Supplementary Information
for

Evaluation of Enzymatic and Magnetic Properties of γ-Glutamyl-[1-^{13}C]glycine and its Deuteration toward the Longer Retention of the Hyperpolarized State

Yohei Kondo,[1] Yutaro Saito,[1] Abdelazim Elsayed Elhelaly,[2] Fuminori Hyodo,[2] Tatsuya Nishihara,[1,†] Marino Itoda,[1] Hiroshi Nonaka,[1,‡] Masayuki Matsuo,[3] and Shinsuke Sando[1,4,]*

[1]*Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*

[2]*Department of Radiology, Frontier Science for Imaging, School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan*

[3]*Department of Radiology, School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan*

[4]*Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*

[†]*Present address: Department of Chemistry and Biological Science, College of Science and Engineering, Aoyama Gakuin University, 5-10-1 Fuchinobe, Chuo-ku, Sagamihara, 252-5258, Japan.*

[‡]*Present address: Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto University Katsura, Nishikyo-ku, 615-8510, Japan*

Contact corresponding author

Prof. Dr. Shinsuke Sando

Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo

7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

Email: ssando@chembio.t.u-tokyo.ac.jp

Table of contents

1. General remarks **3**

2. Figures **4**

Figure S1. Magnetic-field dependence of R_1 of γ-Glu-[1-^{13}C]Gly and γ-Glu-[1-^{13}C]Gly-d_2 in D_2O 4

Figure S2. Dynamic ^{13}C NMR of hyperpolarized γ-Glu-[1-^{13}C]Gly at 1.4 T 5

3. Schemes **6**

Scheme S1. Synthetic scheme of γ-[5-^{13}C]Glu-Gly 6

Scheme S2. Synthetic scheme of γ-Glu-[1-^{13}C]Gly 6

4. Synthesis **7**

5. Reference **10**

1. General remarks

Chemicals used in this study were purchased from commercial suppliers and used without further purification. NMR measurements were conducted with JEOL JNM-ECS 400 (9.4 T), JEOL JNM-ECA 500 (11.7 T), and JEOL JNM-ECA 600 (14.1 T). Hyperpolarized studies were performed with a Hypersense DNP polarizer (Oxford Instruments, UK) and 1.4 T Spinsolve 60 Carbon High Performance benchtop NMR apparatus (Magritek, New Zealand).

Abbreviation

Aq. aqueous solution

Bn: benzyl

CSA: chemical shift anisotropy

Cbz: benzyloxycarbonyl

DMF: *N*,*N*-dimethylformamide

DNP: dynamic nuclear polarization

PBS: Dulbecco's phosphate buffered saline

EDCI: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA: ethylenediaminetetraacetic acid

