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Preparation of (*S*)-1-Cyclopropyl-2-methoxyethanamine by a Chemoenzymatic Route Using Leucine Dehydrogenase

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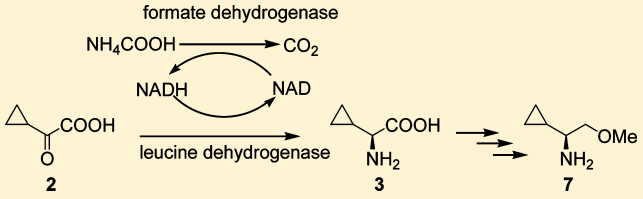
ABSTRACT: (*S*)-1-Cyclopropyl-2-methoxyethanamine is a key chiral intermediate for the synthesis of a corticotropin-releasing factor-1(CRF-1) receptor antagonist. Resolution of the racemic amine by transaminase from *Vibrio fluvalis* gave a 38% yield of the *S*-amine with 53% ee. Resolution by lipase-catalyzed acylation provided the *S*-amine in 35% yield with 91% ee. With limited success of these resolution approaches, an efficient chemo-enzymatic route to (*S*)-1-cyclopropyl-2-methoxyethanamine was devised starting from methylcyclopropyl ketone. Permanganate oxidation of the ketone gave cyclopropylglyoxylic acid, which was converted to (*S*)-cyclopropylglycine by reductive amination using leucine dehydrogenase from *Thermoactinomyces intermedius* with NADH cofactor recycling by formate dehydrogenase from *Pichia pastoris*. Both enzymes were cloned and expressed in recombinant *E. coli*. (*S*)-Cyclopropylglycine obtained from enzymatic reductive amination was isolated as the *N*-Boc derivative and converted to the desired amine by reduction, methylation, and deprotection to give (*S*)-1-cyclopropyl-2-methoxyethanamine in 62% overall yield from cyclopropylglyoxylic acid, with no detectable *R*-enantiomer.



INTRODUCTION

Chiral amines are used for the synthesis of many pharmaceutically interesting compounds. Among the enzymatic processes used to prepare chiral amines are acylation or deacylation using lipases or proteases,[1,2] or oxidation using amine oxidases.[3] Enantioselective acylation combined with chemical racemization of the amines[1,2,4] and enantioselective oxidation combined with nonselective chemical reduction of the imine product[3] have allowed dynamic resolutions giving >50% yields in some cases. Amine transaminases have also been used for the preparation of chiral amines.[5−7] The *S*-specific transaminases are more common than *R*-specific enzymes and can be used to convert a racemic mixture to the *R*-enantiomer and a ketone. An *R*-specific enzyme has been described,[8] and some *R*-specific transaminases are commercially available. Prochiral ketones have also been transaminated to chiral amines when conditions have been devised to overcome the unfavorable equilibrium for this reaction.[9,10]

(*S*)-1-Cyclopropyl-2-methoxyethanamine (**7**) was required for synthesis of a corticotropin-releasing factor-1(CRF-1) receptor antagonist and was previously prepared using an asymmetric Strecker reaction to introduce the chirality.[11,12] Enzymatic approaches were explored to improve atom economy, avoid the use of cyanide, and develop an efficient "green" process. This report describes an efficient chemo-enzymatic route for the preparation of (*S*)-cyclopropylglycine by reductive amination of cyclopropylglyoxylic acid, conversion to the Boc derivative, reduction to the glycinol, O-methylation, and deprotection to give the desired amine.

RESULTS AND DISCUSSION

Resolution of Racemic 1-Cyclopropyl-2-methoxyethanamine (8). An amine transaminase from *Bacillus megaterium* expressed in *Escherichia coli* SC16578 was previously used for conversion of racemic 1-cyclopropylethylamine to the *R*-amine by selective transamination of the *S*-enantiomer.[7] Since (*R*)-cyclopropylethylamine has the same arrangement of cyclopropyl and open-chain substituents as *S*-amine **3**, this transaminase was tested for resolution of racemic 1-cyclopropyl-2-methoxyethanamine (**8**). The enzyme had low enantioselectivity favoring the undesired transamination of *S*-amine with pyruvate as the amino group acceptor, giving an ee of 43% for the undesired *R*-enantiomer.

A screening kit of 17 amine transaminases (Codexis) was also tested for resolution of racemic amine **8** with pyruvate as the amino group acceptor following the screening procedure of the manufacturer. ATA-114 gave 30.5% ee of the *S*-amine, ATA-117 gave 5.6% ee of the *S*-amine and ATA-113 gave 57% ee of the *R*-amine. The remaining 14 enzymes gave no reaction. Transaminase from *Vibrio fluvalis* gave a 38% yield (with a maximum 50%) of *S*-amine with 53% ee.

