SYNTHESIS OF dl-GLUTAMINE-2,5-$^{15}N_2$

William M. Lagna and Patrick S. Callery

Department of Medicinal Chemistry/Pharmacognosy
School of Pharmacy
University of Maryland
Baltimore, Maryland 21201

SUMMARY

A two step synthesis of dl-glutamine-2,5-$^{15}N_2$ is described. 2-Oxapentanedioic acid was reacted with hydrazine sulfate-$^{15}N_2$ to yield 6-carboxy-(2H)-pyridazin-3-one-1,2-$^{15}N_2$, which was then reduced with hydrogen over 10% Pd/C catalyst to dl-glutamine-2,5-$^{15}N_2$. Gas chromatography of the trimethylsilylated derivative of the labeled glutamine was used to establish purity. Mass spectra for dl-glutamine-$^{15}N_2$, dl-glutamine and their intermediates, 6-carboxy-(2H)-pyridazin-3-one-1,2-$^{15}N_2$ and 6-carboxy-(2H)-pyridazin-3-one were consistent with assigned structures.

KEY WORDS: dl-Glutamine-2,5-$^{15}N_2$, Nitrogen-15

INTRODUCTION

Glutamine, consistent with its ubiquitous presence in biological systems, is the object of a growing number of biochemical studies. Several recent articles have dealt with the synthesis and determination of stable isotopes of glutamine as part of

0362-4803/84/040337-09$01.00
© 1984 by John Wiley & Sons, Ltd.
Received December 16, 1983

studies on delineating metabolic pathways (1,2). However, only the amide nitrogen in these studies was labeled. Because certain central nervous system metabolites, such as α-ketoglutaramic acid, are believed to arise from transamination of glutamine catalyzed by glutamine transaminase (glutamine:2-oxo acid amino transferase, EC 2.6.1.15)(3), it is necessary to label both nitrogens in order to more fully characterize the metabolism of glutamine in its key role as nitrogen carrier.

The observation of Kline and Cox (4) that substituted dihydropyridazinones can be reduced catalytically to glutamine analogues in a single step permitted modification of this synthetic pathway for the convenient preparation of dl-glutamine-2,5-$^{15}N_2$.

EXPERIMENTAL

Reagents

2-Oxopentanedioic acid, 10% palladium on carbon, bis-(trimethylsilyl)trifluoroacetamide (BSFTA) and dl-glutamine were obtained from Aldrich and used without further purification. Pyridine, from Matheson, Coleman and Bell, was distilled and dried over KOH prior to use. Hydrazine sulfate-$^{15}N_2$ (98% ^{15}N) was obtained from Cambridge Isotopes.

Methods

Melting points were determined using a Thomas Hoover melting point apparatus and are uncorrected. Gas chromatography was performed on a Varian 2400 GC equipped with flame ionization detectors using a 30 m SE-54 (5% phenyl, 1% vinyl, 94% methylsilicone phase, Supelco, Bellefonte, Pa.) capillary column which was tem-

perature programmed (150-250°C) at 6°/min. Injection port temperature was 250°C, detector temperature was 250°C with a He carrier gas flow of 2.5 cm/sec at a split ratio of 50:1. A Hewlett-Packard 3390A recording integrator was used to calculate peak areas.

Derivatization was accomplished by heating 5 mg of sample in 1 ml of a 1:1 mixture of BSTFA:pyridine at 80°C for 1 hour.

Both electron impact (E.I.) and chemical ionization (C.I., methane) mass spectrometry were performed on an Extranuclear Simulscan quadrupole mass spectrometer interfaced with a Carlo Erba 4160 capillary gas chromatograph and equipped with an Extranuclear 1000 data system. A direct probe inlet was used for sample introduction for isotope enrichment determinations.

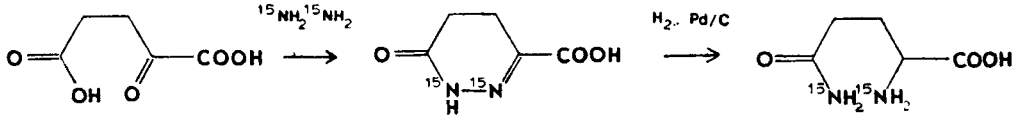
6-Carboxy-(2H)-pyridazin-3-one-1,2-$^{15}N_2$

