The Evidence for the Production Mechanism of *cis-S*-1-Propenylcysteine in Aged Garlic Extract Based on a Model Reaction Approach Using Its Isomers and Deuterated Solvents

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Abstract
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cis-S-1-Propenylcysteine has been identified in aged garlic extract, however, its production mechanism is still unclear. The content of *cis-S*-1-propenylcysteine in aged garlic extract at 22 months of aging was more than twice the molarity of γ-glutamyl-*cis-S*-1-propenylcysteine in raw garlic, a plausible precursor of *cis-S*-1-propenylcysteine. We found that *cis-S*-1-propenylcysteine was generated through the isomerization of *trans-S*-1-propenylcysteine in the model reactions. These results suggested that the isomerization was induced by the electron-releasing conjugative effect of the sulfur atom in *S*-1-propenylcysteine.

Key words

Allium sativum · Amaryllidaceae · aged garlic extract · isomerization · *cis*/*trans-S*-1-propenylcysteine

Abbreviations
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AGE: aged garlic extract
cis-S1PC: *cis-S*-1-propenylcysteine
^{13}C-NMR: carbon 13 nuclear magnetic resonance
D-NMR: deuterium nuclear magnetic resonance
GS1PC: γ-glutamyl-*cis-S*-1-propenylcysteine
^{1}H-NMR: proton nuclear magnetic resonance
HPLC: high performance liquid chromatography
LC: liquid chromatography
LC-MS: liquid chromatography-mass spectrometry
MS: mass spectrometry
NMR: nuclear magnetic resonance
S1PC: *S*-1-propenylcysteine
SAC: *S*-allylcysteine

S-Alk(en)ylcysteine sulfoxides and γ-glutamyl-*S*-alk(en)ylcysteines were found in garlic as its characteristic sulfur storage compounds [1]. Over two decades, many scientists have come to pay more attention to *S*-alk(en)ylcysteines, such as *S*-methylcysteine, *S*-allylcysteine, and *trans*-S1PC, which are produced from γ-glutamyl-*S*-alk(en)ylcysteines [1,2]. Recently, we have reported that *cis*-S1PC is included in AGE [3]. The content of *cis*-GS1PC, a plausible precursor of *cis*-S1PC, in raw garlic was less than 50 % of *cis*-S1PC content in AGE on a molar basis [3,4]. These differences suggested that *cis*-S1PC was produced not only from the precursor, γ-glutamyl peptide, but also from the other compounds. Recently, we demonstrated that *cis*-S1PC was produced from *trans*-S1PC through the isomerization reaction [3]. However, the mechanism of isomerization reaction is unclear. To reveal the production mechanism of *cis*-S1PC, we used the model reaction in which S1PC was incubated in the mixture of deuterated solvents to detect the hydrogen-deuterium exchange within the propenyl group.

Results and Discussion
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SAC is known to be produced from γ-glutamyl-*S*-allylcysteine by an enzymatic reaction [1]. Garlic also contains GS1PC, a plausible precursor compound of S1PC [1]. Lawson et al. showed the existence of *cis*-GS1PC in garlic, and its content was 0.28–0.55 µmol/g (fresh weight) [1,4]. We also confirmed the existence of *cis*-GS1PC in raw garlic and found that its content was 0.54 ± 0.12 µmol/g (dry-garlic) [3]. The qualitative analysis of *cis*-GS1PC in AGE was performed by LC-MS, and we obtained the following mass spectrum of *cis*-GS1PC: authentic *cis*-GS1PC: [M + H]$^{+}$ = *m/z* 291.1001, *cis*-GS1PC in AGE: [M + H]$^{+}$ = *m/z* 291.1002 (○ **Fig. 1 B, C**). The content of *cis*-GS1PC in AGE slightly increased during the aging period but did not exceed 0.8 µmol/g (dry-AGE), whereas the content of *trans*-GS1PC quickly decreased at an early stage in the aging
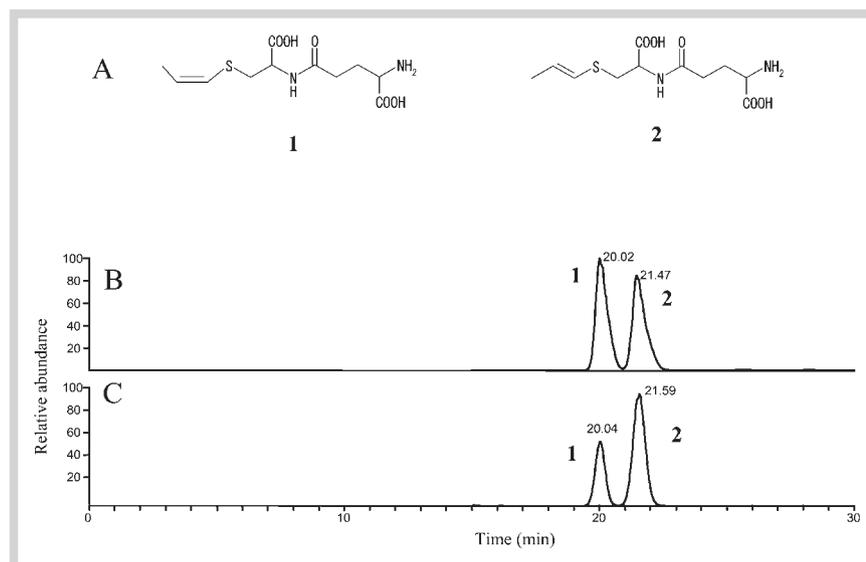
Fig. 1   The chemical structures of *cis*-GS1PC and *trans*-GS1PC, and mass chromatogram of *cis*/*trans*-GS1PC in LC-MS analysis. **A** The chemical structures of *cis*-GS1PC (**1**) and *trans*-GS1PC (**2**). **B** Mass chromatogram of authentic **1** and **2**. **C** Mass chromatogram of **1** and **2** in AGE.

