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Tannins and Related Compounds. XII.[1] Isolation and Characterization of Galloylglucoses from Paeoniae Radix and Their Effect on Urea–Nitrogen Concentration in Rat Serum

MAKOTO NISHIZAWA,[a] TAKASHI YAMAGISHI,[a] GEN-ICHIRO NONAKA,[b] ITSUO NISHIOKA,*[,b] TETSURO NAGASAWA,[c] and HIKOKICHI OURA[c]

Hokkaido Institute of Public Health,[a] N-19, W-12, Kita-ku, Sapporo 060, Japan, Faculty of Pharmaceutical Sciences, Kyushu University,[b] 3–1–1 Maidashi, Higashi-ku, Fukuoka 812, Japan, and Department of Biochemistry, Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University,[c] 2630, Sugitani, Toyama 930–01, Japan

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Together with 1,2,3,6-tetra- and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucoses, homologous series of hexa- and heptagalloylglucoses have been isolated from the tannin fraction of the root of *Paeonia lactiflora* PALL var. *trichocarpa* BUNGE. (Paeoniaceae). Their structures were determined by hydrolytic studies and by comparison of the ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectra with that of pentagalloylglucose. Octa–deca-galloylglucoses were partially purified and their structures characterized mainly by using ^{13}C-NMR spectroscopy. The isolation and structural studies of these galloylglucoses have shown gallotannins in Paeoniae Radix to have a 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose core with depside galloyl groups predominantly at the C-3 and C-6 positions in the glucose residue. Normal-phase high performance liquid chromatographic analysis showed that the gallotannins are composed of tetra–undeca-galloylglucoses having an average molecular weight of 1286, corresponding to 7.3 galloyl groups per glucose molecule.

The effects of hexa–deca-galloylglucoses isolated in this study on the urea–nitrogen (BUN) concentration in rat serum were examined, and all these compounds were shown to have remarkable BUN-decreasing activities.

Keywords——Paeoniae Radix; Paeoniaceae; galloylglucose; HPLC; ^{13}C-NMR; blood urea nitrogen

The root of *Paeonia lactiflora* PALL var. *trichocarpa* BUNGE. (Paeoniaceae) is one of the most important Chinese crude drugs, being contained in many traditional 'Kanpo' prescriptions. With regard to the constituents of this crude drug, Shibata *et al.*[2] isolated and characterized a series of novel monoterpenoid glucosides such as paeoniflorin, albiflorin, oxypaeoniflorin and benzoylpaeoniflorin, and more recently, Shimidzu *et al.*[3] showed the presence of paeoniflorigenone, which is structurally related to the aglycone of paeoniflorin. Extensive pharmacological studies on these compounds, especially on paeoniflorin and paeoniflorigenone, have also been made by Takagi *et al.* and Kimura *et al.*, and it was reported that paeoniflorin shows sedative, hypotensive and weak anti-inflammatory effects and a preventive effect on stress ulcer,[4] while paeoniflorigenone has a relaxing effect on skeletal muscle.[5] In previous papers,[6] we demonstrated that intraperitoneal administration of the aqueous extract of Paeoniae Radix caused a remarkable decrease of urea-nitrogen (BUN) concentration in rat serum. Later, as a result of a search for the active compounds, we found the activity in the tannin fraction, and isolated from this fraction 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose as one of the active compounds.[7,8] As a continuation of our studies on this tannin fraction, we have newly isolated and characterized polygalloylglucoses based on a 1,2,3,4,6-pentagalloylglucose core, and this paper deals with the isolation and structure

elucidation of these compounds. Furthermore, the results of examination of the BUN-decreasing activities of these compounds are also described.

The ethyl acetate-soluble portion of the aqueous acetone extract showed eight peaks corresponding to tetra–undeca-galloylglucoses (G4—G11) in normal-phase high performance liquid chromatography (HPLC) (Fig. 1A).[8-10] Repeated chromatography of this fraction on Sephadex LH-20 using a solvent system of ethanol–water–acetone[8-10] yielded tetra-, penta-, hexa-, hepta-, octa-, nona- and deca-galloylglucoses (G4, G5, G6, G7, G8, G9 and G10).