Hydrazine sulfate-$^{15}N_2$ (1.14 grams, 8.64 x 10^{-3} moles) was titrated to pH 8.0 with a 10% NaOH solution. 2-Oxopentanedioic acid (1.29 grams, 8.84 x 10^{-3} moles) was dissolved in 1.3 ml of H_2O. The hydrazine solution was then added dropwise with stirring to the 2-oxopentanedioic acid solution over a period of 2 minutes. Product was observed to form as a white precipitate within three minutes. The solution was left stirring at room temperature for two hours, then cooled to 8°C and filtered. The crude product was dissolved in a minimum of hot 2M HCl, cooled slowly to 8°C, and the crystals formed were collected by vacuum filtration. The long white needles were dried under vacuum to yield 1.13 grams (7.85 mmoles, 91%) of 6-carboxy-(2H)-pyridazin-3-one-$^{15}N_2$; mp 193-195°C; mass spectrum (E.I.) m/z 142 (2.3%),143

(0.1%), 144 (90.7%), 145 (6.1%), 146 (0.8%); unlabeled standard, m/z 140 (1.4%), 141 (0.4%), 142 (90.3%), 143 (6.9%), 144 (0.9%).

dl-Glutamine-2,5-$^{15}N_2$

6-Carboxy-(2H)-pyridazin-3-one-1,2-$^{15}N_2$ (1.11 grams, 7.82 x 10^{-3} moles) was added to 20 ml of H_2O and deoxygenated 10 minutes with N_2. Pd on carbon (0.40 grams of 10% Pd) was added to the solution, and the system sealed, evacuated then left shaking 24 hours under 50 psi H_2. The solution was then filtered, the catalyst washed three times with 20 ml H_2O, and the combined filtrates were rotary evaporated at 37°C to give an oil. Water was added until the oil dissolved, followed by the dropwise addition of acetone with stirring until a white precipitate formed. The solution was allowed to stand 1 hour, then cooled to 8°C and vacuum filtered. The product was dried under vacuum to yield 0.500 grams (3.38 x 10^{-3} moles, 45%) of dl-glutamine-2,5-$^{15}N_2$; (mp 179-181°C); Mass spectrum (C.I, methane) m/z 147 (1.7%) 148 (2.9%), 149 (90.3%), 150 (4.9%), 151 (0.1%); unlabeled standard m/z 145 (1.1%), 146 (0.1%), 147 (92.9%), 148 (5.7%), 149 (0.1%).



Scheme 1. Overall reaction sequence.

DISCUSSION

A method for the incorporation of labeled nitrogen into glutamine arises from the cyclization reaction of hydrazine-$^{15}N_2$

with 2-oxopentanedioic acid forming 6-carboxy-(2H)-pyridazin-3-one, a substituted $^{15}N_2$-dihydropyridazinone compound. Catalytic hydrogenation then reductively cleaves the azide bond, yielding labeled glutamine directly (4,5).

Formation of 6-carboxy-(2H)-pyridazin-3-one-1,2-$^{15}N_2$ proceeded quickly after initial mixing of reagents. The product was stable to mild acid and was recrystallized from acid solution. Yields were nearly quantitative (90-93% typically). Structure proof was provided by electron impact mass spectrometry. Unlabeled 6-carboxy-(2H)-pyridazin-3-one yields an intense parent ion of m/z 142 (Figure 1). Other major ions include m/z 124 consistent with loss of water and m/z 114 accounted for by loss of carbon monoxide from the parent ion. A mass spectrum of the labeled 6-carboxy-(2H)-pyridazin-3-one-1,2-$^{15}N_2$ showed m/z 144 for the parent ion (Figure 2). Normalized, averaged scans of the respective parent ion peaks indicated greater than 99.5% per atom ^{15}N incorporation.

A comparison of the labeled and unlabeled substituted pyridazinone fragmentation patterns showed $^{15}N_2$ incorporation in peaks m/z 116,126 and 144. The presence of these labeled ions verified the fragmentation patterns proposed for the unlabeled 6-carboxy-(2H)-pyridazin-3-one.

The 6-carboxy-(2H)-pyridazin-3-one-1,2-$^{15}N_2$ was reductively cleaved with H_2 using palladium on carbon as catalyst. dl-Glutamine-2,5-$^{15}N_2$ was formed directly, with typical yields of 40-45 percent. Longer reaction times appeared to increase the amount of glutamic acid-2-^{15}N formed, a particularly undesirable side product for quantitative biochemical applications.

