Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXIX, No.3

SYNTHESIS OF [1,2-$^{13}C_2$] GLY AND [2,2-$^{2}H_2$] GLY GLUTATHIONE

Xiao-Ming Lu, Alan J. Fischman*, Michael Kenneway, Ronald G. Tompkins, and Vernon R. Young
Surgical Service and Nuclear Medicine Division, Massachusetts General Hospital and Harvard Medical School, Boston MA 02114, The Shriners Burns Institute, Boston MA 02114. Department of Biology, Massachusetts Technology of Institute, Cambridge MA 02135
Received on July 28,1996

SUMMARY

[1,2-$^{13}C_2$] Gly and [2,2-$^{2}H_2$] Gly isotopomers of the intracellular tripeptide glutathione were prepared by standard methods of solution phase peptide synthesis. The synthetic products were characterized by gas chromatography/mass spectroscopy (GC/MS) and ^{1}H NMR spectroscopy. Optical purity was determined by hydrolysis, derivatization of the free amino acids with isopropanol-acetyl chloride and pentafluoropropionic anhydride and NCI/MS with a Chirasil-Val Heliflex column. These compounds should serve as useful tracers for the non-invasive study of glutathione synthesis and turnover rates in humans by GC/MS.

Key words: glutathione, isotopomers, GC/MS, [1,2-$^{13}C_2$] Gly GSH, [2,2-$^{2}H_2$] Gly GSH

INTRODUCTION

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) is the most prevalent cellular thiol and plays fundamental roles in the maintenance of tissue oxidant status, detoxification of reactive oxygen species produced by activated neutrophils and in transhydrogenation reactions involved in the formation and maintenance of sulfhydryl groups in other molecules (1). These functions may be compromised in conditions of metabolic stress such as following major burn trauma, possibly due to an inadequate rate of GSH synthesis. However, this is not certain because quantitative aspects of the *in vivo* physiology of GSH metabolism and its interrelationship with sulfur amino acid metabolism in humans, are still poorly defined.

CCC 0362–4803/97/030205–09$17.50

Received 31 July 1996
Revised 1 November 1996

Use of stable isotope labeled GSH as a tracer represents an attractive non-invasive method for studying GSH synthesis and turnover rates in humans, and the interrelationships between GSH and its constituent amino acids (Cys, Glu and Gly) and their metabolic fluxes. However, appropriate tracers and convenient methods for the analysis of isotopically enriched GSH in biological matrices are not currently available (2-6). Hence, we describe here the preparation of [^{2}H] and [^{13}C] GSH isotopomers.

RESULTS AND DISCUSSION

The synthesis of [1,2-$^{13}C_2$] Gly and, [2,2-$^{2}H_2$] Gly GSH was performed by solution phase peptide synthesis (Figure 1)(7-9). [1,2-$^{13}C_2$] glycine **1** was heated with p-toluenesulfonic acid and benzyl alcohol and the resulting [1,2-$^{13}C_2$] Gly-OBzl PTS