Table 1 Content of γ-glutamyl-*cis*/*trans*-*S*-1-propenylcysteine in raw garlic and AGE.

Content (µmol/g-dry AGE)							
Aging period (month)	0[a]	1	2	4	10	16	22
trans-GS1PC	79.34 ± 9.88	41.41 ± 7.40	31.36 ± 1.40	27.35 ± 1.59	6.84 ± 0.25	5.45 ± 0.28	4.13 ± 0.12
cis-GS1PC	0.54 ± 0.12	0.70 ± 0.26	0.52 ± 0.02	0.5 ± 0.1	0.74 ± 0.14	0.72 ± 0.11	0.77 ± 0.05

Data are shown as mean ± SE (n = 3); [a] The content in raw garlic lyophilized (µmol/g-dry garlic); The content of *cis*-GS1PC in raw garlic was reported previously [3]

period, and reached about 5% of content in raw garlic after 22 months of aging (○ **Table 1**) [3]. The content of *cis*-S1PC in AGE gradually increased and reached to more than twice the molarity of *cis*-GS1PC in raw garlic during the 22-month aging period, 1.80 ± 0.01 µmol/g (dry-AGE) [3].

We hypothesized that *cis*-S1PC is produced via isomerization of its *trans* form. As a result of an experiment using the model reaction, we found that *cis*-S1PC was produced from *trans*-S1PC by the isomerization reaction, although its mechanism was unclear [3]. Divalent sulfides have the electron-releasing conjugative effect when an unsaturated group or carbonium ion directly binds to sulfur [5,6]. This causes the cleavage of the π bond between C1 and C2 in the unsaturated group. The isomarization reactions in the deuterated solvents were performed to confirm the cleavage of the C1–C2 bond in the propenyl group. The analyses of reaction mixtures were performed by LC-MS under the selected ion monitoring trace of monodeuterated S1PC (S1PC-d, $C_6H_{10}DO_2NS$; $[M + H]^+ = m/z$ 163.0646 ± 0.0016). The selected ions of S1PC-d, both the *cis* form and *trans* form, were detected in the reaction mixtures, and the ratios of *cis*/*trans* forms were *cis*/*trans* = 24.2/75.8 (○ **Fig. 2 A**) and *cis*/*trans* = 25.1/74.9 (○ **Fig. 2 B**). However, multideuterated compounds, such as dideuterated S1PC, were not observed (data not shown). The mass signal intensity of S1PC-d was less than 10% of that of non-deuterated S1PC. ^{1}H-NMR spectra of isomerization reaction mixtures were measured at day 0, 5, 15, and 30. The proton signals of C1, C2, and C3 in the propenyl group were gradually changed, and the signals of both *cis*- and *trans*-S1PC were detectable in the reaction mixtures after 5 days during the 30-day incubation period, while only one of the signals was observed in each mixture before incubation (○ **Fig. 3**). These results indicated that the isomerizaton between the *cis* form and *trans* form is reversible. We also performed the ^{1}H-NMR and D-NMR analyses of the deuterated S1PC to confirm the deuteraded position of S1PC. However, the change of proton integration by the deuteration was not clearly observed in the ^{1}H-NMR spectra. The deuterium signals of deuterated S1PC were also not detected in the D-NMR spectra (data not shown).

