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Generation of cyclic glutathione via the thiolactonization of glutathione and identification of a new radical scavenging mechanism

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ABSTRACT

Glutathione (GSH) has two important biological activities, GSH conjugation and radical scavenging. In this work, we determined that GSH can participate in an intramolecular cyclization between the glutamic α-carboxylic acid and the cysteinyl thiol moiety under aqueous conditions. Moreover, we showed that the cyclic glutathione (cGSH) product had more potent radical scavenging activity than GSH. The cGSH radical scavenging activity occurred via a mechanism that differed from that of GSH.

Introduction

Glutathione (GSH) is a very important biological tripeptide that is comprised of γ-L-glutamic acid, L-cysteine and glycine (Fig. 1). GSH is intracellularly biosynthesized in the presence of adenosine triphosphate. It is known that GSH has two activities. Firstly, GSH conjugates toxic substances through an enzyme-catalyzed reaction to remove them from the cell [1]. The thiol moiety binds to some substances and the reaction is catalyzed by glutathione S-transferase. Once conjugated, the substances are removed from the cell. This conjugation and removal activity also play a role in anticancer drug resistance [2,3]. However, GSH itself is not removed from cells because it cannot pass through the cell membrane as it has a negative charge and a specific GSH transporter does not exist. After GSH is extracellularly hydrolyzed to its amino acid components, they are transferred into the cell by amino acid transporters. Secondly, GSH acts as a radical scavenger. Quintiliani *et al.* demonstrated the mechanism of the reaction of GSH with OH radicals (OH˙) as follows. First, the thiol moiety is radically cleaved by OH˙ into an H radical (H˙) and S radical (GSH-*S*˙) and then the H˙ traps the OH˙. The GSH-*S*˙ binds with another unit of GSH-*S*˙ through an S—S bond to become a dimer (GSSG, Fig. 1) [1,4]. This reaction occurs in the absence of enzyme [4]. Today, this is known as the general radical scavenging mechanism of GSH. Alvarez-Idaboy et al. reported that GSH has scavenging activity for other radicals, such as ˙OOH or ˙OCH_3 [5]. In this work, we show that GSH can undergo cyclization and form a thiolactone (cyclic glutathione; cGSH) under mild conditions (Fig. 1). Previously, W. H. Koppenol *et al.* suggested that GSH forms a thiolactone under strong acidic conditions [6]. They prepared cGSH in D_2O containing 0.1 M DCl for 6 days and then it was analyzed by NMR. Although the authors published the ^{1}H and COSY NMR data, they did not confirm the structure of the proposed thiolactone and concluded that they could not exclude the possibility that other structures produce identical NMR signals. We obtained cGSH as an almost pure compound in water. In this work, we show that cGSH is the product of GSH thiolactonization. Moreover, we found that cGSH participates in radical scavenging using a different mechanism from GSH.

Results and discussion

Preparation and structure analysis of cGSH [7]

cGSH was prepared as the sole product by stirring GSH in warm water (60 °C) for 72 h (Table 1, entry 1). Under this condition, thiolactonization only occurred between the glutamic α-carboxy moiety and the cysteinyl thiol moiety. Cyclization did not occur between the thiol and glycinyl carboxy moiety. At the start of the reaction, the GSH solution was at pH 3 without the addition of acid. At 37 °C or room temperature, the thiol was oxidized and formed the dimer GSSG. Furthermore, it occurred competitively