Tetra- and pentagalloylglucoses (G4 and G5) each gave a single peak in the reverse-phase HPLC (Fig. 1B), and were identified as 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose (**1**) and 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**2**), respectively, by comparisons of their retention times in HPLC and ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectra with those of authentic samples obtained from Chinese[9] and Turkish galls.[10]

The hexagalloylglucose (G6) fraction contained three components (**3**—**5**) as revealed by reverse-phase HPLC (Fig. 1B). Separation of these compounds was achieved by preparative reverse-phase HPLC on Nucleosil 10C_{18} or Wakogel LC-ODS 30K using the same solvent system as described previously.[9,10] All these compounds gave equimolar amounts of 1,2,3,4,6-pentagalloylglucose and methyl gallate upon mild methanolysis in methanolic acetate buffer (pH 5.5),[8,11] indicating that they have a 1,2,3,4,6-pentagalloylglucose core to which one galloyl group is depsidically attached. In the ^{13}C-NMR spectrum of **3**, the signal due to C-2 in the glucose moiety was shifted downfield by 0.4 ppm as compared with that of **2** (Table I). Similarly in **4** and **5**, the C-3 and C-6 resonances were observed at lower field by 0.6 and 0.4 ppm, respectively. These downfield shifts were similar to those observed in structural studies on Chinese and Turkish gallotannins.[9,10] The identity of **3**—**5** with 2-*O*-digalloyl-1,3,4,6-tetra-*O*-galloyl-*β*-D-glucose, 3-*O*-digalloyl-1,2,4,6-tetra-*O*-galloyl-*β*-D-glucose and 6-*O*-digalloyl-1,2,3,4-tetra-*O*-galloyl-*β*-D-glucose, respectively, was confirmed by HPLC

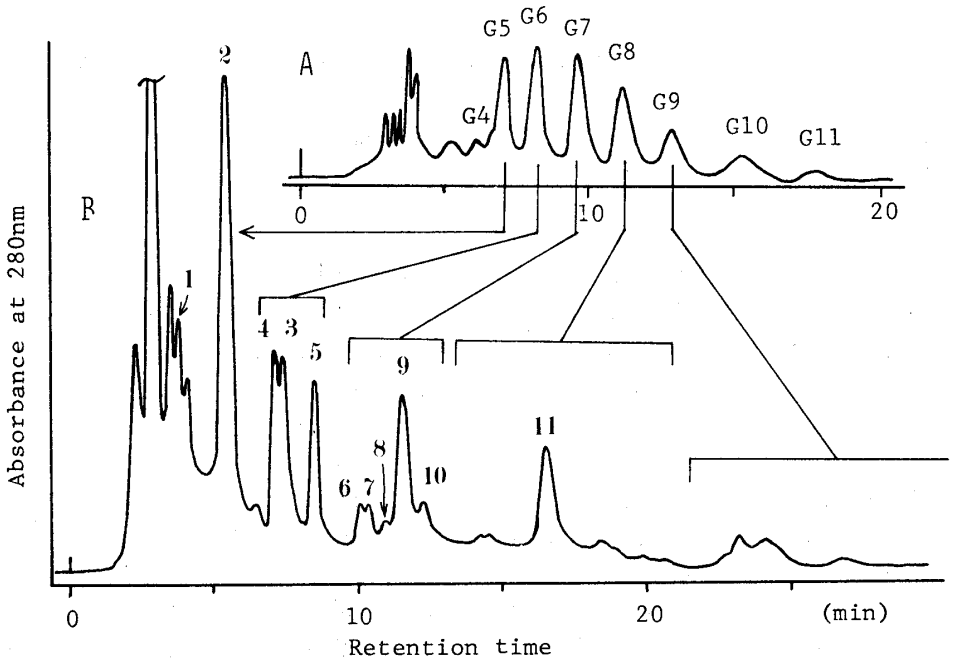


Fig. 1. High Performance Liquid Chromatograms of Gallotannins in Paeoniae Radix

Conditions: A (normal phase) column, Nucleosil 50-10 (3 mm i.d. × 300 mm); solvent, *n*-hexane–methanol–tetrahydrofuran–formic acid (55 : 33 : 11 : 1) (oxalic acid 450 mg/l); flow rate, 1.8 ml/min.

