Stereoselective Synthesis of Stable Isotope-Labeled L-α-Amino Acids: Biosynthesis of ^{2}H-, ^{13}C-, and ^{15}N-Labeled L-Serines.

John Hanners, Rowena Gibson, Karen Velarde, Judy Hammer, Mark Alvarez, Janet Griego, and Clifford J. Unkefer*.

National Stable Isotopes Resource, Group INC-4, MS C345, Los Alamos National Laboratory, Los Alamos, NM 87545 USA.

SUMMARY

We have developed a stereospecific biosynthesis of ^{13}C- and ^{15}N-labeled L-serine which involves the serine-type methylotroph *Methylobacterium extorquens* AM1. In this biosynthesis, C-3 of serine is derived from methanol while C-2, C-1 and the α-amino group are derived from glycine. By starting with the appropriate labeling precursor, we can produce any of the ^{2}H, ^{13}C and/or ^{15}N isotopomers of L-serine. Using a 5-L culture, 40-50 millimoles of L-serine are produced; L-serine and glycine are recovered from the growth medium and then separated chromatographically.

Keywords: L-[1-^{13}C]Serine, L-[2-^{13}C]Serine, L-[3-^{13}C]Serine, L-[2,3-$^{13}C_2$]Serine, L-[3-$^{2}H_2$,^{13}C]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 then resolved using hog kidney acylase. We are developing strategies for the stereoselective synthesis of specifically labeled L-α-amino acids. We report here a biosynthesis of L-serine [(*S*)-2-amino-3-hydroxypropanoic acid] which uses the serine-type methylotrophic bacterium, *Methylobacterium extorquens* AM1. This organism contains large amounts of the enzymes methanol dehydrogenase and serine hydroxymethyltransferase[1]. Methanol dehydrogenase catalyzes the oxidation of

* To whom correspondence should be addressed. Ph. (505)665-2560, FAX (505)665-3166.

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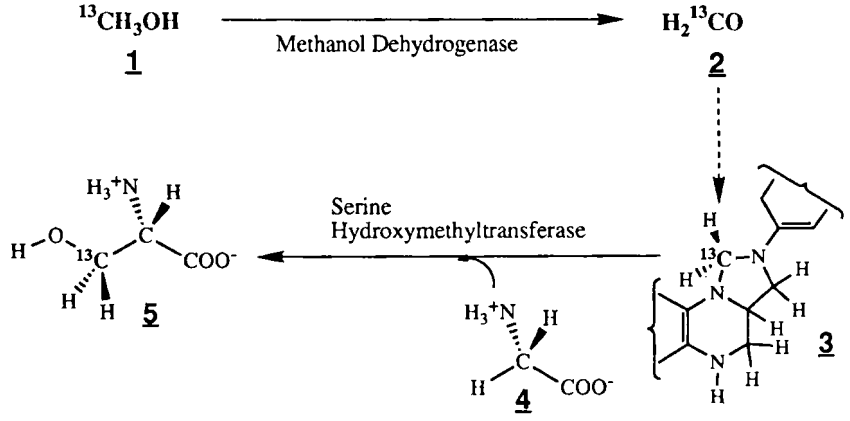
methanol (1) to formaldehyde (2); serine hydroxymethyltransferase catalyzes the stereospecific aldol condensation of formaldehyde (2), as methylenetetrahydrofolate (3), with glycine (4) to produce L-serine (5). In this process C-3 of serine is derived from methanol; C-1, C-2 and the α-amino group of serine are derived from glycine. Therefore, by starting with the appropriate labeled precursors, we can synthesize most of the ^{2}H, ^{13}C, and/or ^{15}N isotopomers of L-serine.

L-serine is an important amino acid because it can serve as a precursor for the synthesis of more complex amino acids; the stereochemistry at the α-carbon produced during the biosynthesis of serine can be retained in the product amino acid. Vederas and coworkers[2,3] have described the synthesis of the N-urethane protected β-lactone of L-serine; the β-lactone can serve as a template for homologation reactions at the β-carbon. Treatment of the L-(N-*t*-boc)-serine-β-lactone with a series of nucleophiles yields α-amino acids with retention of configuration at the α-carbon. In addition, L-serine can serve as substrate for a number of pyridoxal enzymes, which can be used for the net synthesis of aromatic amino acids[4-6]. As will be reported elsewhere, we have used this approach to convert ^{13}C-labeled serine into L-aspartate, L-tryptophan, L-tyrosine, and L-cysteine.