HOBt: 1-hydroxybenzotriazole

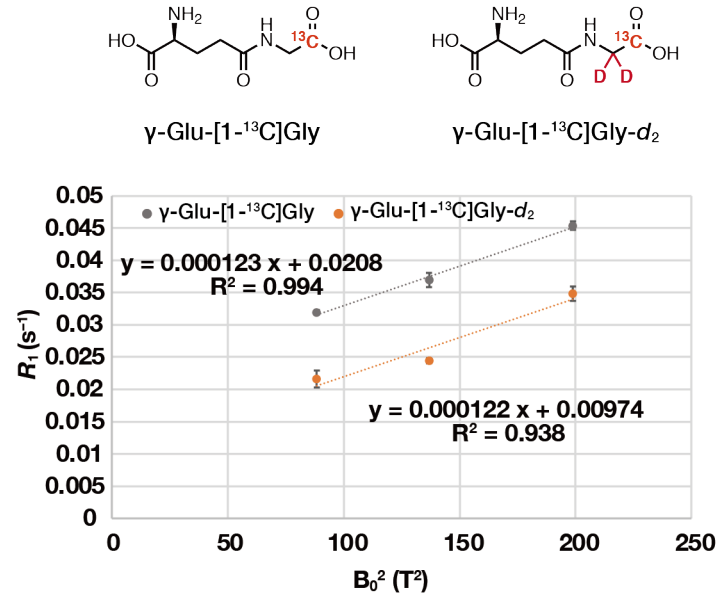
NHS: *N*-hydroxysuccinimide

Su: succinylimidyl

THF: tetrahydrofuran

2. Figures

A



B

Compound	R_{1CSA} at 9.4 T (s^{-1})
γ-Glu-[1-^{13}C]Gly	0.0108
γ-Glu-[1-^{13}C]Gly-d_2	0.0108

Figure S1. R_{1CSA} analysis of γ-Glu-[1-^{13}C]Gly and γ-Glu-[1-^{13}C]Gly-d_2 in D_2O

(A) The external magnetic field dependence of R_1 of γ-Glu-[1-^{13}C]Gly and γ-Glu-[1-^{13}C]Gly-d_2 in D_2O. T_1 values of γ-Glu-[1-^{13}C]Gly and γ-Glu-[1-^{13}C]Gly-d_2 in D_2O were determined at 9.4, 11.7, and 14.1 T. T_1 values were measured by inversion recovery (10 mM, D_2O, 9.4, 11.7, or 14.1 T, 37 °C, pD = 7.4 ± 0.1). Error bars represent standard deviation (n = 3).

(B) R_{1CSA} of γ-Glu-[1-^{13}C]Gly and γ-Glu-[1-^{13}C]Gly-d_2 in D_2O at 9.4 T. See the protocol for the calculation of R_{1CSA} at 9.4 T described in Experimental section.

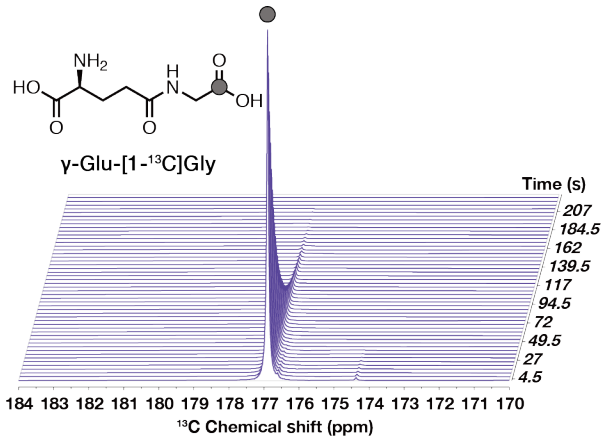


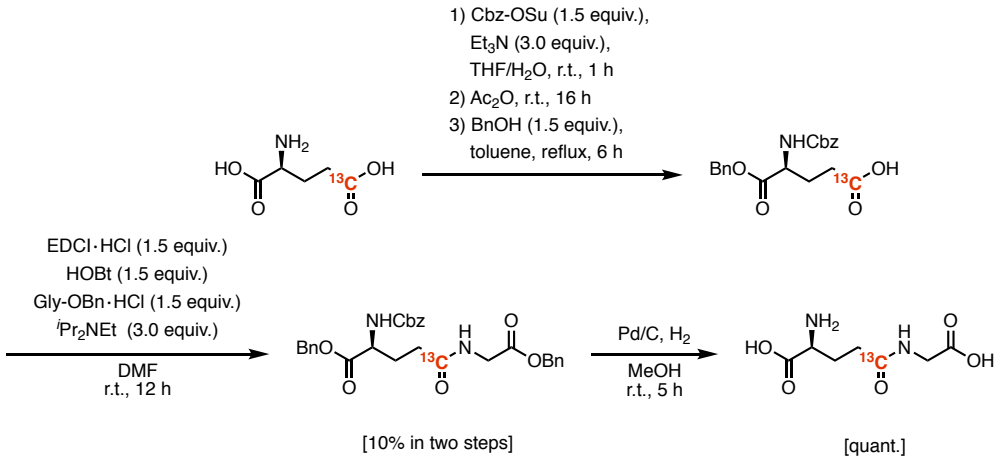
Figure S2. Dynamic ^{13}C NMR spectra of hyperpolarized γ-Glu-[1-^{13}C]Gly acquired using a 1.4 T NMR apparatus.