B (reverse-phase) column, Nucleosil 5C_{18} (4 mm i.d. × 250 mm); solvent, acetonitrile–water (22 : 78) (oxalic acid 2 g/l); flow rate, 1.3 ml/min.

TABLE I. ^{13}C-NMR Chemical Shifts of Gallotannins (δ-Values)

	Glucose						Carboxyl				
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-6
1	93.4	71.7	75.9	69.3	76.0	63.6	165.0	165.7	166.1	—	166.6
2	93.3	71.7	73.3	69.2	73.9	62.8	164.9	165.6	165.8	165.5	166.3
3	93.3	72.1	73.3	69.2	73.9	62.8	164.9	164.8[a]	165.0	165.8	165.5
4	93.3	71.7	73.9	69.2	73.9	62.8	164.8	164.9	165.6[a]	165.3	165.6
5	93.3	71.7	73.3	69.2	73.9	63.2	164.9	165.6	165.9	165.6	165.8[a]
6	93.3	72.0	73.9	69.2	73.9	62.8	164.8	165.0[a]	165.3[a]	165.6	166.3
7	93.3	71.7	73.9	69.2	73.9	62.8	164.8	165.6	165.3[a]	165.6	166.3
9	93.3	71.7	73.9	69.3	73.9	63.1	164.9	165.6	165.3[a]	165.6	165.9[a]
10	93.3	72.1	73.3	69.2	73.9	63.2	164.8	165.0[a]	165.9	165.6	165.8[a]
11	93.3	72.1	73.9	69.2	79.3	63.2	164.8	165.0[a]	165.3[a]	165.6	165.8[a]

a) Carboxyl carbon signal of the proximal galloyl moiety in a galloyl chain.

analysis and by comparison of the ^{13}C-NMR spectra with those of samples obtained previously.[9,10]

The heptagalloylglucose (G7) fraction showed five peaks in reverse-phase HPLC (Fig. 1 B). The components of this fraction corresponding to these peaks (**6**, **7**, **9** and **10**) were isolated by reverse-phase chromatography similar to that described in the case of hexagalloylglucoses. On treatment with aqueous methanol (acetate buffer, pH 5.5), all these isomers gave rise to 1,2,3,4,6-pentagalloylglucose and methyl gallate in the molar ratio 1 : 2. Thus, they were shown to be composed of a 1,2,3,4,6-pentagalloylglucose core with two depsidically linked galloyl groups. In the ^{13}C-NMR spectrum of **9**, the signals due to the C-3 and C-6 carbons in the glucose moiety were observed at lower field by 0.6 and 0.3 ppm, respectively, than those of **2** (Table I), suggesting the presence of depsidically linked galloyl groups at these positions. This was further supported by the fact that boiling of **9** in an aqueous solution furnished, together with gallic acid, partial hydrolysates which were identified as **2**, **4** and **5** by HPLC analysis (Fig. 2A). Accordingly, the structure of **9** was determined to be 3,6-bis-*O*-digalloyl-1,2,4-tri-*O*-galloyl-β-D-glucose. Similarly, the structure of **10** was characterized as 2,6-bis-*O*-digalloyl-1,3,4-tri-*O*-galloyl-β-D-glucose on the basis of ^{13}C-NMR analysis (Table I) and hydrolytic studies. The isomers **6** and **7** were identified by means of ^{13}C-NMR spectroscopy and by HPLC analysis as 2,3-bis-*O*-digalloyl-1,4,6-tri-*O*-galloyl-β-D-glucose and 3-*O*-trigalloyl-1,2,4,6-tetra-*O*-galloyl-β-D-glucose, respectively, which had been isolated previously from Chinese galls.[9] The minor component (**8**) of this fraction could not be isolated in pure form owing to contamination by **9**. However, the structure, 4,6-bis-*O*-digalloyl-1,2,3-tri-*O*-galloyl-β-D-glucose, of this compound could be assigned from the ^{13}C-NMR spectrum, showing downfield shifts of the C-4 and C-6 signals in the glucose moiety (69.6 and 63.1 ppm, respectively).