Strains were selected from soil samples able to grow on the *R*-enantiomer of amine **7** as sole nitrogen source. However when supplied with the racemic amine, the isolates consumed the *R*-amine, but preferentially used the *S*-amine, therefore increasing the ee of *R*-amine. Two D-amino acid oxidases, six L-amino acid transaminases, and two D-amino acid transaminases had no effect on racemic amine **8**. An *N*-Cbz cleaving enzyme[13]

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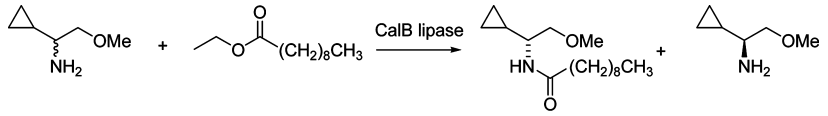
Scheme 1. Resolution of racemic amine 8 using Cal B lipase

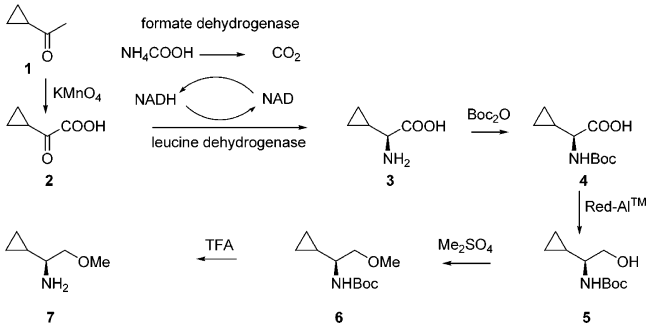


enantioselective for *N*-α-Cbz-L-amino acids had no effect on Cbz-protected racemic amine **8**.

Resolution of racemic amine **8** by acylation using ethyl caprate **9** and Cal B lipase in MTBE gave a 35% solution yield of *S* amine **7** with 91.3% ee (Scheme 1). This approach was previously used to prepare *S*-amines,[14] but in this case the selectivity was not sufficient to achieve the necessary ee (>99%) with good yield.

After initial attempts at resolution of the racemic amine with amine transaminases, lipases and other enzymes met with limited success, a completely different approach using the enzymatic reductive amination of a keto acid to an amino acid was pursued to give the desired enantiomer. We have previously prepared several unnatural *S*-amino acids from the corresponding keto acids using amino acid dehydrogenases[15−18] and explored this approach as an alternative route to the chiral amine. The chemoenzymatic route to the chiral amine **7** is outlined in Scheme 2.

Scheme 2. Route for conversion of cyclopropymethyl ketone (1) to (*S*)-1-cyclopropyl-2-methoxyethanamine (7)



Preparation of Cyclopropylglyoxylic Acid (2). An initial preparation of cyclopropylglyoxylic acid **2** was obtained by addition of cyclopropyl magnesium bromide to diethyl oxalate, followed by hydrolysis of the resulting ethyl ester to give the acid. A better method, oxidation of cyclopropyl methyl ketone (**1**) with potassium permanganate, was subsequently employed. Preparation of cyclopropylglyoxylic acid **2** by oxidation of cyclopropyl methyl ketone **1**, conversion to racemic cyclopropyl glycine, and enzymatic resolution of the Boc-protected methyl ester has been described.[19]

Reductive Amination of Cyclopropylglyoxylic Acid. Leucine dehydrogenases from *Bacillus sphaericus*[15] and from *Thermoactinomyces intermedius* cloned into *E. coli* SC16591, recombinant phenylalanine dehydrogenase from *T. intermedius* (original or modified version),[18] commercially available glutamate dehydrogenase from beef liver, alanine dehydrogenase from *Bacillus subtilis*, and phenylalanine dehydrogenase from *Rhodococcus* species converted the keto acid precursor to (*S*)-cyclopropyglycine (**3**). The *R*-amino acid was produced using each of four *R*-amino acid dehydrogenases (D-AADH-101, -102, -103, -104 from Codexis). All of the amino acid dehydrogenases gave product with 100% ee, except modified phenylalanine dehydrogenase from *T. intermedius* which gave 94% ee.

Leucine dehydrogenase from *T. intermedius* expressed in *E. coli* SC16591 was chosen for preparative purposes. Comparing extract activities with 5 mM α-ketoisovalerate (3282 U/mL) versus 5 mM cyclopropylglyoxylic acid (86 U/mL), leucine dehydrogenase is 38-fold more active with the natural substrate. Formate dehydrogenase from *Pichia pastoris* expressed in *E. coli* SC16556 was used for regeneration of NADH. The construction of the recombinant strains, production of cells by fermentation, and preparation of extracts containing the enzyme activities are described in the Experimental Section.

