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Stereoselective Synthesis of Stable Isotope Labeled L-α-Amino Acids: Synthesis of L-[4-^{13}C] and L-[3,4-$^{13}C_2$]Aspartic Acid

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SUMMARY

We have developed a stereoselective route to isotopically labeled L-aspartic acid using L-serine as a chiral precursor. Labeled serine, prepared biosynthetically was N-protected by conversion to the N-*t*-Boc derivative. (N-*t*-Boc)-[3-^{13}C]Serine is cyclized to its β-lactone which was treated with potassium [^{13}C]cyanide to yield L-β-[3,4-$^{13}C_2$]cyanoalanine. Hydrolysis of the cyanoalanine yielded L-[3,4-$^{13}C_2$]aspartic acid. Similarly, L-[4-^{13}C]aspartate was produced from L-serine and K^{13}CN. Using this route, the L-enantiomer was produced in 96% excess.

Keywords: L-[4-^{13}C]Aspartic Acid, L-[3,4-$^{13}C_2$]Aspartic Acid, and L-[3-^{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 have been resolved using hog kidney acylase. We are developing strategies for the stereoselective synthesis of specifically labeled L-α-amino acids in which 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. For example, Vederas and coworkers[2-4] have described the synthesis of the N-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

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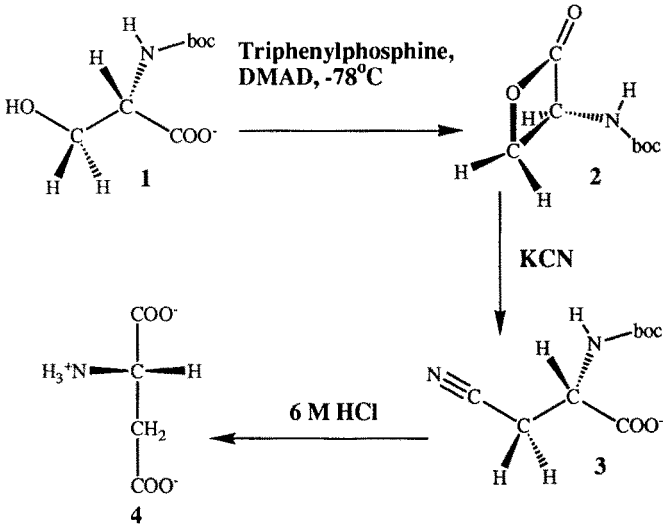
retention of configuration at the α-carbon. We have used this strategy to produce labeled L-aspartic acid from L-serine.

RESULTS AND DISCUSSION

Isotopically labeled aspartic acid was prepared using the synthetic route diagrammed below. Using a modification of the procedure developed by Vederas and coworkers[2-4], L-(N-*t*-Boc)-[3-^{13}C]serine (**1**) was cyclized by treatment with triphenylphosphine (Ph_3P) and dimethyl azodicarboxylate (DMAD) at -78 °C to yield the L-(N-*t*-Boc)-[3-^{13}C]serine-β-lactone (**2**). After isolation from the cyclization reaction mixture by flash chromatography, the β-lactone was, in our hands, prone to oligomerization. We found that this oligomerization reaction was initiated by traces of Ph_3P present in the β-lactone preparations. To assure that all of the Ph_3P was consumed in the Mitsunobu cyclization reaction, we modified the Vederas procedure by adding an excess of DMAD. The excess DMAD resulted in stable preparations of the β-lactone. Using a 1.1 molar excess of DMAD with respect to Ph_3P, we routinely obtained yields of either the L-(N-CBZ)- or L-(N-*t*-Boc)-serine-β-lactone (50%) that are essentially the same as those reported by Vederas[2]; higher yields (70%) were obtained in small scale preparations.



