Association of Tannins and Related Polyphenols with the Cyclic Peptide Gramicidin S

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The association of 10 different tannins and related polyphenols with gramicidin S, a cyclic peptide having a rigid β-turn structure, has been examined using ^{1}H-NMR spectroscopy. In the presence of pentagalloylglucose and epigallocatechin-3-*O*-gallate, the proton signals due to proline and the adjacent phenylalanine moieties selectively shifted to up field, suggesting a regioselective association with the β-turn structure. The association was also supported by the observation of intermolecular nuclear Overhauser effects between epigallocatechin-3-*O*-gallate and the peptide. In contrast, ellagitannins, biogenetically derived from pentagalloylglucose, showed small and non-selective chemical shift changes, suggesting that interaction with these tannins is relatively weak. The hydrophobicity of the tannin molecules and the steric hindrance of the interaction site are thought to be important in the association.

Key words polyphenol; β-turn; hydrophobic; tannin; peptide; epigallocatechin 3-*O*-gallate

Tannins are polyphenols having the capability to cause the precipitation of proteins, and they show various biological activities by inhibiting enzymes. Recently, the preferential association of tannins with proline (Pro) residues in linear peptides was reported.[1,2] Pro residues disrupt both α-helix and β sheet conformation in the peptide secondary structure and commonly occurs on the surfaces of proteins.[3] Since Pro residues are frequently found near protein-protein interaction sites,[4] the preferential interaction of tannins with Pro residues may be related to the broad biological activities of tannins including enzyme inhibition.[5—7] Although a Pro residue is often observed in the β-turn structure and plays an important role for the formation of hairpin structures in peptides,[8] the interaction between the rigid turn structure and tannins has not so far been studied. Hence, in the present study, we have examined the interaction between gramicidin S (**1**) (Fig. 1), a simple cyclic peptide having a rigid β-turn structure,[9,10] and various tannins and related polyphenols by NMR spectroscopic techniques.

Results and Discussion

Association of gramicidin S (**1**) with tannins was visually demonstrated by the formation of precipitates in aqueous solution. We examined the interaction between **1** and various tannins and related polyphenols in detail by NMR technique. The ^{1}H-NMR spectra of **1** (10 mM in 10% DMSO-d_6–D_2O at 20 °C) in the presence of 10 different tannins and related polyphenols [20 mM, pentagalloylglucose (**2**), 1(β)-*O*-galloylpedunculagin (**3**), castalagin (**4**), punicalin (**5**), phillyraeoidin A (**6**), epigallocatechin (**7**), epigallocatechin 3-*O*-gallate (**8**), procyanidin B-2 digallate (**9**), gallic acid (**10**) and a tea flavonol glycoside (**11**) (Fig. 2)] were measured. At this concentration, **1** was expected to aggregate because the concentration was higher than the critical micelle concentration (400 μg/ml in 5% ethanol).[11] Therefore, it is unlikely that **1** formed a simple 1 : 1 complex with tannins. The molecular aggregates of **1** should have the hydrophilic δ-amino groups of ornitine outside facing to the water phase and the hydrophobic alkyl groups of leucine and valine inside. Since the peptide skeleton of **1** was flat and rigid, the Pro and phenylalanine (Phe) residues in the β-turn structure were probably located on the surface of the aggregates as in proteins.[3] As shown in Figs. 3 and 4, tannins **2**, **6**, **8**, and **9** and a flavonol glycoside **11** showed similar selective large up-field shift of the Pro and the adjacent Phe protons. Since the chemical shift changes were caused by the anisotropic effect of the aromatic rings of the tannins, the results indicated that these tannins interacted regioselectively with the β-turn structure of **1**. Ellagitannins **3**, **4**, and **5** and epigallocatechin (**7**) showed small and non-selective chemical shift change of the proton signals of **1**, while **10** gave no significant chemical shift change. Moreover, the comparison of the partition coefficient (P) of the compounds **2**—**11** (*n*-octanol/water at 15 °C, Fig. 2)[12] suggested that polyphenols having a larger P value (less water-soluble) caused the more selective and larger chemical shift change. This was apparent from comparison of three structurally related hydrolysable tannins **2**, **3** and **4** (Fig. 3). They are almost the same in molecular mass but different in the number of biphenyl bonds, and their P values greatly depend on the number of the biphenyl bonds.[12] Formation of the biphenyl bond not only restricted the free movement of the galloyl moiety but also reduced the area of the hydrophobic surfaces of the molecules.[13] These results indicate that the hydrophobic interaction is predominant in the association between tannins and **1**.

