SYNTHESIS OF TRITIUM-LABELED DL-γ-CARBOXYGLUTAMIC ACID AND ITS CYCLIC FORM 5-OXO-2,4-PYRROLIDINE DICARBOXYLIC ACID

Yoshinori Ishikawa and Roy E. Wuthier

Department of Chemistry

University of South Carolina

Columbia, South Carolina 29208

SUMMARY

By condensing diethyl ethoxymethylenemalonate with diethyl acetamidomalonate, 5-hydroxy-2,4-dicarbethoxypyrrole was prepared. Hydrogenation over Pt-C to 5-oxo-2,4-dicarbethoxypyrrolidine, followed by alkaline hydrolysis gave γ-carboxyglutamate. Mild alkaline hydrolysis yielded 5-oxo-2,4-pyrrolidine dicarboxylic acid which was isolated as the cyclohexylamine salt. These procedures were applied to prepare tritium-labeled γ-carboxyglutamic acid (4.9 Ci/mmol) and its cyclic form (6.1 ci/mmol).

Keywords: H-3, γ-Carboxyglutamic acid, 5-Oxo-2,4-Pyrrolidine dicarboxylic acid, Pyroglutamic acid, Glutamic acid

INTRODUCTION

Synthesis of γ-carboxyglutamic acid (Gla) 5 has been reported from several laboratories following the identification of this vitamin K-dependently modified glutamic acid residue in prothrombin.[1-3] The most commonly used procedures begin with malonic acid esters 1, followed by condensation with alanine or serine derivatives 2, or their reaction intermediates, e.g. 2-methyleneglycine derivatives 3. The resulting triesters 4 are subjected to sequential

0362-4803/84/060507-11$01.10

Received October 26, 1983
Revised February 14, 1984

deprotection involving hydrogenolysis, alkali or weak acid treatment.[4-9] Oppliger & Schwyzer[10] have applied a modified Strecker synthesis to prepare the L-Gla derivative with an overall yield of about 10% relative to di-t-butylmalonate. Danishefsky et al.[11] have reported a 5-step synthesis of L-Gla from N-carbobenzoxy L-pyroglutamate by the introduction of a carboxyl group on the 4-position through an enamine intermediate. The primary source of optically pure L-Gla for further synthetic work, however, has been via classical resolution of racemized derivatives.[12,13]

The formation of cyclic Gla-derivatives has been reported as a byproduct in Gla synthesis.[4,6,11] However, no free acid form of pyro-γ-carboxyglutamic acid (<Gla) (5-oxo-2,4-pyrrolidine dicarboxylic acid) 11 has been synthesized. Its presence in animal tissues has been noted by the authors[1]. Attempts to cyclize Gla in water or 0.05 N HCl proved to be unsatisfactory because of the simultaneous formation of pyroglutamic acid (<Glu) (5-oxo-2-pyrrolidine carboxylic acid) 13 in large yield.