Our plan was to use cyanide as the nucleophile and open the L-serine- β-lactone stereoselectively to yield L-β-cyanoalanine which could be hydrolyzed to yield L-aspartic acid. Unfortunately, we were unable to duplicate the high enantiomeric selectivity and high yields reported by Vederas and coworkers. We tried many permutations using KCN,

KCN/18-crown-6, Bu_4NCN, LiCN, NaCN, and TMS-CN as nucleophiles and either L-(N-*t*-Boc)-serine β-lactone or L-(N-CBZ)-serine-β-lactone or ammonium salts of the serine-β-lactone as the substrate. In addition, the reaction was carried out in a number of solvents including DMSO, DMF, THF, MeCN, benzene, and N-methyl-2-pyrrolidinone. We ran the reactions at several temperatures (-15°C to 75°C) and using a variety of addition schemes. In all cases, we experienced difficulty in stabilizing solutions of the β-lactone, and could not prevent side reactions which likely resulted in the oligomerization of serine and/or the formation of α-amino acrylic acid. Oligomerization to the serine polyester resulted in low yields of β-cyanoalanine. Because α-amino acrylate serves as a Michael acceptor and reacts with cyanide to yield racemic β-cyanoalanine, its formation would result in lower enantiomeric selectivity. Indeed both the Cbz- and Boc-protected serine-β-lactones oligomerized in DMSO and DMF even in the absence of added nucleophile. Eventually we found that the addition of dialkyl azodicarboxylate stabilized solutions of L-(N-*t*-Boc)serine-β-lactone.

A solution of L-(N-*t*-Boc)-[3-^{13}C]serine-β-lactone (**2**) and dialkyl azodicarboxylate in DMSO was added to a solution of potassium [^{13}C]cyanide in DMSO to yield L-β-[*cyano*,3-$^{13}C_2$]cyanoalanine (**3**). Acid hydrolysis of the crude β-cyanoalanine yielded L-[3,4-$^{13}C_2$]aspartic acid (**4**) which was purified by ion exchange chromatography. Using this scheme, L-[3,4-$^{13}C_2$]aspartic acid (**4**) was produced with an enantiomeric excess of 96% and in overall yield of 13.3% based on L-(N-t-Boc)-[3-^{13}C]serine, or 26.6% based on K^{13}CN. Similarly, L-[4-^{13}C]aspartate was produced from L-serine and K^{13}CN.

Materials and Methods

NMR Methods- Proton-decoupled ^{13}C FT-NMR spectra were obtained at 50.3 MHz using a Bruker AM-200 WB NMR spectrometer. Acquisition parameters were as follows: 10.869 KHz sweep width, 16 K data points, 0.75 s acquisition time, 10 s relaxation delay, 0.663 Hz/pt data point resolution, and 25°C. Isotopically labeled aspartic acid (100 mg) was dissolved as its hydrochloride salt in D_2O. For the determination of isotopic enrichments, 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 an internal standard.

Chemicals- Potassium [^{13}C]cyanide[5,6] was provided by the National Stable Isotopes Resource and was prepared from [^{13}C]methane as described previously[7]. L-[3-^{13}C]serine was prepared biosynthetically as described previously[1].

Dimethyldiazodicarboxylate (DMAD)- An attractive method for the preparation of dialkyl diazodicarboxylates by N-bromosuccinimide (NBS) oxidation of the corresponding hydrazide has been described[8]. Based on this report we developed the following procedure for the preparation of DMAD. The stable hydrazide precursor was prepared in high yield as reported[9]. It is prudent to make large amounts of this stable precursor and use the NBS oxidation to make DMAD as needed, rather than store large amounts of this unstable material. The procedure, described below, was successfully used for 1 to 37 g scale preparations of DMAD. A mixture of of pyridine (22.65 g, 286 mmol) and of NBS (52.11 g, 293 mmol) in 600 mL of methylene chloride was prepared. The NBS was only partially dissolved before the next step. To this magnetically stirred suspension, 43.0 g (291 mmol) of methylhydrazodicarboxylate[9] was added in one portion. A deep red-orange color developed immediately. The reaction mixture was stirred at room temperature for 30 minutes, during which time all solids dissolved. The red-orange solution was exacted with four 600-mL portions of distilled water, dried with magnesium sulfate, filtered, and evaporated using a rotary evaporator. With an explosion safety shield in place, the residual red-orange oil was distilled under reduced pressure (~1mm Hg pressure, 40 °C, lit. bp 90-91 °C at 15mm Hg[8]). It is necessary to distill DMAD at low pressure to minimized the danger of explosion[9]. The yield of DMAD was 86.7% (36.30 g, 252 mmol).