The octa(G8)-, nona(G9)- and decagalloylglucose (G10) fractions were shown by mild methanolysis to be composed of a 1,2,3,4,6-pentagalloylglucose core. The reverse-phase HPLC of these fractions (Fig. 1B) revealed the presence of many components, of which the major compound **11** was isolated in a chromatographically homogeneous state. The ^{13}C-NMR spectrum of **11** showed the C-2, C-3 and C-6 signals shifted downfield by 0.4—0.6 ppm as compared with those of **2** (Table I). On partial hydrolysis in boiling water, **11** afforded, in addition to gallic acid, a mixture of compounds **2**—**5** and **8**—**10** as examined by reverse-phase HPLC (Fig. 2B). On the basis of these results, the structure of **11** was assigned as 2,3,6-tris-*O*-digalloyl-1,4-di-*O*-galloyl-β-D-glucose. The other minor components of these fractions could

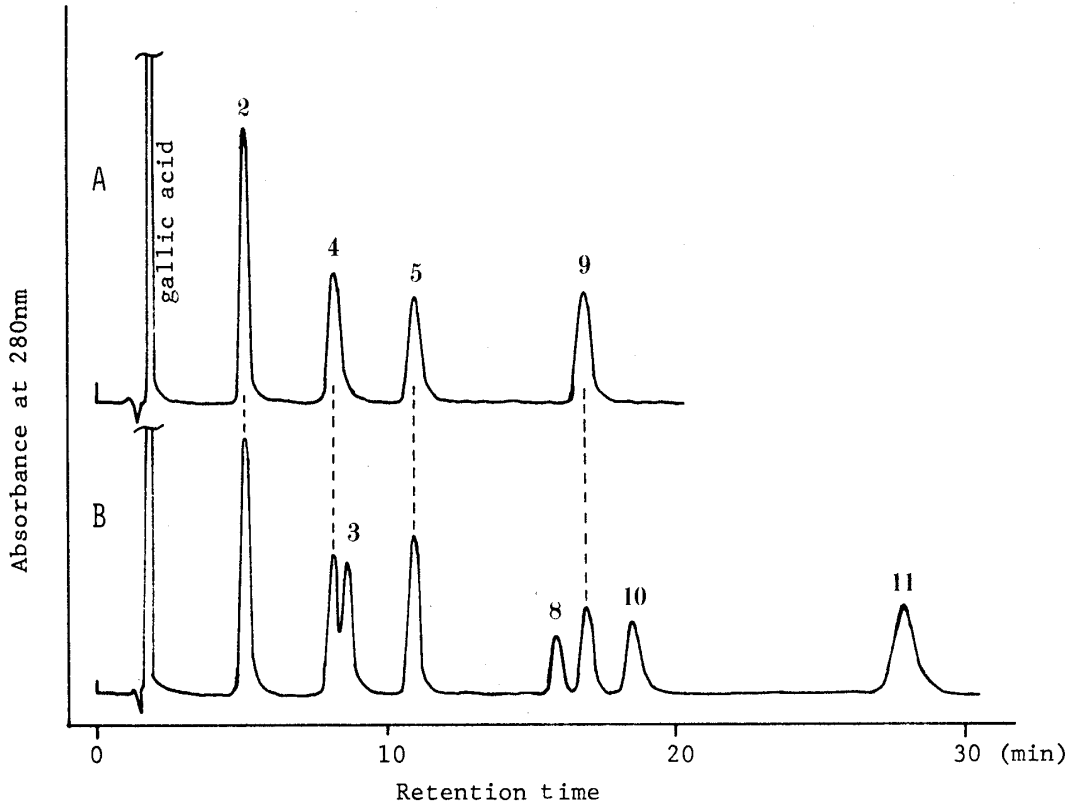


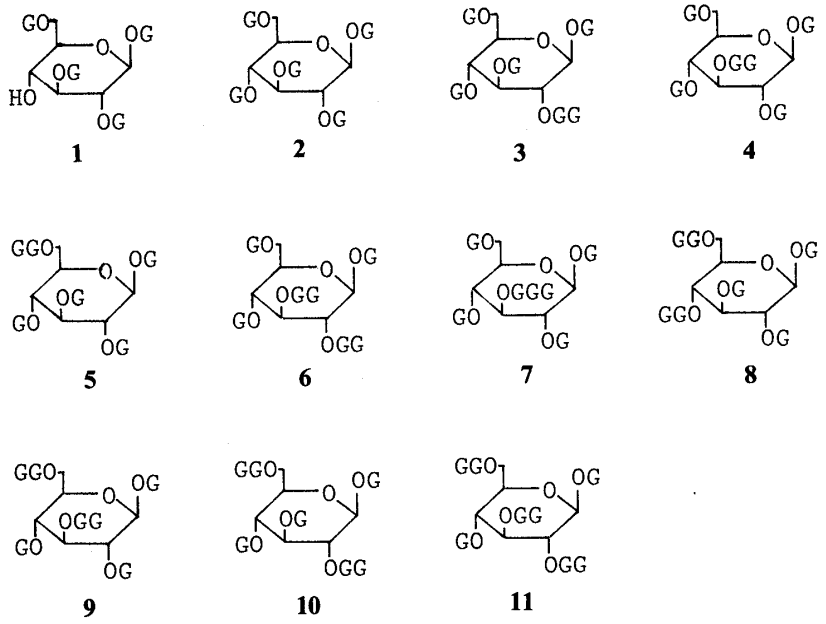
Fig. 2. High Performance Liquid Chromatograms of Partial Hydrolysis Products of **9** (A) and **11** (B)

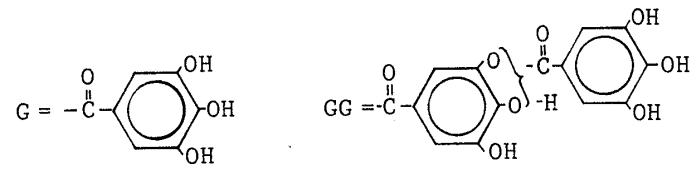
Column, Nucleosil $5C_{18}$ (4 mm i.d. × 250 mm); solvent, acetonitrile–water (21 : 79) (oxalic acid 2 g/l); flow rate, 1.3 ml/min.