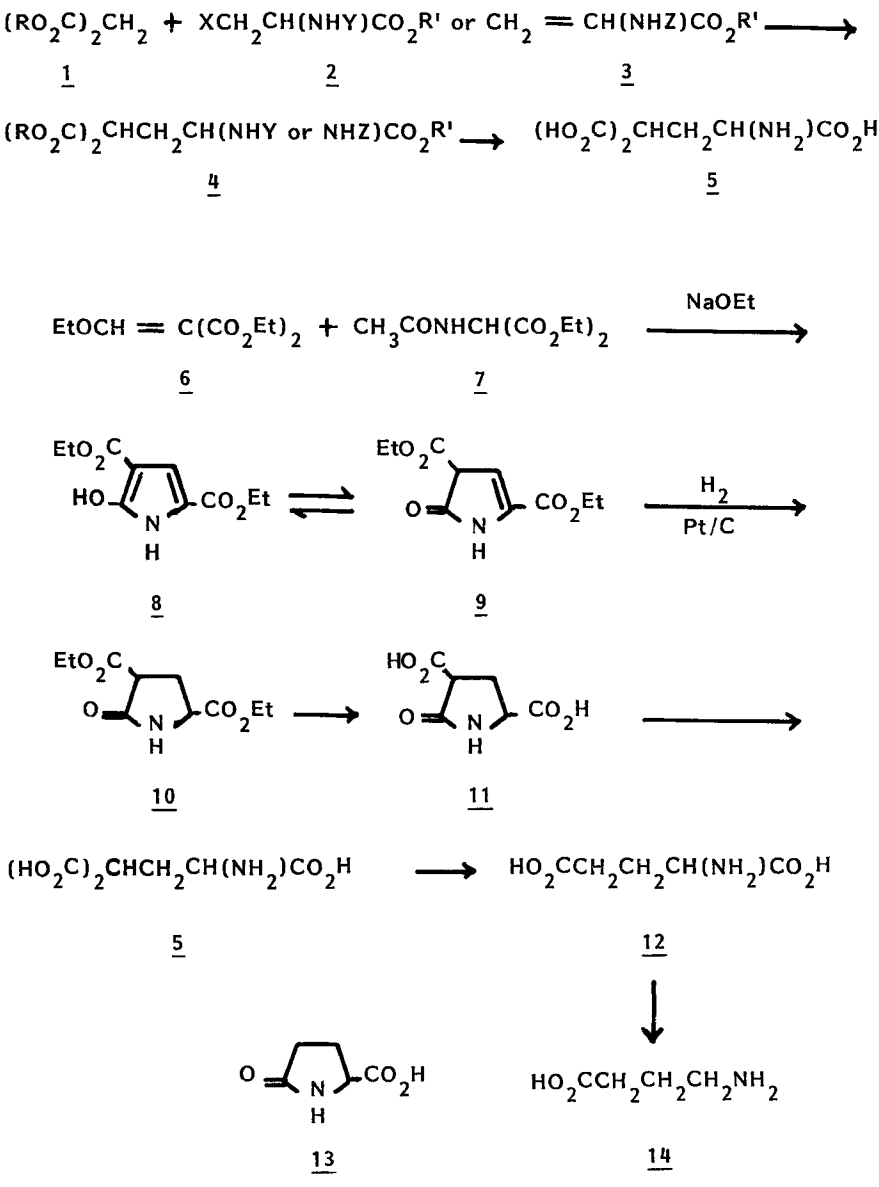
The synthetic routes mentioned above do not provide opportunity for incorporation of high levels of ^{3}H-label which would be essential for biological studies on these compounds. In order that high specific activities of Gla, <Gla, and related compounds could be achieved, we used the method of Stefancich et al.[14] to synthesize a pyrrole intermediate which could be catalytically hydrogenated to diethyl <Gla 10. Mild alkaline treatment produced <Gla, and 2 N KOH hydrolysis yielded Gla from 10, as shown in Scheme 1.

MATERIALS AND METHODS

Diethyl ethoxymethylenemalonate, diethyl acetamidomalonate and 5% platinum on activated carbon were from Aldrich. Hydrogenations were performed at room temperature in a Parr shaker, Type 3911 (H_2-pressure 40-45 psi). The catalytic reduction with tritium gas was carried out at Amersham, Arlington Heights,

---

1. Ishikawa, Y. and Wuthier, R. E. (1981) Bull. S. C. Acad. Sci., 43, 89-90(abstract), presented at the 54th Annual Meeting at the College of Charleston.



Scheme 1: Synthetic routes to γ-carboxyglutamic acid (5) and 5-oxo-2.4-pyrrolidine dicarboxylic acid (11).