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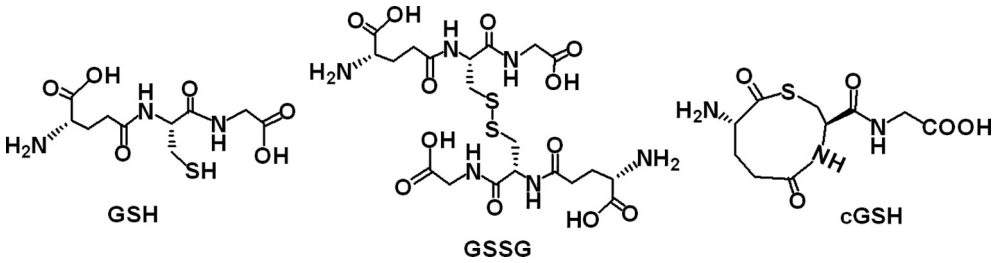
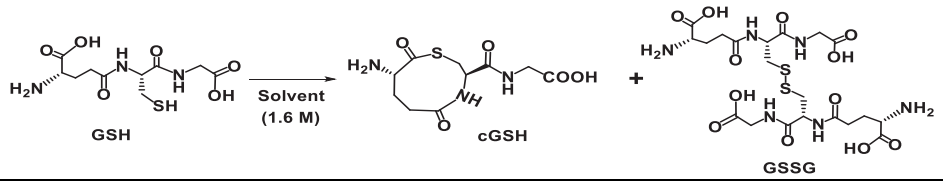


Fig. 1. Structures of GSH, GSSH and cGSH.

Table 1
Generation of cGSH under the tested reaction conditions.



	Reaction condition				Production rate (%) [c]		
entry	Temperature (°C)	Solvent	pH	Reaction time	cGSH	GSSG	GSH (recover)
1	60	H_2O	3	72h	quant. [d]	–	–
2	37	H_2O	3	21d	72	18	10
3	r.t. [a]	H_2O	3	21d	44	15	41
4	60	buffer [b]	6	72h	49	7	44
5	60	DMF	3	72h	n.d. [e]	n.d. [e]	n.d. [e]

[a]The "r.t." means did not control reaction tempruture.
[b]50 mM *N*-Methylmorpholine /50 mM formic acid.
[c]Production rate were calculated by NMR.
[d]The NMR peaks was observed only cGSH.
[e]The reaction did not occur.

with thiolactonization. The reaction was not completed even after 21 days (Table 1, entries 2 and 3). At pH 6 and 60 °C for 72 h, cGSH was produced in an intermediate quantity, half of the initial GSH remained, and GSSG was formed in a small amount (Table 1, entry 4). pH 6 or 37 °C is a condition closer to the biological environment. In DMF solution, GSH did not react (Table 1, entry 5). The cGSH spectral data are shown in Fig. 2 (^{13}C NMR) and Fig. 3 (^{1}H NMR). It was observed that the cysteinyl carbonyl carbon, glutamic α-carbonyl carbon, glutamic α-CH carbon and glutamic γ-carbonyl carbon occurred at different chemical shifts compared with the signals for GSH. In particular, the glutamic α-carbonyl carbon was shifted to low magnetic field (Fig. 2). Moreover, the ^{1}H NMR data showed that the cysteinyl β-CH_2 proton, cysteinyl α-CH proton and glutamic β-CH_2 proton occurred at different chemical shifts from GSH (Fig. 3). Our ^{1}H NMR data coincides with W. H. Koppenol's data [6]. The correlation between the cysteinyl β-CH_2

