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FOUR CAFFEOYL GLYCOSIDES FROM CALLUS TISSUE OF *REHMANNIA GLUTINOSA*

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Key Word Index—*Rehmannia glutinosa*; Scrophulariaceae; callus culture; caffeoyl glycosides.

Abstract—From the methanolic extract of callus tissue of *Rehmannia glutinosa* four phenolic glycosides and one aliphatic glycoside were isolated. Two of the phenolic glycosides were identified as acteoside and forsythiaside and the structures of the other two were elucidated as 3,4-dihydroxy-β-phenethyl-*O*-β-D-glucopyranosyl-(1 → 3)-4-*O*-caffeoyl-β-D-glucopyranoside and 3,4-dihydroxy-β-phenethyl-*O*-β-D-glucopyranosyl-(1 → 3)-*O*-α-L-rhamnopyranosyl-(1 → 6)-4-*O*-caffeoyl-β-D-glucopyranoside.

INTRODUCTION

Iridoids [1, 2], sugars [3] and amino acids [4] have been isolated from the Chinese drug plant *Rehmannia glutinosa* Libosch. var. *purpurea* Makino. In continuing studies on *R. glutinosa*, the clonal propagation of tip tissue in tissue culture has been carried out [5]. Furthermore, two phenolic glycosides were isolated from diseased root and shown to be phytoalexins [6].

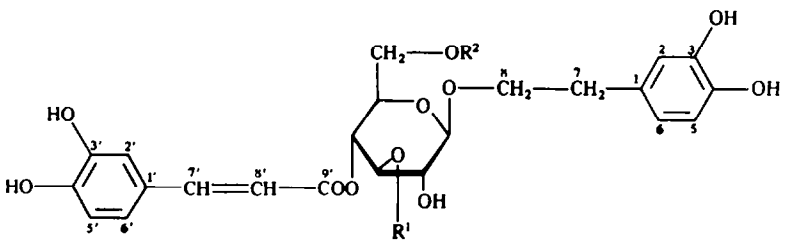
This paper describes the formation of phenolic glycosides in callus culture of *R. glutinosa* and the structure elucidation of two new phenolic glycosides. Their antibiotic activities against various fungi are also discussed.

RESULT AND DISCUSSION

Callus induction from leaf segments was investigated in the media supplemented with combination of 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) under continuous light or dark. The results showed that Murashige–Skoog (MS) medium [7] containing 2,4-D was generally more effective for inducing callus tissue than NAA was. Since the pattern of phenolic glycosides as monitored by TLC was not affected by the environmental conditions, callus tissue was subcultured on the MS media inoculating 2,4-D (1 ppm) under continuous light.

The methanol extract of the cultured tissue was partitioned with organic solvents and then repeatedly chromatographed on Sephadex LH-20 and MCI GEL CHP-20P and compounds **1**–**5** were isolated. Compounds **1** and **2** were identified as acteoside [8, 9] and forsythiaside [10, 11], respectively. Compound **3** was obtained as an amorphous powder, $C_{20}H_{36}O_{16}$, $[\alpha]_D^{19}$ −18°. The FD mass spectra gave the $[M]^+$ at *m/z* 679 $[M+K]^+$ confirming the molecular weight and associated major fragments at *m/z* 663 $[M+Na]^+$, and 502 $[M+Na+H-\text{hexose}]^+$. The UV spectrum of **3** closely resembled those of acteoside and forsythiaside suggesting that **3** contained caffeate and catechol moieties [12]. Acid hydrolysis of **3** with 1 N hydrochloric acid yielded glucose and 3,4-dihydroxyphenethylalcohol. Moreover, alkaline hydrolysis of **3** gave caffeic acid. These components are in good agreement with ^{13}C NMR spectrum (Table 1).

