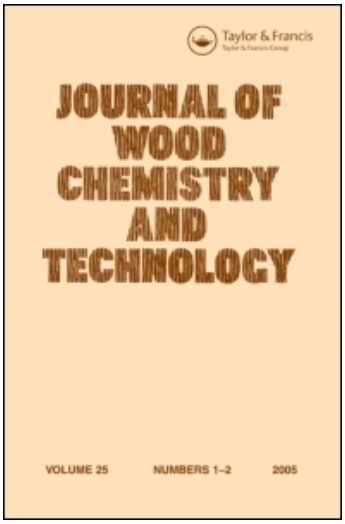

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SYNTHESIS OF CONDENSED TANNIN DERIVATIVES AND THEIR PROTEIN - PRECIPITATING CAPACITY

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ABSTRACT

Four regiospecifically methylated condensed tannin derivatives were synthesized by the condensation of regiospecifically methylated flavan-3,4-diols protected by benzyl groups, and subsequent debenzylation. The monomeric flavan-3,4-diols were obtained *via* four reaction steps starting from phloroacetophenone derivatives and protocatechualdehyde derivatives. Protein - precipitating capacity of these synthetic condensed tannin derivatives was tested and a comparision of these capacity suggests the following results: 1) Phenolic hydroxyl groups in tannins are essential sites for protein precipitation. 2) Although the A-ring has been considered not to be important for tannin - protein interactions, it was proved that the A-ring also plays an important role together with the B-ring. 3) Both hydroxyl groups of A- and B-rings may synergistically interact with proteins.

INTRODUCTION

Tannin is known to have the ability to precipitate soluble protein from aqueous solution.[1] The mechanism of the precipitation has been investigated with natural tannins.[2] Considering that *o*-dihydroxyphenolic groups are commonly found in the B-ring of natural condensed tannins, the sites of tannins interacting with proteins have been thought to be the *o*-dihydroxyphenolic

groups.[3,4] However, details of this mechanism still remain unclear. So, the systematic investigations using the regiospecifically protected condensed tannin derivatives with definite structure may offer useful information on such interactions.

To synthesize the regiospecifically methylated condensed tannin derivatives, it is necessary to obtain the regiospecifically methylated flavan-3,4-diol. The flavan-3,4-diol derivative has been synthesized by the reduction of natural (+)-taxifolin.[5] This synthetic method, however, is not suitable for our purpose, because regioselective methylation of taxifolin is difficult in high yield. Thus, our previously reported synthetic route for flavan-3,4-diol starting from phloroglucinol and protocatechualdehyde[6] is useful for this study.

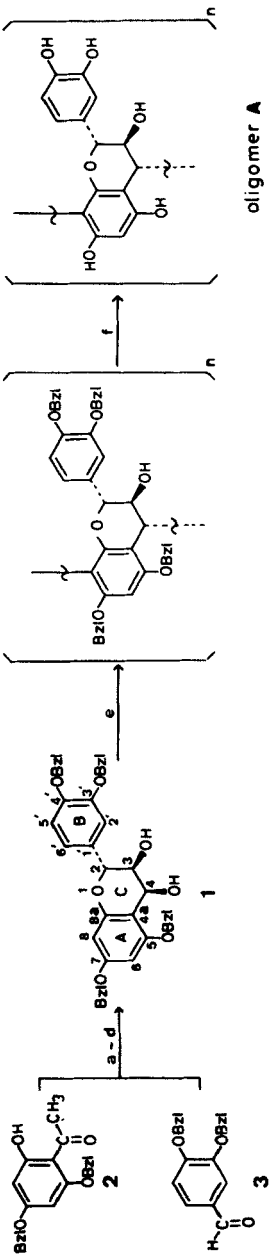
The condensation reaction of the flavan-3,4-diol having free phenolic hydroxyl groups has been reported in the presence of a protic acid,[7] but this reaction system seems to cause undesirable side reactions, because flavan-3,4-diols with free phenolic hydroxyl groups are unstable.[5] On the other hand, the use of the flavan-3,4-diol derivatives protected by benzyl group would reduce such side reactions.

In this paper, a series of regiospecifically methylated condensed tannin derivatives is successfully synthesized from phloroglucinol and protocatechualdehyde, and then the roles of the phenolic hydroxyl groups of the A- and B-rings in the precipitation of proteins are discussed from the results of protein - precipitation tests.

