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# Evaluation of enzymatic and magnetic properties of $\gamma$ -glutamyl-[1- $^{13}\text{C}$ ]glycine and its deuteration toward longer retention of the hyperpolarized state†

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Dynamic nuclear polarization (DNP) is an emerging cutting-edge method of acquiring metabolic and physiological information *in vivo*. We recently developed  $\gamma$ -glutamyl-[1- $^{13}\text{C}$ ]glycine ( $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly) as a DNP nuclear magnetic resonance (NMR) molecular probe to detect  $\gamma$ -glutamyl transpeptidase (GGT) activity *in vivo*. However, the detailed enzymatic and magnetic properties of this probe remain unknown. Here, we evaluate a  $\gamma$ -Glu-Gly scaffold and develop a deuterated probe,  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ , that can realize a longer lifetime of the hyperpolarized signal. We initially evaluated the GGT-mediated enzymatic conversion of  $\gamma$ -Glu-Gly and the magnetic properties of  $^{13}\text{C}$ -enriched  $\gamma$ -Glu-Gly ( $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -[5- $^{13}\text{C}$ ]Glu-Gly) to support the validity of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly as a DNP NMR molecular probe for GGT. We then examined the spin-lattice relaxation time ( $T_1$ ) of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  under various conditions ( $\text{D}_2\text{O}$ , PBS, and serum) and confirmed that the  $T_1$  of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  was maintained for 30 s (9.4 T) and 41 s (9.4 T), respectively, even in serum. Relaxation analysis of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly revealed a significant contribution of the dipole-dipole interaction and the chemical shift anisotropy relaxation pathway (71% of the total relaxation rate at 9.4 T), indicating the potential of deuteration and the use of a lower magnetic field for realizing a longer  $T_1$ . In fact, by using  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  as a DNP probe, we achieved longer retention of the hyperpolarized signal at 1.4 T.

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## Introduction

The cell surface-bound enzyme  $\gamma$ -glutamyl transpeptidase (GGT) catalyzes a reaction that initiates the cellular uptake of the biological antioxidant glutathione (GSH).<sup>1</sup> The  $\gamma$ -glutamyl group of its natural substrate GSH is accurately recognized by GGT, and the  $\gamma$ -peptide

bond is hydrolyzed or glutamic acid (Glu) is transferred to acceptors such as amino acids and peptides. The resulting cysteinyl-glycine (Cys-Gly) undergoes further metabolism by dipeptidases to generate cysteine (Cys) and glycine (Gly). These amino acids are then taken up by cells and resynthesized into intracellular GSH.<sup>2</sup> Thus, GGT is an important enzyme involved in the redox state through GSH homeostasis *in vivo*. In particular, GGT is abundantly expressed in cancers such as soft tissue sarcoma and ovarian adenocarcinoma.<sup>3</sup> Thus, GGT could be a useful cancer biomarker, and the detection of GGT expression and its activity *in vivo* are of biological and medical importance.

The expression and activity of GGT have been detected using various detection modalities. For example, GGT expression *in vivo* can be visualized using positron emission tomography (PET),<sup>4,5</sup> but this approach cannot directly detect GGT activity. Fluorescent molecular probes for detecting GGT activity have been extensively studied, and some have been applied to fluorescence-guided surgery.<sup>6,7</sup> Furthermore, molecular imaging *in vivo* using hyperpolarization has recently attracted attention because it significantly improves the sensitivity of nuclear magnetic resonance and imaging (NMR/MRI).<sup>8</sup>

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Dissolution dynamic nuclear polarization (d-DNP) is a reliable method of hyperpolarization that can increase the detection sensitivity of  $^{13}\text{C}$ -enriched biomolecules, including  $^{13}\text{C}$ -pyruvic acid, and acquire physiological information about metabolism and pH *in vivo*.<sup>9,10</sup> DNP NMR using  $^{13}\text{C}$ -pyruvic acid has also been clinically applied to detect human prostate cancers, indicating the medical and biological value of DNP NMR.<sup>11,12</sup>

We recently developed  $\gamma$ -glutamyl-[1- $^{13}\text{C}$ ]glycine ( $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly) as a DNP NMR molecular probe to non-invasively detect GGT activity *in vivo* (Fig. 1).<sup>13</sup> The structure of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly comprises a  $\gamma$ -Glu group as a GGT recognition moiety and [1- $^{13}\text{C}$ ]Gly as a signaling moiety. The  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly probe was converted to the product, [1- $^{13}\text{C}$ ]Gly, by an enzymatic reaction with GGT. The  $^{13}\text{C}$  chemical shift change between the probe and the product was 4.3 ppm, which is large enough to distinguish *in vivo*. The spin-lattice relaxation time ( $T_1$ ) correlates with the retention time of the hyperpolarized state, and the probe and product had sufficiently long  $T_1$  values of 30 s (9.4 T,  $\text{H}_2\text{O}$ ) and 45 s (9.4 T,  $\text{H}_2\text{O}$ ), respectively, to detect GGT activity in rat kidneys. Since then,  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly has been applied in studies of rat kidneys function, subcutaneous ovarian carcinoma, and MiaPaCa-2 xenografts, as well as glioma models, and it should have further applications *in vivo*.<sup>14–17</sup> However, the structure–activity relationship and detailed magnetic parameters of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly, which are important for DNP MRI studies, have not been investigated.

Here, we report an evaluation of the enzymatic and magnetic properties of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and the development of a deuterated probe,  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ , that can achieve longer lifetime of the hyperpolarized signal than the conventional probe.

