Stereoselective Synthesis of Stable Isotope-Labeled L-α-Amino Acids: Chemomicrobiological Synthesis of ^{13}C- and ^{2}H-Labeled L-Cysteine

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SUMMARY

Tryptophan synthase catalyzes the nucleophilic replacement of the hydroxyl group at C-3 of L-serine. This enzyme can use benzyl mercaptan as a nucleophile in the conversion of serine to S-benzyl-L-cysteine which is deblocked by treatment with sodium in liquid ammonia to yield L-cysteine. A strain of *E. coli* engineered to overproduce tryptophan synthase was used in the conversion of L-serine to L-cysteine. Labeled serine was prepared biosynthetically as described previously.

Keywords: L-[3-^{13}C]cysteine, L-[3-$^{2}H_2$]cysteine, S-benzyl-L-[3-^{13}C]cysteine, S-benzyl-L-[3-$^{2}H_2$]cysteine, L-[3-^{13}C]serine, and L-[3-$^{2}H_2$]serine

INTRODUCTION

Stable isotope-labeled amino acids are required for studies of amino acid metabolism and for studies of peptide and protein structure and dynamics. For many of these applications, the naturally occurring L-configuration of the labeled amino acid is required. In general, specific labels have been introduced into racemic mixtures of α-amino acids which are resolved using hog kidney acylase. We are developing strategies for the stereoselective synthesis of specifically labeled L-α-amino acids in which isotopically-labeled L-serine, produced biosynthetically[1], serves as a template for the synthesis of more complex amino acids. The stereochemistry at the α-carbon produced during the biosynthesis of serine is retained in the product amino acid. In this manuscript we describe the tryptophan synthase-catalyzed conversion of L-[3-^{13}C]serine to S-benzyl-L-[3-^{13}C]cysteine which is readily deblocked to yield L-[3-^{13}C]cysteine.

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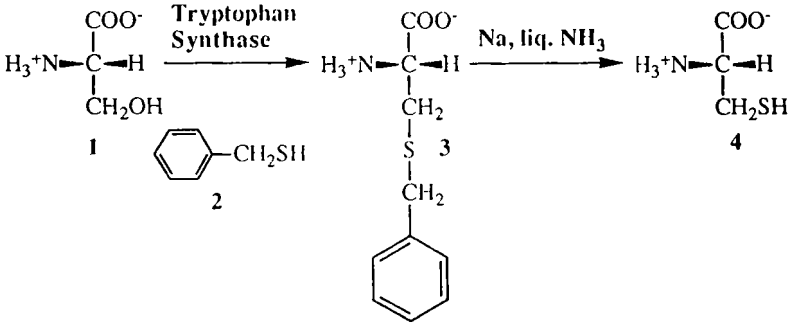
RESULTS AND DISCUSSION

The potential for the tryptophan synthase-catalyzed synthesis of S-benzyl-L-cysteine[2] and Se-benzyl-L-selenocysteine[3] has been recognized by others. In the cell, tryptophan synthase, a pyridoxyl phosphate-requiring enzyme, catalyzes the condensation of indoleglycerol phosphate (InGP) and L-serine to yield L-tryptophan and glyceraldehyde 3-phosphate. Tryptophan synthase effectively catalyzes the α,β-elimination of water from serine, generating a pyridoxyl bound α-aminoacrylate which can serve as a Michael acceptor for nucleophiles[4]. By accepting benzyl mercaptan (2) as a nucleophile, tryptophan synthase catalyzes an overall β-substitution reaction that effectively converts L-serine (1) to S-benzyl-L-cysteine (3). For this synthesis, the enzyme was provided by a genetically engineered strain of *E. coli* which constitutively overproduced tryptophan synthase[5]. Tryptophan synthase was used without purification. Whole cells were incubated in an anaerobic, buffered (pH 7.5) solution that contained isotopically labeled serine and an excess of benzyl mercaptan (2). S-Benzyl-L-cysteine (3) was isolated from the culture filtrate. Incorporation of labeled serine into S-benzyl-L-cysteine (3) was quantitative and proceeded without dilution. S-Benzyl-L-cysteine (3) was deblocked by treatment with sodium in liquid ammonia to yield a mixture of the hydrochloride of L-cysteine (4) and sodium chloride. Under these conditions no racemization of cysteine occurs[6]. Because of its susceptibility to oxidation, L-cysteine (4) was used without purification. Recently we reported the biosynthesis of specifically ^{15}N-, ^{2}H-, and/or ^{13}C-labeled L-serine which, in combination with the procedure outlined above could be used to produce any isotopomer of L-cysteine[1].



