SYNTHESIS OF L-GLUTAMIC ACID STEREOSPECIFICALLY LABELED AT C-4 WITH DEUTERIUM

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SUMMARY

4-[$^2H_2$]-L-glutamic acid was prepared in excellent yield by enzymatic reductive amination of 4-[$^2H_2$]-2-ketoglutaric acid. The synthesis of stereospecifically deuterated (4 R) and (4 S)-[4-$^2H_2$]-L-glutamic acids from (2 RS, 4 S) and (2 RS, 4 R)-4-hydroxyglutamic acids, involving a reduction step by sodium cyanoborodeuteride, was shown to proceed with 75 % inversion of configuration.

Key words : 4-[$^2H_2$]-L-glutamic acid, (4 R)-[4-^2H]-L-glutamic acid, (4 S)-[4-^2H]-L-glutamic acid, stereospecific labeling, cyanoborodeuteride reduction.

There has been considerable interest in recent years about the mechanism of the vitamin K-dependent γ-carboxylation of selected glutamyl residues in synthetic peptides mimicking part of the N-terminal sequence of preprothrombin[1-3]. Several investigations have led to the elucidation of some stereochemical aspects of this reaction, particularly in the associated formation and reduction of vitamin K epoxide[4,5], but little is known about the stereochemistry of the carboxylation reaction itself[6].

In order to elucidate the stereochemistry of proton elimination accompanying the γ-carboxylation reaction, it was necessary to prepare the stereospecifically (4 R) and (4 S) monodeuterated L-glutamic acids. Only a few methods are available for obtaining such molecules[7,8,9] and some of them lead to only one isomer while the others are more adapted to the synthesis of tritiated molecules. In this paper, we report a synthesis of [4-$^2H_2$] L-glutamic acid and of both (4 R) [4-^2H] and (4 S) [4-^2H] L-glutamic acids (4 a and 4 c).

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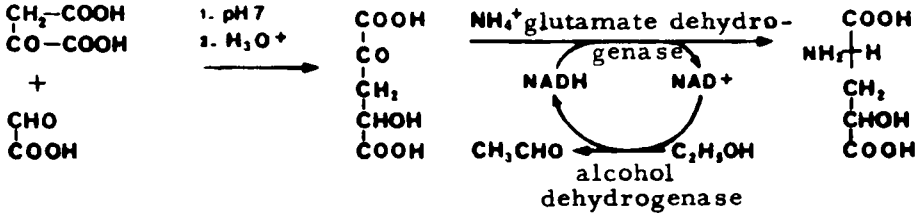
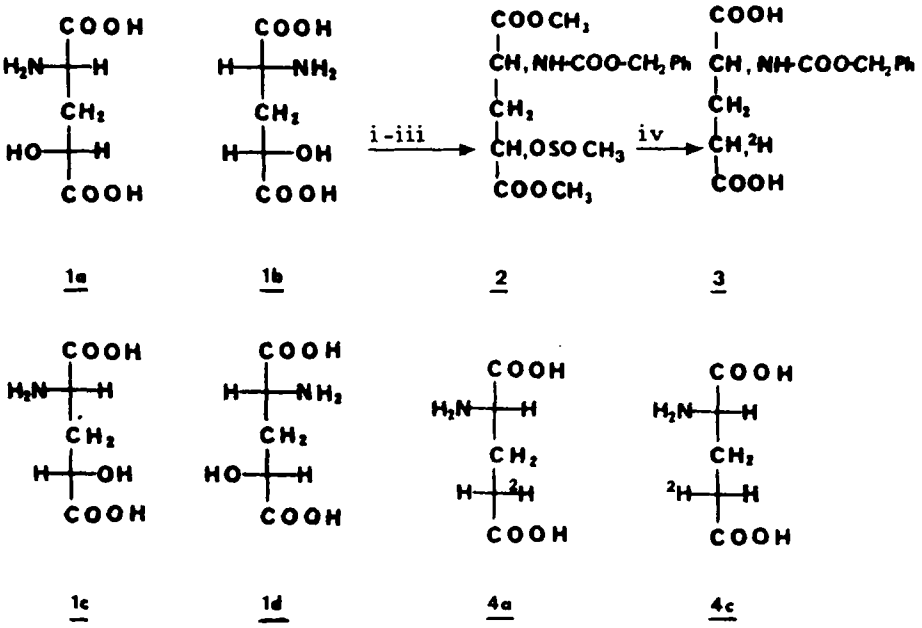
[4-$^{2}H_{2}$] L-Glutamic acid was obtained in nearly quantitative yield by enzymatic reductive amination (glutamate dehydrogenase, E.C. 1.4.1.2) of the corresponding deuterated 2-ketoglutaric acid[10]. The published procedure was modified by introducing a NADH-recycling system involving ethanol and yeast alcohol dehydrogenase (scheme 1). [4-$^{2}H_{2}$] 2-Ketoglutaric acid was prepared (70 % yield) by condensation of the [2,3-$^{2}H_{4}$] diethyl succinate anion (generated by potassium t-butoxide in place of potassium ethoxide-ethanol to avoid deuterium exchange) with ethyl oxalate[11], followed by acidic hydrolysis and decarboxylation in controlled conditions. The resulting glutamic acid was usually 92-95 % deuterated, as found by mass spectrometry and ^{1}H NMR spectroscopy (figure 1).