The pH optimum for leucine dehydrogenase with cyclopropylglyoxylic acid as substrate was found to be 9.0 (Figure 1).

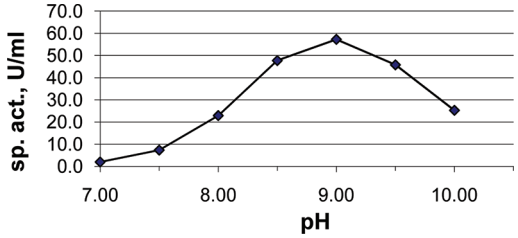


Figure 1. pH optimum for leucine dehydrogenase. The assay mixture contained 5 mg/mL keto acid **2** potassium salt (32.8 mM), 83 mg/mL (1.31 M) ammonium formate, 0.1 mM dithiothreitol, and 0.2 mM NADH. It was adjusted to the indicated pH at 40 °C.

However, since the pH optimum for *P. pastoris* formate dehydrogenase has been reported to be 6.5−7.5,[20,21] the reaction was run at pH 8. The apparent K_m for NADH for leucine dehydrogenase with 33 mM cyclopropylglyoxylic acid at pH 8 was found to be 0.023 mM. Reactions were run with 1 mM NAD, although the low K_m indicates a lower concentration should suffice. Reported K_m's for NAD for formate dehydrogenase are 0.14 mM[20] and 0.27 mM.[21] Batches with 1, 25, and 50 g inputs of keto acid **2** potassium salt (using 10% keto acid salt concentration as described in the Experimental Section) gave 99−100% solution yield of (*S*)-cyclopropyglycine with no detectable *R*-enantiomer.

Conversion of *S*-Amino Acid 3 to *S*-Amine 7. A small quantity of *S*-amino acid **3** was isolated and recrystallized. Treatment of the enzymatic reaction mixture with Boc anhydride gave *N*-Boc-amino acid **4**. Reduction of **4** to Boc-(*S*)-cyclopropylglycinol **5** with various borane and mixed anhydride/borohydride protocols met with varying degrees of success, but reduction with sodium bis(2-methoxyethoxy)-aluminum hydride[22] gave superior results. Boc-(*R*)-cyclopropylglycinol has been reported.[23] Attempts to methylate the alcohol function with alkylating agents and base in homogeneous solution did not give high selectivity for O- versus N-alkylation. However, phase-transfer methylation provided a highly selective means of producing methyl ether **6** in the presence of a Boc-protected primary amine. Deprotection with trifluoroacetic acid gave the desired (*S*)-1-

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cyclopropyl-2-methoxyethanamine **7** in 62% overall yield from cyclopropylglyoxylic acid, with no detectable *R*-enantiomer.

CONCLUSION

Enzymatic resolution approaches to preparation of amine **7** did not give sufficiently high enantiomeric purity. An alternative approach using an *S*-amino acid dehydrogenase to introduce the chirality followed by reduction of the Boc-protected amino acid, then methylation and deprotection gave the desired amine. Since *R*-amino acid dehydrogenases gave the *R*-amino acid, it is expected that this method could also be used to prepare the *R*-amine.

EXPERIMENTAL SECTION

Resolution of Amine 8 by Transaminase. A solution containing 10 mg racemic amine **8**, 10 mg sodium pyruvate, and 0.02 mL ω-transaminase from *V. fluvalis* (Julich) in 1 mL 0.1 M potassium phosphate buffer pH 7.5 was incubated at 28 °C. After 160 h, 43% of the amine remained with 53% ee of the *S*-enantiomer.

Resolution of Amine 8 Using Cal B Lipase. To 1 mL of methyl *tert*-butyl ether solution containing 2 mg of racemic amine **8** were added ethyl caprate **9** (20 μL) and Cal B lipase from *Candida antarctica* (50 mg, from Codexis), and the mixture was shaken at 500 rpm and 25 °C. After 120 h, 35% of the amine remained with 91.3% ee of the *S* enantiomer **7**.