RESULTS AND DISCUSSION

1. Synthesis of condensed tannin derivatives

(+)-Catechin and (-)-epicatechin units are most commonly found in natural condensed tannins.[8] First, we attempted to synthesize the nonmethylated condensed tannin consisting of only



a. NaH (1.2eq) / DMF / r.t. / 2hr / 94.6%.
b. $NaBH_4$ (1eq) / methyl cellosolve / 90°C / 5min.
c. BF_3-Et_2O (0.07eq) / r.t. / 15min.
d. OsO_4 (0.02eq) / N-methylmorpholine-N-oxide (1.1eq) / THF / r.t. / 5.5hr / 64.8% (b-d overall yield).
e. $TiCl_4$ (1eq) / anhydrous CH_2Cl_2 / 0°C / 3hr.
f. 10% Pd-C / dioxane / 90°C / 3hr.

FIGURE 1. Synthetic route for the synthetic condensed tannin, oligomer A.

catechin as a repeating units and named this synthetic tannin as oligomer A. Figure 1 shows the synthetic route for this oligomer A. The key reactions in this synthetic route are the polymerization of flavan-3,4-diol (**1**) and the subsequent debenzylation.

In the previous synthesis of a condensed tannin model compound, flavan-3,4-diol (**1**) was reacted with five equivalents of phloroglucinol in the presence of $TiCl_4$ to yield the expected product, 2,3-trans-3,4-trans-3',4',5,7-tetrabenzyloxy-4-(2,4,6-trihydroxyphenyl)flavan-3-ol, and a mixture of higher condensed products in about 90% and 10% yields, respectively.[6] Accordingly, only a condensation reaction was found to proceed under these reaction conditions. Thus, the polymerization of flavan-3,4-diol (**1**) may proceed under the same reaction conditions as that of the synthesis of the model compound in the absence of phloroglucinol.

Flavan-3,4-diol (**1**) was synthesized via four reaction steps in 61.3% overall yield starting from phloroacetophenone dibenzyl ether (**2**) and protocatechualdehyde dibenzyl ether (**3**) as reported earlier.[6] The condensation of compound (**1**) was conducted using $TiCl_4$ in anhydrous CH_2Cl_2 at 0°C and the reaction was monitored by GPC. Figure 2 indicates that the polymerization rapidly proceeds and that the GPC patterns after 3 hr and 7 hr are similar; namely, the reaction for 3 hr is enough time for the polymerization. The polymerization products were obtained as a light-greenish powder in 99.1 % yield. The number average of degree of polymerization ($\overline{DPn}$) was determined to be 3.7 by vapor pressure osmometry (VPO)[9] and the GPC pattern indicates that the condensation product is polydispersed.

Generally, the benzyl ether is removed efficiently by hydrogenolysis under the neutral conditions. In fact, the complete debenzylation of 3',4',5,7-tetrabenzyloxy-4-(2,4,6-trihydroxyphenyl)flavan-3-ol was accomplished in 99.3% yield using 10% Pd-C and H_2 in methanol at room temperature for 2 hr.[6] However, the debenzylation of the polymerization product of compound (**1**) was found to be extreamely difficult. Even after hydrogenolysis for 28 hr, complete debenzylation was not achieved and ^{1}H-NMR analy-

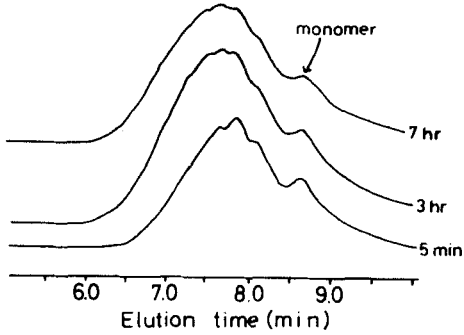


FIGURE 2. GPC analyses: the polymerization reaction of monomer (**1**). Reaction conditions: $TiCl_4$ (1eq) / anhydrous CH_2Cl_2 / 0°C.

sis of the products showed that about one benzyl group per one flavan-3-ol unit still remain. Acetic acid[10] which is generally used as a more drastic solvent for hydrogenolysis, was also not favorable for further debenzylation because the products were unstable under acidic conditions and formed deep - brownish compounds.

