Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXIII, No. 10

Synthesis of D,L-[2-^{15}N,5-^{13}C]Glutamic Acid

John C. LaIuppa and David M. LeMaster

Department of Biochemistry, Molecular Biology and Cell Biology
Northwestern University, Evanston, Illinois 60208

SUMMARY

A one-pot procedure is described for the synthesis of D,L-[2-^{15}N,5-^{13}C]glutamic acid from 2-bromo-4-butyrolactone utilizing potassium ^{15}N-phthalimide and potassium ^{13}C-cyanide as label sources. Following a two column purification procedure, the final product is obtained in 38% yield based on the equimolar label sources.

Key Words: D,L-[2-^{15}N,5-^{13}C]glutamic acid, amino acid synthesis, pK determination

INTRODUCTION

Our studies of the electrostatic interactions on protein surfaces stimulated the need to determine the titration behavior of the carboxylate sidechains. Although the protons on the relevant sidechains generally show titration dependent chemical shifts, they are often strongly affected by ionization of other nearby charged groups as well thus rendering the titration analysis problematic. As the pH dependence of the carboxyl carbon chemical shift is particularly sensitive to its own ionization state, this resonance provides an optimal NMR monitor for titration. 1D ^{13}C NMR titration studies of protein glutamate and aspartate resonances have been carried out (eg. 1,2). On the other hand assignment of these resonances to particular positions in the protein sequence is not always straightforward.

Current protein NMR assignment protocols rely heavily on multi-dimensional ^{1}H-^{13}C heteronuclear correlation experiments in which the

0362–4803/93/100913–07$08.50

Received 26 February, 1993
Revised 11 May, 1993

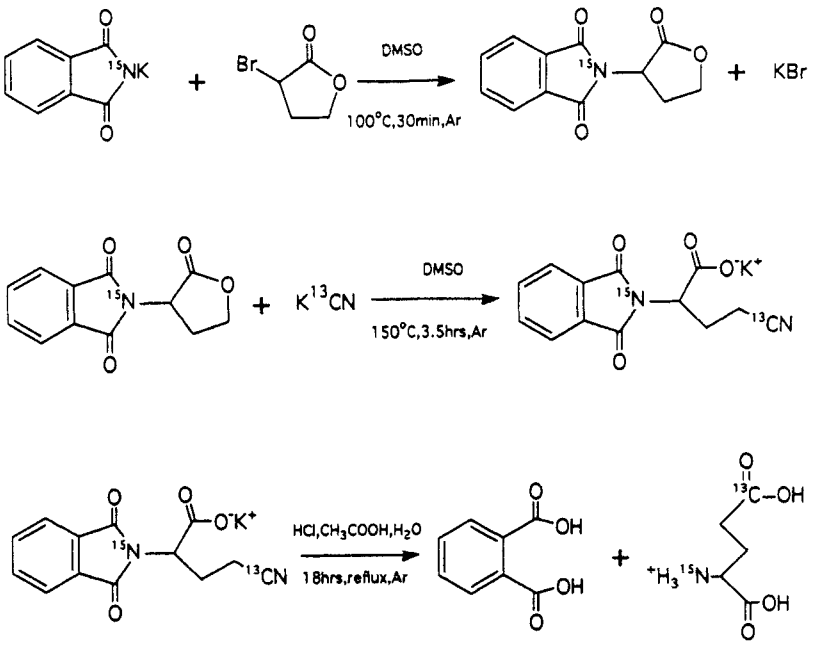
carbon and its directly bonded proton are assigned simultaneously making use of the large one bond spin coupling. As a result, assignment of quaternary carbons is often somewhat more challenging. For the assignment of glutamate C_δ resonances in enriched protein samples, we have chosen to also incorporate ^{15}N so as to facilitate correlation with the mainchain assignments.

Most of the previously reported syntheses of [5-^{13}C] (or [5-^{14}C]) glutamate suffer from multiple steps (3) or else lack an obvious compatibility with the desired ^{15}N labeling (4). An exception to this generalization is the synthesis of glutamate based on the reaction of potassium cyanide with 2-benzamido-4-butyrolactone (5,6). As an analogous starting reagent can be generated via the widely used phthalimide reaction with 2-bromo acid esters to make ^{15}N labeled amino acids (7), we proceeded to examine whether sequential reaction of 2-bromo-4-butyrolactone with potassium ^{15}N-phthalimide followed by K^{13}CN could be efficiently carried out in the same reaction mixture.

RESULTS AND DISCUSSION

Redistilled 2-bromo-4-butyrolactone was reacted with an equivalent of potassium ^{15}N phthalimide in dimethyl sulfoxide at 100°C. Preliminary experiments had demonstrated that the disappearance of 2-bromo-4-butyrolactone, as followed by NMR, was complete after 30 minutes with a resultant 70% yield of the 2-phthalimido derivative. After cooling to room temperature, an equivalent of K^{13}CN was added and reacted at 150°C for 3.5 hours. Earlier studies utilizing purified 2-phthalimido-4-butyrolactone under analogous conditions indicated an 80% yield of potassium 4-cyano-2-phthalimidobutyrate. Dimethyl sulfoxide was chosen as solvent rather than dimethyl formamide as used in the earlier cited synthesis of ^{15}N labeled amino acids (7) due to the substantially higher solubility of potassium phthalimide in dimethyl sulfoxide.