RESULTS and DISCUSSION

D,L-Serine, has been produced by the condensation of formaldehyde with either diethyl acetamidomalonate[7] or copper glycinate[8]; labeled formaldehyde or labeled glycine could be used in this scheme to provide many isotopomers of serine. In addition to the requirement for the resolution of the enantiomers, this procedure is inconvenient because it uses formaldehyde as a precursor for C-3 of serine. The method reported here

produces serine stereospecifically and uses methanol, a convenient starting material, as the precursor of C-3 of serine.

Serine-type methylotrophic bacteria can use methanol as their sole source of carbon and energy[1]. They derive their energy from the methanol dehydrogenase-catalyzed oxidation of methanol to formaldehyde, and assimilate carbon via the serine hydroxymethyltransferase-catalyzed condensation of an equivalent of formaldehyde with glycine to form serine. Because they possess high concentrations of these two enzymes, serine-type methylotrophs have been recognized for their potential for the net synthesis of L-serine[9,10]. Our experiments confirmed that when incubated at pH=8.0 and in the presence of glycine and methanol, stationary cultures of *M. extorquens* AM1 accumulate serine in their culture medium[9]. Under these conditions, most of the methanol was oxidized to carbon dioxide; therefore, the yield from methanol into serine was quite low. Methanol is oxidized to carbon dioxide by *M. extorquens* AM1 in three enzymatic steps; as discussed above, methanol dehydrogenase oxidizes methanol to formaldehyde, which is then oxidized to formate by formaldehyde dehydrogenase. Finally formate dehydrogenase oxidizes formate to carbon dioxide.

In addition to the poor yield from methanol we found that under these conditions, label from [^{13}C]methanol (99.7% ^{13}C) was diluted when incorporated into C-3 of serine (85-92% ^{13}C). This dilution comes from C-2 of natural abundance glycine, as demonstrated in experiments using [2-^{13}C]glycine or [1-^{13}C]glycine to produce labeled serine. This dilution is caused by the action of glycine decarboxylase, which results in the donatation of C-2 of glycine directly to the one-carbon pool as methylene tetrahydrofolate, the substrate of serine transhydroxylmethylase. While glycine decarboxylase is not normally expressed by *M. extorquens* AM1 during growth on methanol[11,12], its expression may be induced during the long incubation periods in the presence of high concentrations of glycine.

The problems of loss of [^{13}C]methanol via oxidation to [^{13}C]carbon dioxide and dilution of label from [^{13}C]methanol were partially overcome by altering conditions during serine production. First, boric acid was added to the culture medium with the glycine. Borate, which inhibits formate dehydrogenase[13], slows the oxidation of formate to carbon dioxide and thus conserves labeled methanol. Also the expression of glycine

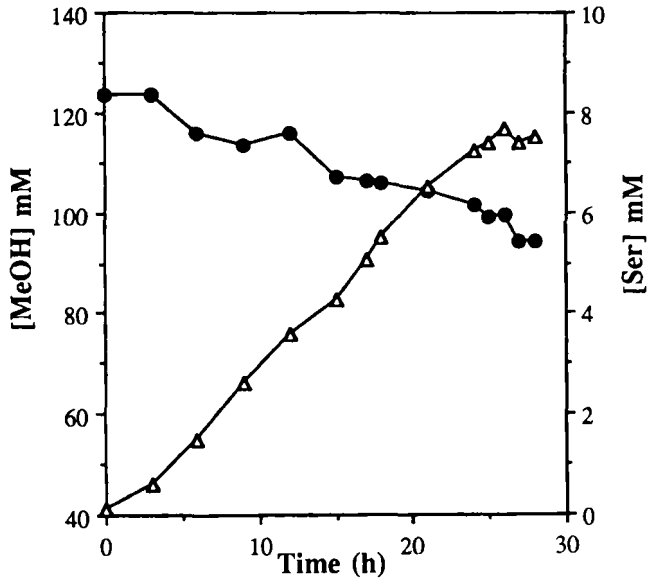


Figure 1. Production of L-[3-^{13}C]serine using *Methylobacterium extorquens* AM1. [^{13}C]Methanol and glycine were added at 0 h; methanol (•) and serine (Δ) concentrations were monitored as described in the Methods.