## Experimental

### Synthesis

**General remarks.** 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 ( $^1\text{H}$ : 400 MHz,  $^{13}\text{C}$ : 100 MHz, JEOL, Japan). All chemical shifts are reported in parts per million (ppm) relative to internal standard. Chloroform- $d_1$  ( $\delta$  7.26 ppm) or  $\text{D}_2\text{O}$  ( $\delta$  4.79 ppm) were used as internal standards for  $^1\text{H}$  NMR. Chloroform- $d_1$  ( $\delta$  77.2 ppm) or 1,4-dioxane ( $\delta$  67.2 ppm) in  $\text{D}_2\text{O}$  were used as internal standards for  $^{13}\text{C}$  NMR. Data are reported as follows: chemical shift, multiplicity (s = singlet, brs = broad singlet, d = doublet, t = triplet, 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- $\gamma$ -Glu(OBn)-OSu.** To a three-necked 300 mL round-bottom flask with a magnetic stirring bar,  $N$ -Cbz- $\gamma$ -Glu(OBn) (6.00 g, 16.2 mmol) was added. The vessel was heated under vacuum for 10 min to remove water. After cooling to room temperature,  $N$ -hydroxyl succinimide (NHS, 3.72 g, 32.4 mmol, 2.0 equiv.) and 4-dimethylaminopyridine (DMAP, 103 mg, 841  $\mu\text{mol}$ , 5.2 mol%) were added. The vessel was purged with  $\text{N}_2$  through evacuation and filling back  $\text{N}_2$  three times. After adding DMF (70 mL) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI·HCl, 4.65 g, 24.2 mmol, 1.5 equiv.), the mixture was stirred at room temperature for 36 h under  $\text{N}_2$  atmosphere. The resulting mixture was added EtOAc (140 mL), and washed with water (200 mL  $\times$  4) and brine (60 mL  $\times$  3). The resulting solution was dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated under reduced pressure. The desired compound was obtained (7.49 g, 99% yield) by recrystallization from hexane/ $\text{CHCl}_3$  as a colorless crystal. Characterization was conducted according to the previous literature.<sup>18</sup>

**Synthesis of  $N$ -Cbz- $\gamma$ -Glu(OBn)-[1- $^{13}\text{C}$ ]Gly- $d_2$ .** To a 50 mL round-bottom flask with a magnetic stirring bar, [1- $^{13}\text{C}$ ]Gly (504 mg, 6.62 mmol), ruthenium on activated carbon (208 mg, 1.6 mol% for Ru), and  $\text{D}_2\text{O}$  (10 mL) were added. The vessel was purged with  $\text{H}_2$  through evacuation of air and filling back  $\text{H}_2$  three times. The mixture was stirred at 90  $^\circ\text{C}$  for 19 h. After cooling to room temperature, the resulting mixture was filtered through Celite®. The filtrate was concentrated under reduced pressure. The product, [1- $^{13}\text{C}$ ]Gly- $d_2$ , was used for the next step without further purification and characterization.

To a 25 mL round-bottom flask with a magnetic stirring bar,  $N$ -Cbz- $\gamma$ -Glu(OBn)-OSu (403 mg, 861  $\mu\text{mol}$ , 1.0 equiv.) was added. After adding THF (2.0 mL),  $\text{H}_2\text{O}$  (2.0 mL), [1- $^{13}\text{C}$ ]Gly- $d_2$  (66.8 mg, 856  $\mu\text{mol}$ ), and  $\text{Et}_3\text{N}$  (349  $\mu\text{L}$ , 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 residue was extracted with EtOAc (30 mL  $\times$  2) and washed with 0.1 M HCl aq. (30 mL  $\times$  2) and brine (30 mL  $\times$  1). The resulting solution was dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated under reduced pressure. The residue was subjected to silica-gel column chromatography ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$ ) to afford  $N$ -Cbz- $\gamma$ -Glu(OBn)-[1- $^{13}\text{C}$ ]Gly- $d_2$  (305 mg, 83% yield based on [1- $^{13}\text{C}$ ]Gly- $d_2$ ) as a white solid.

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.38–7.29 (m, 10H), 6.50 (brs, 1H), 5.66 (d,  $J$  = 7.6 Hz, 1H), 5.21–5.14 (m, 2H), 5.10 (s, 2H), 4.49–4.44 (m, 1H), 4.07–3.94 (m, 0.16H, deuterated), 2.34–2.21 (m, 3H), 2.00–1.91 (m, 1H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  172.7

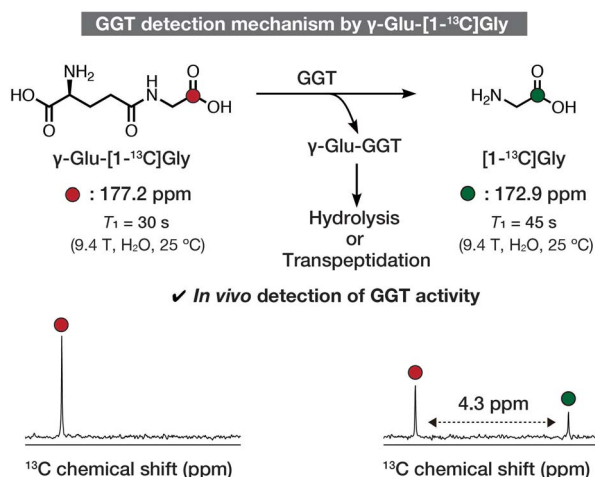


Fig. 1 Schematic illustration of detection of GGT using  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly.  $^{13}\text{C}$  Chemical shift and  $T_1$  values are cited from ref. 13. Colored atoms indicate isotope-enriched  $^{13}\text{C}$ .



( $^{13}\text{C}$ -labeled), 171.9, 170.9, 156.8, 136.1, 135.3, 128.8, 128.8, 128.5, 128.3, 67.7, 67.5, 53.7, 32.2, 28.8, two carbons are not observed due to overlapping, one carbon is not observed due to peak broadening; HRMS (ESI)  $m/z$  calcd for  $\text{C}_{21}^{13}\text{CH}_{22}\text{D}_2\text{N}_2\text{NaO}_7$   $[\text{M} + \text{Na}]^+$ : 454.1635, found 454.1637.

**Synthesis of  $\gamma$ -Glu-[ $1\text{-}^{13}\text{C}$ ]Gly- $d_2$ .** To a 50 mL round-bottom flask with a magnetic stirring bar,  $N$ -Cbz- $\gamma$ -Glu(OBn)-[ $1\text{-}^{13}\text{C}$ ]Gly- $d_2$  (290 mg, 671  $\mu\text{mol}$ ) and palladium on activated carbon (47.0 mg, 6.6 mol% for Pd) were added. The vessel was purged with  $\text{N}_2$  through evacuation and filling back  $\text{N}_2$  three times. After adding MeOH (5.0 mL), the vessel was purged with  $\text{H}_2$ . The reaction mixture was stirred at room temperature for 2 h and filtered with Celite®. The filtrate was concentrated under reduced pressure to afford  $\gamma$ -Glu-[ $1\text{-}^{13}\text{C}$ ]Gly- $d_2$  (137 mg, 99% yield) as a white solid.