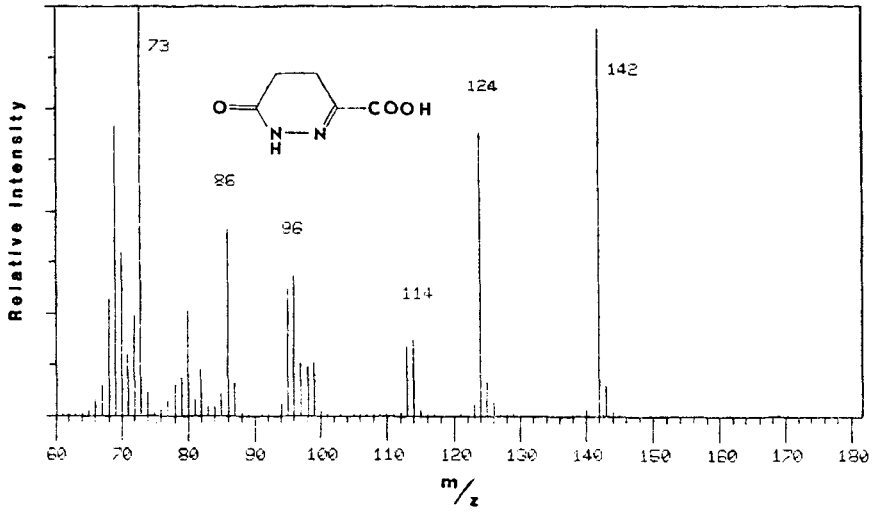


Figure 1. Electron impact mass spectrum of 6-carboxy-(2H)-pyridazin-3-one (sum of 40 scans).

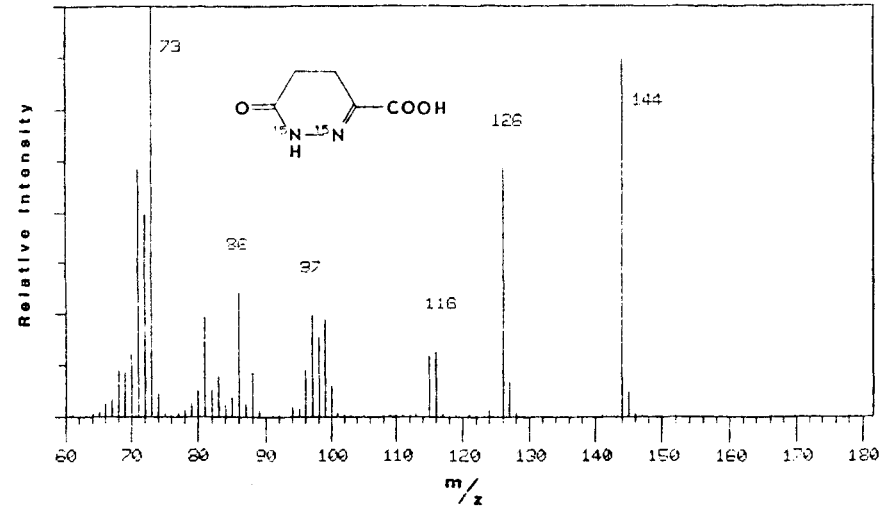


Figure 2. Electron impact mass spectrum of 6-carboxy-(2H)-pyridazin-3-one-1,2-$^{15}N_2$ (sum of 40 scans).

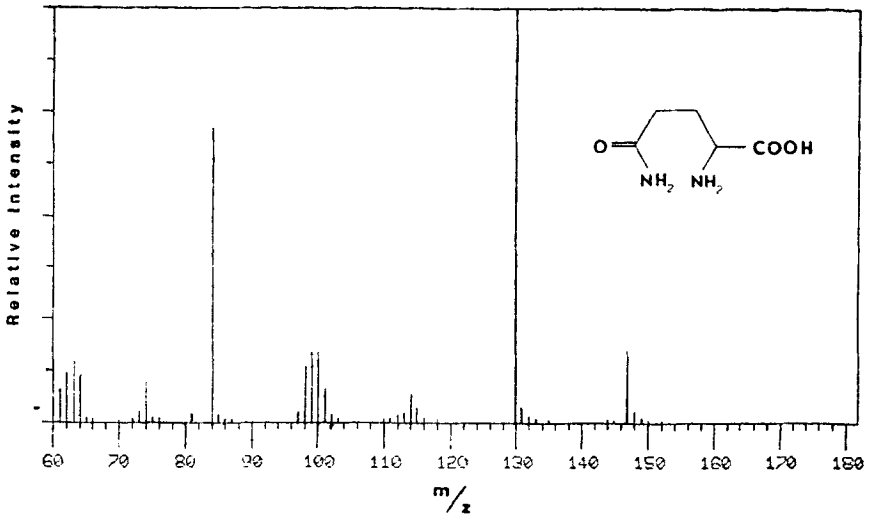


Figure 3. Mass spectrum of dl-glutamine using chemical ionization with methane as reagent gas (sum of 50 scans).