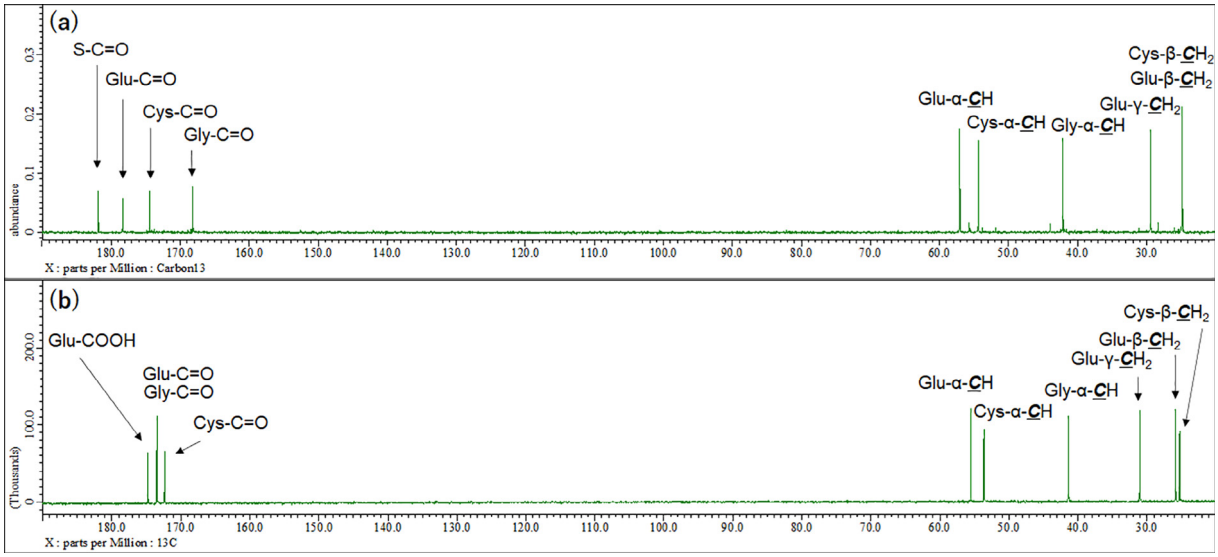


Fig. 2. ^{13}C NMR spectra of cGSH (a) and GSH (b) at 125 MHz in D_2O.

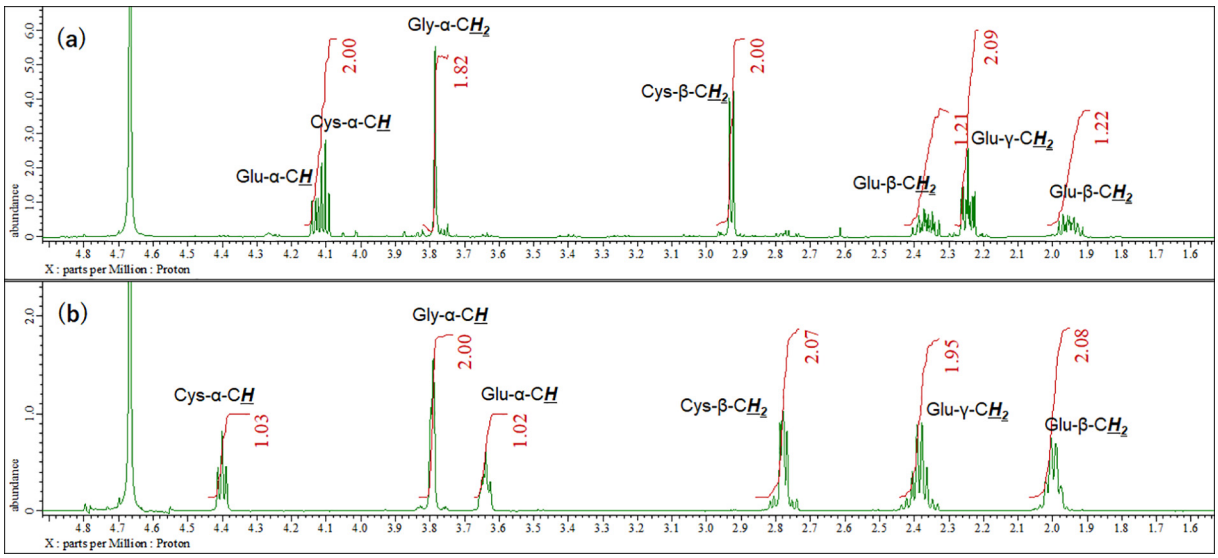


Fig. 3. ^{1}H NMR spectra of cGSH (a) and GSH (b) at 500 MHz in D_2O.