$^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  3.93 (brs, 0.16H, deuterated), 3.83 (t,  $J = 6.2$  Hz, 1H), 2.59–2.46 (m, 2H), 2.25–2.11 (m, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$  177.4 ( $^{13}\text{C}$ -labeled), 175.2, 174.7, 54.9, 43.7 (brs, deuterated carbon), 32.1, 26.9,  $^{13}\text{C}$  NMR was acquired under the neutral condition by adjusting pD using NaOD aqueous solution; HRMS (ESI)  $m/z$  calcd for  $\text{C}_6^{13}\text{CH}_{10}\text{D}_2\text{N}_2\text{NaO}_5$   $[\text{M} + \text{Na}]^+$ : 230.0797, found 230.0799.

### Glu production monitoring with UHPLC

To 100  $\mu\text{L}$  of the substrate solution (5 mM of  $\gamma$ -Glu-Gly,  $\gamma$ -Glu-Gly-Gly, and GSH, or 0, 0.3125, 0.625, 1.25, 2.5, and 5.0 mM of Glu in 0.1 M PB as calibration samples) was added GGT enzyme solution (1 U  $\text{mL}^{-1}$  in 0.1 M PB, 15  $\mu\text{L}$ ) or 0.1 M PB (15  $\mu\text{L}$ ). The reaction mixtures were incubated at 37  $^\circ\text{C}$  for 30 min or 60 min. The reaction was quenched by adding GGT inhibitor solution (10 mM of GGTsTop® in DMSO, 5  $\mu\text{L}$ ). To the vessel containing 80  $\mu\text{L}$  of 50 mM borate buffer (pH 8.0) containing EDTA (20 mM) and 20  $\mu\text{L}$  of 4-fluoro-7-nitrobenzofurazan (20 mM) solution in acetonitrile was added 20  $\mu\text{L}$  of the enzymatic reaction mixture. The resulting mixture was incubated at 60  $^\circ\text{C}$ . The 4-fluoro-7-nitrobenzofurazan labeling reaction was quenched by adding 40  $\mu\text{L}$  of 50 mM HCl aqueous solution. The solution was filtered and used for UHPLC analysis. The concentration of Glu that was produced by GGT-mediated reaction from the substrates was quantified by comparing the fluorescent intensity with that of calibration samples of Glu. One unit of GGT was defined as the amount of the enzyme that generates 1.0  $\mu\text{mol}$  of  $p$ -nitroaniline from  $\gamma$ -Glu- $p$ -nitroanilide per minute at 37  $^\circ\text{C}$  (2.5 mM  $\gamma$ -Glu- $p$ -nitroanilide in 0.1 M PB).

### $T_1$ measurements

$T_1$  values were determined by inversion recovery method using JEOL JNM-ECS 400 (9.4 T), JEOL JNM-ECA 500 (11.7 T), and JEOL JNM-ECA 600 (14.1 T). Measurement conditions:  $\text{D}_2\text{O}$ , PBS, or mouse serum containing 10%  $\text{D}_2\text{O}$ , 10 mM of the compounds, 37  $^\circ\text{C}$ . pD was adjusted to  $7.4 \pm 0.1$  with DCl aq. or NaOD aq. in  $\text{D}_2\text{O}$ . pH was adjusted to  $7.4 \pm 0.1$  with HCl aq. or NaOH aq. in PBS. For the measurements in PBS, glass capillary containing  $\text{D}_2\text{O}$  was used to get the lock signal. All  $T_1$  measurements were conducted under thermal equilibrium state ( $n = 3$ ).

### NMR measurements for $^{13}\text{C}$ chemical shift

$^{13}\text{C}$  chemical shift of probes and products were determined in  $\text{D}_2\text{O}$  using 1,4-dioxane (67.19 ppm) as an internal standard at 9.4 T. Measurement conditions: 5 mM of the substrate, 37  $^\circ\text{C}$ ,  $\text{D}_2\text{O}$ . pD was adjusted to  $7.4 \pm 0.1$  with DCl aq. or NaOD aq. All measurements were conducted at thermal equilibrium state.

### $R_{1\text{DD}}$ analysis

$^1\text{H}$ - $^{13}\text{C}$  dipole-dipole (DD) relaxation is caused by DD interactions mainly due to nearby  $^1\text{H}$  nuclei. The extent of DD relaxation can be denoted using the relaxation rate,  $R_{1\text{DD}}$ .  $R_{1\text{DD}}$  was estimated with a method utilizing NOE. Measurement conditions: 9.4 T, 10 mM, 37  $^\circ\text{C}$ ,  $\text{D}_2\text{O}$ . pD was adjusted to  $7.4 \pm 0.1$  by DCl aq. or NaOD aq. See the detailed protocols described in the previous report.<sup>19</sup>

### $R_{1\text{CSA}}$ analysis

Chemical shift anisotropy (CSA) relaxation is caused by CSA of the nucleus and molecular tumbling. The extent of CSA relaxation can be denoted using the relaxation rate,  $R_{1\text{CSA}}$ .  $R_{1\text{CSA}}$  was estimated by measuring  $T_1$  at various external magnetic field strength (9.4, 11.7, and 14.1 T) under thermal equilibrium state according to the previous reports.<sup>20,21</sup>  $R_{1\text{CSA}}$  contribution at 9.4 T was calculated using the following eqn (1).

$$R_{1\text{CSA}}(9.4 \text{ T}) = R_1(9.4 \text{ T}) - R_1(0 \text{ T}) \quad (1)$$

where  $R_1(0 \text{ T})$  is the y-axis intercept of  $R_1$ - $B_0^2$  plot in Fig. S1.†

### Hyperpolarized experiments

**General information on hyperpolarized studies.** Hyperpolarization was achieved using a HyperSense DNP polarizer (Oxford Instruments, UK) with microwave irradiation at 93.938 GHz and 100 mW at 1.4 K. Subsequent  $^{13}\text{C}$  NMR was acquired with a 1.4 T Spinsolve 60 Carbon High Performance benchtop NMR apparatus (Magritek, New Zealand).