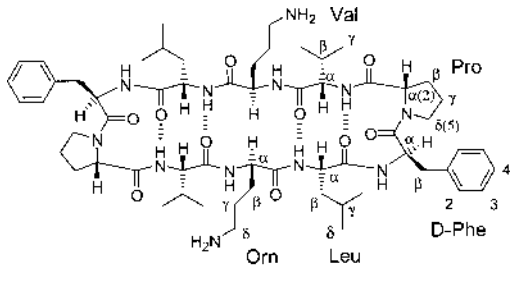

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Fig. 1. Structure of Gramicidin S (**1**)

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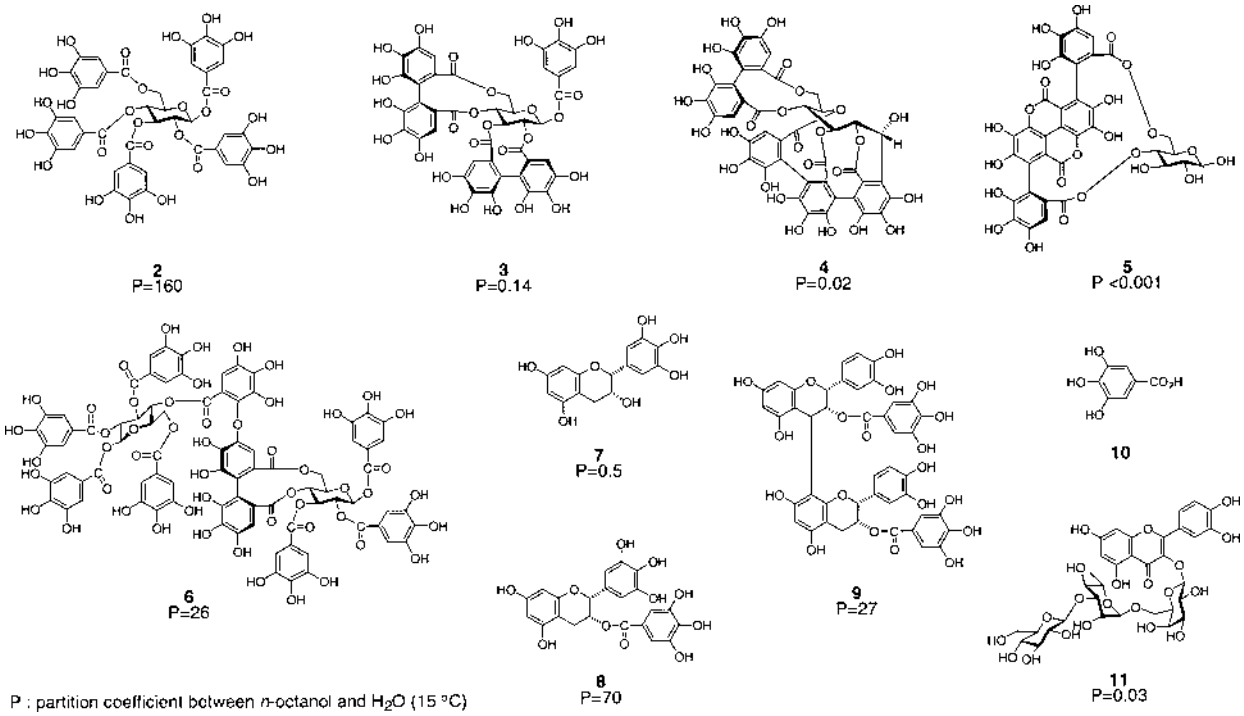