Preparation of Cyclopropylglyoxylic Acid (2). Oxidation of cyclopropyl methyl ketone **1** with potassium permanganate was done essentially as described by Basnak and Farkas[24] except that additional $KMnO_4$ was used to consume unreacted ketone detected by HPLC. A solution of 140 g (1.66 mols) of cyclopropyl methyl ketone **1** and 5.65 g of K_2CO_4 in 1.12 L of water was heated to 50 °C, and a solution of 526 g (3.33 mol) $KMnO_4$ in 11.4 L of water was added over 54 h. After addition was complete, the temperature was maintained at 50 °C for 3 h. At this point only a trace of unreacted cyclopropyl methyl ketone **1** remained, and the solution yield of cyclopropylglyoxylic acid **2** by HPLC was 75%. To remove the excess $KMnO_4$, 250 mL of 3.5% H_2O_2 was added, completely discharging the deep purple color. The solution was cooled to 20 °C and filtered to remove MnO_2. The filtrate was concentrated in vacuo to a solid residue. Recrystallization from methanol (3 L) gave 128 g, 50% yield, of the potassium salt of cyclopropyl glyoxylic acid **2** as a nacreous solid, mp 271.5−273.0 °C (dec). The mother liquor contained 61 g of additional product which was not recovered.

Preparation of (*S*)-Cyclopropylglycine (3). Cyclopropylglyoxylic acid **2** potassium salt (50 g, 0.328 mol), ammonium formate (41.5 g, 0.657 mols) and water (408 mL) were added to a jacketed 1-L reactor. Magnetic stirring was used to dissolve the solids, and the temperature was maintained at 40 °C. The solution was adjusted to pH 8.0 by addition of 4.6 mL of 5 N NaOH. Dithiothreitol (77 mg, 0.5 mmol), NAD (331 mg, 0.5 mmol), formate dehydrogease (814U), and leucine dehydrogenase (2157 U measured with cyclopropylglyoxylic acid **2** as substrate) were added. The stirred solution was maintained at 40 °C, and pH 8.00 was maintained by addition of 5 N NaOH from a pH stat. The initial concentration of keto acid was 100 mg/mL. After 2 h the concentration was 67 mg/mL, and after 17 h cyclopropylglyoxylic acid **2** was not detectable. The solution yield was 37.43 g (*S*)-cyclopropylglycine **3** (99.0%), using L-valine as an HPLC standard (correcting for molecular weight). There was no detectable *R*-enantiomer.

A 57-g portion of the reaction mixture (pH 8.4) containing 3.8 g of the amino acid **3** was adjusted to pH 2 (H_2SO_4) to precipitate protein, adding *n*-BuOH as necessary to break the copious foam. After 1 h the mixture was heated to 80 °C, cooled to room temperature, and filtered. The filtrate was adjusted to pH 7 (NaOH), concentrated in vacuo to a thick slurry (24 g), and filtered. The resulting solid (dry weight 4.2 g) was recrystallized from 20 mL of water to give 1.5 g of **3**, mp >300 °C. The ^{1}H NMR spectrum in D_2O was in agreement with published data.[25]

Isolation of Boc-(*S*)-cyclopropylglycine (4). A 509-g batch of enzymatic reaction mixture derived from 45.9 g (302 mmol) of cyclopropylglyoxylic acid **2** potassium salt was adjusted to pH 10.00 (NaOH), and 143 g of di-*tert*-butyl dicarbonate melt (650 mmol) was added. The mixture was stirred vigorously and the pH maintained between 9.7 and 10.1 for 5 h. Stirring at room temperature was continued for another 16 h. The reaction mixture, 700 mL, pH 9.6, was adjusted to pH 7.9 (H_2SO_4) and stirred with 700 mL of isopropyl acetate, and the pH was adjusted to 2.0 (H_2SO_4). Diatomaceous earth was added and the mixture filtered. The filtrate was adjusted to pH 8.4 (NaOH) and the upper phase (containing Boc-NH_2) discarded. The lower phase (~1000 mL) was stirred with 500 mL of isopropyl acetate and the pH adjusted to 2.0 (H_2SO_4). The lower phase was discarded and the upper concentrated in vacuo to 265 g (about 62 g of product and 230 mL of isopropyl acetate). After seeding and crystallizing overnight at room temperature, 460 mL of heptane was added over 1 h and crystallization continued with ice-bath cooling for 1 h. Filtration and drying gave 53.7 g of Boc-(*S*)-cyclopropylglycine **4**, mp 116−117.6 °C, 83% yield. Concentration of the mother liquor gave an additional 8.2 g of product **4**, mp 108−113.0 °C.