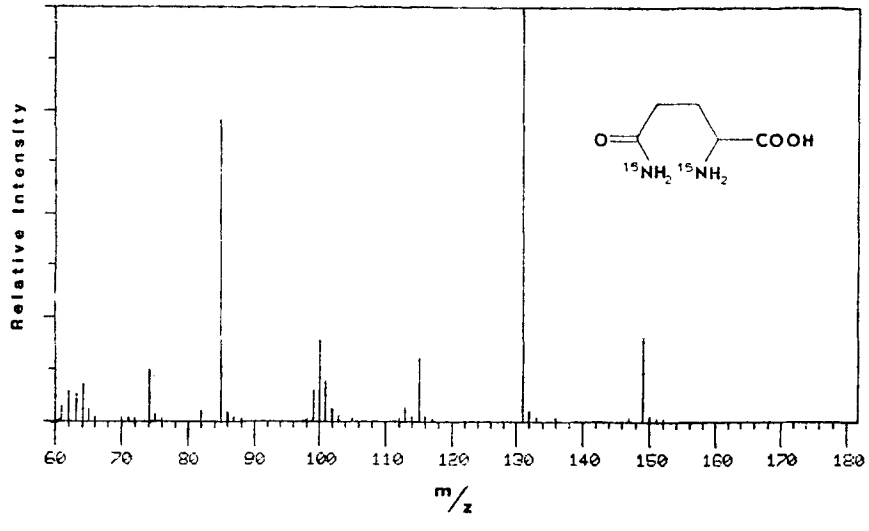


Figure 4. Mass spectrum of dl-glutamine-2,5-$^{15}N_2$ using chemical ionization with methane as reagent gas (sum of 50 scans).

Purity was established by gas chromatography of the trimethylsilylated derivatives. Consistent with the literature (6,7,8,), the trimethylsilylated derivatives of glutamine yielded two peaks with proportions varying from sample to sample. The trimethylsilyl derivative of glutamic acid appears as a distinct peak from the glutamine derivatives, and was not present in the dl-glutamine-2,5-$^{15}N_2$ chromatogram.

Mass spectral analysis of unlabeled glutamine was hindered by the rapid thermal cyclization of glutamine to the lactam pyroglutamic acid (9). Using chemical ionization with methane as reagent gas, a weak parent ion at m/z 147 (MH^+) was identified (Figure 3). The base peak m/z 130 is consistent with loss of ammonia to form pyroglutamic acid. Peak m/z 84 can be accounted for by loss of formic acid from the pyroglutamic acid.

Analysis of the C.I. mass spectrum of the labeled dl-glutamine-2,5-$^{15}N_2$ revealed a parent ion of m/z 149, consistent with the incorporation of two stable isotopes of nitrogen (Figure 4). The base peak of m/z 131 corresponds to loss of $^{15}NH_3$, consistent with protonated pyroglutamic acid-^{15}N. Further loss of HCOOH yields m/z 85, with ^{15}N incorporation evident by comparison with the spectrum of unlabeled compound. Isotope enrichment was 99.2% per atom which is similar to the value determined for the dl-glutamine-2,5-$^{15}N_2$ precursor, 6-carboxy-(2H)-pyridazin-3-one-1,2-$^{15}N_2$.

The methods described afford a facile, inexpensive synthesis of dl-glutamine-2,5-$^{15}N_2$ from its precursor, 6-carboxy-(2H)-pyridazin-3-one-1,2-$^{15}N_2$.

REFERENCES

1. M. Yudoff, I. Nissim and S. Segal, Clin. Chim. Acta **118**, 159 (1982).

2. F. Jahoor, A.A. Jackson and M.H. Golden, Anal. Biochem. **121**, 349 (1982).

3. A. Cooper, A.K. Dhar, H. Kutt and T.E. Duffy, Anal. Biochem. **103**, 118 (1980).

4. G. B. Kline and S.H. Cox, J. Org. Chem. **26**, 1854 (1961).

5. M. Stogniew, L.A. Geelhaar and P.S. Callery, J. Labelled Compds. Radiopharm. **18**, 897 (1981).

6. E. Gajewski, M. Dizdaroglu and M.G. Simic, J. Chromatogr. **249**, 41 (1982).

7. K.R. Leimer, R.H. Rice and C.W. Gehrke, J. Chromatogr. **141**, 355 (1978).

8. C.W. Gehrke and K.R. Leimer, J. Chromatogr. **57**, 219 (1971).

9. G.W. Milne, T. Axenrod and H.M. Fales, J. Am. Chem. Soc. **92**, 5170 (1970).