not be purified owing to the presence of many structural isomers. However, the ^{13}C-NMR spectrum of a mixture of octa–deca-galloylglucoses (G8-G10) indicated that all these components have more than one depsidically linked galloyl group at the C-3 and C-6 positions of the glucose moiety since the signals corresponding to the C-3 (73.9 ppm) and C-6 (63.2 ppm) carbons were observed at lower positions than those of **2** (Fig. 3). On the other hand, signals due to C-2 and C-4 were split into two peaks; one was shifted downfield due to the presence of depside galloyl group(s) while the other remained unchanged. This implied that the proportion of the isomers having depsidically linked galloyl group(s) at the C-2 position was one-half judging from the intensities of these split signals. Similarly, the proportion at the C-4 position was estimated to be *ca.* one-tenth.

In previous studies on Chinese and Turkish gallotannins,[9,10] we have demonstrated that the depsidically linked galloyl groups with *m*- and *p*-depside linkages are attached to a carbohydrate core. This was based on the fact that the ^{13}C-NMR spectra of these gallotannins showed two sets of aromatic carbon signals arising from both *m*- and *p*-depside galloyl groups. As in those cases, the observation of the signals at δ 151.2, 132.2 and 128.7 derived from *p*-depside galloyl group(s) and at δ 143.3, 121.6, 117.5 and 114.5 from *m*-depside galloyl group(s) suggested that the depside galloyl groups in Paeonia gallotannin are located at both the *m*- and *p*-hydroxyl groups of proximal galloyl groups.