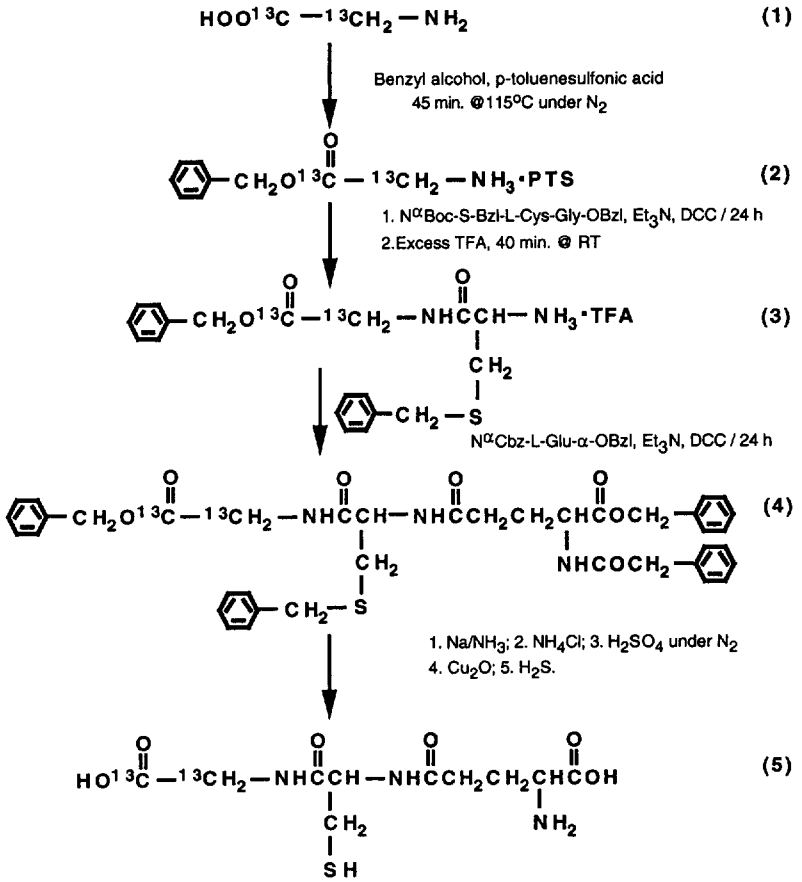

Figure 1: Reaction scheme for the solution phase peptide synthesis of [1,2-$^{13}C_2$] Gly GSH. [2,2-$^{2}H_2$] Gly GSH was prepared by the identical procedure.

salt **2** was crystallized by addition of ether and recrystallized from methanol/ether. **2** and triethylamine were dissolved in CH_2Cl_2 at 0°C; N$^{\alpha}$-t-Boc-S-Bzl-L-Cys was added and coupling was performed by addition of dicyclohexylcarbodiimide. After 12 hours at room temperature, dicyclohexylurea was removed by filtration and the filtrate was washed with 0.5 N HCl, 5% $NaHSO_4$, and water. The solvent was evaporated and the fully protected dipeptide was crystallized from ethyl acetate / petroleum ether. Treatment with excess anhydrous trifluoroacetic yielded S-Bzl-L-Cys-[1,2-$^{13}C_2$]-Gly-OBzl TFA salt **3**. Using the same procedure N$^{\alpha}$-Cbz-L-Glu-α-OBzl was coupled with **3** to produce N$^{\alpha}$-Cbz-L-Glu(α-OBzl)-(S-Bzl)-L-[1,2-$^{13}C_2$]Cys-Gly-OBzl **4**. The benzyl and benzyl oxycarbonyl protecting groups were removed in a single step by treatment with Na in liquid NH_3 and the deprotected peptide was precipitated with Cu_2O. The copper salt was treated with H_2S to yield the final product, [1,2-$^{13}C_2$] Gly GSH **5**. [2,2- $^{2}H_2$] Gly GSH **5a** was prepared by an identical procedure starting with [2,2- $^{2}H_2$] Gly **1a**. Optical purity was evaluated by hydrolyzing the peptide and analyzing the resulting mixture of amino acids by chromatography on a Chirasil-val Heliflex GC column followed by NCI/MS.

EXPERIMENTAL

[1,2-$^{13}C_2$] Gly (99%) and [2,2-$^{2}H_2$]Gly (98%) were obtained from the Cambridge Isotope Laboratories, Inc. (Andover, MA). Benzyl alcohol, anhydrous ammonia, hydrogen sulfide gas, sodium metal and anhydrous trifluoroactic acid were obtained from Aldrich Chemical Company (Milwaukee, WI). Cuprous oxide was a product of the Fluka Inc. (Ronkonkoma, NY). Ammonium chloride was from Fisher Scientific (Springfield, NJ). N,N'-dicyclohexylcarbodiimide and triethylamine were the products of Sigma Chemical Company (St. Louis, MO).