Illinois. Spectral measurements were made as follows: ultraviolet, GCA/McPherson EU-701; NMR, proton in D_2O, Varian EM 390, by William Fobare; mass (70 ev, solid probe), Finnigan 4021C, by Michael Walla. Amino acid analyses were carried out on a Beckman Model 119C analyzer.[15] Elemental analyses were performed by the Robertson Laboratory, Florham Park, NJ. Stereospecific L-glutamate decarboxylase (EC 4.1.1.15, Sigma Type V from E. coli)[16] was used to determine the stereoisomers of Glu derived from Gla or <Gla. The reaction was carried out as follows: Gla monoammonium salt, or <Gla cyclohexylamine salt, (1 μmol) was heated 18 h in 0.2 ml of 6 N HCl at 105°C. Hydrolysates were evaporated in a vacuum desiccator over KOH pellets. The residue was dissolved in 0.9 ml of 0.2 M acetate buffer, pH 4.5, and 0.1 ml (10 units) of L-glutamate decarboxylase, reconstituted in 0.01 M KH_2PO_4 buffer, pH 7.0, was added to initiate decarboxylation (37°C). After 15, 30 and 60 min, aliquots (0.1 ml, 100 nmol) of the reaction mixture were added to 0.9 ml of cold 0.2 M sodium citrate buffer (pH 2.2) to stop the reaction. The citrate solution (200 μl), containing the 4-amino-n-butyric acid 14 so produced, was injected into the amino acid analyzer. The compound 14 eluted just ahead of tyrosine (91.0-91.4 min), the ninhydrin color factor being about 80% of Glu (39.0-39.6 min).

SYNTHESIS

5-Hydroxy-2,4-dicarbethoxy pyrrole (8). Diethyl ethoxymethylenemalonate (6, 10.80 g), diethyl acetamidomalonate (7, 10.85 g) and 1.15 g of sodium metal in 150 ml absolute ethanol were refluxed for 4 h. After addition of 3 ml of acetic acid, the solvent was removed in vacuo; the residue was dissolved in a mixture of 100 ml of ethyl acetate and 200 ml of ethyl ether, washed with 100 ml of water several times, and evaporated to a crystalline solid. The residue was recrystallized from 80 ml of ethyl acetate. The final product, 5.8 g (51%) of white shiny plates, had a m.p. of 185-187°C (literature, m.p. 186-187°C).[14,17] Longer reaction time yielded a more colored product in somewhat higher yield.

Spectral data: λmax 289-290 nm (ε20,200, keto form 9) in acetic acid; λmax 350-352 nm (ε19,600, enol form 8) in dimethylformamide; λmax 285-286 nm (ε21, 100), in dioxane; λmax 289-290 nm (ε14,400) and 335-336 nm (ε9,900), in ethanol; λmax 291-292 nm (ε16,800) and 340-341 nm (ε5,700) in tetrahydrofuran. Mass spectrum: m/e 227 (parent ion), 181 (-EtOH, base peak), 153 (-CO), 135 ($-H_2O$).

Hydrogenation of keto ester (9). To 908 mg (4 mmol) of 8 in 120 ml of warm glacial acetic acid was added 1 g of 5% platinum on activated carbon. The mixture was hydrogenated for 24 h, or until the absorption at 290 nm in acetic acid disappeared completely. The catalyst was filtered off and washed with acetic acid. The filtrate was concentrated to a syrup in a rotary evaporator, evaporation being repeated twice after adding 100-ml portions of water to remove acetic acid. The residue was extracted with 100 ml of ethyl ether, the small insoluble portion being discarded. An aliquot of the ether extract was acid hydrolyzed, followed by amino acid analysis. Yields of 10 calculated from Glu formation, were 82-88% of expected. Hydrogenation in other solvents, under the same conditions described above, yielded less Glu after hydrolysis: ethanol, 36%; tetrahydrofuran, 11%; 2-methoxyethanol, 4%; dioxane, 3%; and dimethylformamide, 0.4% of expected value. The ether extract above was used for spectral determinations of 10, and to prepare Gla and <Gla, without further purification. Mass spectrum of 10: m/e 229 (parent ion), 156 ($-C_2H_5COO$, base peak), 128, 110.