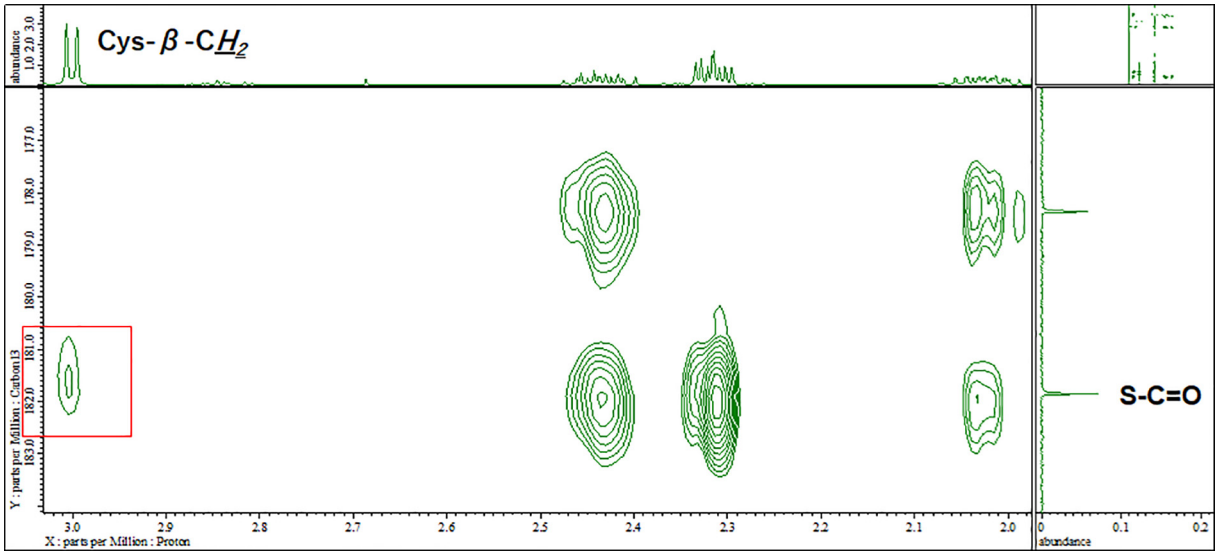


Fig. 4. HMBC data of cGSH at 500 MHz in D_2O, long range coupling J = 4 Hz.

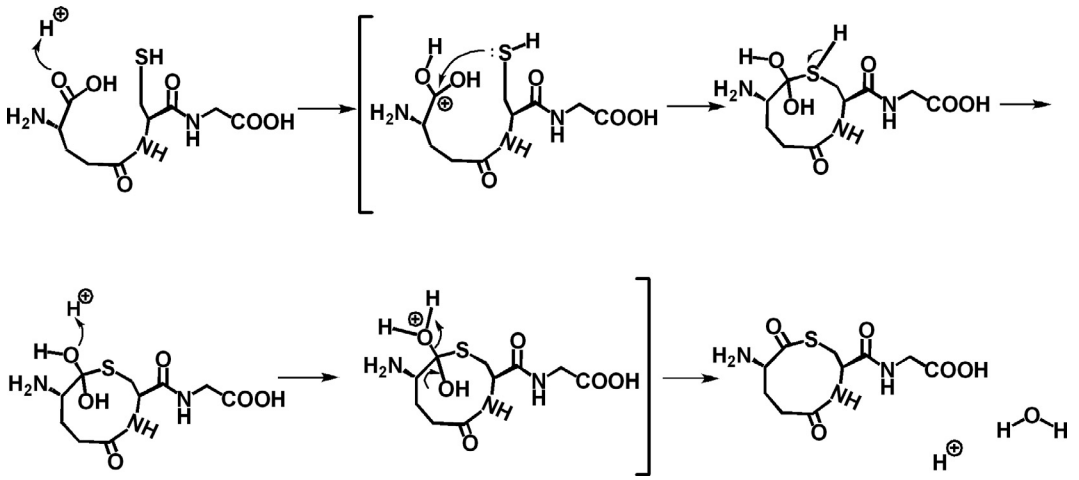
proton and glutamic α-carbonyl carbon (S–C═O) was weakly observed as a long range coupling of J = 4 Hz in the HMBC data (Fig. 4). The HMBC data for the long-range coupling of J = 8 Hz is shown in Fig. S1. Moreover, also the 2D NMR spectra (COSY and HSQC) are shown in Fig. S1. From the IR analysis, the peak at 2,550 cm^{-1} (SH) was not detected for cGSH although the SH peak was detected for GSH (Fig. S2). The ESI-HR/MS analysis showed that the m/z was 290.0805 (calcd for $C_{10}H_{15}N_3O_5S$ $[M + H]^+$= 290.0811).