### Sample preparation for hyperpolarized experiments

To 82.5 mg of  $\gamma$ -Glu-[ $1\text{-}^{13}\text{C}$ ]Gly was added 38  $\mu\text{L}$  of 5 M NaOH aqueous solution, 60  $\mu\text{L}$  of  $\text{H}_2\text{O}$ , and 15  $\mu\text{L}$  of 10 M NaOH aqueous solution, successively. Similarly, to 81.6 mg of  $\gamma$ -Glu-[ $1\text{-}^{13}\text{C}$ ]Gly- $d_2$  was added 38  $\mu\text{L}$  of 5 M NaOH aqueous solution, 60  $\mu\text{L}$  of  $\text{H}_2\text{O}$ , and 15  $\mu\text{L}$  of 10 M NaOH aqueous solution, successively. After dissolving the probes, the final volumes became 180  $\mu\text{L}$  for both  $\gamma$ -Glu-[ $1\text{-}^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[ $1\text{-}^{13}\text{C}$ ]Gly- $d_2$ . Therefore, the final concentration of NaOH and the probes were 1.9 M and 2.2 M, respectively.

### Hyperpolarized dynamic $^{13}\text{C}$ NMR

25  $\mu\text{L}$  of 2.2 M  $\gamma$ -Glu-[ $1\text{-}^{13}\text{C}$ ]Gly or  $\gamma$ -Glu-[ $1\text{-}^{13}\text{C}$ ]Gly- $d_2$  in 1.9 M NaOH aqueous solution with 15 mM OX063 was hyperpolarized using the HyperSense DNP polarizer for 1 h. Hyperpolarized probe solution was acquired by the dissolution process with 3 mL of PBS containing 0.3 mM EDTA. 600  $\mu\text{L}$  of hyperpolarized probe solution was used for acquiring dynamic  $^{13}\text{C}$  NMR (flip



angle = 10°, repetition time = 4.5 s) with a 1.4 T Spinsolve 60 Carbon High Performance benchtop NMR apparatus.

## Results and discussion

### Validation of $\gamma$ -Glu-Gly scaffold and $^{13}\text{C}$ -enrichment of C1 in Gly residue

**Evaluation of GGT-mediated Glu production of peptide substrates.** We initially evaluated Glu production by GGT from GSH,  $\gamma$ -Glu-Gly-Gly, and  $\gamma$ -Glu-Gly *in vitro*. We added beef kidney GGT to 5 mM GSH,  $\gamma$ -Glu-Gly-Gly, and  $\gamma$ -Glu-Gly in phosphate buffer (PB), which we assumed was close to the local probe concentration in DNP MRI. The samples were incubated at 37 °C for 30 or 60 min. We then determined the amounts of Glu generated as a hydrolytic product of GGT by mixing with 4-fluoro-7-nitrobenzofurazan and comparing the results of UHPLC with a calibration curve (Fig. 2). Each of GSH,  $\gamma$ -Glu-Gly-Gly, and  $\gamma$ -Glu-Gly produced Glu *via* hydrolysis catalyzed by GGT. We found that  $\gamma$ -Glu-Gly-Gly generated more Glu than GSH, a natural substrate of GGT and that  $\gamma$ -Glu-Gly-Gly and  $\gamma$ -Glu-Gly produced similar amounts of Glu. This implied that  $\gamma$ -Glu-Gly is a good dipeptide substrate that can achieve hydrolytic conversion comparable to that of tripeptides. The hyperpolarized state realized by d-DNP immediately decays back to thermal equilibrium after dissolution with  $T_1$  as the time constant. Therefore, practical DNP molecular probes need to react fast enough with the target enzyme to produce a measurable amount of product within a few minutes. The finding that  $\gamma$ -Glu-Gly produced more Glu than the natural substrate GSH under our experimental conditions supports the notion that  $\gamma$ -Glu-Gly is a useful DNP probe from the viewpoint of enzymatic conversion.

**Evaluation of magnetic properties of  $\gamma$ -[5- $^{13}\text{C}$ ]Glu-Gly,  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly, and their GGT-hydrolysis products.** We investigated the appropriate  $^{13}\text{C}$ -enrichment position for  $\gamma$ -Glu-Gly as

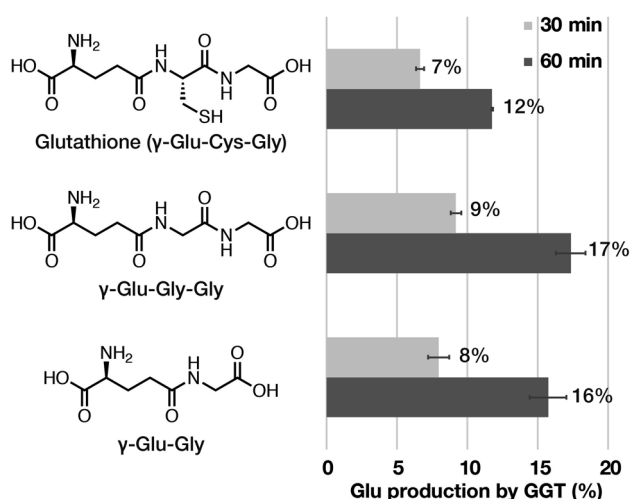


Fig. 2 Comparison of GGT-mediated enzymatic reaction among GSH ( $\gamma$ -Glu-Cys-Gly),  $\gamma$ -Glu-Gly-Gly, and  $\gamma$ -Glu-Gly. Glu production (%) was calculated as  $100 \times [\text{Glu}]/[\text{initial substrate concentration, 5 mM}]$  at the time point of 30 min and 60 min. Error bars represent standard deviation ( $n = 3$ ).

a DNP probe. To realize hyperpolarized metabolic analysis *in vivo*, an isotope-enriched position needs to have a long  $T_1$  and a large chemical shift change before and after the reaction. Many DNP probes utilize  $^{13}\text{C}$ , which is a hetero nucleus with a small gyromagnetic ratio and thus a long  $T_1$ . Furthermore, since  $^1\text{H}$  is not directly attached, quaternary carbons are useful for realizing long  $T_1$  by avoiding relaxation due to DD interactions with nearby  $^1\text{H}$ , which is generally the main relaxation source for  $^{13}\text{C}$ .<sup>22,23</sup>  $\gamma$ -Glu-Gly has the three quaternary carbonyl carbons that would realize a long  $T_1$ . Among them, C5 of the Glu residue and C1 of the Gly residue are thought to induce a large chemical shift change upon GGT-mediated structural changes (Fig. 3A). Therefore, we focused on  $\gamma$ -[5- $^{13}\text{C}$ ]Glu-Gly,  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly (the conventional probe), and their GGT-hydrolysis products ([5- $^{13}\text{C}$ ]Glu and [1- $^{13}\text{C}$ ]Gly).