DL-Monoammonium γ-carboxyglutamate. The reduction product (10 in ethyl ether solution) from 4 mmol of 9 was hydrolyzed in 15 ml of 2 N KOH at 105°C for 22 h. The hydrolysate was diluted with 45 ml of water and applied onto 50 ml of AG 50WX8 (NH_4^+-form, 50-100 mesh). Ammonium γ-carboxyglutamate was eluted with 600 ml of water, concentrated in vacuo, washed with ethyl acetate, and crystallized from water-ethanol, pH 2.8.[4] Yield: 445 mg (2.1 mmol, 54%); m.p. 167-169°C (d). Anal. Calcd. for $C_6H_{12}N_2O_6$ (208): C, 34.62; H, 5.77; Found: C, 34.77; H, 5.41. Column chromatography of the synthetic amino acid at pH 3.25[15] gave the same retention time and peak shape as that of authentic DL-Gla. Analysis for monoammonium Gla, 101.9±0.7% of calcd. value; after acid hydroly-

sis, Glu in 101.5% of expected value. When the acid hydrolysate was treated with excess of L-glutamate decarboxylase, 51.3% of the total Glu was converted to 4-amino-n-butyrate 14 within 1 h.

5-Oxo-2,4-pyrrolidinedicarboxylic acid (11). The ethyl ether extract of 10 from 908 mg of 9 was evaporated to a syrup, and 15 ml of 1 N KOH in 80% methanol added. After 1 h at room temperature, 15 ml of water was added, the clear solution being applied onto 20 ml of AG 50WX8 (H^+-form, 50-100 mesh), and free <Gla 11 being eluted with 200 ml of water. The eluate was evaporated under reduced pressure to a nearly colorless syrup, 10 ml of acetone was added, and the small amount of insoluble residue was removed. The acetone extract was concentrated and lyophilized to 0.6 g (87%) of hygroscopic powder. Proton NMR: 4.05-4.35 (m, α-CH), 3.45-3.75 (m, γ-CH), and 2.35-3.05 (m, β-CH_2). The γ-CH showed fast exchangeability with deuterons in D_2O.

5-Oxo-2,4-pyrrolidine dicarboxylic acid cyclohexylamine salt. To an aqueous ethanol solution of 3.33 mmol of 11 was added 1 ml of cyclohexylamine. The mixture was evaporated to a solid, washed with ethyl ether, and recrystallized from 95% ethanol and ethyl ether to yield 1.22 g (98%), m.p. 187-189°C (d). Anal. Calcd. for $C_{18}H_{33}N_3O_5$ (371): N, 11.32; Found: N, 11.17. Alkaline hydrolysis formed Gla 1 in 94.6±0.8% yield. Acid hydrolysis formed DL-Glu 12 in 99.5±0.3% yield. With L-glutamate decarboxylase, 51.3% of the Glu was converted to 14.

[2,3-^{3}H] 5-Oxo-2,4-pyrrolidine dicarboxylic acid (pyroGla). To a solution of 23 mg of 5-hydroxy-2,4-dicarbethoxypyrrole in 5 ml of ethyl acetate, 50 mg of 5% Pt/C catalyst and 25 Ci of tritium gas were added and the reaction mixture was stirred overnight at room temperature. Labile tritium and catalyst were removed and an aliquot of the product, 150 mCi in 25 ml ethanol, was shipped in dry ice to the authors. The ethyl ester solution was evaporated to dryness and extracted with 5 ml of ethyl ether. The ether was removed under N_2 and the <Gla ester was hydrolyzed in 1 ml of 1 N KOH in 80% methanol at room temperature for 1.5 h. To the reaction mixture, 2 ml of water was added and the clear solution

was applied to 3 ml of AG50W X8 (hydrogen form) resin column. ^{3}H-PyroGla was eluted with 60 ml of water.

[2,3-^{3}H]-Gamma carboxyglutamic acid (Gla). One half of the ^{3}H-pyroGla solution was evaporated to dryness, dissolved in 2 N KOH (1 ml) and transferred to a small polypropylene tube. After hydrolysis of 105°C overnight[15], water was added and the solution applied onto 5 ml of AG50WX8 (NH_4^+ form) resin. ^{3}H-Gla ammonium salt was eluted with 100 ml of water, evaporated to dryness, and redissolved in 30 ml of water for storage.