From the findings mentioned above, it has become clear that polygalloylglucoses in Paeoniae Radix are exclusively based on a 1,2,3,4,6-pentagalloylglucose core, and that the depside galloyl groups are attached predominantly to the C-3 and C-6 positions in the glucose moiety through *m*- and *p*-depside linkages. The relative compositions of galloylglucoses in Paeoniae Radix as determined by normal-phase HPLC are listed in Table II. The average





GGG = trigalloyl (= galloyl–galloyl–galloyl)

Chart 1

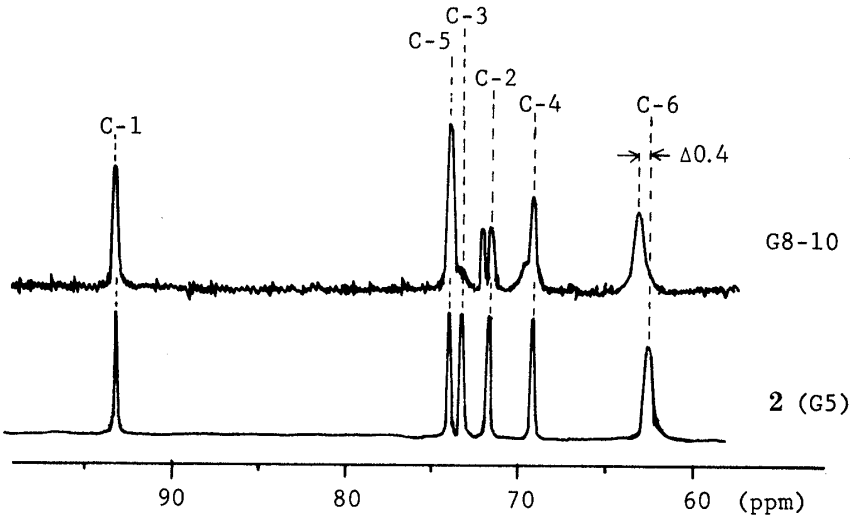


Fig. 3. ^{13}C-NMR Spectra of **2** and G8-10 (Glucose Carbon Region, in Acetone-d_6)

molecular weight was calculated to be 1286, which corresponds to 7.3 galloyl groups per glucose molecule.

Finally, the BUN-decreasing activities of galloylglucoses isolated in this study were examined. As shown in Table III, all the components caused, after intraperitoneal administration, remarkable decreases of BUN concentrations in rat serum, and the activities of hexa–

Table II. Relative Compositions of Galloylglucoses in Paeoniae Radix

	Ratio (%)[a]	Components
Tetra-GG[b]	1	**1**
Penta-GG	13	**2**
Hexa-GG	19	**3—5**
Hepta-GG	23	**6—10**
Octa-GG	22	**11** and more than 5 components
Nona-GG	14	More than 5 components
Deca-GG	6	
Undeca-GG	2	

a) Determined by normal-phase HPLC analysis.
b) GG represents galloylglucose.

Table III. Effect of Galloylglucoses on Blood Urea Nitrogen (BUN) Concentration in Rat Serum