Repetition time = 4.5 s, flip angle = 10°. See the detailed protocol in Experimental section.

3. Schemes

3-1. Synthetic scheme of γ-[5-^{13}C]Glu-Gly

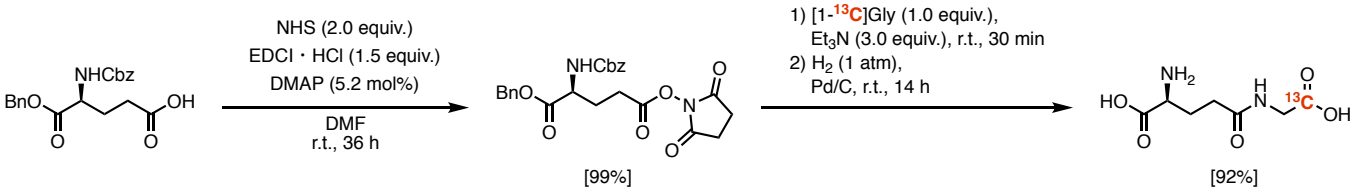
Synthesis of γ-[5-^{13}C]Glu-Gly was performed according to Scheme S1. See the Synthesis section for the detailed procedures.



Scheme S1. Synthetic scheme of γ-[5-^{13}C]Glu-Gly.

3-2. Synthetic scheme of γ-Glu-[1-^{13}C]Gly

Synthesis of γ-Glu-[1-^{13}C]Gly was performed according to Scheme S2. See the Synthesis section for the detailed procedures.



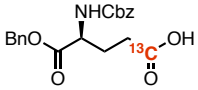
Scheme S2. Synthetic scheme of γ-Glu-[1-^{13}C]Gly.

4. Synthesis

General information on synthesis

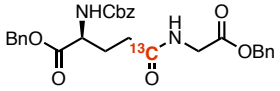
Reagents and solvents were purchased from standard suppliers and used without further purification. NMR spectra for characterization were acquired with a JNM-ECS 400 spectrometer (^{1}H: 400 MHz, ^{13}C: 100 MHz, JEOL, Japan). All chemical shifts are reported in parts per million (ppm). Chloroform-d_1 (δ 7.26 ppm) or D_2O (δ 4.79 ppm) were used as internal standards for ^{1}H NMR. Chloroform-d_1 (δ 77.2 ppm) or 1,4-dioxane (δ 67.2 ppm) in D_2O were used as internal standards for ^{13}C NMR. Data are reported as follows: chemical shift, multiplicity (s = singlet, brs = broad singlet, d = doublet, t = triplet, td = triplet of doublets, m = multiplet), coupling constant (Hz), and integration. High resolution mass spectrometry (HRMS) was acquired using micrOTOF II (ESI, Bruker Daltonics, USA).

Synthesis of *N*-Cbz-γ-[5-^{13}C]Glu(OBn)



To a 25-mL round-bottom flask with a magnetic stirring bar, [5-^{13}C]Glu (100 mg, 675 μmol, 1.0 equiv.) was added. After adding THF (1.0 mL), H_2O (1.0 mL), Et_3N (280 μL, 2.01 mmol, 3.0 equiv.), and Cbz-OSu (256 mg, 1.03 mmol, 1.5 equiv.), the reaction mixture was stirred at room temperature for 1 h. The resulting mixture was then evaporated to remove the solvent. The residue was acidified with 0.1 M HCl aq. and extracted with EtOAc (30 mL × 3). The organic phase was washed with brine (30 mL × 1), dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to give a colorless oil. After adding Ac_2O (5 mL, excess) to the oil, the resulting mixture was stirred for 16 h at room temperature and evaporated to remove the solvent. To the residue was added BnOH (96.0 μL, 923 μmol, 1.4 equiv.) and toluene (3.0 mL). The mixture was refluxed for 6 h. After cooling to room temperature, saturated $NaHCO_3$ aq. was added. The aqueous phase was washed with Et_2O and acidified with 2 M HCl aq., and extracted with EtOAc (30 mL × 3). The resulting organic solution was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product containing regioisomers was used for the next step without further purification and characterization.