Reduction of Boc-(*S*)-cyclopropylglycine (4) to Boc-(*S*)-cyclopropylglycinol (5). A solution of 2.02 g (9.39 mmol) of *N*-Boc-(*S*)-cyclopropylglycine **4** in 10 mL of dry THF (DriSolv, EMD Chemicals) was added dropwise over 15 min to 9.7 mL of a 66% solution of sodium bis(2-methoxyethoxy)aluminum hydride in toluene (Red-Al, 33 mmol) under nitrogen with stirring and cooling in an ice bath. After an additional 2 h, 2.5 mL (62 mmol) of MeOH was added dropwise, releasing 630 mL of hydrogen (very vigorous evolution). Finally, a solution of 20 g of potassium sodium tartrate tetrahydrate (Rochelle's salt) in 20 mL of water was added, releasing an additional 40 mL of hydrogen. The mixture was stirred at room temperature for 15 min, and the upper of three phases was separated. The combined lower phases were extracted with three 10-mL portions of isopropyl acetate. The combined organic extract (upper phase) was washed with two 10-mL portions of 5% $NaHCO_3$ and two 10-mL portions of water. The extract was concentrated in vacuo, giving 1.64 g of solid, which was warmed gently in vacuo until it just melted. After cooling, the viscous liquid was scraped thoroughly with a seeded spatula and warmed a little while the solid crystallized in vacuo, giving 1.62 g (8.07 mmol, 86% yield) of **5**, mp 62−64.5 °C. This material was essentially pure and satisfactory for further use without additional purification. Product from a different preparation (reduction of the mixed anhydride from isobutylchloroformate with sodium borohydride) was recrystallized from heptane to give **5** as crystalline solid (ultrafine needles) melting at 65.5−66.5 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.13 (m, 1H), 0.25 (m, 2H), 0.37 (m, 1H), 0.82

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(m, 1H), 1.37 (s, 9H), 2.97 (m, 1H), 3.38 (m, 2H), 4.52 (t, 1H, J = 5.6 Hz, rapid exchange with methanol-d_4), 6.10 (br, 0.1 H, slow exchange with methanol-d_4, minor rotamer), 6.47 (br d, 0.9H, J = ~7 Hz, slow exchange with methanol-d_4).

Phase Transfer O-Methylation of *N*-Boc-(*S*)-cyclopropylglycinol 5. A solution of 1.47 g of Boc-(*S*)-cyclopropylglycinol **5** (7.30 mmol) and 4.8 mg of benzyltriethylammonium chloride (0.021 mmol) in 14 mL of dichloromethane (DCM) was cooled in an ice bath and stirred, and 3.5 mL of 50% NaOH (65 mmol) was added followed by 1.04 mL of dimethyl sulfate (11.0 mmol). The mixture was stirred with cooling for 0.5 h and then at room temperature for 17 h. HPLC indicated that about 1% of the starting material remained. To destroy the remaining dimethyl sulfate, 1 mL of 15 M ammonium hydroxide was added, and the mixture was stirred for 1 h at room temperature. The upper (organic) phase was separated, washed with water. and concentrated gently (140 mm, 20 °C) in vacuo to avoid volatilization of the product. The residue was redissolved in ethyl acetate and concentrated (40 mm, 20 °C) twice to remove water azeotropically, giving 2.3 g of crude **6**, as an oil.

Preparation of *O*-Methyl-(*S*)-cyclopropylglycinol (7). Crude *N*-Boc-*O*-methyl-(*S*)-cyclopropylglycinol (**6**), 2.3 g, was dissolved in 7 mL of TFA. After 10 min the solution was concentrated in vacuo, giving 2.71 g of oil. The oil was dissolved in 10 mL of DCM and shaken with 14 mL of 5 M NaOH. The lower (organic) phase was separated and stirred with 2 mL of water, and the pH (12) was adjusted to 5−6 with 1.00 N HCl (6.80 mL, sharp end point). The basic aqueous phase was extracted with an additional 2 mL of DCM, the extract was added to the first extract mixture, and the pH was readjusted to 5−6 with 1 N HCl (0.20 mL). The upper phase was separated and concentrated in vacuo, giving 1.06 g of crude HCl salt of **7**, mp 192.5−193.0 °C. Recrystallization from a mixture of dichloromethane (in which the salt is soluble) and isopropyl acetate gave 0.97 g of *O*-methyl-(*S*)-cyclopropylglycinol **7**, HCl salt (hygroscopic), mp 193.0−194.0 °C, yield 88% from Boc-(*S*)-cyclopropylglycinol. Anal. Calcd for $C_6H_{14}ClNO$: C, 47.52; H, 9.30; N, 9.23; Cl 23.38. Found: C, 47.49; H, 9.58; N, 9.43; Cl, 23.22.