Melting points were determined with a Thomas Hoover capillary melting point apparatus and are uncorrected. TLC was performed on EM Science 250 µ Silica Gel F254 plates with fluorescent indicator and spots were detected with UV light. Preparative column chromatography was done using the flash chromatography technique on Silica Gel (40-63 µ, E. Merck. No. 9385). Elemental analysis were performed by Galbraith Laboratories, Inc. (Knoxville, TN). All samples were determined in duplicate. NMR spectra were recorded using an Analogic Spectrometer,

FAB MS was performed at the Mass Spectrometry Laboratory of the Department of Chemistry, Harvard University. Degree of racemization was determined with HP GC-MS (4890/5988): pre-column, 5 X 0.53 mm ID Phenyl-Methyl, deactivated; analytic column, Chirasil-Val Heliflex (25 m X 0.25 mm ID, Alltech).

[1,2-$^{13}C_2$] Gly-OBzl PTS salt (2) A mixture of [1,2-$^{13}C_2$] glycine (99%, 10 g, 0.129 mol, **1**), p-toluenesulfonic acid (24.7 g, 0.129 mol), benzene (50 mL) and benzyl alcohol (53.4 mL, 0.516 mol) was vigorously stirred and heated at 115°C for 45 min. A brisk nitrogen stream was directed over the reaction mixture to remove liberated water and prevent oxidation. The reaction mixture was diluted with benzene and crude Gly-OBzl PTS salt was crystallized by addition of ether. The final product was recrystallized from methanol/ether in 88% yield. MP: 135 - 140 °C; TLC on silica gel (CH_2Cl_2:MeOH = 100:10 (v/v)) revealed a single spot (R_f = 0.41). ^{1}H NMR (300 MHz, $^2H_6$-DMSO): δ 2.29 (s, 3H, $CH_3C_6H_4$), 3.65 & 4.15 (dd, 2H, $COCH_2NH$), 5.24 (d, 2H, $OCH_2C_6H_5$), 7.13 & 7.30-7.54 (m, 9H, $CH_2C_6H_5$ and $CH_3C_6H_4$). MS (N-acetyl derivative prepared by treatment with acetic anhydride and triethylamine at 60°C for 1 hour); MW calculated: 209, fragmentation pattern (M/z) 209, 180, 102, 91,73 (207, 179, 100, 91, 72 for the unlabeled compound); Anal. Calcd. for $C_{16}H_{19}NO_5S$: C, 57.21, H, 5.64, N, 4.13. Found, C, 55.50, H, 5.59, N, 4.74.

[2,2-$^2H_2$]-Gly-OBzl PTS salt (2a) This compound was prepared from [2,2- $^2H_2$] Gly (**1a**) using the identical procedure. Yield: 85%. MP: 135 - 140 °C; TLC: R_f = 0.41. ^{1}H NMR: δ 2.29 (s, 3H, $CH_3C_6H_4$), 5.22 (s, 2H, $OCH_2C_6H_5$), 7.13 & 7.30-7.54 (m, 9H, $CH_2C_6H_5$ and $CH_3C_6H_4$). MS: MW calculated; 209, fragmentation pattern (M/z) 209, 181, 102, 91, 74. Anal. Calcd. for $C_{16}H_{19}NO_5S$: C, 56.62, H, 6.27, N, 4.13. Found, C, 55.54, H, 5.67, N, 4.68.