Although the deuterated position in S1PC under the model reaction using deuterated solvents was not confirmed by NMR analysis, the detection of a mass signal of monodeuterated *cis*/*trans*-S1PC suggested that more than half a portion of *cis*-S1PC in AGE is produced from the *trans* form by the electron-releasing conjugative effect of a sulfur atom in the *S*-propenyl group (○ **Fig. 4**) [5, 6]. These results can help us understand the complex sulfur chemistry in the processing of garlic such as the aging process.

Materials and Methods
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Chemicals and reagents
Chemicals for synthesis were obtained from Tokyo Chemical Industry. Solvents for LC, LC-MS, and NMR analyses were purchased from Wako Pure Chemicals Industry. γ-Glutamyl-*cis*/*trans*-*S*-1-propenylcysteines were synthesized and purified according to previous reports [7–10].

Analyses of γ-glutamyl-*cis*/*trans*-*S*-1-propenylcysteines in raw garlic and aged garlic extract
AGE was prepared according to a previous report [11]. The contents of *cis*/*trans*-GS1PC were analyzed according to a previous report [3]. Identification of *cis*/*trans*-GS1PC was performed by LC-MS.

Isomerization reaction of *S*-1-propenylcysteine
cis-S1PC and *trans*-S1PC were dissolved in deuterium oxide separately, and acetic acid-d_4 was added to each solution to adjust the pH to be between 3 and 5. The solutions were dispensed into glass sample vials and NMR tubes, and stored at 60 °C. The solutions in the glass vials were lyophilized after incubation for 30 days. The isomers of S1PC in the solution were isolated by preparative HPLC according to the previous report [3], and analyzed by LC-MS and NMR. ^{1}H-NMR analyses of the solutions in the NMR tubes were performed on days 0, 5, 15, and 30.

### Liquid chromatography-mass spectrometry analysis
The LC-MS analysis was carried out on a system consisting of an Ultimate 3000 (Dionex/Thermo Fisher Science) coupled to a Q-Exactive (Thermo Fisher Scientific). The column utilized for separation was a Cadenza CD-C18 column (2.0 mm×150 mm, 3 µm, Imtakt Corporation) with a flow rate of 0.2 mL/min. The chromatographic separation of *cis*/*trans*-GS1PC was achieved using a mobile phase consisting of water containing 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B) with isocratic elution (A : B = 97 : 3, v/v). The qualitative analysis of S1PC-d was performed using a mobile phase consisting of water containing 0.3% (v/v) heptafluorobutyric acid (solvent A) and 80% (v/v) methanol containing 0.3% (v/v) heptafluorobutyric acid (solvent B) with isocratic elution (A : B = 73 : 27, v/v). The MS analysis was carried out using the following conditions: ionization mode: ESI[+] (positive mode), mass range: m/z 50–750, resolution: 70 000, maximum IT: 200 ms, isolation width: 4.0 m/z. The quantitative analysis of *cis*/*trans*-GS1PC was carried out by the LC method according to a previous report [3].