It is necessary for the progress of hydrogenolysis that the benzyl group comes into contact with the surface of the catalyst.[11] For the condensed tannin molecule it is known that strong rotational hinderance[12] (15-20 kcal/mol[13] for dimers) exists around the interflavanoid linkage and E. Haslam *et. al.* reported[13] that a high temperature (above 170°C) is required for the free rotation. Thus, such steric hinderance should be reduced as much as possible for the complete debenzylation.

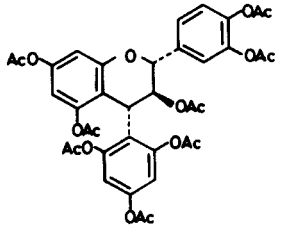
For reducing such rotational hinderance, debenzylation was carried out at 90°C. This reaction was monitored by the measurement of ion differential spectrum which has been used for the quantitative analysis of the phenolic hydroxyl groups in lignin.[14] The absorbance at 295 nm in this spectrum leveled off within 3 hr suggest that the hydrogenolysis was finished by this reaction time. Complete debenzylation was also confirmed by the

inspection of ^{1}H-NMR spectrum of the hydrogenolysis products measured in DMSO-d_6; the signals derived from benzyl aromatic protons appeared at the magnetic field less than 7.3 ppm were not observed in this spectrum. Thus, hydrogenolysis conditions were established with Pd-C in dioxane at 90°C for 3 hr and the condensed tannin (oligomer A) which had strong astringency was obtained quantitatively as a colorless powder.

However, the several earlier reports[15,16,17] suggest that the structure of the condensed tannins is altered during hydrogenolysis by possible side reactions such as the following: 1) the reduction of the hydroxymethyne group at C_4 position to a methylene group,[18] 2) the reductive ring - opening reaction of the C-ring,[16] 3) the epimerization at C_2 position,[15] and 4) the cleavage of the interflavanoid linkage.[16] For these reasons, we examined simple model compounds to determine whether such side reactions take place or not during hydrogenolysis.

Two model compounds were selected; flavan-3,4-diol (**1**) as a model of the lowest terminal unit in the condensed tannin molecule and 2,3-trans-3,4-trans-3',4',5,7-tetrabenzyloxy-4-(2,4,6-trihydroxyphenyl)flavan-3-ol[6] as a model of the procyanidin chain extender units. After hydrogenolysis of these model compounds under the same reaction conditions as the above synthesis of the condensed tannins. The structures of the products were confirmed by ^{1}H-NMR analyses of their acetates. The latter model compound gave the expected compound (**4**) in about 95% yield, while the former model compound (**1**) gave the product (**5**) whose hydroxymethyne group at C_4 position was reduced to methylene group in about 90% yield. The ring - opening product (**6**), 1-(3,4-diacetoxyphenyl)-2-acetoxy-3-(2,4,6-triacetoxyphenyl)propane, was detected in only a few percent yield based on TLC analysis. Neither products epimerized at C_2 position nor products cleaved at the interflavanoid linkage were detected.

Considering that the condensation products with higher molecular weight generally have lower reactivity than simple model compounds, other than the reduction of the hydroxymethyne group at C_4 position, side reactions would not be expected on the hydrogenolysis.



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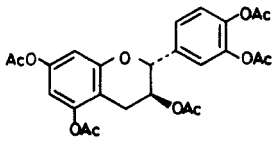

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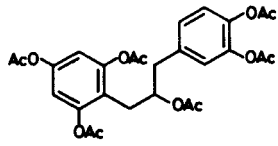

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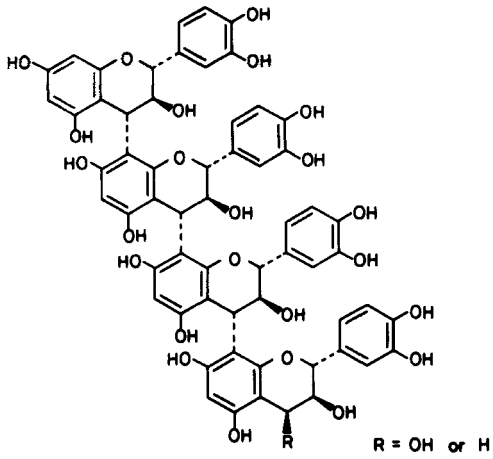


FIGURE 3. A proposed structure for the synthetic condensed tannin, oligomer A.

