STEREOSPECIFIC SYNTHESIS OF L-[1,4-$^{13}C_2$]ASPARTIC ACID, L-β-([^{13}C]CYANO)ALANINE AND L-[4-^{13}C]ASPARTIC ACID

Klaus H. Röhm and Robert L. Van Etten
Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

SUMMARY

A convenient stereospecific synthesis of L-aspartic acid, ^{13}C-labelled in both carboxyls, is described. The crucial step of the procedure is the conversion of [1,4-$^{13}C_2$]fumaric acid to L-aspartic acid utilizing the enzyme L-aspartase. In addition, an improved synthesis of L-β([^{13}C]cyano)alanine was developed. This compound is easily converted to L-[4-^{13}C]aspartic acid.

Key Words: L-[4-$^{13}C_2$]Aspartic acid, L-β([^{13}C]Cyano)alanine, L-[4-^{13}C]Aspartic acid, L-Aspartase, Acylase I

INTRODUCTION

For NMR studies on the mechanism of action of L-asparaginase, we needed L-aspartic acid that was ^{13}C-labeled in both C_1 and C_4, and some labeled exclusively in the C_4-position. The 1,4-labeled material allowed us to study the regiospecificity of the asparaginase-catalyzed oxygen exchange between aspartic acid and water by utilizing the ^{18}O isotope shift on ^{13}C-NMR (1). For kinetic studies of ^{18}O exchange, singly-labelled [4-^{13}C]aspartic acid was preferable because it does not exhibit line-splitting due to long range 1,4-coupling (2). In addition, we were interested in obtaining L-β-([^{13}C]cyano) alanine, an asparagine analog and also an asparaginase substrate.

A variety of synthetic routes to aspartic acid may be found in the literature. However, none of them were well suited for our purposes, either because the necessary ^{13}C-labeled precursors were not available at a reasonable cost, or because too many steps were involved to afford the product in acceptable yields. Furthermore, most published syntheses resulted in racemic D,L-

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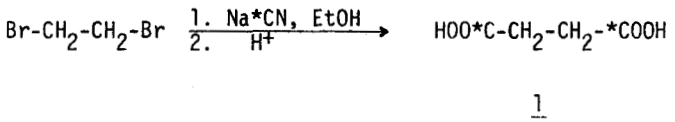
aspartic acid. This is difficult to resolve by typical procedures. For example, the enzyme acylase I shows very weak activity against N-acylated aspartic acid derivatives. For this reason, procedures yielding racemic aspartic acid were considered impractical. By combining favorable approaches from published procedures and by including a stereospecific enzyme-catalyzed reaction, we devised a route providing L-[1,4-$^{13}C_2$]aspartic acid from Na^{13}CN in only four steps and with an overall yield of 15-20%.

RESULTS AND DISCUSSION

The synthesis of labelled L-aspartic acid is outlined in Scheme I. The label was introduced in a symmetric fashion by reaction of dibromoethane with Na^{13}CN, followed by acid hydrolysis to give succinic acid. This intermediate was converted to fumaric acid by a two-step procedure involving bromination to meso-dibromosuccinate and elimination of Br_2 via the diiodo-derivative (4,5). Fumaric acid was transformed to L-aspartic acid by the enzyme L-aspartase in good yield (6). Purification of the product by ion exchange chromatography afforded the labeled amino acid in high enantiomeric purity.

The synthesis of L-β-([^{14}C]cyano)alanine has been described by Giza and Ressler (7). Their procedure is based in turn on a method described by Atkinson (8). However, in agreement with other authors (9), we found that the methoiodide intermediate employed by Atkinson was very difficult to purify and gave poor yields when reacted with NaCN. We therefore introduced the label by condensing [1-^{13}C]bromoacetonitrile with sodium acetamidomalonic ester according to Hellmann and Folz (9). Labeled bromoacetonitrile is conveniently prepared from Na^{13}CN via cyanomethyl piperidine (10,11; see Scheme II). N-Acetyl-D,L-β-cyanoalanine was obtained after decarboxylation and partial hydrolysis. This intermediate was enzymatically resolved to afford L-cyanoalanine, which may be degraded to L-asparagine or L-aspartic acid (7). An advantage of using cyanoalanine as an intermediate is that ^{18}O may be introduced in high yield into the β-carboxyl group by carrying out the nitrile hydrolysis in $H_2{}^{18}O$.