We prepared  $\gamma$ -[5- $^{13}\text{C}$ ]Glu-Gly according to Scheme S1.† The  $^{13}\text{C}$  chemical shift of each probe/product in  $\text{D}_2\text{O}$  under neutral conditions was determined at 9.4 T using 1,4-dioxane (67.19 ppm) as an internal standard for  $^{13}\text{C}$  NMR (Fig. 3B). The chemical shift differences between the probe and the product were 6.8 ppm for  $\gamma$ -[5- $^{13}\text{C}$ ]Glu-Gly/[5- $^{13}\text{C}$ ]Glu and 4.4 ppm for  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly/[1- $^{13}\text{C}$ ]Gly. The chemical shift change given by  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly/[1- $^{13}\text{C}$ ]Gly was distinguishable *in vivo*. The chemical shift change of 6.8 ppm by  $\gamma$ -[5- $^{13}\text{C}$ ]Glu-Gly/[5- $^{13}\text{C}$ ]Glu was even larger, suggesting that both pairs can clearly distinguish the hyperpolarized signals of the probe and the product.

We then evaluated the  $T_1$  values of each probe and product at 9.4 T using inversion recovery (Fig. 3B). The  $T_1$  of  $\gamma$ -[5- $^{13}\text{C}$ ]Glu-Gly was  $13 \pm 1$  s (9.4 T, 10 mM,  $\text{D}_2\text{O}$ , 37 °C), and that of the corresponding product, [5- $^{13}\text{C}$ ]Glu, was  $35 \pm 1$  s (9.4 T, 10 mM,  $\text{D}_2\text{O}$ , 37 °C). The  $T_1$  value of the conventional probe,  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly, was  $31 \pm <1$  s (9.4 T, 10 mM,  $\text{D}_2\text{O}$ , 37 °C), and that of the corresponding product, [1- $^{13}\text{C}$ ]Gly, was  $56 \pm 4$  s (9.4 T, 10 mM,  $\text{D}_2\text{O}$ , 37 °C). The results of measuring the  $T_1$  of  $\gamma$ -[5- $^{13}\text{C}$ ]Glu-Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly revealed that the  $T_1$  of the carbonyl carbons in the same molecule can differ  $\sim 2$ -fold depending on the  $^{13}\text{C}$ -labeling position ( $13 \pm 1$  vs.  $31 \pm <1$  s). These results indicated that the chemical shift changes of both pairs of the probe and the product were sufficient (6.8 vs. 4.4 ppm), whereas the  $T_1$  of  $\gamma$ -[5- $^{13}\text{C}$ ]Glu-Gly was much shorter than that of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly ( $13 \pm 1$  vs.  $31 \pm <1$  s). Therefore,  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly with the longer  $T_1$ , is the preferred DNP probe for detecting GGT because the probe must retain the hyperpolarized signal long enough to generate an obvious enzymatic reaction product.

### Relaxation mechanism analysis of $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$

**Synthesis of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ .** A detailed evaluation of the enzymatic reaction and the magnetic parameters of  $\gamma$ -Glu-Gly showed that  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly is a promising DNP probe for sensing GGT. In addition, in the previous report, we could detect the enzymatic conversion of a probe even *in vivo* using  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly. However, if the distance from the polarizer to the MRI or NMR apparatus is far, or if longer retention of the hyperpolarized signal is required for measurements, a deuterated  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly with an extended  $T_1$  would be appropriate





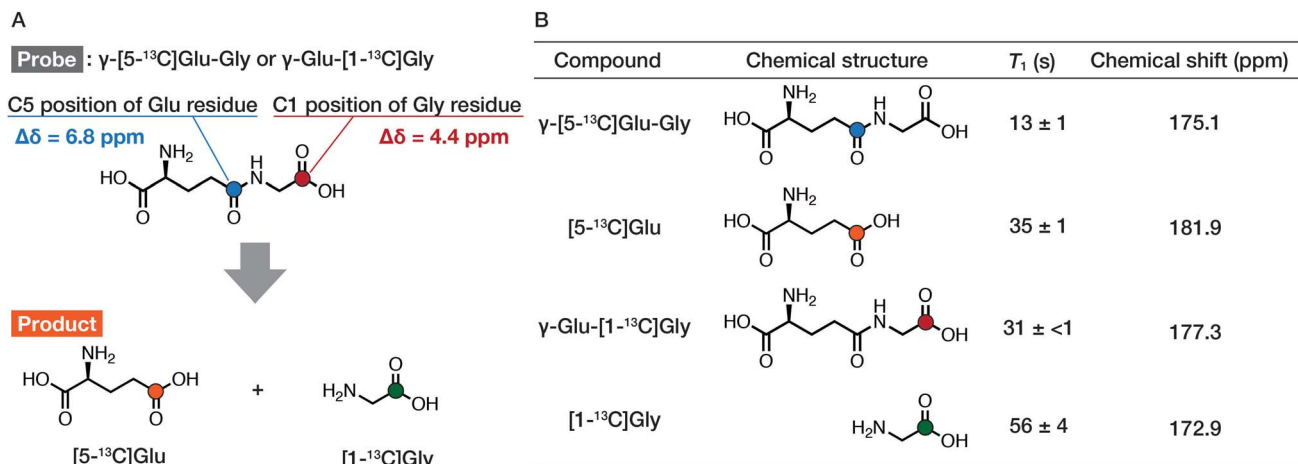


Fig. 3 Potential  $^{13}\text{C}$ -enriched positions of  $\gamma$ -Glu-Gly and its hydrolysis products, and the magnetic parameters of probes and products. (A) Potential  $^{13}\text{C}$ -enriched positions of  $\gamma$ -Glu-Gly and its hydrolysis products, [5- $^{13}\text{C}$ ]Glu and [1- $^{13}\text{C}$ ]Gly. Colored atoms indicate isotope-enriched  $^{13}\text{C}$ .  $\Delta\delta$  is  $^{13}\text{C}$  chemical shift difference between the probe and the product. (B) Chemical structures,  $T_1$  values, and  $^{13}\text{C}$  chemical shifts of probes and products.  $T_1$  values were measured by inversion recovery (9.4 T, 10 mM,  $\text{D}_2\text{O}$ , 37 °C,  $\text{pD} = 7.4 \pm 0.1$ ). Error bars represent standard deviation ( $n = 3$ ).  $^{13}\text{C}$  Chemical shift was determined using 1,4-dioxane (67.19 ppm) as an internal standard (9.4 T, 5 mM,  $\text{D}_2\text{O}$ , 37 °C,  $\text{pD} = 7.4 \pm 0.1$ ).

to further extend the hyperpolarization lifetime of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly.