decarboxylase is inhibited by the addition of borate. In addition, the temperature of the fermentor was lowered to 20°C during serine production; this minimized loss of serine by inhibiting further growth of *M. extorquens* AM1 and by inhibiting possible contamination of the fermentor by other organisms. When incubated with glycine and [^{13}C]methanol, stationary cultures of *M. extorquens* AM1 maintained at 20°C in alkaline culture medium (pH=8.0) that contained borate (100 mM) produced L-[3-^{13}C]serine; label was incorporated from [^{13}C]methanol into the C-3 of serine without significant dilution. During the production phase of the fermentation, methanol concentration was monitored by gas chromatography and serine concentration was monitored by amino acid analysis. A profile of methanol utilization and serine production is plotted in Figure 1. The serine concentration increases linearly for approximately 24 h to a steady state concentration of 7.5 to 10 mM. At this time the optimal yield of serine from [^{13}C]methanol is achieved. Unused [^{13}C]methanol can be recovered from the culture medium by fractional distillation. Longer incubation times resulted in further loss of labeled methanol with no net increase in the serine concentration. Decreasing the the starting concentration of labeled methanol (125 to 12.5 mM) significantly decreased the total amount of serine (0.03 mM) produced.

TABLE 1
Isotopomers of Serine Biosynthesized using *M. extorquens* AM1

Isotopomer of Serine	[a]Labeled Starting Material	[b]Isotopic Purity of Serine	% Yield of Isotope in Serine	[c]Total Recovery of Isotope
[3-^{13}C]	[^{13}C]Methanol	97.6%	31%	78%
[2-^{13}C]	[2-^{13}C]Glycine	99%	47%	91%
[1-^{13}C]	[1-^{13}C]Glycine	99%	45%	88%
[2,3-$^{13}C_2$]	[^{13}C]Methanol,	99%	29%	75%
	[2-^{13}C]Glycine	99%	44%	76%
[3-$^{2}H_2$,^{13}C]	[$^{2}H_4$,^{13}C]Methanol	97% ^{13}C 98% ^{2}H	32%	75%
[^{15}N]	[^{15}N]Glycine	97%	44%	87%

[a]Enrichment of starting materials : ^{13}C, 99.2%; ^{2}H; 98%; ^{15}N, 98 %. [b]Isotopic purity of products were determined by NMR as described in the Methods. [c]Total recovery of isotope is the sum of the labeled products and labeled starting materials recovered.

By starting with labeled glycine, we have produced other isotopomers of L-serine (Table 1). During production of serine from labeled glycine, glycine was used as a limiting reagent. Because glycine decarboxylase activity is not expressed in the presence of borate, there are no other pathways for the bacterium to degrade glycine; therefore, the total yield of label in serine is typically greater from glycine (44-47%) than from methanol (29-31%).

METHODS

Chemicals-- [^{13}C] and [$^{2}H_4$,^{13}C]methanol were prepared by the reduction of [^{13}C]carbon monoxide[14] using the apparatus described by Ott and coworkers[15]. The [1-^{13}C], [2-^{13}C], and [1,2-$^{13}C_2$]acetic acids were prepared by the ruthenium-catalyzed carbonylation of methanol[16]. Carbon-13 Labeled glycines[17] were prepared from the corresponding bromoacetic acids[18]. [^{15}N]glycine[19] was prepared from potassium [^{15}N]phthalimide[20].

NMR Methods--Proton and proton decoupled ^{13}C FT-NMR spectra were obtained at 300.13 and 50.3 MHz respectively using Bruker (WM-300 WB and AM-200 WB) NMR spectrometers. Acquisition parameters were as follows: ^{1}H NMR --- 3 KHz sweep width, 32 K data points, 5.11 s acquisition time, 0.196 Hz/pt data point resolution, 256 scans, and 25°C. Proton decoupled ^{13}C NMR --- 10.869 KHz sweep width, 32 K data points, 1.51 s acquisition time, 5 s relaxation delay, 0.663 Hz/pt data point resolution, 1024 scans, and

25°C. Because of relatively short relaxation times and Nuclear Overhauser Enhancement, ^{13}C NMR specta obtained using normal acquisition parameters grossly over-estimated the relative concentration of protonated species in samples of L-[3-$^{2}H_2$,^{13}C]serine. Therefore, ^{13}C NMR specta of L-[3-$^{2}H_2$,^{13}C]serine were obtained with the ^{1}H-decoupler gated on during the acquisition and gated off during a long delay (60 s) between acquisitions; this acquisition sequence is designed to yield a proton decoupled spectrum with minimum NOE; in addition, the long delay will minimize intensity differences due to partial saturation of deuterated species. Samples (100 mg) were dissolved as zwitterions in $^{2}H_2O$ for ^{13}C NMR analysis. Samples (50 mg) for ^{1}H NMR were dissolved in $^{2}H_2O$ (pH = 11.0) or converted to their N-acetylated derivative and dissolved in [$^{2}H_4$]methanol; in all cases the spectrometer was locked on the deuterium signal. 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 external TMS=0 ppm.