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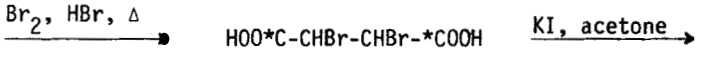

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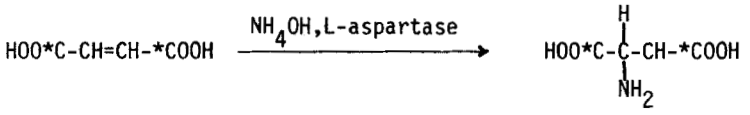

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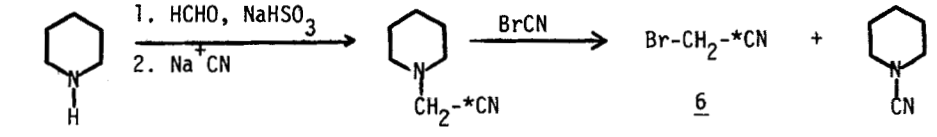
Scheme I: Synthetic route to L-[1,4-$^{13}C_2$]aspartic acid



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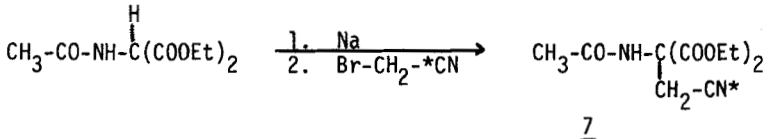

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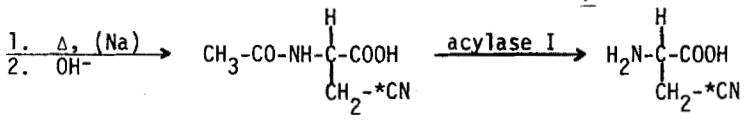

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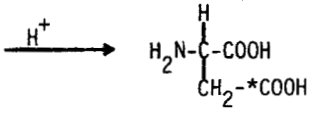

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Scheme II: Synthetic routes to D- and to L-β-cyanoalanines and L-[4-^{13}C]aspartic acid.

EXPERIMENTAL

NMR spectra were recorded with a Varian CFT-20 or a Varian XL-200 spectrometer. Chemical shifts are given relative to TMS. Thin-layer chromatograms were obtained using Merck silica gel plates and 1-butanol:acetic acid:water (4:1:1) as solvent. Melting points were taken in open capillaries and are uncorrected.

[1,4-$^{13}C_2$]Succinic Acid (1) - Labeled succinic acid (1) was synthesized according to ref. 3 via the dinitrile without isolating that intermediate. Yield: 84%; m.p. 180°C; reported, 185°C (13); ^{13}C-NMR (H_2O/D_2O): 178.0 ppm.

[1,4-$^{13}C_2$]2,3-Dibromosuccinic Acid (2) (cf. ref. 4) - Compound 1 (5.6 g, 47 mmol), 4 ml H_2O, 1.2 ml 48% HBr and 5 ml (15.5 g, 97 mmol) bromine were placed in a long-necked 5 x 20 cm Pyrex tube having a volume of 260 ml. The tube contents were frozen in liquid nitrogen, the tube sealed and heated to 105°C for 4.5 days. (Caution, internal pressure develops!) After cooling first to room temperature, the tube was cooled in liquid nitrogen and opened. It was connected to a vacuum line (1 mm Hg) and evacuated at room temperature until the contents were dry and almost colorless (3 h). The dry residue was extracted with ether (4 x 50 ml), and the ether extract was evaporated to afford 2 as a slightly yellow solid. Yield: 10.3 g (79%); m.p. > 250°C (sealed capillary); reported meso, 255°C (sealed tube) D,L, 171°C (13) . ^{13}C-NMR (H_2O/D_2O): 172.2 ppm (meso-form, ∿75%); 167.9 ppm (D,L-form, ∿25%).

[1,4-$^{13}C_2$]Fumaric Acid (3) (cf. ref. 5) - Compound 2 (4.3 g, 16 mmol; meso plus D, L) in 60 ml acetone and 8.3 g (50 mmol) KI in 40 ml acetone were mixed together and refluxed for 2 h. After cooling, the iodine that was formed was reduced by titration with 1 M $Na_2S_2O_3$ until the solution became colorless. Water (180 ml) and 20 ml glacial acetic acid were added, and the mixture was continuously extracted with ether for 36 h. The extract was evaporated to dryness, and 3 was crystallized from a few ml of boiling 1 N HCl. Yield: 0.93 g (51%); m.p. 287-290°C (sealed capillary); reported, 286-287°C (sealed capillary) (5,13). ^{13}C-NMR (H_2O/D_2O): 170.2 ppm.