The number average of molecular weight ($\overline{Mn}$) of the acetylated synthetic condensed tannin was measured by VPO;[9] $\overline{Mn}$ proved to be 1850, which corresponds to $\overline{DPn}$ of 3.7.

^{13}C-NMR spectrum of the acetylated synthetic condensed tannin is in good agreement with that of the acetyl derivative of natural condensed tannin extracted from Cryptomeria japonica bark. The reaction mechanism[6] of polymerization suggests the 2,3-trans-3,4-trans configuration.

Thus, we temporarily present the structure of the present synthetic condensed tannin as shown in Fig. 3, although natural condensed tannins have been reported to contain mainly C_4 - C_8 linkage together with C_4 - C_6 one.[19]

Further investigation is necessary for the structural estimation. The synthetic condensed tannin has strong astringency. According to this synthetic route, it is possible to synthesize condensed tannin derivatives with several kinds of substituents.

2. Protein - precipitating capacity of the synthetic condensed tannin derivatives.

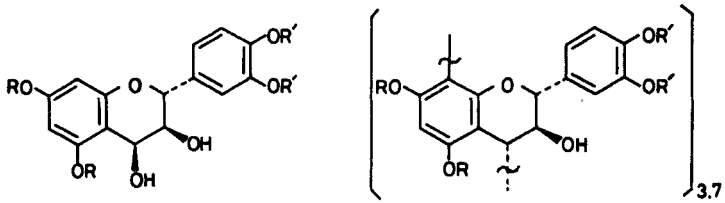
It has been believed that the sites of tannins interacting with proteins is mainly o-dihydroxyphenolic groups corresponding to the B-ring in condensed tannin molecules, but that the A-ring hydroxylation is not important in these interactions.[3] J. P. McManus et. al. studied the association of several simple phenols (resorcinol, catechol, pyrogallol and methyl gallate) with BSA (bovine sereum albumin) using equilibrium dialysis and microcalorimetry, and obtained the results supporting the above suggestion.[20] However, direct experimental proof using condensed tannins has not yet been obtained. To clarify this point, we synthesized three methylated condensed tannin derivatives, oligomer B, C and D,(Table), and measured their BSA - precipitating capacity.

These three regiospecifically methylated condensed tannin derivatives (oligomers B-D) were easily prepared by the above synthetic method from the monomers (**7**)-(**9**), respectively. The monomeric compounds (**7**)-(**9**) were synthesized in high yield by the same method as that of monomer (**1**) from phloroacetophenone derivatives and protocatechualdehyde derivatives.

Since it was reported that only polyphenols with the molecular weights of more than 500[21] have protein - precipitating capacity, monomer without such capacity[22] should be removed from the synthetic condensed tannin mixture before testing their astringency. As to oligomer A and natural condensed tannins, monomeric compounds could be completely removed using Sephadex LH-20,[23] and this was confirmed by GPC analysis. The removal of the monomeric compounds from oligomers B-D was carried out by TLC

TABLE. Four regiospecifically methylated condensed tannin derivatives, oligomer A-D, selected for the protein - precipitating test.

Monomer	R	R′	Condensed tannin derivative	R	R′
1	Bzl	Bzl	oligomer **A**	H	H
7	CH_3	Bzl	oligomer **B**	CH_3	H
8	Bzl	CH_3	oligomer **C**	H	CH_3
9	CH_3	CH_3	oligomer **D**	CH_3	CH_3



Monomer Condensd tannin derivative

before debenzylation; Rf values of condensation products were smaller values (almost zero) than those (Rf: 0.16-0.33) of monomeric compounds on TLC (EtOAc : n-hexane = 1:2, v/v).

L. J. Porter et. al. reported that the predominant factor affecting astringency of natural condensed tannins is the molecular weight.[24] The degrees of polymerization determined by GPC analyses were found to be about 3.7 for the four synthetic condensed tannin derivatives (oligomers A-D) and about 3-5 for natural condensed tannins of Acacia mollisima bark and Cryptomeria japonica bark, respectively.