Synthesis of *N*-Cbz-γ-[5-^{13}C]Glu(OBn)-Gly-OBn

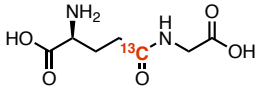


To a two-necked 25-mL round-bottom flask with a magnetic stirring bar, the crude product containing *N*-Cbz-γ-[5-^{13}C]Glu(OBn) (obtained above, 64.9 mg, 174 μmol), HOBt (35.4 mg, 262 μmol, 1.5 equiv.), and EDCI·HCl (51.4 mg, 268 μmol, 1.5 equiv.) were added. The vessel was purged with N_2 through evacuation and filling back N_2 three times. After adding DMF (3.0 mL), Gly-OBn·HCl (51.6 mg, 256 μmol, 1.5 equiv.), and $^{i}Pr_2NEt$ (91.0 μL, 522 μmol, 3.0 equiv.), the mixture was stirred at room temperature for 12 h. The resulting

mixture was evaporated to remove the solvent. The residue was added 0.1 M HCl aq. and extracted with EtOAc (30 mL × 4). The organic phase was washed with 0.1 M HCl aq. (30 mL × 2), saturated $NaHCO_3$ aq. (30 mL × 2), and brine (30 mL × 1). The resulting solution was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was subjected to silica-gel column chromatography (Hexane/EtOAc) to afford *N*-Cbz-γ-[5-^{13}C]Glu(OBn)-Gly-OBn (14.0 mg, 10% yield in two steps based on [5-^{13}C]Glu) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39–7.28 (m, 15H), 6.35 (brs, 1H), 5.68 (d, *J* = 7.7 Hz, 1H), 5.16 (s, 4H), 5.10 (s, 2H), 4.51–4.43 (m, 1H), 4.11–3.95 (m, 2H), 2.34–2.19 (m, 3H), 2.06–1.93 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.2, 172.2 (^{13}C-labeled), 169.9, 156.5, 136.3, 135.4, 135.3, 128.8, 128.7, 128.6, 128.5, 128.4, 128.3, 67.5, 67.4, 67.3, 53.6, 41.6, 32.2 (d, *J* = 48.9 Hz), 28.8, three carbons are not observed due to overlapping; HRMS (ESI) *m/z* calcd for $C_{28}{}^{13}CH_{30}N_2NaO_7$ $[M+Na]^+$: 542.1979, found 542.1996.

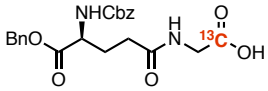
Synthesis of γ-[5-^{13}C]Glu-Gly



To a 25-mL round-bottom flask with a magnetic stirring bar, *N*-Cbz-γ-[5-^{13}C]Glu(OBn)-[1-^{13}C]Gly-OBn (14.0 mg, 26.9 μmol) and palladium on activated carbon (9.3 mg, 32 mol% for Pd) were added. The vessel was purged with N_2 through evacuation and filling back N_2 three times. After adding MeOH (3.0 mL), the vessel was purged with H_2. The reaction mixture was stirred at room temperature for 5 h and filtered with Celite®. The filtrate was concentrated under reduced pressure. After adding H_2O, the resulting solution was lyophilized to afford γ-[5-^{13}C]Glu-Gly (quant.) as a white solid.