### Nuclear magnetic resonance analysis
^{1}H-, D-, and ^{13}C-NMR spectra of the compounds were taken in D_2O, D_2O containing acetic acid-d_4, and H_2O on a VNMRS-500 spectrometer (VARIAN, Inc.) at 500 MHz and 125 MHz, respectively.
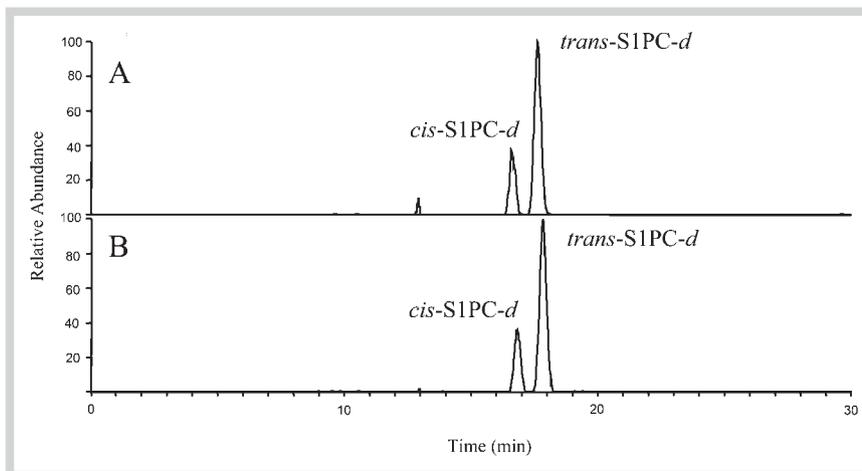
Fig. 2 Chromatogram of LC-MS analysis for model reaction samples using deuteride solvent. **A** Mass chromatogram of monodeuterated *cis*/*trans*-S1PC on the isomerization sample using the *trans* form. **B** Mass chromatogram of monodeuterated *cis*/*trans*-S1PC on the isomerization sample using the *cis* form.
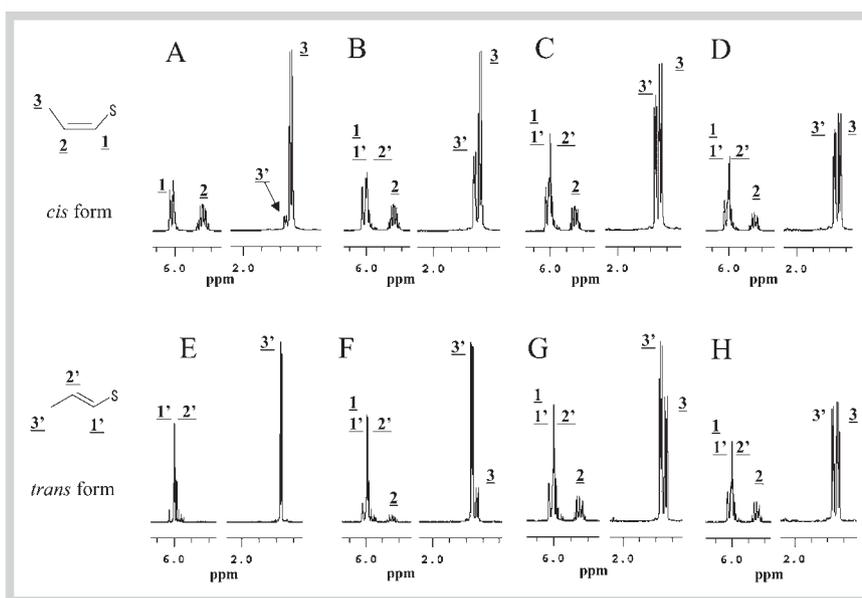
Fig. 3 Change of ¹H-NMR signals of *S*-propenyl groups in *cis*/*trans*-S1PC. **A–D** ¹H-NMR for C1, C2, and C3 in the *S*-propenyl groups of *cis*/*trans*-S1PC in the reaction mixture using *cis*-S1PC (**A** 0 days, **B** 5 days, **C** 15 days, **D** 30 days). **E–H** ¹H-NMR for C1, C2, and C3 in the *S*-propenyl groups of *cis*/*trans*-S1PC in the reaction mixture using *trans*-S1PC (**E** 0 days, **F** 5 days, **G** 15 days, **H** 30 days).
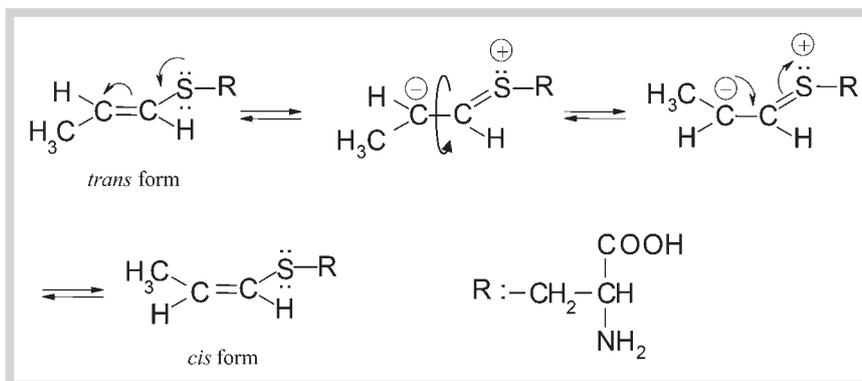
Fig. 4 Production mechanism of *cis*-S1PC through isomerization from the *trans* form.

Acknowledgements
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Conflict of Interest
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The authors report no conflict of interest.

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