Fig. 2. Structures and Partition Coefficients of Tannins and Polyphenols

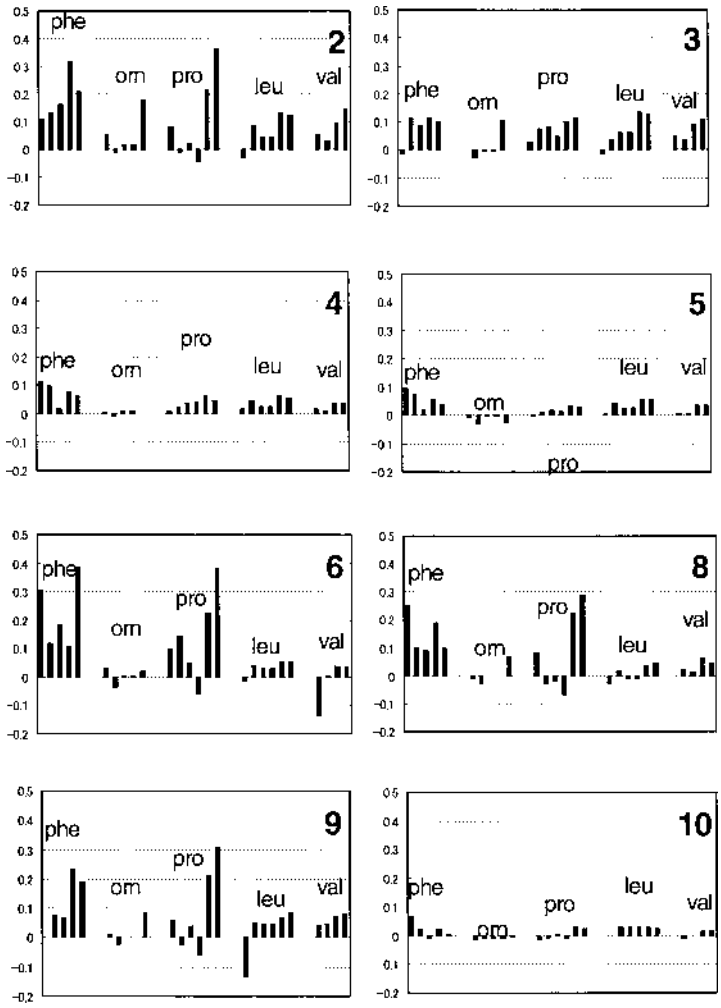

Fig. 3. Chemical Shift Changes (ppm) of **1** in the Presence of Tannins and Polyphenols

Phe: α, β1, β2, Bz-2,6, Bz-3,4,5; Orn: α, β1, β2, γ, δ; Pro: α, β1, β2, γ, δ1, δ2; Leu: α, β1, β2, γ, δ1, δ2; Val: α, β, γ1, γ2. Positive value: up field shift.

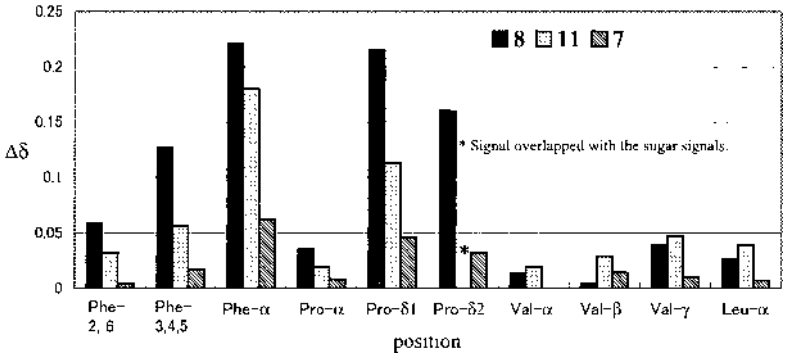

Fig. 4. Chemical Shift Changes (ppm) of **1** in the Presence of Compounds **7**, **8**, and **11**