Culture Conditions--M. extorquens AM1 (*Pseudomonas* AM1, ATCC 14718) cultures were maintained on agar plates that contained the following: KH_2PO_4 (1.0 g/L); K_2HPO_4 (2.12 g/L); $CH_3NH_2 \cdot HCl$ (6.75 g/L); $MgSO_4 \cdot 7H_2O$ (100 mg/L); $CaCl_2$ (10 mg/L); $FeSO_4 \cdot 7H_2O$ (10 mg/L); $MnSO_4 \cdot H_2O$ (0.5 mg/L); $Na_2MoO_4.2H_2O$ (0.5 mg/L); and Bacto agar (Difco) (15 g/L). The organism was cultured in standard liquid medium[21] using methanol (5 ml/l) as the carbon source; the medium contained the following: $(NH_4)_2SO_4$ (0.2 g/L); NH_4Cl (1.6 g/L); Na_2HPO_4 (3.0 g/L); KH_2PO_4 (2.72 g/lL); $MgSO_4$ (98 mg/L); $CuSO_4$ (70.0 mg/L); $MnSO_4 \cdot H_2O$ (35.0 mg/L); $ZnCl_2$ (24.0 mg/L); $CaCl_2$ (1.0 mg/L); $CoCl_2$ (18.0 mg/L); H_3BO_4 (7.0 mg/L); $(NH_4)_6Mo_7O_{24}$ (60.0 mg/L); $FeSO_4$ (6.0 mg/L); and citric acid (600 mg/L). The organism was transferred from the agar plate to a 1-L fluted flask that contained 200 ml of the liquid medium and was cultured at 30°C on a rotary shaker (200 rpm). After 48 h, the culture was diluted (7 mL/200 mL) into fluted flasks and again allowed to grow for 48 h; these flasks provided an inoculum for the fermentor (10 vol%). *M. extorquens* AM1 was cultured at 30 °C in liquid medium in commercial fermentors (1-L, 5-L, or 20-L,); during the growth phase, the pH was maintained at 6.8 with the addition of NaOH (1 M), and cultures were aerated by bubbling air (1.5 L/m) and stirring

(200 rpm). Methanol in the culture medium was monitored by gas chromatography as follows. A sample (1 mL) was removed from the fermentor and centrifuged to remove the bacteria. The supernatant (20 μL) was diluted with water (180 μL) that contained ethanol (56 μl ethanol/100 mL H_2O) as an internal standard. The sample (1μL) was injected on to a Porapac QS column (Alltech Associates, 80-100 mesh, 2 mm ID X 4 ft, glass) at 128 °C and monitored using a flame ionization detector. When the methanol was exhausted from the medium (24-48 h, ~4.0 OD @ 560 nm), the culture was prepared, as described below, for the production of serine.

Biosynthetic Production of L-[3-^{13}C] or L-[3-$^{2}H_2$,^{13}C]Serine--In order to deplete intracellular metabolite pools of natural abundance carbon-13 intermediates, *M. extorquens* AM1 cultures which had exhausted the methanol from their growth medium were incubated under growth conditions for 4 h (pH = 6.8, 30 °C, 1.5 L/m air bubble rate, and 200 rpm stir rate.) Following the 4 h incubation, the temperature of the culture was lowered to 20 °C. Then boric acid (6.2 g/L) and glycine (10 g/L) were added to the culture medium as solids, and the pH of the medium was adjusted to pH 8.0 with the addition of NaOH (1 M). The production of L-serine was initiated with the addition of [^{13}C]methanol (4 g/L), and the serine concentration in the medium was monitored as follows. Samples (1 mL) were removed from the fermentor and centrifuged to remove cells; the supernatant was analyzed for serine using a Beckman model 6300 automated amino acid analyzer. When the serine concentration reached a maximum (24-72 h), the cells were removed from the culture medium by centrifugation (8000 X G) to produce a clarified culture medium. L-[3-^{13}C]Serine was recovered from the clarified medium. L-[3-$^{2}H_2$,^{13}C]Serine was produced by simply substituting [$^{2}H_4$,^{13}C]methanol for [^{13}C]methanol.