The proposed thiolactonization mechanism

Previously, Schmir *et al.* reported that bicyclic mercapto acid was formed via a thiolactonization mechanism under an acidic catalyzed condition [8]. Herein, it was shown that thiolactonization of GSH also occurred easily under mild acidic conditions. Moreover, the reaction did not occur in an aprotic solvent such as DMF. The proposed reaction mechanism is shown in Scheme 1. First, the glutamic α-carboxylic oxygen was protonated, and then the dihydroxy cation was attacked by the thiol. Finally, cGSH was produced through the formation of an oxonium ion.

DPPH radical scavenging activity

The DPPH radical reagent is often used as a method to evaluate radical scavenging activity. In the case of GSH, the S and H in the thiol moiety become GSH-S· and H· (radical cleavage) via reaction with the DPPH radical. The H· reacts with the DPPH radical, and further generates DPPH. By comparison, the S· forms an S—S bond, and GSH becomes GSSG (oxidized GSH). We used a modification of the methods of Takebayashi *et al.* to determine the DPPH radical scavenging activity of GSH and cGSH [9,10]. Using DPPH, a decrease in the absorbance value indicates an increase in the radical scavenging activity. As shown in Fig. 5a, at pH 6 cGSH showed the most potent radical scavenging activity at 5 min while GSH required at least 30 min to show equivalent radical scavenging activity (Fig. 5a). The radical scavenging activity of cGSH was almost constant for 90 min. In terms of their overall radical scavenging activities, GSH was higher than that of cGSH. At both pH 3 and pH 6, the



Scheme 1. Proposed mechanism of thiolactoniztion.

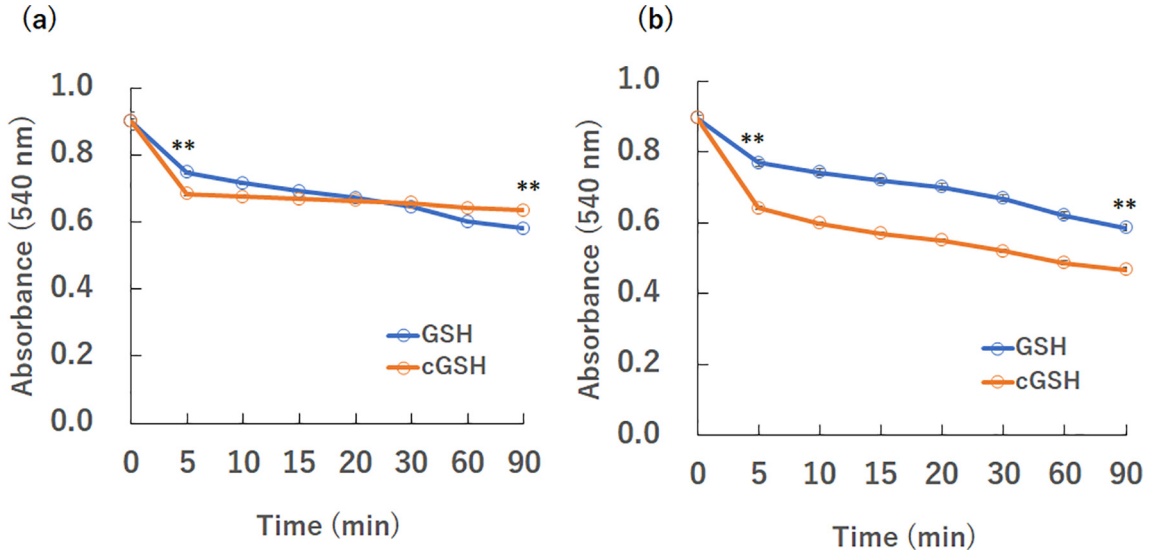


Fig. 5. DPPH radical scavenging activities of cGSH and GSH. The final concentration of cGSH and GSH was 20 μM. The DPPH final concentration was 100 μM. $^{**}p < 0.01$ [11] (a) Comparison of the DPPH radical scavenging rate of cGSH and GSH at pH 6, (b) Comparison of the DPPH radical scavenging rate of cGSH and GSH at pH 3.