L-[1,4-$^{13}C_2$]Aspartic Acid (4) (cf. ref. 6) - Compound 3 (580 mg) was suspended in 2.5 ml H_2O and 0.1 ml 10 mM EDTA and 0.25 ml 50 mM $MgSO_4$ were added. After adjusting the pH to 8.5 with 58% NH_4OH, the solution was placed in a 40°C water bath and 10 mg L-aspartase from *Hafnia alvei* (Sigma Chem. Co.) in 0.5 ml 10 mM phosphate buffer, pH 7, was added. Formation of aspartic acid was complete after 3 h. The reaction mixture was boiled for a few minutes to inactivate the enzyme, diluted to 30 ml, and the pH adjusted to 1.5 with conc. HCl. The resulting solution was applied to a 3.5 x 15 cm column of Dowex 50W-X2 (200-400 mesh), equilibrated with HCl at pH 1.5. The column was washed with 50 ml HCl, pH 1.5, before eluting the product with 3 N HCl. Evaporation of the solution at reduced pressure afforded crude aspartic acid hydrochloride. Final purification was achieved by rechromatography on a 1 x 30 cm column of Dowex 50W-X2 equilibrated with pH 1.5 formic acid. The crude hydrochloride was dissolved in the minimum volume of formic acid, pH 1.5, applied to the column, eluted with a linear gradient from 0.2 M pyridinium formate, pH 3.0 to 1 M pyridinium formate, pH 5.0. Ninhydrin-positive fractions were pooled, evaporated at reduced pressure, taken up in H_2O, and evaporated again. The residue was crystallized from water/ethanol to give 4. Yield: 290 mg (44%). TLC: Rf 0.19, homogeneous, identical with aspartic acid. ^{13}C-NMR (H_2O/D_2O, pH 7): doublets due to long-range 1,4 coupling (J_{14}=3.3 Hz) C4: 178.3 ppm; C1: 175.0 ppm. No fumaric acid was present.

Enzymatic determination with a stereospecific test employing glutamic-oxalacetic transaminase and malate dehydrogenase (12) showed the product to be > 90% pure L-aspartic acid.

1([^{13}C]Cyanomethyl)piperidine (5) - Compound 5 was synthesized according to ref. 10, using Na^{13}CN (91.5 atom % ^{13}C, MSD Isotopes). The fraction distilling between 100-105°C/20 mm Hg was collected; reported b.p. 99-100°C/16 mm Hg (10). Yield: 81%; colorless oil.

[1-^{13}C]Bromoacetonitrile (cf. 11) (6) - Compound 5 (4.8 g, 38 mmol) and 4.5 g (42 mmol) of cyanogen bromide were placed in a small Teflon-lined autoclave and heated to 110°C for 4 h. After cooling, the liquid formed was taken up in ether.

The ether phase was thoroughly washed with water, dried over $MgSO_4$ and evaporated. The remaining yellow oil was fractionally distilled at 16 mm Hg. Compound 6 was obtained as the fraction distilling at 50-95°C; reported b.p. 46°C/13 mm Hg (11). Yield: 3.0 g (65%); slightly yellow oil.

Diethyl Acetamido ([^{13}C]cyanomethyl)malonate (7) - Diethyl acetamidomalonate (4.35 g, 20 mmol) was converted to the Na-salt as described in ref. 9. To a solution of the compound in 50 ml boiling benzene 2.25 g of 6 (10 mmol) in 5 ml benzene was added dropwise. The mixture was refluxed for 2 h and allowed to cool. NaBr was filtered off, the reaction mixture concentrated to about 10 ml and the title compound 7 was crystallized by careful addition of petroleum ether. It was recrystallized from 2-propanol/hexane. Yield: 2.9 g (60%); m.p. 83-84°C; reported m.p. 85°C (9); ^{13}C-NMR ($CDCl_3$): 115.7 ppm.

L-β-([^{13}C]cyano)alanine (9) - Compound 7 (800 mg, 4.4 mmol) was dissolved in 10 ml absolute ethanol containing a trace of sodium metal. The solution was refluxed for 30 min and evaporated at reduced pressure. The residue was taken up in 10 ml water and titrated to pH 10.0 with NaOH. Subsequently, pH was kept at 10.0 by automatic titration with NaOH until base consumption ceased. Enough solid NaH_2PO_4 was added to bring pH to 7.0 and the reaction mixture was incubated with 5 mg porcine kidney acylase I (Sigma, grade III) for 5 h at room temperature. It was acidified to pH 1.5 with formic acid and applied to a column of Dowex 50W-X2 (200-400 mesh). The title compound 9 is bound to the resin under these conditions, while unreacted N-acetyl-D-cyanoalanine passes through the column. (Hydrolysis of this N-acetyl-D-cyanoalanine in 6 N HCl yields D-[4-^{13}C]aspartic acid.) Compound 9 was eluted with a pyridinium formate gradient and purified as described for aspartic acid (4). Yield: 130 mg (72%) m.p. 214°C (dec.); reported m.p. 215-218°C (dec) (7). TLC: Rf 0.27 (identical to an authentic standard), homogeneous, characteristic green color with ninhydrin. ^{13}C-NMR (H_2O/D_2O pH 7): 118.7 ppm.

L-[4-^{13}C]Aspartic acid (10) - Hydrolysis of 9 in 6 N HCl (110°C, 16 h) afforded 10 in quantitative yield. Enzymatic assay as for 4 established that 10 was

> 90% pure L-aspartic acid. This is also consistent with the conclusion that no racemization occurs during the hydrolysis of 9.

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