Scheme 1

As both diastereoisomers of 4-monodeuterated L-glutamic acid were required, a general application stereospecific reduction reaction was wanted. The selective reduction of halogenated or sulfonate ester derivatives in the presence of reactive carboxylate ester and amide groups, without elimination reactions, has been described using sodium cyanoborohydride as the source of hydride ions[12,13]. Moreover, in one case, the displacement reaction by the deuterated hydride was shown to be mostly c curring by a $S_{N}2$ process, in polar aprotic solvent, affording a highly stereoselective method for the introduction of deuterium[13].

4-Hydroxy L-glutamic acids were easily obtained by enzymatic reductive amination of (4 RS) 4-hydroxy 2-ketoglutaric acid[10,14], but the diastereoisomers could be resolved by ion exchange chromatography[15] only on a limited scale (0.1-0.2 g). We thus prefered a chemical preparation[16] of the mixture of the four 4-hydroxyglutamic acid isomers, from which diastereoisomeric racemates 1 a + 1 b and 1 c + 1 d were separated by fractional crystallization[15].



Scheme 2

i, $PhCH_2OCOCl$, MgO, water-toluene ; ii, CH_2N_2, ether ;
iii, CH_3SO_2Cl, pyridine-toluene ; iv, NaB^2H_3CN, HMPT, 100°C.

Both racemates were carbobenzoxylated, then converted to methyl diesters and O-mesylated. The conditions for a successful reduction of 2 by sodium cyanoborohydride necessitated a careful optimization, because of the unexpected formation of unidentified by-products during the long heating period in HMPT necessary for completing the reaction. Best conditions, 18 h at 100°C with excess hydride, resulted in 55 % yield of glutamate derivatives 3, containing approximately 1 deuterium atom in the 4-position (from 90-95 % deuterated $NaBD_3CN$). After isolation of 3, mild alkaline hydrolysis and Pd/C catalyzed hydrogenolysis, the presumably racemic 4-deuterated glutamic acids were acetylated and submitted to hydrolysis by hog kidney acylase I[17, 18]. The presumed (4 S) [4-^{2}H] and (4 R) [4-^{2}H] L-glutamic acids 4 a and 4 c were purified by ion exchange chromatography and analyzed with regard to their asymmetric centers.

HPLC of the free aminoacids 4 a and 4 c in the presence of Cu^{2+} and a chiral ligand[19] as well as GC of their N-trifluoroacetyl O-isopropyl esters on a chiral phase[20,21] indicated an optical purity at C-2 higher than 98 %.