The formation of the precipitates of BSA with oligomers A-D was carried out in 0.2 M acetate buffer (PH 4.5[25]) at 20°C. The protein remaining in the solution was measured by the ninhydrin method.[26] The precipitated BSA was estimated by subtracting the residual BSA in the supernatant solution from the total BSA used. The results are summarized in Fig. 4.

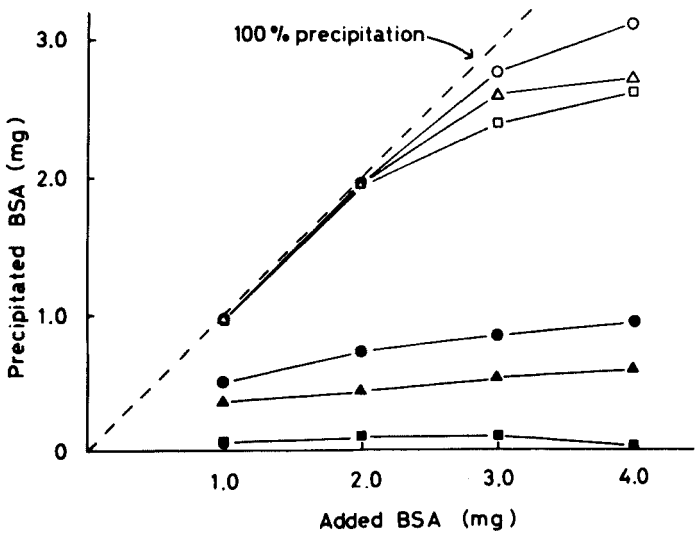


FIGURE 4. BSA- precipitating capacity of natural condensed tannins and the synthetic condensed tannins, oligomer A-D. ○: Acacia mollisima bark tannin, △: Cryptomeria japonica bark tannin, □: oligomer A, ●: oligomer B, ▲: oligomer C, ■: oligomer D.

Oligomer A consisting of catechin as a repeating unit shows almost the similar BSA - precipitating capacity as that of natural condensed tannins from Acacia mollisima bark and Cryptomeria japonica bark. Since the natural condensed tannins are optically active different from the synthetic tannins, optical activity of the C-ring was little effect on complexation ability. Porter et. al. discussed the effect of configuration of C-ring on the protein - precipitating capacity.[24]

The capacity of oligomer B and oligomer C was found to be 35% and 23% of that of oligomer A, respectively, and oligomer D had little complexation ability (less than 3% of that of oligomer A) when 2, 3 and 4 mg of BSA were used for the test. The comparision of the complexation ability of these oligomers indicates following results: 1) phenolic hydroxyl groups in tannins are essential sites for the precipitation of proteins. 2) Al-

though the A-ring has been considered not to be important for tannin - protein interactions, this investigation, using oligomers B-D is the first proof that the A-ring also plays an important role together with the B-ring. 3) Furthermore, since total precipitated BSA of oligomer B and C is only about 60% of that of oligomer A, both the hydroxyl groups of the A- and B-rings may synergistically interact with proteins.

EXPERIMENTAL

1. Synthesis of condensed tannin derivatives

The melting point are uncorrected. A SHIMADZU UV-365 spectrometer was used for UV spectra and the molar absorptivity was calculated on the assumption that the number average of molecular weights of all synthetic condensed tannin derivatives were 3.7. ^{1}H-NMR spectra were taken with a Varian XL-200 FT-NMR (200 MHz), using TMS as an internal standard. Mass spectra (MS) were obtained by the use of a SHIMADZU GCMS-QP1000 (70eV) spectrometer; the relative abundance of each peak is designated in parentheses. GPC analyses were carried out on SHIMADZU LC-4A and Shodex KF-802.5 column, using THF distilled before use and the flow rate was 1.0 ml/min. VPO was conducted in $CHCl_3$ on peracetates with KNAUER Vapour Pressure Osmometer. Preparative TLC was done on silica gel plate (Kieselgel 60 F_{254}, Merck). Oligomers A-D may contain a trace amounts of each monomer.

Synthesis of oligomer A

Monomer (1), 2,3-trans-3,4-cis-3',4',5,7-tetrabenzyloxyflavan-3,4-diol[6] obtained in 61.3% yield *via* four reaction steps from phloroacetophenone dibenzyl ether (2) and protocatechualdehyde dibenzyl ether (3) was used for a starting material.

