Note

Molecular Capture and Conformational Change of Diketopiperazines Containing Proline Residues by Epigallocatechin-3-O-gallate in Water

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The addition of an aqueous solution of diketopiperazine cyclo(Pro-Xxx) (Xxx: amino acid residue) to an aqueous solution of (−)-epigallocatechin-3-O-gallate (EGCg) led to precipitation of the complex of EGCg and cyclo(Pro-Xxx). The molecular capture abilities of cyclo(Pro-Xxx) using EGCg were evaluated by the ratio of the amount of cyclo(Pro-Xxx) included in the precipitates of the complex with EGCg to that of the total cyclo(Pro-Xxx) used. Stronger hydrophobicity of the side chain of the amino acid residue of cyclo(Pro-Xxx) led to a higher molecular capture ability. Furthermore, the molecular capture ability decreased when the side chain of the amino acid residue had a hydrophilic hydroxyl group. When diketopiperazine cyclo(Pro-Xxx), excluding cyclo(D-Pro-L-Ala), was taken into the hydrophobic space formed by the three aromatic A, B, and B′ rings of EGCg, and formed a complex, their conformation was maintained in the hydrophobic space. Based on nuclear Overhauser effect (NOE) measurement, the 3-position methyl group of cyclo(D-Pro-L-Ala) in D2O was axial, whereas that of cyclo(L-Pro-L-Ala) was equatorial. When cyclo(D-Pro-L-Ala) was taken into the hydrophobic space of EGCg and formed a 2 : 2 complex, its 3-position methyl group changed from the axial position to the equatorial position due to steric hindrance by EGCg.

Key words (−)-epigallocatechin-3-O-gallate; diketopiperazine; proline residue; hydrophobic space; molecular capture ability; quantitative 1H-NMR

Introduction

When an aqueous solution of caffeine is poured into an aqueous solution of catechin (−)-epigallocatechin-3-O-gallate (EGCg), which is the most abundant catechin in tea, precipitation due to the creaming-down phenomenon is observed.1) Crystallization of this precipitate and X-ray crystal structure analysis revealed that it was a 2 : 2 complex of caffeine and EGCg2–4) (Fig. 1). As shown in Fig. 1, the caffeine moieties of the 2 : 2 complex were located in the space surrounding the bottom and top walls of the B′ rings of EGCg moieties, and left and right walls of the A and B rings of other EGCg moieties. As a result, caffeine molecules were captured by the hydrophobic space formed by the three aromatic A, B, and B′ rings of the EGCg in the 2 : 2 complex. Therefore, the hydrophobicity of the 2 : 2 complex of caffeine and EGCg increased, whereas its solubility in water decreased rapidly compared with EGCg and caffeine alone, causing precipitation due to the creaming-down phenomenon.

We previously investigated the higher-order functions of tea gallate-type catechins.5) As part of this study, we are developing the ability using tea gallate-type catechins and their derivatives to selectively capture the desired compound from an aqueous solution. We evaluated the molecular capture abilities of heterocyclic compounds using EGCg by the ratio of the amount of heterocyclic compounds included in the precipitates to that of the total heterocyclic compounds used.6) However, no correlation was found between the chemical structures of the heterocyclic compounds and the molecular capture ability.

Therefore, similar to the chemical structure of caffeine, several types of diketopiperazine cyclo(Pro-Xxx) (Xxx: amino acid residue) consisting of 6- and 5-membered rings were investigated as a substrate of EGCg. Cyclo(Pro-Xxx) is advantageous as because its side chain can be freely altered by substituting amino acid residues (Fig. 2).

The crystals obtained by pouring the aqueous solutions of cyclo(D-Pro-Gly) and cyclo(L-Pro-Gly) into an aqueous EGCg

![Fig. 1. Structure of (−)-Epigallocatechin-3-O-gallate (EGCg), Caffeine, and 2:2 Complex of Caffeine and EGCg](image)
solution were analyzed by X-ray crystal structure analysis, and found to be a 2:2 complex of cyclo(-Pro-Gly) or cyclo(Pro-Pro-Gly) and EGCg, respectively. Similar to caffeine moieties in the 2:2 complex of caffeine and EGCg, cyclo(Pro-Gly) moieties in the 2:2 complex of cyclo(Pro-Gly) and EGCg were taken into the hydrophobic spaces formed by the three aromatic A, B, and B’ rings of EGCg (Fig. 3).