Material	Dose (mg/rat)	No. of rats	BUN (mg/100 ml)[a]	(%)
Exp. I. Control[b]	—	17	15.9±1.9	100
1,2,6-Trigalloyl glucose*	1.0	6	14.0±1.2[d]	88
	2.5	6	12.6±2.6[e]	86
	5.0	5	12.5±1.5[e]	78
1,2,3,6-Tetra-galloylglucose	1.0	6	13.8±3.2	86
	5.0	5	11.1±1.7[f]	70
	10.0	5	11.7±1.1[f]	74
Exp. II. Control[c]	—	12	14.6±2.1	100
1,2,3,4,6-Penta-galloylglucose (**2**)	0.5	5	13.2±1.5	90
	1.0	10	11.9±1.8[e]	82
	2.5	9	10.9±1.3[f]	75
	5.0	5	10.9±1.0[e]	75
Exp. III. Control[b]	—	7	18.0±2.2	100
Hexagalloyl-glucose (G6)	0.5	5	14.3±1.1[e]	79
	1.0	6	14.0±1.5[e]	78
	2.5	6	12.7±0.5[f]	71
Heptagalloyl-glucose (G7)	0.5	5	13.2±1.1[e]	73
	1.0	4	12.0±0.4[f]	67
	2.5	5	12.7±1.7[e]	71
Exp. IV. Control[b]	—	17	14.3±2.1	100
Octagalloyl-glucose (G8)	0.5	10	10.6±0.7[f]	74
	1.0	10	10.7±1.0[f]	75
	2.5	11	12.6±1.1[d]	88
Nonagalloyl-glucose (G9)	0.5	12	12.1±1.2[e]	85
	1.0	11	10.7±1.1[f]	75
	2.5	10	10.4±0.9[f]	73
Decagalloyl-glucose (G10)	0.5	5	12.3±1.1	86
	1.0	4	9.4±1.2[e]	66
	2.5	4	9.0±1.1[e]	63

a) Data are expressed as means+S.D. *b*) Saline. *c*) 5% EtOH–Saline.
d) $p<0.05$. *e*) $p<0.01$. *f*) $p<0.001$.
* This compound was isolated from rhubarb.[12]

deca-galloylglucoses were somewhat higher than that of previously reported 1,2,3,4,6-pentagalloylglucose.

Further studies on the mechanism of the action of this BUN-decreasing activity are in progress, and the results will be reported elsewhere in the near future.

Experimental

Optical rotations were measured on a Perkin–Elmer model 243 digital polarimeter. ^{13}C-NMR spectra were recorded on a JEOL FX-100 spectrometer at 25.05 MHz in acetone-d_6 with tetramethylsilane as an internal standard. HPLC was performed on a Hitachi model 638 liquid chromatograph equipped with a Hitachi variable-wavelength spectrophotometric detector. For normal-phase HPLC analysis a Nucleosil 50-10 (Macherey–Nagel) column (3 mm i.d. × 300 mm, glass) was used with a solvent system of *n*-hexane–methanol–tetrahydrofuran–formic acid (55 : 33 : 11 : 1) containing oxalic acid (450 mg/l). A Nucleosil $5C_{18}$ (Macherey-Nagel) column (4 mm i.d. × 250 mm) was used for reverse-phase HPLC, and the mobile phase was prepared by dissolving 2.0 g of oxalic acid in 1000 ml of acetonitrile–water (21 : 79). Preparative-scale HPLC was carried out on a machine constructed from a Milton–Rhoy pump, a Pharmacia UV-2 dual-path monitor and a Nucleosil $10C_{18}$ (Macherey–Nagel) column (8 mm i.d. × 250 mm), or on an instrument consisting of a Fluid Metering low pressure pump with a Wakogel LC-ODS 30K (Wako Pure Chemical Co. Ltd.) column (15 mm i.d. × 300 mm).