radical scavenging activity trend of GSH was comparable with those reported by Takebayashi *et al.* [10]. However, at pH 3, cGSH had higher radical scavenging activity than GSH (Fig. 5b). These results suggested that the radical scavenging mechanism of cGSH is different from that of GSH.

Proposed radical scavenging mechanism of cGSH

The DPPH radical reagent was added to a water solution of cGSH at pH 3 to examine the radical scavenging mechanism of cGSH (the pH was not controlled. Therefore, the cGSH solution was at pH 3). After the reaction finished, the mixture was washed with $CHCl_3$, and the cGSH in the water layer was analyzed by NMR after lyophilization. When the DPPH radical reagent was added at two times the molar concentration of cGSH, the reaction was completed. The results showed that cGSH scavenges two radicals. Moreover, the obtained cGSH derivative structure was different from that of GSSG (Fig. 6). Using a 1:1 mixture of cGSH and DPPH, approximately 50% of the cGSH derivative was consumed according to the NMR spectrum (Fig. S3). Moreover, in the absorbance assay, using the pH 3 condition, the results of the radical scavenging assay showed that cGSH scavenged about two radicals while GSH scavenged one radical (Scheme S1). The 1D and 2D NMR data (Fig. S5) showed that the cGSH derivative had two glutamic acid residues and they were bonded in a symmetrical fashion. Moreover, HMBC between glycinyl-carbonyl carbon and cysteinyl-β-CH_2 proton was not observed (Fig. S5). This shows that the cGSH derivative does not have a cyclic structure. On the other hand, the cysteinyl-β-CH_2 was similar coupling constant to that of GSSG. We infer that cGSH derivative has S—S bond like a GSSG. Taken together, we proposed the cGSH derivative structure as shown in Fig. 7. This structure led us to suggest that the thiolactone was opened by a radical reaction, and the glutamic acid was removed from the tripeptide. Furthermore, it was inferred that the cutting and binding of glutamic acid is due to the radical transfer reaction following ring opening, because the glutamic acid is not removed in GSH. There is no report on ring-opening of thiolactone or lactone by radical reaction. We believe that this may be a new radical reaction. In this report, we could not confirm the structure because the compound was not detected by ESI-MS. We concluded that although cGSH was difficult to ionize, the cGSH derivative was not ionized sufficiently for detection by ESI-MS.