Therefore, instead of the glycine residue of cyclo(Pro-Gly), diketopiperazine cyclo(Pro-Xxx) having amino acid residues with different side chains was synthesized. We next evaluated the molecular capture ability of cyclo(Pro-Xxx) by EGCg, and the correlation between the chemical structures of cyclo(Pro-Xxx) and the molecular capture ability was investigated.

Upon the complex formation of EGCg, the conformational change of cyclo(Pro-Xxx) was also investigated.

**Experimental**

**NMR Experiments**  
^1H-NMR spectra were recorded at 35°C on a JEOL JMN-LA500 (Tokyo, Japan) operating at 500 MHz using a 5 mmø sample tube. In general, ^1H-NMR experiments were performed with 32 K data points covering a spectral width of 10000 Hz with an approx. 3.7 s pulse delay time and 16 scan times. D₂O (99.9 atom% D; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a measuring solvent. Chemical shift values are expressed in ppm downfield using deuterated sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS-d₆, Wako Pure Chemical Industries, Ltd.) as an internal standard.

Nuclear Overhauser effect (NOE) spectroscopy was typically conducted with 32K data points covering a spectral width of 10000 Hz and an approx. 5 s presaturation time, with the other parameters the same as for ^1H-NMR spectra.

Rotating frame nuclear Overhauser effect (ROE) spectroscopy was performed with a mixing time of 250 ms, approx. 5 s pulse delay time, 80 scan times, and 8 dummy scan times, with the other parameters the same as for ^1H-NMR spectra.

**General Preparation for Diketopiperazine Cyclo(Pro-Xxx) (Xxx: Amino Acid Residue)**  
To a solution of tert-butoxycarbonyl (Boc)-Pro (5.0 mmol) and N-methyl-morpholine (NMM) (0.61 mL, 5.5 mmol) in CH₂Cl₂ (40 mL), isobutyl chlorocarbonate (0.65 mL, 5.0 mL) was added at −15°C. To the solution of resulting mixed anhydride, a solution of Xxx-OBzl·p-tosylate (or Xxx-OBzl·HCl) (5.0 mmol) and NMM (0.61 mL, 5.5 mmol) in CH₂Cl₂ or CH₂Cl₂ and N,N,N-dimethyl-formamide (DMF) (40 mL) was added at −15°C. The mixture was stirred at −15°C for 1 h and at room temperature for 2 h, and then evaporated under reduced pressure. The residue was dissolved in EtOAc and washed with water, 10% aqueous NaHCO₃ solution, water, and saturated saline solution. The ethyl acetate layer was evaporated under reduced pressure to give Boc-Pro-Xxx-OBzl in a quantitative yield.

Boc-Pro-Xxx-OBzl was added 4 M HCl·1,4-dioxane (approx. 30 mL) and stirred at room temperature for 1 h. The solution was evaporated under reduced pressure, and the resulting residue was washed with ether several times to afford HCl·Pro-Xxx-OBzl in a quantitative yield.

HCl·Pro-Xxx-OBzl was dissolved in 2-butanol containing 0.1 M acetic acid (15 mL per 1 mmol of HCl·Pro-Xxx-OBzl) and NMM (15 times the molar amount of HCl·Pro-Xxx-OBzl). While monitoring the product formation on the TLC, the mixture was refluxed for 4 h–1 d and evaporated under reduced pressure. The resulting residue was purified by column chromatography (SiO₂, CHCl₃–MeOH = 10 : 1 or 100 : 1) to afford diketopiperazine cyclo(Pro-Xxx) in a moderate yield.