METHODS

Chemicals--L-[3-^{13}C]Serine (97.6% enrichment) and L-[3-$^{2}H_2$]serine (98.5% enriched) were prepared biosynthetically as described previously[1].

NMR Methods-- Proton decoupled ^{13}C FT-NMR spectra were obtained at 50.3 MHz using a Bruker AM-200 WB NMR spectrometer. For ^{13}C analysis acquisition parameters were as follows: 10.869 KHz sweep width, 32 K data points, 1.51 s acquisition time, 10 s relaxation delay, 0.663 Hz/pt data point resolution, and 25°C. For analysis of deuterium analysis the aquisition parameters were the same except the decoupler was gated off durng a long relaxation delay (120 s) between pulses and on during data aquisition. This aquisition sequence minimized NOE and relaxation effects that would over estimate the proton content at C-3 of S-benzyl-L-[3-^{2}H]cysteine. S-Benzyl-L-cysteine (100 mg) was dissolved as its hydrochloride in D_2O for NMR analysis. L-Cysteine was dissolved as its zwitter ion in D_2O. Signal intensities were determined by Lorentzian line shape analysis carried out on a MicroVax II using a modified Levenberg-Marquardt algorithm implemented by the NMR1 software package supplied by the National Institutes of Health Resource for NMR Data Analysis (Syracuse, NY). Chemical shifts are reported in ppm downfield from TMS (0 ppm) using dioxane (67.86 ppm) as a internal standard.

Culture Conditions-- A strain of *E.coli* that constitutively overproduces tryptophan synthase was obtained from Professor Charles Yanofsky of Stanford University (Stanford, CA 94305-5020). This organism contains a plasmid, pWS1 that encodes for *E. coli trp* C^+, B^+, A^+ and a chloramphenicol resistance marker; the parent strain, *E. coli* W3310 Δ[*tonB trpBA17*] *his*, produced tryptophan synthase constitutively[5]. The organism was maintained on nutrient agar plates with chloramphenicol (200 μg/L) and cultured in liquid medium that contained the following: NH_4OH (16.75 mM); K_2HPO_4 (10.0 g/L); $NaH_2PO_4 \cdot H_2O$ (2.32 g/L); $MgSO_4$ (98 mg/L); $FeCl_2$ (1.9 mg/L); citric acid (2 g/L); glucose (2 g/L); casamino acids (Difco) (5 g/L); and chloramphenicol (200 μg/L).

S-Benzyl-L-[3-^{13}C]- or S-Benzyl-L-[3-$^{2}H_2$]Cysteine (2)-- Using a sterile loop, the organism was transferred from an agar plate to a 50-mL fluted flask that contained 5 mL of sterile liquid culture medium. The organism was cultured at 37°C on a rotary shaker (200 rpm). After 24 h, the 5 mL culture was diluted into 1.5 L of sterile liquid culture medium in a fermentor at 37°C. The fermentor was oxygenated by bubbling air (1.5 L/m) and stirring

(200 rpm). After 24 h, the cells were harvested from the culture broth by centrifugation. For the production of S-benzyl-L-cysteine, the cells were resuspended in a fermentor that contained labeling medium. The labeling medium was prepared as follows: TRIZMA base (Sigma Chemical Co., tris(hydroxymethyl)aminomethane, 100mM) and KH_2PO_4 (20 mM) were dissolved to their final concentrations and the pH adjusted to 7.5 with 1 M HCl. Cells were incubated in production medium at 20°C with stirring (200 rpm). Helium was bubbled through the cell suspension for 1 h. Then isotopically labeled L-serine (15 mM) and benzyl mercaptan (20 mM) were added. The conversion of L-serine to S-benzyl-L-cysteine was monitored quantitatively using a Beckman model 6300 automated amino acid analyzer. When serine had disappeared from the production medium, oxygen was purged through the cell suspension for several hours to oxidize residual benzyl mercaptan to its insoluble disulfide. Cells and dibenzyl disulfide were removed by centrifugation and discarded. Isotopically labeled S-benzyl-L-cysteine was isolated from the culture broth as described below.

Purification of Labeled S-Benzyl-L-Cysteine-- The culture broth was concentrated using a rotary evaporator, redissolved in water (300 mL), and deionized using a column (5X75 cm) that contained Dowex AG 50-X8 (200-400 mesh) in the H^+ form. After loading the sample, the column was washed with water until chloride was no longer detected in the eluent with $AgNO_3$. S-Benzyl-L-cysteine bound to the column and was eluted with 1.0 M NH_4OH (3.5 L). Column fractions (200 mL) were monitored for free amino groups by their colorimetric reaction with ninhydrin as described previously[1]. Column fractions that gave a positive ninhydrin reaction were monitored for amino acids by quantitative amino acid analysis. Isotopically labeled S-benzyl-L-cysteine was crystallized as its zwitterion from water. The yield of labeled S-benzyl-L-cysteine from labeled L-serine was nearly quantitative (96%).