S-Bzl-L-Cys-[1,2-$^{13}C_2$]-Gly-OBzl TFA salt (3) [1,2-$^{13}C_2$]-Gly-OBzl PTS **2** (40 g, 0.112 mol) and triethylamine (15.6 mL, 0.112 mol) were dissolved in CH_2Cl_2 (200 mL) and stirred at 0°C. Ice-cold N$^{\alpha}$-t-Boc-S-Bzl-L-Cys (34.8 g, 0.112 mol in 200 mL of CH_2Cl_2) was added and coupling was started by addition of dicyclohexylcarbodiimide (23 g, 0.112 mol). After 12 hours at room temperature, dicyclohexylurea was removed

'by filtration and washed with CH_2Cl_2 (100 mL X 2). The filtrate was washed with 0.5 N HCl (200 mL), 5% $NaHSO_4$ (200 mL), and Milli-Q water (200 mL X 2) (10). The solvent was evaporated, trace water was removed azeotropically at 65°C and the product was crystallized from ethyl acetate / petroleum ether. The t-Boc group was removed by treatment with excess anhydrous trifluoroacetic acid at room temperature for 1 hour under nitrogen. After evaporation at reduced pressure, a slightly yellow oil was obtained in 90% yield. TLC on silica gel ($CHCl_3$:MeOH = 93:7 (v/v)) revealed a single spot (R_f = 0.72). ^1H NMR ($^2H_6$-DMSO): δ 2.79-2.81 & 2.87-2.89 (dd, 2H, CHCH_2S), 4.12 (m, 1H, CHCH$_2$S), 3.75 (s, 2H, SCH_2C$_6$H$_5$), 3.80 & 4.28 (dd, 2H, COCH_2NH), 5.12 (d, 2H, OCH_2C$_6$H$_5$), 7.20-7.40 (m, 10H, OCH$_2$C$_6$*H$_5$* and SCH$_2$C$_6$*H$_5$*), 1.39 (no t-Boc protons were detected).

S-Bzl-L-Cys-[2,2-^{2}H$_2$]-Gly-OBzl TFA salt (3a) This compound was prepared from **2a** using the identical procedure.

N$^\alpha$-Cbz-L-Glu(α-OBzl)-(S-Bzl)-L-Cys-[1,2-^{13}C$_2$] Gly-OBzl (4) This compound was prepared by coupling S-Bzl-L-Cys-[1,2-$^{13}C_2$] Gly-OBzl (51 g, 0.108 mol., **3**) and N$^\alpha$-Cbz-L-Glu-α-OBzl (40.1 g, 0.108 mol) in the same manner as above. Yield, 90%. MP: 145 - 147°C. TLC on silica gel ($CHCl_3$:MeOH = 93:7 (v/v)) revealed a single spot (R_f = 0.20). ^1H NMR (300 MHz, $^2H_6$-DMSO): δ 1.80-1.90 & 2.00-2.15 (m, 2H, NHCOCH$_2$CH_2CH), 2.30 (m, 2H, NHCOCH_2CH$_2$CH), 2.45-2.55 & 2.70-2.80 (m, 2H, CHCH_2S), 3.68 & 4.15 (m, 2H, COCH_2NH), 3.73 (s, 2H, SCH_2C$_6$H$_5$), 4.15 (m, 1H, CHCOOCH$_2$C$_6$H$_5$), 4.60 (m, 1H, CHCH$_2$S), 7.20-7.40 (m, 20H, 4 C$_6$H$_5$), 5.04 (d, 2H, CHCOOCH_2C$_6$H$_5$), 5.14 (m, 4 H, CH$_2$COOCH_2C$_6$H$_5$ and CHNHCOOCH_2C$_6$H$_5$). Anal. Calcd. for $C_{39}H_{41}N_3O_8S$: C, 65.90, H, 5.79, N, 5.89, S, 4.49. Found, C, 65.48, H, 5.92, N, 6.11, S, 4.40.

N$^\alpha$-Cbz-L-Glu(α-OBzl)-(S-Bzl)L-Cys-[2,2-^{2}H$_2$]-Gly-OBzl (4a) This compound was prepared from **3a** using the identical procedure. The ^1H NMR spectrum **4a** was identical to that of the ^{13}C labeled compound with exception that resonances at 3.68 and 4.15 were not detected. This confirmed that the resonances at 3.73 (s, 2H) and 4.15 (m, 1H) correspond to SCH_2C$_6$H$_5$ and HCOOCH$_2$C$_6$H$_5$ respectively. Anal. Calcd.

for $C_{39}H_{41}N_3O_8S$: C, 65.90, H, 5.79, N, 5.89, S, 4.49. Found, C, 65.83, H, 6.01, N, 6.05, S, 4.58.