L-(N-*t*-Boc)-[3-^{13}C]Serine (1)- L-[^{13}C]Serine was converted to its N-*tert*-butyloxycarbonyl derivative (1) by treatment with BOC-ON™ (Aldrich Chemical Co.). A mixture consisting of L-[3-^{13}C]serine (3.46 g, 32.6 mmol), triethylamine (6.85 mL), BOC-ON™ (8.83 g, 35.85 mmol), 20 mL of dioxane and 20 mL of water was stirred at room temperature. The initially turbid yellow reaction mixture turned clear yellow after 30 min. The solution was stirred an additional 2.5 hr. Then 50 mL of water was added, and the resulting mixture was extracted with two 75-mL portions of ethyl acetate. After cooling in an ice bath, the aqueous phase was acidified to approximately pH 2 with cold HCl (12 M).

The cool, acidified solution was extracted three times with ethyl acetate (100 mL). The organic phases were pooled and dried with magnesium sulfate, filtered and evaporated by rotary evaporation to yield 6.38 g (31 mmol crude) of clear viscous oil. Compound **1** (4.34 g, 21 mmol, 64%) was obtained as white crystals from ethyl acetate/hexane. The mother liquor was evaporated, and the residue was placed in a desiccator over phosphorus pentoxide and stored at reduced pressure overnight. The crude residue (0.67 g, 4 mmol, 11%), a slightly-yellow tinted solid, was combined with the crystalline product and used as described below for the preparation of **2**. ^{13}C NMR ($CDCl_3$) C1, 173.61; C2, 55.36; C3, 62.84; Boc 156.2, 80.48, 28.2.; $^1J_{C3\text{-}C2}$ 37 Hz

L-(N-*t*-Boc)-, and L-(N-CBZ)-Serine-β-lactone (2)- As discussed above, this preparation is a modification of that reported by Vederas[2]. Triphenylphosphine (Ph_3P) (6.57 g. 25 mmol) was dried overnight in a vacuum dessiccator over phosphorus pentoxide. The dried Ph_3P was dissolved in 170 mL of anhydrous THF (SurSeal™, Aldrich Chemical Co.) and placed in a 500-mL, 3-neck flask that was fitted with a mechanical stirrer, an addition funnel, and a gas inlet. While being flushed with a slow stream of argon, the reaction flask was cooled to -78°C with a dry ice/acetone bath. Freshly prepared DMAD (4.02 g, 27.5 mmol) was dissolved in 5 mL of anhydrous THF, and this solution was added dropwise to the cold stirring Ph_3P solution over 12 min. The resulting solution was stirred at -78°C for an additional 10 min; a precipitate began to appear after about 2 min into this period. Compound **1** (5.16 g, 25.0 mmol) was dissolved in 40 mL of anhydrous THF and added dropwise over 30 min to the cold Mitsunobu complex. The slurry was stirred an additional 20 min at -78°C, followed by removal of the bath. Stirring was continued at room temperature for 2.5 h. As the reaction mixture warmed up, its turbidity cleared to a pale yellow solution. At the end of this time, the THF was removed by rotary evaportation, leaving a viscous yellow oil. The oil was diluted with an equal volume of ethyl acetate and loaded on a flash chromatography column that contained 125 g of Silica Gel (Merck, grade 60, 230-400 mesh). The β-lactone (**2**) was eluted using ethyl acetate:hexane (15:85 v/v) as the mobile phase. After the first six fractions (125 mL), a number of ~30 mL fractions were collected. Fractions that contained the β-lactone (**2**) were identified by TLC analysis (ethyl acetate:hexane, 15:85); characteristically, the β-lactone gave a yellow color with bromocresol green spray[2]. Fractions that contained **2** (7-26) were pooled and the solvent evaporated, leaving 2.59 g