We first investigated the  $T_1$  relaxation effect of the  $\alpha$ - $^1\text{H}$  of the Gly residue of the probe (Fig. 4A). We synthesized  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  as shown in Scheme 1. The quantitative *N*-hydroxyl succinimide (NHS) esterification proceeded using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI·HCl) as a coupling agent. The NHS ester can be purified by extraction and recrystallization, and isolated in 99% yield. By coupling of the NHS ester with [1- $^{13}\text{C}$ ]Gly- $d_2$ , *N*-Cbz- $\gamma$ -Glu(OBn)-[1- $^{13}\text{C}$ ]Gly- $d_2$  was synthesized.  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  was then obtained *via*

hydrogenation to deprotect Cbz and Bn groups. Details of the synthetic procedure are described in the Experimental section. This scheme can provide sufficient amounts (gram scale) of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ .

#### $T_1$ measurements of $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ under various solvent conditions

The  $T_1$  of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  was evaluated at 9.4 T under various solvent conditions. In the previous report, only the  $T_1$  at 25 °C in  $\text{H}_2\text{O}$  has been reported. However,  $T_1$

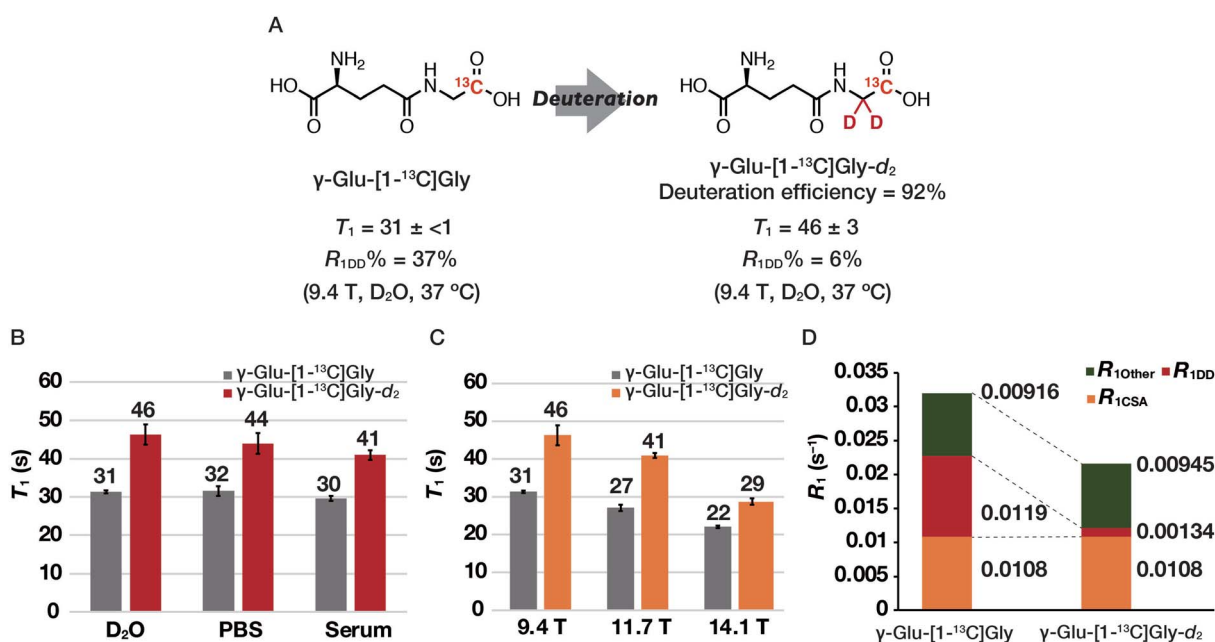
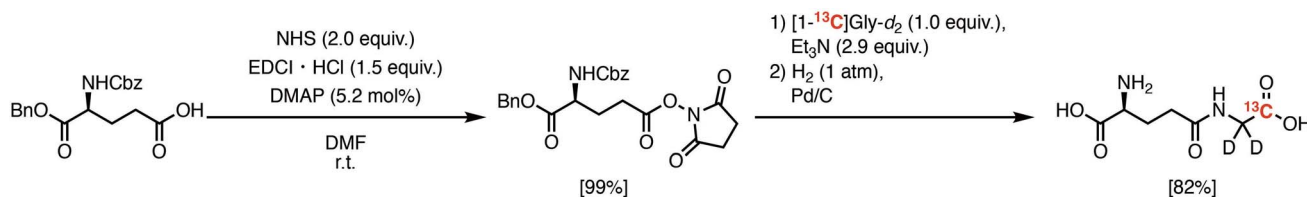


Fig. 4 Chemical structures,  $T_1$  values, and  $T_1$  relaxation analysis of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ . (A) Chemical structures of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ . (B)  $T_1$  measurements of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  in  $\text{D}_2\text{O}$ , PBS, and serum containing 10%  $\text{D}_2\text{O}$  (9.4 T, 10 mM, 37 °C). (C)  $T_1$  measurements of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  in  $\text{D}_2\text{O}$  at 9.4, 11.7, and 14.1 T. Error bars represent standard deviation ( $n = 3$ ). (D)  $T_1$  relaxation analysis of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  in  $\text{D}_2\text{O}$  at 9.4 T.