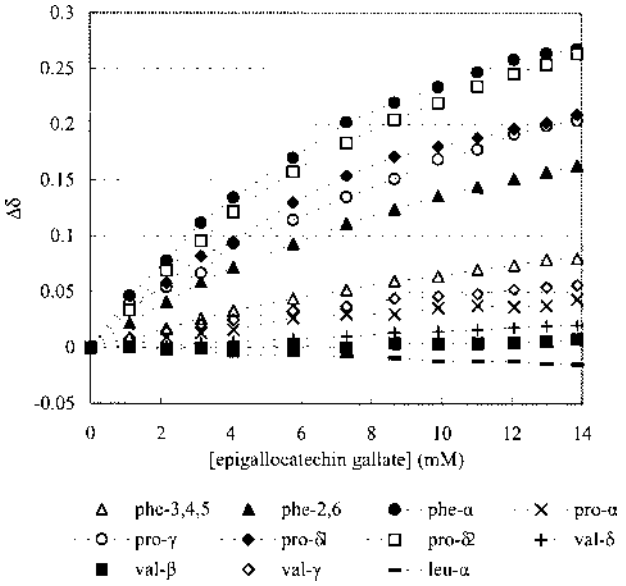

Fig. 5. Chemical Shift Changes (ppm) of **1** in the Presence of Different Concentrations of **8**

The ^{1}H-NMR spectra were measured in 10% DMSO-d_6–D_2O at 20 °C. The concentration of **1** was 10 mM and the concentration of **8** was increased successively from 0 to 1.1, 2.2, 3.2, 4.1, 5.8, 7.3, 8.7, 9.9, 11.0, 12.1, 13.0, and 13.9 mM.

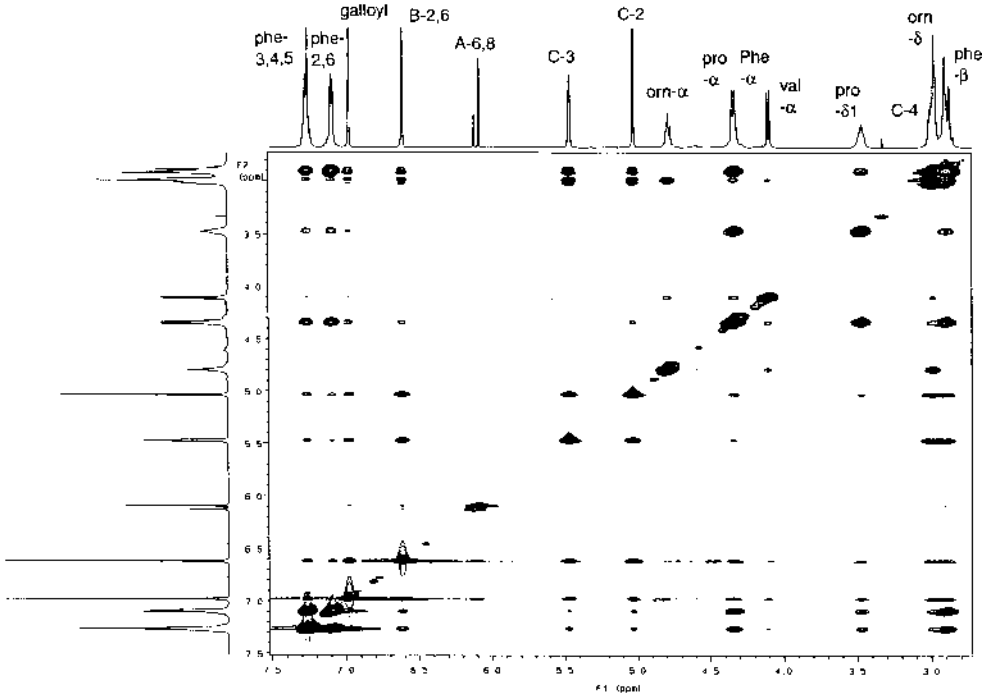

Fig. 6. NOESY Spectrum of a Mixture of **1** and **8**

1 (10 mM) and **8** (20 mM) in 10% DMSO-d_6–D_2O at 40 °C.