The preparation of *O*-methyl-(*S*)-cyclopropylglycinol **7** by an alternate route has been reported.[11,12]

Cloning of the *T. intermedius* Leucine Dehydrogenase (TILDH) Gene. A polymerase chain reaction (PCR) was carried out using primers homologous to the 5′ and 3′ ends of the published TILDH DNA sequence[26] that also contained restriction endonuclease recognition sites for *Nde*I and *Bam*HI, respectively. The reaction used *T. intermedius* chromosomal DNA as template and Z-Taq DNA polymerase (Takara) with included buffer and dNTP solution. Thermocycling conditions were the followiong: 94 °C, 2 min (1 cycle); 94 °C, 30 s; 50 °C, 30 s; 72 °C, 1 min (30 cycles); 72 °C, 5 min (1 cycle). A small aliquot of the reaction was electrophoresed on a 1.0% TAE agarose gel to verify amplification of a 1.1 kb fragment. The remainder of the reaction was extracted with an equal volume 1:1 phenol/$CHCl_3$ and centrifuged at 13,500*g* for 2 min. The upper layer was removed to a new microfuge tube and DNA precipitated by addition of 0.1 vol 3 M sodium acetate pH 4.8 and 2 vol. 100% EtOH. DNA was pelleted by centrifugation at 13,500*g* for 5 min. The pellet was washed with 200 μL 70% EtOH, the liquid was removed, and the tube was placed in a SpeedVac unit for 3 min with heat. DNA was resuspended in 34 μL water and digested with 7.5 U each *Nde*I and *Bam*HI (Invitrogen) at 37 °C for 2 h in a 40 μL final volume. The entire reaction was electrophoresed on a 1.0% TAE agarose gel, and the amplified fragment was excised for purification using the Qiagen Gel Isolation Kit.

The digested PCR fragment was ligated to *Nde*I + *Bam*HI-digested expression vector pBMS2004 in a 5:1 molar ratio using the FastLink ligation kit (Epicentre) for 15 min at room temperature. DNA was precipitated by addition of 10 volumes of 1-butanol and pelleted by centrifugation for 5 min, 13,500*g*. The liquid was removed and the pellet washed with 200 μL 70% EtOH before drying in a SpeedVac for 3 min with heat. The pellet was resuspended in 4 μL water to which 40 μL of electrocompetent DH10B cells (Invitrogen) was added. Electroporation was carried out in a 0.2-cm gap cuvette (Invitrogen) at 25 kV, 25 μF, 200 Ω. SOC medium (960 μL) was added, and the suspended cells were shaken at 37 °C, 225 rpm for 1 h. Cells were concentrated by centrifugation at 13,500*g*, and most of the medium was removed. Cells were resuspended in the remaining liquid and spread for single colonies on LB agar plates containing 50 μg/mL of kanamycin sulfate (Sigma), which were then incubated at 37 °C for 20 h. Correct ligation was confirmed by PCR of Km colonies using primers included in the original amplification reaction.

Cloning of the *P. pastoris* Formate Dehydrogenase (PPFDH) Gene. Initial isolation and cloning of the PPFDH gene into expression vector pBMS2000 was previously described.[18] The gene[27] was subsequently modified by preparing a synthetic version whose codons usage reflects that found in *E. coli* ("Ecobias") and included an amino acid change at amino acid 245 from serine to alanine (S245A). These two modifications led to substantial increases in titer and stability of the recombinant PPFDH protein.

Heterologous Expression of TILDH. Plasmid DNA was prepared from a culture confirmed to contain pBMS2004-TILDH using the Fast Plasmid kit (Eppendorf) and transformed into electrocompetent *E. coli* BL21 cells as described above. A single kanamycin-resistant colony was inoculated into MT5-M2 medium containing 50 μg/mL kanamycin and grown at 30 °C, 250 rpm, for 20 h. The optical density of the culture at 600 nm was measured and diluted to OD = 0.3 into 25 mL MT5-M2 Km medium in a 250-mL flask. The flask was incubated at 30 °C, 250 rpm, until the $OD_{600\ nm}$ was 0.8−1.0. The culture was divided into two 10-mL aliquots in 250-mL flasks and IPTG to 50 μM or 1 mM added from a 1 M sterile stock in water. The flasks were placed on a 30 °C shaker for an additional 22 h. Cultures were transferred to a 15-mL disposable polypropylene tube, and cells were pelleted in a Beckman JA 5.3 swinging bucket rotor for 7 min, 5,000*g*, 23 °C. Medium was removed, and the cells were resuspended in a buffer consisting of 50 mM sodium phosphate buffer pH 7.0 + 1 mM dithiothreitol (DTT). Cells were pelleted as above, and buffer was removed. The wet cell weight was recorded, and cells were resuspended in 10 vol of the same buffer. An 0.8-mL aliquot of the cell suspension was placed into a 1.5-mL microfuge tube and lysed by sonication using a Fisher Sonic Dismembrator, (setting "15" with microtip; 3 × 15 s). The samples were centrifuged 13,500*g* for 5 min, and the lysate was placed in a new centrifuge tube. Overexpression of a soluble 40,000 Da protein was confirmed by SDS-polyacryamide electrophoresis of a 1-μL aliquot of the sonicate followed by staining using the Simply Blue reagent (Invitrogen). Slightly better expression was observed after induction with the 1 mM IPTG, but growth of the culture was greatly retarded as

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compared to the lower level of inducer tested. Further optimization indicated that 100 μM IPTG gave the preferred level of growth and activity.

