, M.; Syldatk, C. In *Enzyme Catalysis in Organic Synthesis*, 2nd ed.; Drauz, K., Ed.; Wiley-VCH: Weinheim, 2002; Vol. 2, p 761.
(7) Komeda, H.; Asano, Y. *Eur. J. Biochem.* **2000**, *267*, 2028.
(8) Alexandre, F.-R.; Pantaleone, D. P.; Taylor, P. P.; Fotheringham, I. G.; Ager, D. J.; Turner, N. J. *Tetrahedron Lett.* **2002**, *43*, 707.
(9) Vedha-Peters, K.; Gunawardana, M.; Rozzell, J. D.; Novick, S. J. *J. Am. Chem. Soc.* **2006**, *128*, 10923.
(10) Hanson, R. L.; Davis, B. L.; Goldberg, S. L.; Johnston, R. M.; Parker, W. L.; Tully, T. P.; Montana, M. A.; Patel, R. N. *Org. Process Res. Dev.* **2008**, *12*, 1119.
(11) Taylor, P. P.; Pantaleone, D. P.; Senkpeil, R. F.; Fotheringham, I. G. *Trends Biotechnol.* **1998**, *16*, 412.
(12) Cassidy, M.; Chen, C.-K.; Goswami, A.; Guzikowski, S.; Hanson, R.; Johnston, R.; Lai, C.; Okuniewicz, F.; Parker, W.; Razler, T.; Randazzo, M.; Reiff, E.; Xu, X. Manuscript in preparation.
(13) Chen, A. M.; Wang, Y.; Wenslow, R. M. *Org. Process Res. Dev.* **2008**, *12*, 271.
(14) Urata, H.; Ishii, Y.; Fuchikami, T. *Tetrahedron Lett.* **1989**, *30*, 4407.
(15) Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. D.; Smith, J. A.; Struhl, K. *Current Protocols in Molecular Biology*; John Wiley and Sons: New York, NY, 1990; Vol. *1*, Supplement 27, section 2.4.1.
(16) Goswami, A.; Goldberg, S. L.; Johnston, R. M.; Hanson, R. L.; Parker, W. L. Mutant amino acid dehydrogenase and its use in preparing amino acids from keto acids. Patent Application No. WO/2012/106579, 2012. *Chem. Abstr.* **2012**, *157*, 354578.