Radiopurities and specific activities. The radiopurities of ^{3}H-pyroGla and Gla were determined by paper chromatography on Whatman No. 1 paper using n-butanol-acetic acid-water (4:1:2, v/v) as a solvent system. ^{3}H-PyroGla (R_f 0.37) was approximately 97% pure; ^{3}H-Gla (R_f 0.15) was greater than 99% pure. The radioactivities of both compounds were determined by using 100 µl of sample solution, or standard ^{3}H-water (New England Nuclear, 2.54 x 10^6 dpm/ml, 4-5-82), to calculate counting efficiency. To determine the amount of pyroGla and Gla, the stock solution (100 µl) was hydrolyzed in 6 N HCl at 110°C overnight and the product, glutamic acid, was determined using a Beckman 190C amino acid analyzer[15]. The specific activities of ^{3}H-pyroGla and Gla were 6.1 and 4.9 Ci/mmol, respectively. The ^{3}H-Gla ammonium salt solution was stable at 5° for at least 3 months. ^{3}H-PyroGla, in free acid solution, gradually formed ^{3}H-pyroglutamic acid.

DISCUSSION

The new synthetic scheme described here provides a facile route to radiolabeled <Gla, Gla and its related compounds. Catalytic reduction of the keto ester with $^{3}H_2$, which is the most satisfactory procedure for tritium labeling, was used to produce diethyl [2,3-^{3}H]pyroGla. From this mother compound, [^{3}H]-pyroGla, Gla, Glu, <Glu, and enzymatically, γ-amino n-butyrate (GABA), have been easily prepared with high specific activity.

Radiolabeled Gla with such high specific activity should be of great value not only for metabolic studies, but also for analysis of Gla in biological and clinical samples. A suitable internal standard has been long sought for accurate assessment of Gla recovery. Fernlund[18] quantitated urinary Gla excretion using an anion-exchange concentration step and automated amino acid analysis. His study did not, however, use an internal standard so that recovery of Gla in each sample could not be precisely assessed. For such purposes, Hauschka[19] mentioned using [^{14}C]alanine or norleucine, and Gundberg et al.[20] and Levy and Lian[21] used [γ-^{14}C]Gla of low specific activity (1.6 mCi/mmol). We have used [^{14}C]cysteic acid prepared from [U-^{14}C]cystine (318 mCi/mmol).[15] High specific activity radiolabeled Gla, the best type of standard, has not been available, mainly because the commonly used synthetic routes to Gla start from a [^{14}C]-malonic acid ester of low specific activity ($\sim$10 mCi/mmol).

Free Gla and Gla-containing peptides are present in abnormally high amounts in the urine of patients with renal stones.[22] Most Gla in urine appears to be derived from the degradation of blood coagulation factors and of other Gla-containing proteins. The catabolism of vitamin K-dependent proteins, and the metabolic fate of released Gla residues, however, are not well understood. Shah et al.[23] have reported that in the rat, Gla is metabolically inert and is quantitatively excreted in urine after 48 h. They have also shown that DL-Gla labeled with ^{14}C in the γ-carboxy group (8.46 mCi/mol) is effectively concentrated by kidney slices, but not by preparations from brain, intestine or liver. They observed apparent transient accumulation of Gla in liver, muscle and bone, but that only the kidney appeared to be capable of concentrating Gla from the plasma. Because of the low specific activity of the DL-Gla, these authors were forced to administer 2 mg (10 μmol, 0.07 μCi) of the monoammonium salt to 150-165 g rats intracardially. However, from recent data on urine analysis, Gla excretions by normal human adults (70 kg) averaged only 40 μmol in 24 h.[21] Thus, Shah's studies were conducted under conditions which appear to be far from physiological. Their data, however, are the sole evidence upon which the assump-

tions have been made that Gla is not metabolized in humans, and that urinary Gla excretion is closely related to blood clotting status and to bone metabolism. It would be highly desirable to re-examine the metabolism of Gla under physiological conditions.