After removal of solvent by distillation, acid hydrolysis, and removal of the crystalline phthalic acid, the glutamate was purified by cation



exchange followed by anion exchange displacement chromatography (8). Most amine containing byproducts, such as homoserine, passed directly through a Dowex 1 column in acetate form. The sidechain pK of 4.25 (9) for glutamate causes it to displace the acetate (pK of 4.75) and bind to the column. A formic acid (pK of 3.75) solution was then used to displace the bound glutamate. Rotary evaporation yields the pure crystalline product in 38% yield.

Correlation of a specific protein glutamate carboxyl resonance with its corresponding amide nitrogen can be made utilizing their mutual spin couplings to the intervening protons. Experiments to observe these long range heteronuclear couplings have been used to observe correlations between proton and carbonyl resonances in protein spectra (10).

A 1D analog of these heteronuclear correlation experiments is illustrated in Figure I for [2-^{15}N,5-^{13}C]glutamate in which specific heteronuclear coupling interactions have been selectively enhanced. In these experiments the correlation is observed between the Hβ resonances and the 2-^{15}N (panel A) or 5-^{13}C (panel B) resonances, respectively. The chemical shifts of the

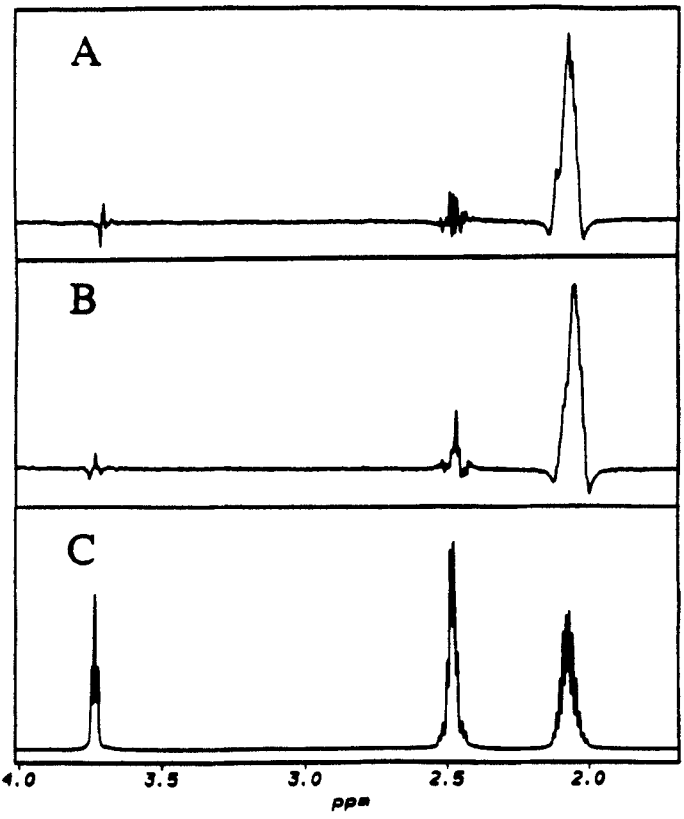


Figure 1. Heteronuclear edited ^{1}H NMR spectra of [2-^{15}N,5-^{13}C]glutamate. In panel C is given the ^{13}C and ^{15}N broadband decoupled spectrum of the α (3.75 ppm), β (2.05 ppm) and γ (2.50 ppm) resonances. Panel A contains the first t_1 point of an ^{1}H-^{15}N heteronuclear correlation experiment tuned for 4 Hz couplings with broadband ^{13}C decoupling. Panel B contains the corresponding ^{1}H-^{13}C experiment also tuned for 4 Hz couplings with broadband ^{15}N decoupling.

correlated ^{13}C and ^{15}N resonances can be obtained from the corresponding 2D experiment.

These heteronuclear spin coupling experiments can be enhanced by elimination of ^{1}H homonuclear spin couplings which both broaden the resulting resonances as well as give rise to antiphase modulation. Solvent exchange procedures have been developed which provide [2-^{2}H], [3,3-$^{2}H_2$] and [4,4-$^{2}H_2$]glutamate (11) as well as chiral deuteration of the β position (Homer, R. J., Kim, M. S. and LeMaster, D. M., manuscript submitted).

EXPERIMENTAL

$(^{15}NH_4)_2SO_4$ was obtained from Mound Laboratories. K^{13}CN was obtained via a grant from the Stable Isotopes Division of Los Alamos National Laboratories. 2-bromo-4-butyrolactone was obtained from Aldrich Chemical Co. and redistilled. All other chemicals were reagent grade. Dimethyl sulfoxide was dried over molecular sieves. The ^{1}H spectra were obtained on the Bruker AMX 600 at the Northwestern 600 MHz NMR facility.