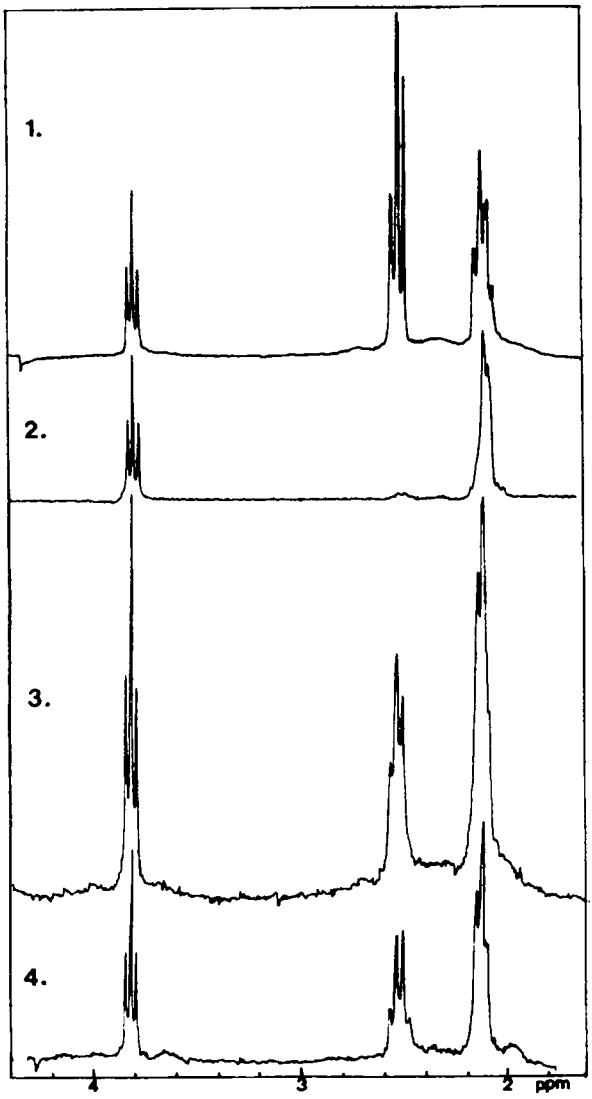


Fig. 1 - 250 MHz ^{1}H NMR spectra in D_2O of : 1. L-Glu 2. [4-$^{2}H_2$] L-Glu 3. (4R)-[4-^{2}H]-L-Glu 4. (4S)-[4-^{2}H]-L-Glu.

Determination of the absolute configuration of the deuterated 4-carbon atom required the elaboration of a new method[22,23], involving conversion of samples of glutamic acids to succinic acids, the 2-pro S hydrogen (deuterium) atoms of which were exchanged with water in the presence of glyoxylate and Pseudomonas indigofera isocitrate lyase[24] (E.C. 4.1.3.1.). Analysis of succinic acids as their dimethyl esters by chemical ionization (NH_3)-mass

Table I : Deuterium content of dimethyl succinates derived from 4 a and 4 c, determined by GC/C.I. -Mass Spectrometry[a], before and after enzymatic proton exchange of the corresponding succinic acids.

[2-^{2}H] dimethyl succinate	% abundance[b] of molecular ions 147 (MH^+)	148 (MH^+ + 1)
From 4a : before exchange	22.5	77.5
after exchange	79.9	20.1
From 4c : before exchange	18.9	81.1
after exchange	37.2	62.8

(a) Ion abundances were averaged for the whole chromatographic peak to avoid error arising from isotopic separations.

(b) Normalized and relative values corrected for natural isotope abundance.

spectrometry allowed an accurate determination of the original deuterium distribution between positions 4-pro R and 4-pro S of glutamic acid (Table I). The results show that, besides undeuterated molecules (22.5 % and 18.9 %), reflecting probably the high isotope effect of the reduction reaction, 4a and 4c contain respectively 57.4 % and 62.8 % isotope in the expected position. We conclude that the introduction of deuterium by displacement of the mesyl group by $NaBD_3CN$ has occurred with 76 ± 2 % inversion.

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EXPERIMENTAL SECTION

^{1}H NMR spectra were recorded in $CDCl_3$ with Me_4Si as reference, or in D_2O, on a R-32 Perkin Elmer (90 MHz) or a Cameca (250 MHz) spectrometer. Mass spectra were carried out at the Centre de Spectrométrie de Masse du C.N.R.S. (Lyon - France), on a VG 70 - 70 F mass spectrometer coupled with a Perkin Elmer Sigma 3 gas chromatograph equipped with a

25 m OV-101 fused silica capillary column operated at 60-200°C with helium gas; ion source temperature was 200°C and positive chemical ionization was obtained with NH_3 gas (0.3 - 0.5 torr). Optical rotations were measured on a Perkin Elmer 141 polarimeter in a 1.0 dm cell. HPLC analysis were performed on a 25 cm x 4.6 mm RSIL C 18 Alltech column. Medium pressure column chromatography was carried out with Merck Silicagel H 60. Analytical TLC was performed on Merck Silicagel 60 F-254 precoated plates (0.2 mm) and spots were made visible in U.V. light or by spraying with 10 % phosphomolybdic acid in ethanol and heating to 120°C. Hexamethylphosphorotriamide (HMPT) was a Merck product.