of white solid (13.8 mmol, 55%). Occasionally, not all of the DMAD was removed during flash chromatography, as evidenced by the pale yellow color of the early flash fractions. The yellow colored DMAD could be removed from **2** *in vacuo*. The lactones isolated from the flash chromatography fractions were used without further purification. Commercial N-carbobenzoxy-L-serine (Sigma Chemical Co.) was lactonized using the same procedure. Melting points: L-(N-CBZ)-serine-β-lactone 130.4-131.4°C(d) (lit. 133-134°C[5]); L-(N-*t*-Boc)-serine-β-lactone 119.2-120.0°C(d) (lit. 119.5-120.5°C[5]). $^{13}C\{^1H\}$ NMR L-(N-CBZ)-serine-β-lactone C1, 168.69; C2, 59.63; C3, 67.82; CBZ 155.20, 135.41, 128.6, 128.56, 128.375, 66.34; L-(N-*t*-Boc)-[3-^{13}C]serine-β-lactone ($CDCl_3$): C1, 169.21; C2, 59.44; C3, 66.56; Boc 154.45, 81.40, 28.14; $^1J_{C3\text{-}C2}$ 33.2 Hz.

L-[4-^{13}C]Aspartic Acid (4)- Labelled K^{13}CN (99+% ^{13}C) (6.72 g, 102 mmol) was ground in an agate mortar. The resulting fine powder was added to 400 mL DMSO (fresh SurSeal™, Aldrich Chemical Co.) in a 2-L flask and stirred for 24 hr under a dry argon atmosphere. N-CBZ-L-serine-β-lactone (20.43 g, 92.4 mmol) and redistilled diethyldiazodicarboxylate (DEAD) (Sigma Chemical Co., DMAD could also be used) (16.10 g, 92.4 mmol) were dissolved in 100 mL DMSO. While under an argon atmosphere and stirring, the K^{13}CN/DMSO slurry was cooled to 18-19°C. The L-(N-CBZ)-serine-β-lactone/DEAD solution was added dropwise over 100 min. After addition was complete, the dark orange reaction mixture was stirred an additional 15 min. At the end of this time, the reaction mixture was frozen and lyophilized for 42 h to remove the DMSO. Water (500mL) was added to the resulting dark tar. The resulting turbid yellow mixture was extracted once with 500 mL of diethyl ether, followed by 2X250 mL portions of diethyl ether. The aqueous phase was acidified with concentrated HCl. A brown oil came out of solution, and was taken up in 2x250 mL of methylene chloride. The pooled extract was dried with magnesium sulfate and the solvent evaporated, leaving an orange oil. The flask was connected through a trap to a vacuum pump, and volatiles were removed at reduced pressure until the pressure stabilized (0.2 mm Hg), leaving a clear red-amber residue (22.64 g). The residue was refluxed in 100 mL of 6M HCl for 10 h. The turbid yellow reaction mixture was cooled and extracted with 2x50 mL methylene chloride. The clear yellow aqueous phase was evaporated (rotary evaporation), leaving 21.0 g of a yellow viscous oil. L-[4-^{13}C]Aspartate was recovered from this oil by chromatography on Dowex 1 in the acetate form. Aspartate bound to the column and was eluted with acetic acid (1 M).

Column fractions (30 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 were monitored for amino acids by quantitative amino acid analysis. The aspartic acid was converted to its zwitterion by chromatography on Dowex 50 H^+; the aspartic acid bound to the resin and was eluted with ammonia (1 M). Fractions that contained aspartate were pooled and solvents evaporated (rotary evaporator). L-Aspartic acid was crystallized from water. L-[4-^{13}C]aspartate (3.55 g, 26.7 mmol) was obtained in 26.2% yield based on the labelled $K^{13}CN$. $^{13}C\{^1H\}$ NMR (D_2O): C1, 172.05; C2, 50.15; C3, 34.64; C4, 174.12; $^1J_{C4\text{-}C3}$ 55.5 Hz.