Scheme 1 Synthetic scheme of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ .

values in PBS and serum are also important for DNP NMR/MRI experiments because hyperpolarized probes are often dissolved in aqueous buffers and circulate in biological media to reach a target site after administration *in vivo*. Thus, we measured the  $T_1$  of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  at 9.4 T in  $\text{D}_2\text{O}$ , PBS, and serum (Fig. 4B). The results showed that even in mouse serum, the  $T_1$  was  $30 \pm 1$  s for  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $41 \pm 1$  s for  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  (9.4 T, 10 mM, mouse serum with 10%  $\text{D}_2\text{O}$ , 37 °C), which was comparable to that in  $\text{D}_2\text{O}$ . These results suggested that the hyperpolarization lifetime of these probes should be sufficient, even in a biological environment.

### $T_1$ and $R_{\text{DD}}$ analysis of $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$

We measured the  $T_1$  of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  to determine the DD relaxation effect of the  $\alpha$ - $^1\text{H}$  of the Gly residue (Fig. 4A). The deuteration efficiency of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  was 92% and the  $T_1$  was  $46 \pm 3$  s (9.4 T, 10 mM,  $\text{D}_2\text{O}$ , 37 °C). Deuteration of the  $\alpha$ - $^1\text{H}$  of the Gly residue in  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly resulted in a 1.5-fold longer  $T_1$  ( $46 \pm 3$  vs.  $31 \pm <1$  s).

We investigated how  $\alpha$ -deuteration of the Gly residue reduced the contribution of DD relaxation of the probe to overall  $T_1$  relaxation (Fig. 4A). The relaxation rate of the nucleus,  $R_1$  ( $=1/T_1$ ), is described as the sum of the contributions from independent relaxation factors as denoted in eqn (2).<sup>25,26</sup>

$$\frac{1}{T_1} = R_1 = R_{\text{DD}} + R_{\text{ICSA}} + R_{\text{IOther}} \quad (2)$$

where  $R_{\text{DD}}$  is a relaxation due to DD interaction mainly caused by nearby  $^1\text{H}$ ,  $R_{\text{ICSA}}$  is a relaxation due to CSA, and  $R_{\text{IOther}}$  is a component from all other relaxations including scalar coupling, spin rotation, and relaxation by paramagnetic agents.  $R_{\text{DD}}$  and  $R_{\text{ICSA}}$  typically account for most of the  $R_1$  of  $^{13}\text{C}$  nuclei at high magnetic fields. We then estimated the  $^1\text{H}$ - $^{13}\text{C}$   $R_{\text{DD}}$  contributions of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  using the nuclear Overhauser effect.<sup>19,26–30</sup> We found that 37% of the total  $R_1$  of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly was responsible for DD relaxation at 9.4 T, whereas only 6% of that of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  was responsible for DD. Because the  $R_{\text{DD}}$  of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ , in which only the  $\alpha$ - $^1\text{H}$ s of the Gly residue were deuterated, decreased to 6%, most of the DD relaxation of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly was caused by the  $\alpha$ - $^1\text{H}$  of the Gly residue. The findings that the  $\alpha$ - $^1\text{H}$  near the  $^{13}\text{C}$  nucleus of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly accounted for most of the DD relaxation is consistent with the theoretical explanation that the  $R_{\text{DD}}$  is inversely proportional to the sixth power of the distance between the  $^1\text{H}$  and  $^{13}\text{C}$  nuclei.<sup>25,26</sup> These findings showed that  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  suppressed most of the

DD relaxation and resulted in a 1.5-fold longer  $T_1$  than the conventional probe at 9.4 T.

### Analysis of $R_{\text{ICSA}}$ and $T_1$ measurements of $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ at various magnetic field strengths

We measured the  $T_1$  of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  at 9.4, 11.7, and 14.1 T to determine the contribution of the magnetic field-dependent relaxation pathway. The CSA relaxation mechanism increases in proportion to the square of the external magnetic field strength ( $B_0$ ).<sup>20,21</sup> Assuming that magnetic field-dependent relaxation mechanisms other than CSA are negligible,  $R_{\text{ICSA}}$  can be estimated by examining the magnetic field dependence of  $T_1$ . The results of the  $T_1$  measurements of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  in  $\text{D}_2\text{O}$  at 9.4, 11.7, and 14.1 T showed that  $T_1$  tends to become shorter with increasing magnetic field strengths of 9.4, 11.7, and 14.1 T ( $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly:  $31 \pm <1$ ,  $27 \pm 1$ , and  $22 \pm <1$  s respectively;  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ :  $46 \pm 3$ ,  $41 \pm 1$ , and  $29 \pm 1$  s, respectively; Fig. 4C). This is because relaxation due to CSA increases at higher magnetic field strength, and results in a shorter  $T_1$ . The contribution of  $R_{\text{ICSA}}$  to total  $R_1$  calculated from the  $R_1$ - $B_0^2$  plot in  $\text{D}_2\text{O}$  was 34% for  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly at 9.4 T (Fig. 4D and S1†). This result is consistent with the fact that carbonyl  $^{13}\text{C}$  generally exhibits a large relaxation due to the CSA mechanism.<sup>22</sup> Together with the results of the  $R_{\text{DD}}$  analysis, 71% of the relaxation pathway of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly at 9.4 T was accounted for by the DD and CSA relaxation mechanisms (Fig. 4D).

Relaxation due to the DD and CSA mechanisms must be reduced to further extend the hyperpolarization signal lifetime of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly. The DD relaxation can be reduced to 6% by deuterating the  $\alpha$ - $^1\text{H}$  of the Gly residue of  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly. In addition, the contribution of  $R_{\text{ICSA}}$  can be reduced by a lower magnetic field strength, meaning that the hyperpolarization signal lifetime of both  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  should be extended in a lower magnetic field along with the longer  $T_1$ . Especially,  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  should realize the longer  $T_1$  and thus the longer retention of the hyperpolarized signal at lower magnetic field by reducing both  $R_{\text{DD}}$  and  $R_{\text{ICSA}}$ .