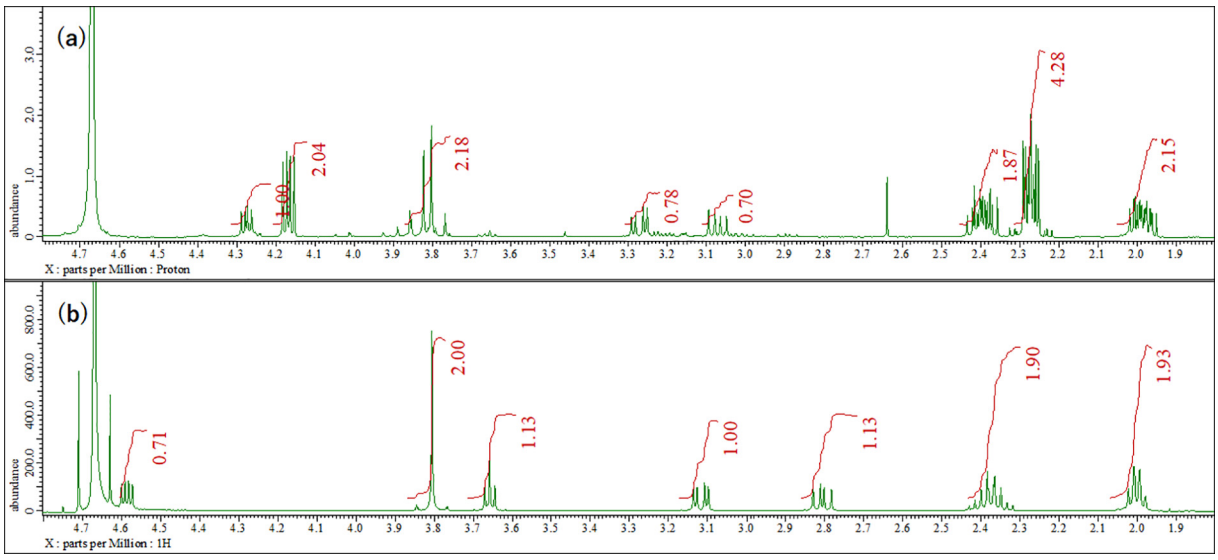


Fig. 6. Comparison of the ^{1}H NMR of the cGSH derivative in the presence of DPPH at two times the molar concentration of cGSH (a) and commercial GSSG (b) at 500 MHz in D_2O.

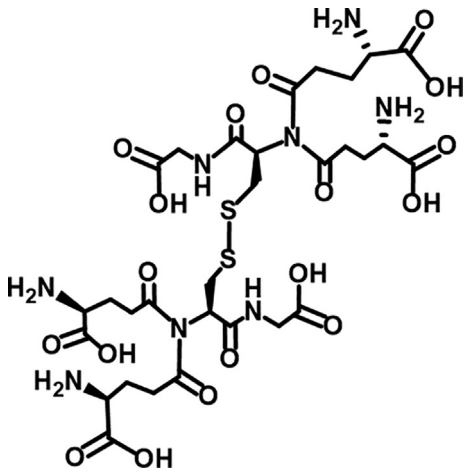


Fig. 7. Proposed structure of the cGSH derivative after the DPPH radical reaction.

Conclusion

We determined the cGSH chemical structure. The results revealed that cGSH shows radical scavenging activity similar to GSH; however, the mechanism is different. This suggests that cGSH may also be involved in other previously observed biological activities of GSH.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tetlet.2021.152836.

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[7] Preparation of cGSH; GSH (100 mg, 0.3 mmol) was dissolved in water (2 ml, 0.2 M). The GSH solution was lyophilized after stirring at 60 °C for 72 h. ^{1}H NMR (500 MHz, D_2O): δ4.13 (dd, 1H, *J*=3.4, 9.2 Hz, Glu-α-C***H***), 4.10 (t, 1H, *J*=5.7 Hz, Cys-α-C***H***), 3.79 (d, 2H, *J*=1.7 Hz, Gly-α-C$\boldsymbol{H_2}$), 2.93 (d, 2H, *J*=5.7 Hz, Cys-β-C$\boldsymbol{H_2}$), 2.37 (m, 1H, Glu-β-C$\boldsymbol{H_2}$), 2.23–2.26 (m, 2H, Glu-γ-C$\boldsymbol{H_2}$), 1.96 (m, 1H, Glu-β-C$\boldsymbol{H_2}$); ^{13}C NMR (125 MHz, D_2O): δ181.9 (S-***C***=O), 178.4 (Glu-***C***=O), 174.4 (Cys-***C***=O), 168.2 (Gly-***C***=O), 57.1 (Glu-α-***C***H), 54.4 (Cys-α-***C***H), 42.1 (Gly-α-***C***H), 29.4 (Glu-γ-***C***H_2), 24.9 (Cys-β-***C***H_2), 24.8 (Glu-β-***C***H_2); ESI-HR/MS m/z 290.0805 (calcd for $C_{10}H_{15}N_3O_5S$ [M+H]+ =290.0811).
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[11] Data are expressed as average values with standard deviations. Student's t test was used for statistical analysis. Statistical analysis was carried out using the statistical package R (version 4.0.1).