Isolation of Galloylglucoses——Air-dried Paeoniae Radix (1.8 kg) collected from plants cultivated at the National Institute of Hygienic Sciences, Hokkaido Station of Medicinal Plants, Nayoro, was extracted three times with aqueous acetone at room temperature for 12 h. The combined extracts were concentrated *in vacuo* to *ca.* 1 l, and the aqueous solution was extracted three times with ethyl acetate (500 ml each). The ethyl acetate layer was washed with water saturated with NaCl (500 ml × 2), dried over Na_2SO_4, and evaporated to give the tannin fraction (33.7 g, 1.9%). A part (13.3 g) of this fraction in ethanol was applied to a Sephadex LH-20 column, and fractionated using a solvent system of ethanol–water–acetone as described previously.[8-10] Each fraction was analyzed by normal-phase HPLC. On repeated chromatography on Sephadex LH-20, tetra (G4)-, penta (G5)-, hexa (G6)-, hepta (G7)-, octa (G8)-, nona (G9)-, and decagalloylglucose (G10) fractions were obtained; G4, 35 mg; G5, 290 mg; G6, 542 mg; G7, 640 mg; G8, 464 mg; G9, 210 mg; G10, 73 mg. Hexa- (150 mg), hepta- (250 mg) and octagalloylglucose (150 mg) fractions were separately subjected to preparative reverse-phase HPLC, three isomers (**3**—**5**) of hexagalloylglucose, four isomers (**6**, **7**, **9** and **10**) of heptagalloylglucose and one octagalloylglucose (**11**) were obtained as light brown amorphous powders. The hexagalloylglucoses **3**—**5**, and the heptagalloylglucoses **6** and **7**, were identified as 2-*O*-digalloyl-1,3,4,6-tetra-*O*-galloyl-*β*-D-glucose, 3-*O*-digalloyl-1,2,4,6-tetra-*O*-galloyl-*β*-D-glucose, 6-*O*-digalloyl-1,2,3,4-tetra-*O*-galloyl-*β*-D-glucose, 2,3-bis-*O*-digalloyl-1,4,6-tri-*O*-galloyl-*β*-D-glucose and 3-*O*-trigalloyl-1,2,4,6-tetra-*O*-galloyl-*β*-D-glucose, respectively, by HPLC analysis and by direct comparison of the ^{13}C-NMR spectra with those of samples obtained previously.[9,10] 3,6-Bis-*O*-digalloyl-1,2,4-tri-*O*-galloyl-*β*-D-glucose (**9**), $[\alpha]_D^{20}$ +33.6° (*c* = 0.54, acetone). *Anal.* Calcd for $C_{55}H_{40}O_{34}\cdot 2H_2O$: C, 50.20; H, 3.43. Found: C, 50.20; H, 3.68. 2,6-Bis-*O*-digalloyl-1,3,4-tri-*O*-galloyl-*β*-D-glucose (**10**), $[\alpha]_D^{20}$ +48.6° (*c* = 0.40, acetone). *Anal.* Calcd for $C_{55}H_{40}O_{34}\cdot 2H_2O$: C, 50.20; H, 3.40. Found: C, 52.16; H, 3.40. 2,3,6-Tris-*O*-digalloyl-1,4-di-*O*-galloyl-*β*-D-glucose (**11**), $[\alpha]_D^{20}$ +39.3° (*c* = 0.82, acetone). *Anal.* Calcd for $C_{62}H_{44}O_{38}\cdot H_2O$:C, 52.62; H, 3.28. Found: C, 52.45; H, 3.30.

General Procedure for Methanolysis——A solution of gallotannin (1 mg) in 0.05 M acetate buffer (pH 5.5, 1 ml) and methanol (2 ml) was kept for 24 h at room temperature. The reaction products were analyzed by reverse-phase HPLC, and the ratio of methyl gallate and **2** was determined by measuring the peak heights.

Partial Hydrolysis of 9—11——A solution of each gallotannin in water (1 mg/ml) was heated for 30 min on a boiling water bath. After cooling, the products were examined by reverse-phase HPLC; the results are described in the text.

BUN Assay——1) Animals: Male Wistar rats weighing about 140 g were maintained in an air-conditioned room (25 ± 1 °C, 60% relative humidity) with lighting from 6 a.m. to 6 p.m., and were fed a pellet diet (CLEA Japan Inc., Tokyo) and tap water freely.

2) Preparation of Serum: Each gallotannin in saline or 5% ethanol–saline was administered intraperitoneally to rats at 10 a.m. while control rats were treated with an equal volume of saline or 5% ethanol–saline. Blood samples were collected by decapitation at 6 p.m., 8 h after treatment. The blood was kept in a cold room (4 °C) for several hours, and then serum was separated by centrifugation (1000 × *g*, 10 min, 4 °C). During the experiment, the pellet diet was removed from the rat cage at 9 a.m. but water was given freely.

3) Estimation of BUN Concentration:[13] BUN concentration in serum was estimated using the commercial reagent, BUN "EIKEN" (Eiken Chemical Co., Tokyo), in a DSA-560 discrete sample analyzer (Beckman Instrument Inc., U.S.A.).

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