Growth of *E. coli* BL21(pBMS2004-TILDH) SC16591 Cells Expressing Leucine Dehydrogenase. Two frozen vials of *E. coli* SC16591 were thawed, and the contents were transferred to two 500-mL flasks containing 100 mL of MT5-A 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 (filter-sterilized). The flasks were incubated at 30 °C and 250 rpm for 24 h, after which 5 mL was transferred to four 4-L flasks containing 1 L of the same medium. These second-stage flasks were then incubated at 30 °C and 230 rpm for 22 h. A sufficient quantity (1.4 L) to yield a starting OD_{600} of ∼0.1 AU/cm in 100 L of MT5-M2 medium (2.0% Quest Hy-Pea, 1.85% Tastone 154, 4.0% glycerol, 0.6% Na_2HPO_4, 0.125% ammonium sulfate, 0.04% Ucon LB625, and 0.005% Km sulfate, filter-sterilized) was then pooled and transferred to the fermentor. Process conditions for the 147-L fermentor containing 100 L of medium were as follows: 30 °C; no pH control; agitation, 500 rpm; aeration, 100 lpm; back pressure, 10 psi; foam controlled by the addition of UCON LB625 on demand.

At an OD_{600} of ∼1.6 AU/cm, which occurred 4.5 h following inoculation, a filter-sterilized IPTG solution was added to a final level of 100 μM. The run was completed after 20 h, at which time OD_{600} was ∼40 AU/cm, and off-gas CO_2 had declined from a peak of 2.0% to 0.28%. The whole broth was cooled to 4−10 °C, after which the cells were recovered by centrifugation and washed with 50 mM pH 7 phosphate buffer. A total of 4.3 kg of cell paste was recovered and stored at −75 °C.

Growth of *E. coli* JM110(pBMS2000-Ecobias FDH*S245A*) SC16556 Cells Expressing Formate Dehydrogenase. Plasmid pBMS2000-Ecobias FDH*S245A* was transformed into *E. coli* strain JM110 by electroporation as described above, forming strain SC16556. Two frozen vials of *E. coli* SC16556 were thawed, and the contents 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 (added separately), and 0.005% Km sulfate (filter-sterilized). Flasks were incubated at 30 °C and 250 rpm for 8 h, followed by a 5 mL transfer of broth to four 4-L flasks containing 1 L of the same medium. These second-stage flasks were incubated at 30 °C and 230 rpm for 28 h and then pooled and transferred to a fermentor containing 250 L of MT5-M2 medium (2.0% Quest Hy-Pea, 1.85% Tastone 154, 4.0% glycerol, 0.6% Na_2HPO_4, 0.125% ammonium sulfate, 0.04% Ucon LB625, and 0.005% Km sulfate, the latter filter-sterilized and added separately). The resulting OD_{600} at the start of the run was ∼0.034 AU/cm. Process conditions for the 380-L fermentor containing 250 L of medium were as follows: 30 °C; no pH control; agitation, 550 rpm; aeration, 250 lpm; back pressure, 10 psi; foam controlled by the addition of UCON LB625 on demand.

Three hours following inoculation, a filter-sterilized IPTG solution was added to a final level of 50 μM. The run was completed after 20 h, at which time OD_{600} was 56 AU/cm, and off-gas CO_2 was still climbing. The whole broth was cooled to 4−10 °C, after which the cells were again recovered by centrifugation and washed with 50 mM pH 7 phosphate buffer. A total of 13.3 kg of cell paste was recovered and stored at −75 °C.

Preparation of Formate Dehydrogenase from *E. coli* SC16556 and Leucine Dehydrogenase from *E. coli* SC16591. Cells were suspended in 50 mM potassium phosphate buffer pH 7 containing 1 mM dithiothreitol at a 20% w/v concentration. Cells were disrupted by two passages through a microfluidizer. Poly(ethyleneimine) (PEI, Sigma) was added to the extracts to 0.25% w/v. After 30 min on ice to allow coagulation of debris, the extracts were clarified by centrifugation for 12 min at 15000*g*. The supernatants were stored at −20 °C until use. There was no loss of activity for either enzyme after more than 4 months storage at −20 °C. The formate dehydrogenase activity was 32.6 U/ml. Leucine dehydrogenase activity was 3282 U/mL measured with α-ketoisovalerate as substrate and 86 U/mL measured with cyclopropylglyoxylate as substrate. Activities used for the preparative batches are reported in units with 5 mM cyclopropylglyoxylic acid as the substrate.