Potassium 4-^{13}C-Cyano-2-^{15}N-phthalimidobutyrate

Potassium ^{15}N-phthalimide (prepared from $(^{15}NH_4)SO_4$ (12)) (18.6g, 100 mmol) was dissolved in 425 ml of dry dimethyl sulfoxide at 100°C under argon, and 2-bromo-4-butyrolactone (16.5g, 8.3mL, 100mmol) was dripped in over 10 minutes with stirring, then stirred for an additional 20 minutes. The mixture was cooled to room temperature, potassium ^{13}C-cyanide (6.6g, 100mmol) was added, and the solution was then heated to 150°C. The reaction was stirred at temperature for 3.5 hrs under argon. The solvent was then removed at 75°C under vacuum to yield the crude product and residual salt.

[2-^{15}N, 5-^{13}C]Glutamic acid

The crude potassium 4-^{13}C-cyano-2-^{15}N-phthalimidobutyrate was dissolved in a 750 ml solution of water, concentrated hydrochloric acid and glacial acetic acid in equal proportions and then refluxed under argon for 18 hours. The solution was rotary evaporated to a brown powder. The residue was resuspended in water and rotary evaporated. This process was repeated twice more to remove residual acid. After resuspension in 2 l of water, the phthalic acid crystals were removed by filtration. The filtrate was loaded onto a 150 ml column (2.5 x 30 cm) of Dowex 50 X-8 resin in H^+ form. After washing with several column volumes of water, the column is eluted with 0.15M ammonium hydroxide. The effluent is rotary evaporated to dryness, resuspended in 2 l of water and loaded onto a 100ml column (2.5

x 20 cm) of Dowex 1 X-8 in acetate form (converted from chloride form with 2M sodium acetate). After washing with water, six column volumes of 0.25 M formic acid was passed into the column. The effluent was rotary evaporated to dryness and vacuum dried against P_2O_5 to yield 5.6g (38%) of [2-^{15}N, 5-^{13}C]glutamic acid.

NMR Methods

The first t_1 point of a 2D heteronuclear correlation experiment (13) was recorded for each spectrum. For the ^{1}H-^{13}C correlation a 62.5 msec correlation delay was used (optimizing for a 4 Hz coupling) with broadband ^{15}N decoupling throughout. Similarly a 62.5 msec correlation delay and ^{13}C broadband decoupling was used for the ^{1}H-^{15}N correlation experiment. Sixteen scans were collected for the 50 mM sample of [2-^{15}N, 5-^{13}C] glutamate.

ACKNOWLEDGMENT

This work was supported by Project Grant GM-38779 from the National Institutes of General Medical Sciences, the Presidential Young Investigator Award Grant DMB-8957336 from the National Science Foundation and the NIH (RR02231) Stable Isotope Resource Program at Los Alamos. J. C. L. wishes to acknowledge support from the NIH Molecular and Cellular Biology Predoctoral Training Grant GM-08061.

The 600 MHz NMR facility used in these studies was funded by the W. M. Keck Foundation (Grant 891040), the National Institutes of Health Shared Instrumentation Program (Grant 1 S10 RR05880), the National Science Foundation Instrumentation and Instrument Development Program (Grant 8908093), the State of Illinois Technology Challenge Grant Program (Grant 90-82115) and Northwestern University.

REFERENCES

1. Shindo, H., Egan, W. and Cohen, J. S. - J. Biol. Chem. 253: 6751 (1978).
2. Niu, C. H., Matsuura, S., Shindo, H. and Cohen, J. S. - J. Biol. Chem. 254: 3788 (1979)
3. Cappon, J. J., Baart, J., van der Walle, G. A. M., Raap, J. and Lugtenburg, J. - Recl. Trav. Chim. Pays-Bas 110: 158 (1991)
4. Synder, H. R., Shekleton, J. F. and Lewis, C. D. - J. Am. Chem. Soc. 67: 310 (1945)
5. Pichat, L., Mizon, J. and Herbert, M. - Bull. Chim. Soc. France 1787 (1963)
6. Unny, V. K. P., Thyagarajan, S. and Viswanathan, K. V. - Proc. Nucl. Chem. Radiochem. Symp. 1981 334-336 (1983)
7. Ott. D. G. - Synthesis with Stable Isotopes, John Wiley and Sons, New York 1981. p. 40.
8. LeMaster, D.M. and Richards, F.M. - Analyt. Biochem. 122: 238 (1982)
9. Weast, R. C., ed. - Handbook of Chemistry and Physics, CRC Press, Boca Raton, 1984. p. D-164.
10. Bax, A., Sparks, S. W. and Torchia, D. A. - J. Am. Chem. Soc. 110: 7926 (1988)
11. LeMaster, D.M. and Richards, F.M. - J. Labelled Compds. Radiopharm. 19: 639 (1982)
12. Ott. D. G. - Synthesis with Stable Isotopes, John Wiley and Sons, New York 1981. p. 110.
13. Bodenhausen, G. and Ruben, D. J. - Chem. Phys. Lett. 69: 185 (1980)