From the preceding discussion it is apparent that a crucial factor for such a study is the availability of radiolabeled Gla, and related compounds of high specific activity. With such highly labeled compounds, metabolism and transport could be studied more precisely, even at physiological levels. Used as an internal standard, Gla could be monitored and estimated with less background contribution, and recoveries could be more accurately calculated. Availability of high specific activity compounds should also facilitate the isolation and indentification of Gla and its related compounds in biological samples. Thus, the newly described synthetic scheme should open up opportunities to better study the metabolism of these interesting and important biological molecules. Because of its structural relationship to <Glu, it would be interesting to study the biochemical behavior of <Gla on 5-oxoprolinase (ATP-hydrolysing) (EC 3.5.2.9).[24)]

ACKNOWLEDGEMENTS

This study was supported in part by funds provided by Grant AM-18983 from the United States Public Health Service, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases and by NIH Biomedical Research Support Grant 08-57RR07160 E08.

Portions of these results were reported at the Annual Meeting of the Federation of American Societies for Experimental Biology, April 15-23, 1982 in New Orleans, Louisiana.

REFERENCES

1) Nelsestuen, G.L., Zytkovicz, T.H., & Howard, J.B. - J. Biol. Chem. 249, 6347 (1974).

2) Magnusson, S., Sottrup-Jensen, L., Petersen, T.E., Morris, H.R., & Dell, A. - FEBS Letters, 44, 189 (1974).

3) Stenflo, J., Fernlund, P., Egan, W., & Roepstorff, P. - Proc. Natl. Acad. Sci. USA 71, 2730 (1974).

4) Fernlund, P., Stenflo, J., Roepstorff, P., & Thomsen, J. - J. Biol. Chem. 250, 6125 (1975).

5) Morris, H.R., Thompson, M.R., & Dell, A. - Biochem. Biophys. Res. Commun. 62, 856 (1975).

6) Marki, W., & Schwyzer, R. - Helv. Chim. Acta 58, 1471 (1975).

7) Boggs, N.T.,III, Gawley, R.E., Koehler, K.A., & Hiskey, R.G. - J. Org. Chem. 40, 2850 (1975).

8) Weinstein, B., Watrin, K.G., Loie, H.J., & Martin, J.C. - J. Org. Chem. 41, 3634 (1976).

9) Marki, W., Oppliger, M., & Schwyzer, R. - Helv. Chim. Acta 59, 901 (1976).

10) Oppliger, M., & Schwyzer, R. - Helv. Chim. Acta 60, 43 (1977).

11) Danishefsky, S., Berman, E., Clizba, L.A., & Hirama, M. - J. Am. Chem. Soc. 101, 4385 (1979).

12) Marki, W., Oppliger, M., Thanei, P., & Schwyzer, R. - Helv. Chim. Acta 60, 798 (1977).

13) Boggs, N.T.,III, Goldsmith, B., Gawley, R.E., Koehler, K.A., & Hiskey, R.G. - J. Org. Chem. 44, 2262 (1979).

14) Stefancich, G, Nacci, V., Filacchioni, G., Giuliano, R., & Artico, M. - Farmaco. Ed. Sci. 30, 917 (1975). [C.A. 84, 43746z (1976)].

15) Ishikawa, Y., and Wuthier, R.E. - Anal. Biochem. 114, 388 (1981).

16) Gale, E.F. - Biochem J. 39, 46 (1945).

17) Kaneko, T., and Nakayama, S. - Nippon Kagaku Zasshi 77, 1054 (1956). [C.A. 53, 5146h (1959)].

18) Fernlund, P. - Clin. Chim. Acta 72, 147 (1976).

19) Hauschka, P.V. - Anal. Biochem. 80, 212 (1977).

20) Gundberg, C.M. Lian, J.B., and Gallop, P.M. - Anal. Biochem. 98, 219 (1979).

21) Levy, R.J., and Lian, J.B. - Clin. Pharmacol. Ther. 25, 562 (1979).

22) Hauschka, P.V., Lian, J.B., and Gallop, P.M. - Trends in Biochemical Sciences 3, 75 (1978).

23) Shah, D.V., Tews, J.K., Harper, A.E., & Suttie, J.W. - Biochim. Biophys. Acta 539, 209 (1978).

24) Van der Werf, P., Orlowski, M., & Meister, A. - Proc. Natl. Acad. Sci. USA, 68, 2982 (1971).