[^{2}H] Sodium cyanoborohydride ($NaBD_3CN$) was prepared by three successive exchanges at pH 2[25] of $NaBH_3CN$ (Aldrich) with 99.5 % D_2O. The final product was crystallized and stored as the dioxane complex. Analysis by ^{1}H NMR of the residual proton content was effected on a 30-50 mg sample, dried at 80° (0.5 torr) during 24 h, dissolved in D_2O (0.5 mL) containing glycine as internal standard, and compared to the hydride amount, estimated by iodometric determination. The hydride was similarly dried immediately before use in reduction reactions.

(2 RS, 4 RS) 4-Hydroxyglutamic acid 1 was prepared and the diastereoisomeric racemates (1a + 1b and 1c + 1d) separated according to the described method[15,16]. The purity of each diastereoisomer ($\geq$ 98 %) was tested by high voltage paper electrophoresis at pH 1.9[26] and by ion exchange chromatography on a Multichrom Beckman aminoacid analyzer (pH 3.1 to 4.0).

(2 S) [4-$^2H_2$] Glutamic acid. To a stirred suspension of t-Bu OK (25.4 mmoles) in ether (30 mL) at 0°C was added ethyl oxalate (25.4 mmoles) then, by portions [2,3-$^2H_2$] ethyl succinate (~98 % deuterated). After 15 min, 1 N HCl (50 mL) was added and the mixture extracted with ether. After drying (Na_2SO_4), [4-$^2H_2$] ethyl oxalosuccinate (25 mmoles, >98 % deuterated) was obtained as a colorless oil, and was refluxed in 0.2 N HCl (100 mL) during 4 h. The resulting solution of [4-$^2H_2$] 2-ketoglutaric acid (15 mmoles, estimated by the glutamate dehydrogenase reaction) was neutralized with

concentrated ammonia and converted quantitatively to (2 S) [4-$^2H_2$] glutamic acid in the conditions described for 1a and 1c preparation. After crystallization from water-EtOH, mp = 197-8 °C dec ; $[\alpha]_D^{20}$ = + 31.5° (c = 1, 1N HCl) ; ^{1}H NMR (D_2O, 90 MHz) : 3.82 (t, J = 6 Hz, CH), 2.54 (m, 4-CH_2, 0.1 H), 2.16 (enlarged d, J = 6 Hz, 3 - CH_2, 2 H).

(2 S, 4 S) and (2 S, 4R) 4-hydroxyglutamic acids (1 a and 1 c).

4-Hydroxy 2-ketoglutaric acid[10] (7.5 mmoles), adjusted at pH 7.5 with ammonia, bovine serum albumin (200 mg), NAD^+ (0.2 mmoles) and ethanol (5 mL) in 0.05 M phosphate buffer pH 7.5 (200 mL) containing 0.1 mM EDTA, was incubated with beef liver glutamate dehydrogenase (Boehringer, 500 U) and yeast alcohol dehydrogenase (Sigma, 1000 U). Residual hydroxy 2-ketoglutarate was spectrometrically estimated on 10 µL aliquots treated with 5 % trichloroacetic acid and converted to 4-hydroxyglutamate in the presence of excess glutamate dehydrogenase, NADH and NH_4Cl. After 24-30 h (>95 % conversion) solid trichloroacetic acid (5 g) was added, precipitated proteins removed by centrifugation and the supernatant, diluted to 1 L with water, filtered on a 30 x 1.6 cm Dowex 50 X 2 (H^+) column ; after washing with water (300 mL), 4-hydroxyglutamic acid was eluted with 0.25 N HCl. The pooled fractions were evaporated, taken up into water (1 L) and chromatographed on a 50 x 3 cm Dowex 1 X 4 (AcO^-) column eluted with 0.5 N AcOH.

Small amounts (100-200 mg) of (2S, 4S) an (2S, 4R) 4-hydroxyglutamic acids (1 a and 1 c) underwent nearly complete separation on a 45 x 3, 5 cm AG 1 X 8 (AcO^-, < 400 mesh) column[15].

1a: $[M]_D^{20}$ = -23.5° (c = 1, water) ; + 4.4° (c = 1, 5 N HCl) (lit : [14] - 22.3° ; + 5°)

1c: $[M]_D^{20}$ = + 31.6° (c = 1, water); + 61.5° (c = 1, 5 N HCl) (lit : [14] + 31.8° ; + 61.6°)

N-Carbobenzoxy 4-methane sulfonyloxy glutamic acid dimethyl ester 2.