Polymerization of monomer (1)

Monomer (**1**) (1.0 g, 1.50 mM) was dried over P_2O_5 in a vacuum desiccator before use. To a stirred solution of monomer (**1**) in 90 ml of anhydrous CH_2Cl_2, $TiCl_4$ (165 µl, 1.50 mM) in 10 ml of anhydrous CH_2Cl_2 was added dropwise at 0°C over a period of 5 min and the resulting brown solution was stirred for 3 hr. The reaction mixture was diluted with EtOAc and washed successively with water, $NaHCO_3$ solution and brine. The organic layer was dried over Na_2SO_4 and evaporated *in vacuo* to yield benzylated oligomer A as a greenish solid (967 mg, 99.1% recovery). UV λ_{max}^{MeCN}nm (log ε): 281 (4.29). Anal. Calcd. for $(C_{43}H_{36}O_6)_{3.7}OH \cdot 3H_2O$: C, 77.3; H, 5.7. Found: C, 77.3; H, 5.5, hereafter OH including in the molecular formula means the hydroxyl group of the lowest terminal unit.

Oligomer A

To a stirred solution of the polymerization product of monomer (**1**) (980 mg) in 98 ml of dioxane passed through on Alumina column (ICN Alumina B-Akt-1, ICN BIOMEDICALS), 10% Pd-C (980 mg) was added and the reaction mixture was stirred under hydrogen at 90°C for 3 hr. Pd-C was removed from the reaction mixture by centrifugation at 3000 rpm for 10 min and twice washed with dioxane (50 ml). The combined supernatant solution was evaporated *in vacuo* below 40°C to about 2 ml. The solution obtained was poured into 50 ml of n-hexane and the resulting precipitate was filtered and dried over P_2O_5 in a vacuum desiccator to yield oligomer A as a colorless powder (531 mg). Although more than the theoretical yield was obtained, this was due to the hydrated water because oligomer A was rather hygroscopic. UV λ_{max}^{MeOH} nm (log ε):282 (4.04). Anal. Calcd. for $(C_{15}H_{13}O_6 \cdot 0.2H_2O)_{3.7}$: C, 61.5; H, 4.6. Found: C, 61.6; H, 5.3.

The removal of the monomer from oligomer 1 (531 mg) was carried out on Sephadex LH-20 column (20 g, 6.5 cm x 4 cm) eluted first with EtOH for the elution of the monomer and then acetone / H_2O (7 : 3, v/v) to yield oligomer 1 without the monomer (460 mg).

Synthesis of oligomers B-D

Oligomers B-D were obtained according to a similar procedure as described for the synthesis of oligomer A. The recoveries in all reaction steps were almost quantitative. The removal of the monomers was carried out by TLC (EtOAc / n-hexane = 1 : 2, v/v) before debenzylation.

Oligomer B

Benzylated oligomer B: UV λ_{max}^{MeCN} nm (log ε): 280 (4.26). Anal. Calcd. for $(C_{31}H_{28}O_6)_{3.7}OH \cdot 3.1H_2O$: C, 72.1; H, 5.6. Found: C, 72.1; H, 5.6.

Oligomer B: UV λ_{max}^{MeOH} nm (log ε): 282 (4.12). Anal. Calcd. for $(C_{17}H_{17}O_6 \cdot 0.3H_2O)_{3.7}$: C, 63.3; H, 5.5. Found: C, 63.2; H, 5.3.

Oligomer C

Monomer (8) was obtained from phloroacetophenone dibenzyl ether (2) and protocatechualdehyde dimethyl ether in 45.1% overall yield. Mp 212-213°C; UV λ_{max}^{MeOH} nm (log ε): 278 (3.59); ^{1}H-NMR ($CDCl_3$) δ: 2.56 (1H, d, J=7.5, C_3-OH), 2.74 (1H, d, J=2.8, C_4-OH), 3.90, 3.91 (6H, 2s, $-OCH_3$), 4.01 (1H, ddd, J=10.3, 7.5, 4.1, C_3-H), 4.91 (1H, d, J=10.3, C_2-H), 5.00 (2H, s, -Bzl), 5.06, 5.13 (2H, 2d, J=11, -Bzl), 5.12 (1H, dd, J=2.8, 4.1, C_4-H), 6.20, 6.29 (2H, 2d, J=2.2, aromatic proton (A-ring)), 6.88-7.00 (3H, m, aromatic proton (B-ring)), 7.28-7.48 (10H, m, aromatic proton); MS m/z (%): 514 (M^+, 0.5), 496 (3.3), 468 (5.2), 377 (4.6), 181 (4.7), 180 (24.1), 178 (6.0), 165 (4.5), 151 (4.3), 92 (8.6), 91 (100). Anal. Calcd. for $C_{31}H_{30}O_7$ $0.3H_2O$: C, 71.6; H, 5.9. Found: C, 71.6; H, 5.8.