Enzyme Assays. Assays were done using 1-cm path length cuvets. The leucine dehydrogenase assay contained in 1.0 mL at 40 °C: 0.2 mM NADH, 5 mM sodium α-ketoisovalerate or 5 mM potassium cyclopropylglyoxylate, 0.75 M NH_4OH adjusted to pH 8.75 with HCl. Absorbance decrease was monitored at 340 nm. Blanks contained enzyme but no keto acid (to correct for background NADH oxidation). Enzyme activity units were calculated as μmol/min based on the rates of absorbance change.

The formate dehydrogenase assay contained in 1.0 mL at 40 °C: 1 mM NAD, 100 mM sodium formate, 100 mM potassium phosphate buffer, pH 8.0. Absorbance increase was monitored at 340 nm. Blanks contained no enzyme. Enzyme activity units were calculated as μmol/min based on the rates of absorbance change.

HPLC Methods. For the Cal B resolution of racemic amine, the amount of remaining amine and enantiomeric composition were determined by conversion to the dansyl derivative[28] followed by analysis on C18 and chiral columns. Analysis on a YMC pack Pro C18 150 × 4.6 mm 3 μm column used a gradient of solvent A (0.05% TFA in water:methanol 80:20) and solvent B (0.05% TFA in acetonitrile:methanol 80:20) starting from 0% B to 100% B in 20 min at a flow rate of 1 mL/min at 40 °C, and UV detection at 220 nm whereby the dansyl derivative of 1-cyclopropyl-2-methoxyethanamine showed a peak at 12.5 min. Analysis on a Chiralpak AS-RH, 150 × 4.6 mm, 5 μm column (Chiral Technologies Inc.) was carried out using an isocratic mixture of 76% solvent A and 24% solvent B at a flow rate of 0.5 mL/min at 30 °C and detection by UV at 220 nm. The peaks for the dansyl derivatives of the S- and R-enantiomers of 1-cyclopropyl-2-methoxyethanamine were at 20.2 and 23.6 min, respectively.

To measure the ee of (*S*)-1-cyclopropyl-2-methoxyethanamine **7** for all other experiments, Marfey's reagent was used to generate diasteromeric derivatives. A sample of 10 μL containing about 0.1 mg amine, 8 μL 1 M $NaHCO_3$, and 40 μL 1% w/v Marfey's reagent (*N*-α-[2,4-dinitrophenyl-5-fluorophenyl]-L-alanine amide, Pierce) in acetone were combined and heated for 1 h at 40 °C. The samples were cooled to room temperature; then 8 μL of 1 N HCl and 934 μL of 40% acetonitrile/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 μm column. The mobile phase was 35% acetonitrile/65% water (each containing 0.05% trifluoroacetic acid), flow rate was 1 mL/min, detection was at 340 nm, temperature was 40 °C, and injection volume

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was 20 μL. Retention times were *S*-enantiomer (**7**), 17.1 min; *R*-enantiomer, 21.2 min.

Marfey's reagent was used as described above to determine the ee of (*S*)-cyclopropylglycine **3**. Samples were analyzed with a YMC Pak Pro C18 15 cm × 0.46 cm 3 μm column. The mobile phase was 32% acetonitrile/68% water (each 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 (**3**), 8.7 min; *R*-enantiomer, 14.4 min

The ee of **3** was also determined with a Regis Davankov Ligand Exchange 15 cm × 0.46 cm chiral column. Samples of 0.02 mL were diluted with 0.98 mL water and placed in a boiling water bath for 2 min. For quantitation, L-valine was used as a standard, assuming the same molar absorbance for the copper complexes of the amino acids. The mobile phase was 0.5 mM $CuSO_4$ in water, 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 (**3**) 2.2 min, *R*-enantiomer 4.9 min.

Cyclopropylglyoxylic acid (**2**) was determined in the same samples using a YMC Pak Pro C18 15 cm × 0.46 cm 3 μm column. The mobile phase was 5% acetonitrile/95% water (each containing 0.05% trifluoroacetic acid), flow rate was 1 mL/min, detection was at 220 nm, temperature was 40 °C, and injection volume was 10 μL. The retention time for **2** was 4.5 min.

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Notes

The authors declare no competing financial interest.

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