### Dynamic $^{13}\text{C}$ NMR spectra of hyperpolarized $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$ at 1.4 T

We acquired dynamic  $^{13}\text{C}$  NMR spectra of hyperpolarized  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly and  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly- $d_2$  at 1.4 T to demonstrate the utility of the deuterated probe for further  $T_1$  elongation by reducing both  $R_{\text{DD}}$  and  $R_{\text{ICSA}}$ . We hyperpolarized  $\gamma$ -Glu-[1- $^{13}\text{C}$ ]Gly under the condition without glassing reagents (2.2 M  $\gamma$ -Glu-



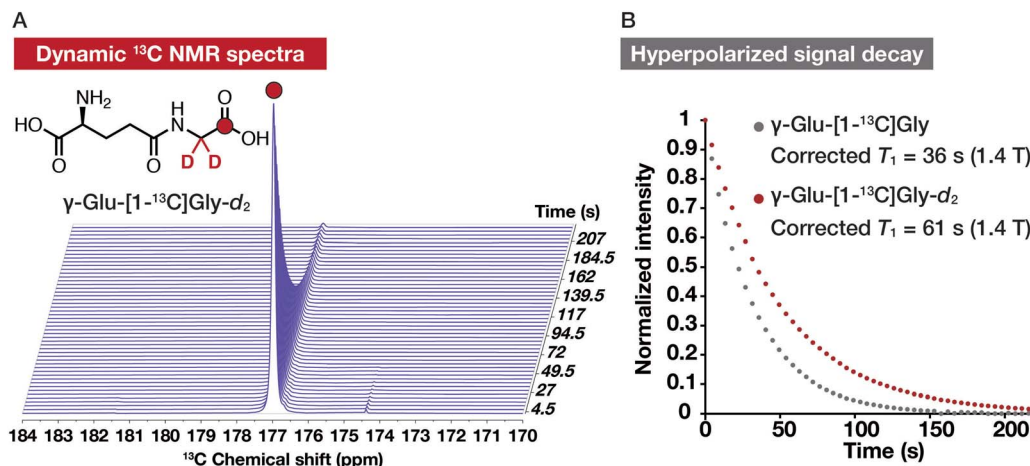


Fig. 5 Monitoring of the hyperpolarized  $^{13}\text{C}$  NMR signal decay of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  and  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  at 1.4 T. (A) Dynamic  $^{13}\text{C}$  NMR spectra of hyperpolarized  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  at 1.4 T. Colored atoms indicate isotope-enriched  $^{13}\text{C}$ . (B) Hyperpolarized signal decays of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  and  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  and their corrected  $T_1$  at 1.4 T.

$[1\text{-}^{13}\text{C}]$ Gly in 1.9 M NaOH with 15 mM OX063) using the HyperSense DNP polarizer. After dissolving the cryogenic sample by 3 mL of PBS containing 0.3 mM EDTA, hyperpolarized  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  solution (600  $\mu\text{L}$ ) was used to acquire dynamic  $^{13}\text{C}$  NMR spectra with a 1.4 T benchtop NMR apparatus.  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  was also hyperpolarized with the same procedure. Both of the probes gave the enhanced  $^{13}\text{C}$  NMR signals (Fig. 5A and S2†).  $T_1$  of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  calculated from the hyperpolarized signal decay was 61 s at 1.4 T (corrected  $T_1$  considering magnetic loss during successive acquisition pulses), which was longer than that of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  (36 s, Fig. 5B). As we expected,  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  showed the longer  $T_1$  at 1.4 T than 9.4 T ( $44 \pm 3$  s at 9.4 T vs. 61 s at 1.4 T). The experimentally obtained  $T_1$  value of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  (61 s at 1.4 T) was shorter than the extrapolated value from the  $R_1\text{-}B_0^2$  plot in  $\text{D}_2\text{O}$  (101 s at 1.4 T, Fig. S1†). The reason the experimentally determined  $T_1$  (61 s at 1.4 T, corrected) calculated from the signal decay curve is shorter than the extrapolated  $T_1$  (101 s at 1.4 T) might be explained by coexisting radicals, which are included during the hyperpolarization process. These results, however, indicated that  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  is a DNP probe that has a longer  $T_1$  than  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  and thus achieves a longer hyperpolarization signal lifetime at 1.4 T.

## Conclusions

In this study, we evaluated the enzymatic and magnetic properties of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  and developed the deuterated probe,  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  that can show the longer  $T_1$  and thus the longer lifetime of the hyperpolarized signal. We found that (1)  $\gamma\text{-Glu-Gly}$  showed a rapid enzymatic reaction rate with GGT, (2)  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  showed a longer  $T_1$  than  $\gamma\text{-[5-}^{13}\text{C]Glu-Gly}$ , another potential  $^{13}\text{C}$ -enriched  $\gamma\text{-Glu-Gly}$ , (3) 71% of the  $T_1$  relaxation of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  can be accounted for by the DD and CSA relaxation pathway at 9.4 T, thus allowing  $T_1$  elongation by deuteration and lower magnetic field strength, and that (4)  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  indeed showed longer retention of the hyperpolarized signal at 1.4 T. These results confirmed the

value of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  as a new DNP probe for detecting GGT with a longer lifetime of the hyperpolarized signal. Furthermore, the molecular properties of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  determined herein suggested that  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  could be useful for biological studies of GGT.

Although the extended hyperpolarization lifetime was demonstrated *in vitro*, *in vivo* verification experiments were not tried in this study. The feasibility of the elongated hyperpolarized signal of the deuterated probe *in vivo* should be investigated in future studies. In addition, there may still be room for further improvement in the molecular design of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  before the applications of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  could be more widened and significantly contribute to hyperpolarized MR studies. For example, the GGT enzymatic reaction rate of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  might still be able to be improved, because  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  provided only a few percent of the product/probe ratio in the previous *in vivo* experiments using tumor-bearing xenografts.<sup>15,17</sup> DNP probes that can detect GGT with better conversion could be developed by understanding the enzymatic reaction mechanism of GGT.

We believe that the enzymatic properties and various magnetic parameters of  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly}$  and  $\gamma\text{-Glu-[1-}^{13}\text{C]Gly-d}_2$  determined herein will provide useful information for widespread use as a DNP probe for GGT detection in biological studies, and insights for the development of DNP probes designed *de novo* in the future.

## Author contributions

S. S. conceived and designed the project; Y. K., T. N., and M. I. synthesized compounds with the help of Y. S. and H. N.; Y. K. conducted *in vitro* enzymatic reaction assay and NMR measurements for  $T_1$ , chemical shift, and relaxation analysis with the help of Y. S.; A. E. E., F. H., and M. M. organized and/or performed the hyperpolarized experiments; Y. K., Y. S., and S. S. wrote the manuscript, which was edited by all co-authors.



## Conflicts of interest

There are no conflicts of interest to declare.

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