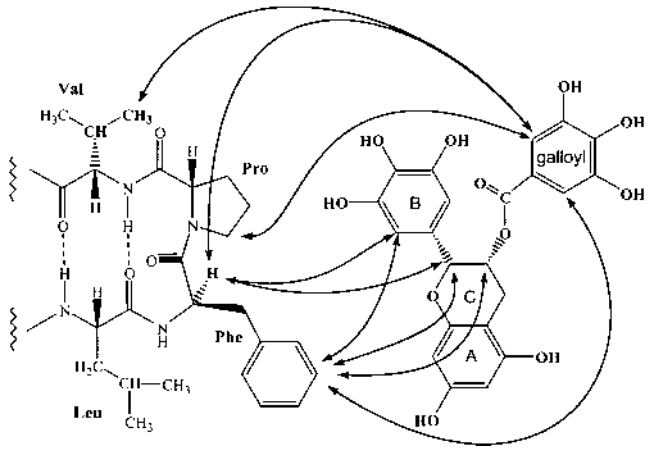

Fig. 7. Intermolecular NOESY Correlations between **1** and **8**

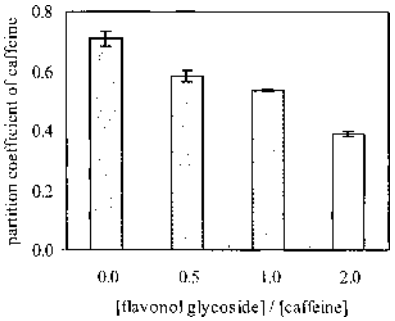

Fig. 8. Partition Coefficients of Caffeine in the Presence of **11**

The association of **1** with **8**, the major green tea polyphenol, was further studied in detail. As shown in Fig. 5, the chemical shift change of **1** depended on the concentration, and the degree of the shift change became smaller at higher concentrations of **8**. The nuclear Overhauser effect spectroscopy (NOESY) spectrum of the mixture (Fig. 6) revealed intermolecular nuclear Overhauser effects (NOEs): the Pro-δ protons of **1** was correlated with the galloyl protons of **8**, and the Phe-α and the aromatic protons of **1** showed cross peaks with the H-2, H-3, galloyl and B-ring protons of **8** (Fig. 7). This result indicated that the molecule of **8** preferentially interacted with the Phe and Pro residues of **1**. In addition, epigallocatechin (**7**), the desgalloyl derivative of **8**, induced only a small chemical shift change for **1**, revealing that the galloyl group in **8** plays an essential role in the interaction (Fig. 4), probably because of the hydrophobicity around the galloyl ester.

A tea flavonol glycoside (**11**) caused selective large chemical shift changes in **1** similar to those caused by **8** (Fig. 4), despite its high water-solubility (P=0.03) owing to the presence of a hydrophilic trisaccharide group. However, the hydrophobic interaction of **11** with caffeine was somewhat different from that of **8**. When caffeine was partitioned between *n*-octanol and water, the distribution of caffeine to the organic layer decreased in the presence of **11** (Fig. 8), and the decrease depended on the concentration of **11**. In contrast, a similar experiment using **8** did not show such a decrease in the distribution of caffeine, probably because **8** migrated to the organic layer together with the caffeine. Furthermore, the precipitates formed by adding **8** to the aqueous solution of caffeine were dissolved by addition of **11**. These behaviors of **11** could be explained by the high water-solubility and amphipathic nature of **11**, which possesses a highly hydrophobic aglycone and large hydrophilic sugar moieties. In an experiment using **1** instead of caffeine, both **8** and **11** increased the turbidity of the solution of **1**. This was probably because polyphenols associate with the aggregates of **1** in a manner different from that with caffeine.