Benzylated oligomer C: UV λ_{max}^{MeCN} nm (log ε): 279 (4.21). Anal. Calcd. for $(C_{31}H_{28}O_6)_{3.7}OH \cdot H_2O$: C, 73.6; H, 5.8. Found: C, 73.6; H, 5.7.

Oligomer C: UV λ_{max}^{MeOH} nm (log ε): 278 (4.12). Anal. Calcd. for $(C_{17}H_{17}O_6 \cdot 0.8H_2O)_{3.7}$: C, 61.5; H, 5.7. Found: C, 61.5; H, 5.3.

Oligomer D

Monomer (9) was obtained from phloroacetophenone dimethyl ether and protocatechualdehyde dimethyl ether in 60.5% overall yield. Mp 167-169°C; UV λ_{max}^{MeOH} nm (log ε): 278 (3.58); ^{1}H-NMR ($CDCl_3$) δ: 2.58 (1H, d, J=7.2, C_3-OH), 2.79 (1H, d, J=2.5, C_4-OH), 3.80, 3.91, 3.94, 3.96 (12H, 4s, $-OCH_3$), 4.00 (1H, ddd, J=4.1, 7.2, 10.3, C_3-H), 4.91 (1H, d, J=7.2, C_2-H), 5.04 (1H, dd, J=2.5, 4.1, C_4-H), 6.10 (2H, s, aromatic proton (A-ring)), 6.84-7.10 (3H, m, aromatic proton (B-ring)); MS m/z (%): 362 (M^+, 5.8), 344 (16.2), 316 (46.7), 301 (21.0), 183 (19.8), 181 (15.0), 180 (100), 178 (17.9), 165 (23.1), 164 (9.6), 163 (14.7), 151 (20.2). Anal. Calcd. for $C_{19}H_{22}O_7 \cdot 0.2H_2O$: C, 62.3; H, 6.2. Found: C, 62.3; H, 6.1.

Oligomer D: UV λ_{max}^{MeOH} nm (log ε): 278 (4.13). Anal. Calcd. for $(C_{19}H_{20}O_6)_{3.7} \cdot 2.9H_2O$: C, 62.9; H, 6.0. Found: C, 62.9; H, 5.7.

2. Measurement of the protein - precipitating capacity of condensed tannins

Materials

The natural condensed tannins were extracted from Acacia mollisima bark and Cryptmeria japonica bark with MeOH and acetone / H_2O (7 : 3, v/v), respectively, and purified by Sephadex LH-20 column chromatography. Acacia mollisima bark tannin; Anal. calcd. for $(C_{15}H_{13}O_6 \cdot 0.9H_2O)_n$: C, 59.0; H, 4.9. Found: C, 59.1; H, 5.2. Cryptomeria japonica bark tannin; Anal. Calcd. for $(C_{15}H_{13}O_6 \cdot H_2O)_n$: C, 58.6; H, 4.9. Found: C, 58.7; H, 4.7. BSA (F-V) was purchased from Armour Pharmaceutical Co..

Determination of the precipitated BSA

Each condensed tannin (1.0 mg) in 200 μl of MeOH was mixed with the solution of BSA (1.0-4.0 mg) in 1.4 ml of 0.2M acetate buffer (PH 4.5) and each solution was left for 1 hr at 20°C. After the resulting precipitates were separated by centrifugation for 10 min at 3000 rpm, the amounts of BSA in the supernatant

solutions were measured by the ninhydrin method.[26] The precipitated BSA was estimated by subtracting the residual BSA in the supernatant solution from the total BSA used.

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