Characterization of S-Benzyl-L-cysteine-- The enantiomeric purity of labeled S-benzyl-L-cysteine was determined by gas chromatography using a fused silica capillary column (25 meter) with a chiral stationary phase (Chrasil-Val III, Alltech Associates) and flame ionization detection. S-Benzyl-L-cysteine was chromatographed as its N-heptafluorobutyric amide isopropyl ester[7] which was prepared as follows. Sample (1-2 mg) was treated with 2M HCl in n-propanol and heated at 100°C for three hours.

Volatiles were evaporated from the sample at 40°C under a stream of argon. The dried sample was dissolved in 300 µl of N-heptafluorobutyric anhydride (Sigma Chemical Co.) and incubated at room temperature for 30 min. Again, volatiles were evaporated at 40°C under a stream of argon. The sample was dissolved in chloroform for GC analysis. Isotopically labeled S-benzyl-L-cysteine eluted with a retention time (34.2 min) identical to commercial S-benzyl-L-cysteine; no evidence was obtained for the D-isomer. Based on this analysis, preparations of labeled S-benzyl-cysteine contained the L-isomer in enantiomeric excess of >99.5%. Isotopic purities were determined by proton-decoupled ^{13}C NMR; the intensity of ^{13}C satellites on the α-carbon resonance was used to estimate the enrichment of S-benzyl-L-[3-^{13}C]cysteine. Label was incorporated into S-benzyl-L-[3-^{13}C]cysteine (97.6% enrichment) from L-[3-^{13}C]serine (97.6% enrichment) without dilution. The deuterium content at C-3 of deuterium-labeled S-benzyl-L-cysteine was determine from the relative intensities of the five line mutiplet from the $^{2}H_2$ and the triplet from the $^{2}H_1$ species; no evidence for $^{1}H_2$ species was obtained. Based on this spectrum, the S-benzyl-L-[3-$^{2}H_2$]cysteine was >98.2% enriched with deuterium at C-3. Preparations of labeled S-benzyl-L-cysteine were characterized and shown to be pure by quantitative amino acid analysis. Melting points, determined using a Thomas Hoover capillary melting point apparatus, were as follows: commercial S-benzyl-L-cysteine, 215 °C (d);S-benzyl-L-[3-$^{2}H_2$]cysteine, 215 °C (d); S-benzyl-L-[3-^{13}C]cysteine, 214 °C (d). $^{13}C\{^{1}H\}$ NMR: C-1, 171.56; C-α, 53.33; C-β, 31.80; benzyl carbon, 36.72; ring 138.85, 130.34, 130.26, 128.95 ppm; $^{1}J_{C\text{-}\alpha\text{-}C\text{-}\beta}$ = 34.87 Hz. Elem. Anal. Calcd. for S-benzyl-L-[3-^{13}C]cysteine: C, 57.05; H, 6.17; N, 6.60; Found: C, 57.38; H, 6.03; N, 6.39. Elem. Anal. Calcd. for S-benzyl-L-[3-$^{2}H_2$]cysteine: C, 56.32; H, 7.07; N, 6.57; Found: C, 56.01; H, 6.75; N, 6.37.

L-[3-^{13}C]- or L-[3-$^{2}H_2$]Cysteine (2)-- S-Benzyl-L-cysteine (2 g) and a magnetic stir bar were placed in a 100-mL, three-neck round bottom flask fitted with a Dry Ice condenser, a ground glass stopper and a septum. While under positive nitrogen pressure, the reaction flask and condenser were brought to -78 °C with Dry Ice/acetone. Ammonia gas was then condensed into the reaction flask via syringe needle, until 35-40 mL of liquid ammonia had collected. After the S-benzyl-L-cysteine was dissolved, sodium metal was added in small pieces. The addition of sodium to liquid ammonia resulted in a blue color which cleared as the reaction proceeded. Sodium was added until the blue color persisted. Excess sodium was then destroyed by adding a small amount of

ammonium chloride. The reaction flask was removed from the Dry Ice/acetone bath, and the ammonia was allowed to slowly evaporate. Aqueous HCL (1 M) was added until all the remaining solid dissolved. The resulting solution was evaporated to dryness giving a mixture of cysteine • hydrochloride and NaCl. $^{13}C\{^1H\}$ NMR: C-1, 171.95; C-α, 57.45; C-β, 27.3 ppm.

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