**Quantitative NMR Experiments**  
Quantitative ^1H-NMR was performed with the following optimized parameters: probe temperature, 25°C; spinning, off; number of scans, 8; spectral width, 20 ppm; relaxation delay, 64 s; pulse angle, 90°; internal standard, DSS-d₆ (Wako Pure Chemical Industries, Ltd.). Dimethyl sulfoxide (DMSO)-d₆ (99.9 atom% D; Wako Pure Chemical Industries, Ltd.) was used as a measuring sol-
Preparation of a Solid Formed by Cyclo(Pro-Xxx) and EGCg, and Calculation of the Molecular Capture Ability

Diketopiperazine cyclo(Pro-Xxx) (1.09 × 10^{-3} mmol) and EGCg (1.09 × 10^{-2} mmol) were dissolved in distilled water (80 and 30 µL, respectively). The aqueous solution of cyclo(Pro-Xxx) was poured into the aqueous solution of EGCg, and the mixture was heated at 90°C to dissolve completely, and then left at room temperature for 1 h followed by 4°C for 1 d to obtain a supernatant liquid and sticky precipitate. After removing the supernatant liquid, the sticky precipitate was evaporated under reduced pressure to create a solid.

The solid was dissolved in DMSO-d_{6} (520 µL) containing DSS (1.44 × 10^{-3} mmol). The contents of the resulting DMSO-d_{6} solutions were measured by the integrated value of the methine proton signal for H_{3} of cyclo(Pro-Xxx) and singlet (9H) of the three methyl groups of DSS as an internal standard in the quantitative 1H-NMR spectrum. The molecular capture ability of cyclo(Pro-Xxx) using EGCg was evaluated by the ratio of the amount of cyclo(Pro-Xxx) included in the solid to the amount of cyclo(Pro-Xxx) (1.09 × 10^{-3} mmol) used.

Results and Discussion

Molecular Capture of Diketopiperazine Cyclo(Pro-Xxx)

Using EGCg An aqueous solution of diketopiperazine cyclo(Pro-Xxx) (Xxx: amino acid residue) was poured into an aqueous solution of EGCg. The resulting precipitate was dried to obtain a solid of the complex of cyclo(Pro-Xxx) and EGCg.

The obtained solid of the complex was measured by quantitative NMR. The proton signals for H_{1} and H_{2} did not overlap with other proton signals. However, H_{2} may have an abnormally long relaxation time and not be detected as a proton signal. Therefore, the integrated value of the proton signal of H_{2} was used to assess the molecular capture ability.

The molecular capture ability of cyclo(Pro-Xxx) using EGCg was evaluated by the ratio of the amount of cyclo(Pro-Xxx) (Xxx: amino acid residue such as Phe and Tyr) was higher than that of their diastereomer cyclo( D-Pro-L-Www), and the molecular capture ability of cyclo(Pro-Ser(Bzl)) was 0%, whereas that of cyclo(Pro-Ser) (D-Pro-L-Trp), cyclo(Pro-Ser(Bzl)), cyclo(Pro-Ser(Tyr(Bzl))), and cyclo(Pro-Ser(Tyr(Bzl))) were insoluble in water (80 µL) under this experimental condition.

Conformational Change of Cyclo(Pro-Ala) upon Formation of a Complex with EGCg 1H-NMR spectra of a solution of cyclo(Pro-Ala) and an equimolar solution of cyclo(Pro-Ala) and EGCg in D_{2}O are shown in Fig. 4. Based on comparison of the proton signals in 1H-NMR spectra of cyclo(Pro-Ala) alone, those of an equimolar solution of cyclo(Pro-Ala) and EGCg were broad, suggesting that cyclo(Pro-Ala) was taken into the three aromatic A, B, and C rings of EGCg, and formed the 2:2 complex with EGCg, resulting in strong restriction of their molecular motion.

Upon the formation of the 2:2 complex of cyclo(Pro-Ala) with EGCg, the proton signal for the H_{1} and H_{2} of cyclo(Pro-Ala) moiety in the axial position exhibited an upfield shift due to magnetic anisotropic shielding of the ring current from the B’ ring of the EGCg moiety (Table 2). On the other hand, the methyl proton signal of cyclo(Pro-Ala) moiety exhibited a downfield shift due to magnetic anisotropic deshielding of the ring current from the B’ ring of EGCg, suggesting that the methyl group existed outside the hydrophobic space.