^{1}H NMR (400 MHz, D_2O) δ 3.92 (d, *J* = 4.0 Hz, 2H), 3.86–3.83 (m, 1H), 2.57–2.51 (m, 2H), 2.29–2.15 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 177.6, 175.1 (^{13}C-labeled), 174.7, 54.9, 54.9, 44.0, 32.1 (d, *J* = 49.8 Hz), 26.9, ^{13}C NMR was acquired under the neutral condition with adjusting pD using NaOD aqueous solution, high concentration induces peak splitting at 54.9 ppm; HRMS (ESI) *m/z* calcd for $C_6{}^{13}CH_{12}N_2NaO_5$ $[M+Na]^+$: 228.0672, found 228.0668.

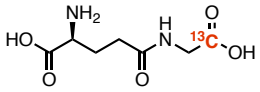
Synthesis of *N*-Cbz-γ-Glu(OBn)-[1-^{13}C]Gly



To a 50-mL round-bottom flask with a magnetic stirring bar, *N*-Cbz-γ-Glu(OBn)-OSu (400 mg, 852 μmol) was added. After adding THF (2.0 mL), H_2O (2.0 mL), [1-^{13}C]Gly (64.3 mg, 845 μmol, 1.0 equiv.), and Et_3N (349 μL, 2.50 mmol, 2.9 equiv.), the reaction mixture was stirred at room temperature for 30 min. The resulting mixture was evaporated to remove the solvent. After acidifying with 0.1 M HCl aq., the mixture was extracted with EtOAc (30 mL × 3), and the organic phase was washed with 0.1 M HCl aq. (30 mL × 3) and brine (30 mL

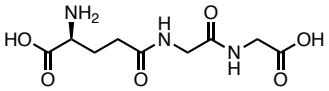
× 1). The resulting solution was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford *N*-Cbz-γ-Glu(OBn)-[1-^{13}C]Gly (347 mg, 95% yield) as a white solid. Characterization was conducted according to the previous literature.[1]

Synthesis of γ-Glu-[1-^{13}C]Gly



To a 50-mL round-bottom flask with a magnetic stirring bar, *N*-Cbz-γ-Glu(OBn)-[1-^{13}C]Gly (344 mg, 801 μmol) and palladium on activated carbon (39.1 mg, 4.6 mol% for Pd) were added. The vessel was purged with N_2 through evacuation and filling back N_2 three times. After adding MeOH (5.0 mL), the vessel was purged with H_2. The reaction mixture was stirred at room temperature for 14 h and filtered with Celite®. The filtrate was concentrated under reduced pressure to afford γ-Glu-[1-^{13}C]Gly (151 mg, 92% yield) as a pale brown solid. Characterization was conducted according to the previous literature.[1]

Synthesis of γ-Glu-Gly-Gly



γ-Glu-Gly-Gly was synthesized according to Scheme S2 using Gly-Gly instead of [1-^{13}C]Gly with minor modification. γ-Glu-Gly-Gly (157 mg, 35% yield in two steps) was obtained as a white solid.

^{1}H NMR (400 MHz, D_2O) δ 3.98 (s, 2H), 3.94 (s, 2H), 3.83–3.80 (m, 1H), 2.54 (td, J = 7.6 Hz, J = 3.0 Hz, 2H), 2.21–2.15 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 175.9, 174.7, 174.3, 172.6, 54.5, 43.1, 42.2, 31.8, 26.5; HRMS (ESI) *m/z* calcd for $C_9H_{15}N_3NaO_6$ $[M+Na]^+$: 284.0853, found 284.0865.

5. Reference

1 T. Nishihara, H. A. I. Yoshihara, H. Nonaka, Y. Takakusagi, F. Hyodo, K. Ichikawa, E. Can, J. A. M. Bastiaansen, Y. Takado, A. Comment and S. Sando, *Angew. Chem. Int. Ed.*, 2016, **55**, 10626–10629.