[1,2-$^{13}C_2$] Gly GSH (5) and [2,2-$^2H_2$] Gly GSH (5a) Benzyl and benzyloxy-carbonyl protecting groups were removed in a single step by treatment with Na in liquid NH_3 (11-12) Protected **4** or **4a** (1 g) was dissolved in liquid ammonia (20 mL) under a nitrogen stream and small pieces (~1 x 1 x 5 mm) of freshly cut Na metal were slowly added until a dark blue color persisted for 10 minutes. The color was discharged with a minimal amount of solid ammonium chloride and the ammonia was evaporated at room temperature with nitrogen. To minimize racemization, protonation of carboxylate and thiolate groups was carefully performed at 0°C by addition of 0.1 N H_2SO_4 in small portions to a final pH of 2.5. The cuprous salts of **5** or **5a** were precipitated by addition of Cu_2O and the free peptides were released by bubbling with H_2S. The final product was crystallized from ethanol/water (70/30, v/v).

Optical purity of GSH isotopomers

[1,2-$^{13}C_2$-L-Gly] GSH was hydrolyzed by treatment with 6 N HCl for 24 hours at 110°C and the resulting mixture of amino acids was analyzed with a Chirasil-val Heliflex GC column followed by NCI/MS. To determine the degree of racemization that occurs during hydrolysis, optically pure samples (1 mg) of [3,3-$^2H_2$]-L-Cys and unlabeled glutathione (Sigma) were analyzed in parallel.

After hydrolysis, the reaction mixtures were derivatized with excess isopropanol-acetyl chloride (5/1 = v/v, 250 µL) at 80°C for 30 minutes followed by evaporation of excess reactants under a stream of nitrogen at 65°C and further derivatization with pentafluoropropionic anhydride (50 µL) in CH_2Cl_2 (250 µL) at room temperature for 1 hour. The samples were then dried, redissolved in CH_2Cl_2 (1 mL) and ~0.5 µL of aliquots were applied to the column.

When [3,3-$^2H_2$]-L-Cys was derivatized without exposure to hydrolysis condition, no D-Cys was detected by chromatography / (NCI/MS). However, two types of Cys derivatives were identified; a major peak of N,S-dipentafluoropropionyl isopropyl ester [retention time: 16.3 min.; MW (calculated: 457): found 437 = M - HF] and a small peak of N-pentafluoropropionyl isopropyl ester [retention time: 14.0 min., MW (calculated: 311): found 271 = M - 2HF]. Peaks with identical retention times were detected with