The major difference in the 1H-NMR spectra between cyclo(Pro-Ala) and cyclo(Pro-Ala) was the chemical shift value of the methine proton signal for H_{1}. Although the
The methine proton signal for H₃ of cyclo(L-Pro-L-Ala) was at 4.301 ppm, that of cyclo(D-Pro-L-Ala) was at 4.066 ppm, with a difference of 0.235 ppm (Table 2).

NOE was performed to investigate the difference in conformation between cyclo(L-Pro-L-Ala) and cyclo(D-Pro-L-Ala) in D₂O. As a result, characteristic NOE was observed between the 3-position methyl proton and methine proton H₉ of cyclo(D-Pro-L-Ala) (Fig. 5). On the other hand, no NOE was observed between those of cyclo(L-Pro-L-Ala). As the methine proton H₉ took an axial position, the 3-position methyl group of cyclo(D-Pro-L-Ala) was suggested to be in the axial position, whereas that of cyclo(L-Pro-L-Ala) was in the equatorial position (Fig. 5).

ROE measurement of cyclo(D-Pro-L-Ala) in the presence of...
of EGCG in D$_2$O was next performed. As a result, no ROE was observed between the 3-position methyl proton and the methine proton H$_{9}$ of cyclo(o-Pro-l-Ala) moiety. When cyclo(o-Pro-l-Ala) was taken into the hydrophobic space formed by the three aromatic A, B, B’ rings of the EGCG, forming the 2:2 complex of cyclo(o-Pro-l-Ala) and EGCG, the 3-position methyl group in the axial position, which was highly sterically hindered by EGCG, was considered to conformationally change to the equatorial position (Fig. 6).

Along with above change of the methyl group, the methine H$_{3}$ changed from the equatorial position to the axial position and its proton signal exhibited a downfield shift from 4.07 to 4.19 ppm (Table 2). As a result, the chemical shift value of the methine proton signal of the H$_{3}$ (4.194 ppm) of cyclo(o-Pro-l-Ala) moiety in the 2:2 complex became almost the same as that for the H$_{3}$ (4.139 ppm) of cyclo(l-Pro-l-Ala) moiety in the 2:2 complex with EGCG. Therefore, a conformational change of the 3-position methyl group of cyclo(o-Pro-l-Ala) in the axial position to the equatorial position was considered to have occurred upon the 2:2 complex formation with EGCG, whereas its diastereomer cyclo(l-Pro-l-Ala) was taken into the hydrophobic space formed by the three aromatic A, B, and B’ rings of EGCG with no conformational change. Upon complex formation with EGCG, the conformational change that occurred in cyclo(o-Pro-l-Ala) did not occur in other diketopiperazine cyclo(Pro-Xxx).

**Conflict of Interest** The authors declare no conflict of interest.

**References**

1) Maruyama N., Suzuki Y., Sakata K., Yagi A., Ina K., *Proc. International Symposium Tea Science*, 145–149 (1991).
2) Tsutsu H., Sato T., Ishizu T., *Chem. Lett.*, 41, 1669–1671 (2012).
3) Ishizu T., Tsutsu H., Kinoshita Y., Mukaida H., Sato T., Kajitani S., *Chem. Pharm. Bull.*, 62, 552–558 (2014).
4) Ishizu T., Tsutsu H., Sato T., *Chem. Pharm. Bull.*, 64, 676–686 (2016).
5) Ishizu T., *Chem. Pharm. Bull.*, 68, 1143–1154 (2020).
6) Tsutsu H., Sato A., Fuji S., Fujioka Y., Ishizu T., *Chem. Pharm. Bull.*, 67, 501–504 (2019).
7) Ishizu T., Tsutsu H., Yokoyama A., *Tetrahedron Lett.*, 56, 1111–1114 (2015).
8) Ishizu T., Tsutsu H., Yokoyama E., Tanabe H., Yokoyama A., *Chem. Pharm. Bull.*, 64, 142–149 (2016).
9) Vaughan J. R. Jr., Osato R. L., *J. Am. Chem. Soc.*, 74, 676–678 (1952).
10) Suzuki K., Sasaki Y., Endo N., Mihara Y., *Chem. Pharm. Bull.*, 29, 233–237 (1981).