L-[3,4-$^{13}C_2$]Aspartic Acid (4)- A 250-mL flask was charged with a magnetic stirring bar, 50 mL of fresh anhydrous DMSO (SurSeal™, Aldrich Chemical Co.), and finely ground (agate mortar) $K^{13}CN$ (0.991 g, 15.0 mmol) and sealed with a rubber septum. The suspension was dissolved by stirring under an argon atmosphere for 2 h. Using a syringe pump, a solution containing L-(N-*t*-Boc)-[3-^{13}C]serine-β-lactone (2.57 g, 13.6 mmol) (2) and freshly prepared DMAD (1.98 g, 13.6 mmol) in DMSO (10 mL) was added over 2.25 h to the rapidly stirred cyanide solution. At the end of this time, the reaction mixture was transferred to a 500 mL flask, frozen and lyophilized overnight to remove the DMSO. After lyophilization, 60 mL of water was added to the dark viscous residue, resulting in a turbid yellow mixture which was extracted 3X with 50 mL portions of diethyl ether. The aqueous phase was carefully acidified with HCl (12 M), giving a orange-brown oil which was extracted 2X with 50 mL portions of methylene chloride. The pooled organic phases were dried with magnesium sulfate. After filtration and removal of the solvent, 2.58 g of a glassy solid was obtained. The solid was refluxed in 30 mL of 6M HCl for 6 hr. The volatiles were removed under reduced pressure (rotary evaporator) and the residue was purified by chromatography as described above. L-[3,4-$^{13}C_2$]Aspartic acid was prepared in overall yield of 26.6% based on $K^{13}CN$ or 29.3% based on L-(N-*t*-Boc)-[3-^{13}C]serine-β-lactone. $^{13}C\{^1H\}$ NMR (D_2O): C1, 172.05; C2, 50.24; C3, 34.73; C4, 174.12; $^1J_{C4\text{-}C3}$ 55.5 Hz; $^1J_{C3\text{-}C2}$ 38.0 Hz.

Characterization of Labeled L-Aspartic Acid- The L-isomer of aspartic acid was produced in enantiomeric excess of 96% as determined by gas chromatography using a

fused silica capillary column (25 meter) with a chiral stationary phase (Chirasil-Val III, Alltech Associates). Aspartate was chromatographed as its N-heptafluorobutyric amide diisopropyl ester[10] and monitored using a flame ionization detector. The L-enantiomer (17.9 m) eluted after the D-enantiomer (17.5 m). Isotopic purities were determined by ^{1}H and proton decoupled ^{13}C NMR; the intensity of ^{13}C satellites on the β-proton resonances was used to estimate the enrichment at C-3 of L-[3,4-$^{13}C_2$]aspartate. The ^{13}C satellites on the ^{13}C resonance of C-3 were used to estimate the enrichment at C-4 of L-[4-^{13}C] and L-[3,4-$^{13}C_2$]aspartate. Preparations of ^{13}C-labeled aspartate were characterized and shown to be pure by quantitative amino acid analysis. Elem. Anal. Calcd. for L-[4-^{13}C]aspartate: C, 36.57; H, 5.26; N, 10.45; Found: C, 36.51; H, 5.17; N, 10.45. Calcd. for L-[3,4-$^{13}C_2$]aspartate: C, 37.04; H, 5.22; N, 10.37; Found: C, 37.11; H, 5.17; N, 10.52.

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References

1. Hanners, J. L., Gibson, R., Velarde, K., Hammer, J., Alvarez, M., Griego, J. and Unkefer, C., - J. Labelled Comp. Radiopharm. 16: in press (1991).
2. Arnold, L. D., Kalantar, T. H., and Vederas, J. C. - J. Am. Chem. Soc. 107: 7105-7109 (1985).
3. Arnold, L. D., Drover, J. C. G., and Vederas, J. C. - J. Am. Chem. Soc. 109: 4649-4659 (1987).
4. Arnold, L. D., May, R.G., and Vederas, J. C. - J. Am. Chem. Soc. 110: 2237-2241 (1988).
5. Ott, D. G. in *Synthesis with Stable Isotopes of Carbon, Nitrogen, and Oxygen*, pp. 23, John Wiley and Sons, New York (1981).
6. Walker, T. E., Unkefer, C. J., and Ehler, D. S. - J. Carbohydrate Chem. 7: 115-132 (1988).
7. Ott, D. G. in *Synthesis with Stable Isotopes of Carbon, Nitrogen, and Oxygen*, pp. 124-25, John Wiley and Sons, New York (1981).
8. McKay, D. and McIntrye, D. D. - Can. J. Chem. 62: 355-360 (1984).
9. Kauer, J.C., - Org. Syn. Coll. Vol. IV: pp 411, Wiley: New York (1963).
10. Knapp, D. R. in *Handbook of Analytical Derivatization Reactions*, pp 255, John Wiley and Sons, New York (1979).