Biosynthetic Production of L-Serine Labeled from Glycine--L-[1,^{13}C], L-[2-^{13}C], L-[α-^{15}N]Serine, and were produced from the corresponding labeled glycines by modification ot the procedure outlined above. In these incubations the starting concentration of labeled glycine was lowered (0.5 to 2 g/L) so that glycine was the limiting reagent. Both the product (labeled serine) and the remaining starting material (labeled glycine) were recovered from the culture medium. L-[2,3-$^{13}C_2$]serine was produced by using both [^{13}C]methanol (4 g/L) and [2-^{13}C]glycine (2 g/L) during the production phase of the fermentation.

Purification of L-Serine--The clarified culture broth was concentrated *in vacuo* using a rotary evaporator; labeled methanol was recovered from the condensate by fractional distillation. The concentrated medium was dissolved 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 $AgNO_3$. L-Serine and glycine bound to the column and were 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 follows. The sample (20 µL) was spotted on paper (Whatman 3 mm), dried with a heat gun and then sprayed with a ninhydrin solution (0.5% in 1-butanol). The colorimetric reaction was developed at 100 °C. Column fractions that gave a positive ninhydrin reaction were pooled and concentrated *in vacuo* to remove water and ammonia. L-Serine and glycine were then separated by column (130X160 cm) chromatography on Dowex AG 50-X8 (200-400 mesh) in the H^+ form[22]. The sample was dissolved in water (75 mL) and loaded on the column; L-serine and glycine bound to the resin and were eluted with 0.85 M HCl. Column fractions (10 mL) were collected and samples (100 µL) removed for analysis. The samples were dried in a vacuum oven at 100 °C, redissolved in water (100 µL) and assayed using the ninhydrin spot test. Ninhydrin-positive fractions were analyzed for amino acids using a Beckman model 6300 automated amino acid analyzer. Fractions that contained serine (130 to 160) and glycine (240 to 310) were pooled separately and dried *in vacuo* to remove water and HCl. Serine·HCl and glycine·HCl were converted to their zwitterionic forms as follows. The hydrochloride was dissolved in water and applied to a Dowex AG 50-X8 H^+(200-400 mesh) column. After loading the sample, the column was washed with water until chloride was no longer detected in the eluent. The zwitterionic form of the amino acids was eluted from the column with 1.0 M NH_4OH and recovered from the eluent by rotary evaporation of water and ammonia. Serine and glycine were crystallized as their zwitterions from ethanol.

Characterization of L-Serine--The enantiomeric purity of labeled serines was determined by gas chromatography using a fused silica capillary column (25 meter) with a chiral stationary phase (Chrasil-Val III, Alltech Associates). Serine was chromatographed as its N-pentafluoropropionyl amide isopropyl ester[23] and monitored using a flame ionization detector. Labeled serine eluted with a retention time (13.5 m) identical to

authentic L-serine; no evidence was obtained for the D isomer (retention time 13.0 m). Based on this result and the optical rotation (L-[3-^{13}C]serine, $[\alpha]_D$ = -6.85 (0.505 g in 5 ml H_2O, lit $[\alpha]_D$ = -6.83), preparations of labeled serine contained the L-isomer in enantiomeric excess of >99.5%. Isotopic purities reported in Table 1 were determined by ^{1}H and proton decoupled ^{13}C NMR; the intensity of ^{13}C satellites on the α- or β-proton resonances was used to estimate the enrichment of L-[2-^{13}C] and L-[3-^{13}C]serine, respectively. The ^{13}C or ^{15}N satellites on the natural abundance ^{13}C resonance of C-2 were used to estimate the enrichment of L-[1-^{13}C] and L-[2-^{15}N]serine. Deuterium content of L-[3-$^2H_2$,^{13}C]serine was determined ^{13}C NMR. Preparations of labeled serine were characterized and shown to be pure by quantitative amino acid analysis. $^{13}C\{^1H\}$: (δ ^2H_2O pH =7.0: C1, 173.1; C2, 57.4; C3, 61.3; ^{1}J Hz: C1-C2, ; C2-C3,). Elem. Anal. Calcd. for L-[3-$^2H_2$,^{13}C]serine: C, 34.25; H, 8.39; N, 12.96; Found: C, 34.30; H, 8.87; N, 13.18. Calcd. for L-[3-^{13}C]serine: C, 34.90; H, 6.60; N, 13.20; Found: C, 35.13; H, 6.75; N, 13.44. Calcd. for L-[2-^{13}C]serine: C, 34.90; H, 6.60; N, 13.20; Found: C, 34.87; H, 7.32; N, 13.55.

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