The diastereoisomeric 4-hydroxyglutamic acids 1 a + 1 b and 1 c + 1 d were treated in aqueous solution, in the presence of MgO, with excess 50 % solution of benzylchloroformate in toluene (Merck) and the N-carbobenzoxylated 4-hydroxyglutamic acid, extracted in EtOAc after acidification (85 % yield) was methylated in ether with diazomethane. The resulting dried colorless oil (1 mmole), in order to avoid spontaneous lactonization, was immediately treated with methane sulfonylchloride (2 mmoles) and pyridine (1 mL) in

toluene (3 mL). After one night at room temperature, treatment with water, extraction with CH_2Cl_2, washing and drying, the residual oil was rapidly chromatographed on a silicagel column and eluted with CH_2Cl_2-EtOAc (85 : 15) to give pure mesylate 2 (85 % yield) ; ^{1}H NMR ($CDCl_3$, 90 MHz) : 7.34 (s, C_6H_5), 5.12 (s, CH_2O), 4.48 (m, CH), 4.32 (m, CHO), 3.13 - 3.14 (2 s, CH_3 - SO), 3.75 and 3.73 (2 s, CH_3O), 2.44 (m, CH_2).

N-Carbobenzoxy [4-^{2}H] glutamic acid dimethyl ester 3.

A solution of 2 (1 mmole) in HPMT (4 mL) was added to 6-7 mmoles of dried $NaBD_3CN$ and the mixture was stirred under an argon atmosphere at 100°C during 18 h. After cooling, water (20 mL) was added and the solution, acidified at pH 1 with 2 N HCl, was extracted with ether. After drying and removal of the solvent, the residual oil was submitted to column chromatography on silicagel. Compound 3 was eluted with CH_2Cl_2 - EtOAc (85 : 15) (55 % yield) : ^{1}H NMR ($CDCl_3$, 250 MHz) 7.32 (s, C_6, H_5), 5.55 (d, J = 7.5 Hz, NH), 5.10 (s, CH_2O), 4.40 (2 t, J = 5 Hz, CH), 3.72 and 3.64 (2 s, CH_3 O), 2.4 (m, 4-CH_2, 1 H), 2.09 (2 m, 3-CH_2, 2 H).

[4-^{2}H] Glutamic acid 4.

Each racemic 3 (1 mmole) was saponified during 1 h at room temperature in MeOH (30 mL) and 4 % Li OH in water (12 mL). It was verified (with ^{14}C labeled [4-^{3}H] glutamate) that no hydrogen exchange occurred in these conditions. After acidification at pH 2 with H Cl, MeOH was removed under reduced pressure and the oily residue extracted with ether to give crystallized N-carbobenzoxy [4-^{2}H] glutamic acid (mp 114°C). After hydrogenation under atmospheric pressure in AcOH (5 mL) in the presence of 10 % Pd on charcoal (50 mg) during 5 h, the resulting product was crystallized in water-ethanol (75 % yield).

(2 S, 4 R) and (2 S, 4S) [4-^{2}H] glutamic acids (4a and 4c).

Each racemic [4-^{2}H] glutamic acid (1, 5 mmole) was dissolved in water (10 mL) at 0°C and acetic anhydride (3 mmoles) was added by portions while the pH was maintained at 8. 5 ± 0. 5 with 4 N NaOH additions. When free glutamic acid became undetectable by colorimetric reaction with ninhydrin, the titration was continued for 2 h. Phosphate buffer (0. 05 M) pH 7. 5 containing

1 mM $CoCl_2$ (35 mL) and hog kidney acylase I (Sigma grade II, 30 mg) were added and the solution was incubated at 37°C. Enzymatic hydrolysis was followed by colorimetric reaction with ninhydrin and stopped at about 50 % (2 h) by acidification to pH 3 with 2 N HCl ; precipitated proteins were filtered with Celite and the filtrate, diluted to 300 mL, chromatographed on a Dowex 50 X 2 (H^+) column. Glutamic acid was eluted with 0.25 N HCl and the pooled fractions were evaporated, taken up in water (500 mL), adjusted to pH 8 with dilute NaOH and chromatographed on a Dowex 1 X 8 (AcO^-) column. Elution with 0.5 N AcOH gave pure 4a or 4c, which were crystallized from EtOH-water (75 % yield).

4a : $[\alpha]_D^{20}$ = + 10.5° (c = 1, water) ; + 29.1° (c = 2, 5N HCl)

4c : $[\alpha]_D^{20}$ = + 28.4° (c = 2, 5N HCl).

^{1}H NMR (D_2O, 250 MHz) : 3.80 (t, J = 6 Hz, 2-CH), 2.54 (m, 4-CH_2, 1 H), 2.14 (m, 3-CH_2, 2H).

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