Previously, we have examined the regioselectivity of the hydrophobic association between tannins and various crude drug constituents, such as paeoniflorin, amygdalin, aconitine, and liquiritin, and pointed out that the association occurred at the sterically unhindered site of the molecules.[12] In the present experiments, the preferential interaction of polyphenols with the β-turn structure of **1** suggested that the flat and hydrophobic Pro residue on the surface of the molecular aggregates of **1** provides the vacant space for the association with tannins.

In conclusion, our results showed that the interaction of tannins with **1** depended on their structure and hydrophobicity. It was also suggested that the unique characteristics of the Pro residue, its flat and rigid structure and hydrophobicity, is important in the tannin-protein interactions at the β-turn structure and not only in linear peptides.[1,2]

Experimental

Material Gramicidin S hydrochloride was purchased from Sigma. Pentagalloylglucose was prepared from tanninc acid.[14] Galloylpedunculagin,[15] castalagin,[16] punicalin,[17] phillyraeoidin A[18] were isolated from *Platycalya strobilacea*, *Castanea crenata*, *Punica granatum*, and *Quercus phillyraeoides*, respectively. Epigallocatechin 3-*O*-gallate, epigallocatechin[19] and quercetin 3-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside[20] were isolated from green tea.

^{1}H-NMR Measurements The ^{1}H-NMR spectra were measured with a Varian Gemini 300 spectrometer at 20 °C. Gramicidin S (**1**) (0.008 mmol, final concentration, 10 mM) was dissolved in 75 μl of DMSO-d_6 and diluted with 675 μl of D_2O or tannin solution (final concentration, 20 mM). Assignments of the signals of **1** were made by ^{1}H–^{1}H correlation spectroscopy. In the concentration dependent experiments (Fig. 5), the solution containing **8** (36 mM) and **1** (10 mM) was added stepwise to the solution of **1** (10 mM) and the ^{1}H-NMR spectrum measured at each concentration. The NOESY spectrum of a mixture of **1** (10 mM) and **8** (20 mM) at 40 °C was obtained by using a Varian Unity plus 500, and the experiment was performed using standard Varian pulse sequences (mixing time 0.5 s).

Partition of Polyphenols and Caffeine An aqueous solution (1.0 ml) containing caffeine (5.2 mM) and the flavonol glycoside **11** (0, 2.6, 5.2, 10.4 mM) was partitioned with *n*-octanol (1.0 ml) at 18 °C. Caffeine in the organic and aqueous layer was analyzed by HPLC performed on a Cosmosil 5C_{18}-AR (Nacalai Tesque Inc., Japan) column (4.6 mm i.d.×250 mm) (mobile phase, CH_3CN–50 mM H_3PO_4, gradient elution from 10 to 50% CH_3CN within 60 min; flow rate, 0.8 ml/min, detection: UV absorption at 275 nm). The partition coefficient was calculated based on the peak area (peak area of organic layer/peak area of aqueous layer).

Dissolution of Precipitates of 1 and 8 Transparency (%) at 750 nm of the solution containing caffeine (13.5 mM), **8** (13.5 mM) and **11** (1.3, 2.0, 2.7, 3.4 mM) was compared: caffeine (98.3%), **8** (98.6%), caffeine+**8** (0.13%), caffeine+**11** (3.4 mM) (97.7%), **8**+**11** (3.4 mM) (97.2%), caffeine+**8**+**11** (1.3 mM) (0.25%), caffeine+**8**+**11** (2.0 mM) (14.3%), caffeine+**8**+**11** (2.7 mM) (92.0%), and caffeine+**8**+**11** (3.4 mM) (96.33%).

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