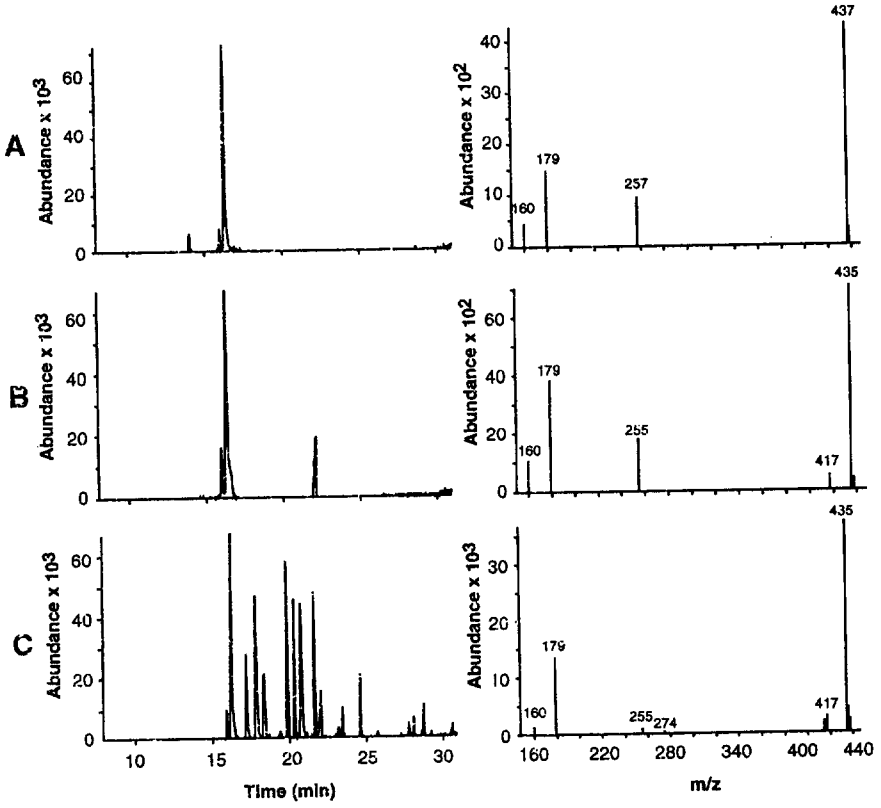

Figure 2: Racemization determinations for [3,3-$^{2}H_2$]-L-Cys (A), unlabeled GSH (B) and [1,2-$^{13}C_2$-Gly] GSH (C). The left and right panels illustrate the total ion chromatography and mass spectra respectively. Instrument: HP GC-MS (5890/5988A). Pre-column: 5 m x 0.53 mm ID Phenyl-Methyl deactivated column; analytic column: Chirasil-Val Heliflex (25 m x 0.25 mm ID, Alltech). Temperatures: Injection port (cool on column), interface: 220°C, source: 100°C. The column temperature was: maintained at 80°C for 4 minutes, increased to 190°C at 4°C / min., increased to 220°C at 30°C/min. and maintained at 220°C for 1.5 min. All samples were scanned with negative chemical ionization (using methane) from 150 to 750 M/z. Carrier gas: helium, 2 psi. Peak 1 (14.0 min): N-pentafluoropropionyl isopropyl ester of L-Cys (D-Cys can be detected at 13.80 min. when large amounts of material are injected), peak 2 (16.0 min.): N,S-dipentafluoropropionyl isopropyl ester of D-Cys, peak 3 (16.3 min): N,S-dipentafluoropropionyl isopropyl ester of L-Cys, peak 4 (22.1 min): N-pentafluoro-propionyl isopropyl ester of L-Glu. D-Glu was not detected.

unlabeled L-Cys [MW (calculated: 455), found: 435 = M - HF and MW (calculated: 309), found: 269 = M - 2HF]. When [3,3-$^{2}H_2$]-L-Cys was derivitized after exposure to hydrolysis condition, 5.9% D-Cys was detected by chromatography / (NCI/MS). As

Figure 2 A illustrates, the peaks for D- and L-Cys were well resolved with retention times of 16.0 and 16.3 min. respectively. Similar degrees of racemization (6.0%) were measured when optically pure (>99%) samples of unlabeled D- and L-Cys were analyzed. These results indicate that all of the racemization of the Cys residue results from the hydrolysis procedure and chemical derivatization makes no contribution.

Using the same procedures, analysis of hydrolyzed unlabeled GSH indicated that the extents of racemizations for L-Cys and L-Glu residues (retention time for N-pentafluoropropionyl diisopropionyl Glu ester: 22.1 min.; MW (calculated 377) found: 357 = M - HF) were 9.6% and 0%, respectively (Figure 2 B). Racemization of the L-Cys residue of synthetic [1,2-$^{13}C_2$] Gly GSH was determined to be 7.8% and no D-Glu was detected (Figure 2 C). These results indicate that the chiral purity of synthetic [1,2-$^{13}C_2$] Gly GSH was ~98%.

Acknowledgments

This work was largely supported by Grants (Nos: 18430 and 18470) from the Shriners Hospitals for Crippled Children

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