Partial hydrolysis of **3** with 0.05 N hydrochloric acid gave desrhamnosyl acteoside [13] and glucose establishing that **3** may have the same partial structure as acteoside with two glucose moieties in the molecule. Therefore, the linkage of the terminal glucose was determined by the ^{13}C NMR spectrum. Comparison of the



1 R^1 = rhamnosyl, R^2 = H; acteoside
2 R^1 = H, R^2 = rhamnosyl; forsythiaside
3 R^1 = glucosyl, R^2 = H
4 R^1 = glucosyl, R^2 = rhamnosyl

Table 1. ^{13}C NMR chemical shifts (pyridine-d_5) of caffeoyl esters

		Desrhamnosyl acteoside	Forsythiaside	3	4
3,4-Dihydroxy phenethyl moiety	1	130.2	130.3	130.2	130.3
	2	116.4	116.5	116.5	116.5
	3	146.3	146.4	146.3	146.4
	4	145.4	145.4	145.5	145.4
	5	117.3	117.4	117.4	117.4
	6	120.3	120.5	120.4	120.5
	7	35.9	36.1	36.1	36.2
	8	71.2	72.5	71.1	71.3
Caffeate moiety	1′	126.5	126.7	127.0	127.0
	2′	115.5	115.7	115.8	115.8
	3′	146.5	146.9	147.0	147.0
	4′	150.1	150.4	150.4	150.4
	5′	116.5	116.5	116.7	116.5
	6′	122.2	122.1	122.2	122.1
	7′	147.1	149.5	147.5	147.6
	8′	114.5	114.6	115.1	115.1
	9′	167.2	167.0	167.1	166.8
Glucose (inner)	1	104.1	104.5	103.9	104.0
	2	74.9	74.6	76.2	76.2
	3	75.5	75.7	84.8	84.4
	4	72.3	71.4	70.5	70.4
	5	75.9	75.0	76.2	76.2
	6	62.0	67.5	62.0	67.3
Rhamnose	1		102.4		102.5
	2		72.0		72.1
	3		72.4		72.7
	4		73.8		73.9
	5		69.8		69.9
	6		18.5		18.6
Glucose	1			106.6	106.5
	2			74.6	74.5
	3			78.2	78.3
	4			71.4	71.5
	5			78.0	78.1
	6			62.5	62.7

^{13}C NMR spectrum of **3** with that of β-laminaribiose(3-*O*-β-glucopyranosyl-D-glucopyranose) [14], indicated that the ^{13}C resonance of the terminal glucose moiety showed no glycosidation shift. On the other hand, the signal of C-3 of the inner glucose was located at 1.9 ppm higher field relative to the C-3 signal (86.7 ppm) of β-laminaribiose and this was due to the β-effect of the neighboring caffeate group. Finally, the coupling constants of the anomeric proton signals [δ4.22, (*d*, *J* = 8 Hz), 4.3 (*d*, *J* = 8 Hz)] in the ^{1}H NMR spectrum of **3** indicated that two glucose moieties in **3** were β-linked and the same linkage occurred between the aglycone and the glucobiose unit. From these results the structure of **3** was established as 3,4-dihydroxy-β-phenethyl-*O*-β-D-glucopyranosyl-(1 → 3)-4-*O*-caffeoyl-β-D-glucopyranoside.

Compound **4**, $C_{35}H_{46}O_{20}$, $[\alpha]_D^{19} - 14^c$ was an amorphous substance. The FD mass spectrum showed peaks due to $[M + Na]^+$ at *m/z* 809, $[M + Na - 146]^+$ at *m/z* 663 and $[M + Na - \text{hexose}]^+$ at *m/z* 647, suggesting that **4** may be triglycoside of 3,4-dihydroxyphenethylalcohol with rhamnose and a hexose as terminal moieties. This suggestion was confirmed by acid hydrolysis giving glucose, rhamnose and 3,4-dihydroxyphenethylalcohol. The ^{1}H NMR spectrum of **4** was similar to that of **3**, except for a new anomeric proton signal at δ5.02 (*br s*) and methyl proton signals at 1.86 (*d*, *J* = 8 Hz) due to the rhamnose. The ^{13}C NMR spectrum of **4** clearly showed that no low field shifts were observed on the resonances of rhamnose and one of the two glucose moieties, but the resonances of C-3 and C-6 of the inner glucose moiety showed glycosidation shifts. Moreover, the resonances of **4** were similar to the addition of resonance of forsythiaside and those of **3** (Table 1) suggesting that **4** was the 6-rhamnoside of **3**. This was confirmed when partial hydrolysis of **4** with 0.05 N hydrochloric acid gave desrhamnosyl acteoside, **3** and forsythiaside which were identified by TLC and HPLC comparisons with authentic samples. Since the coupling constants of the anomeric proton signals [δ4.40 (*d*, *J* = 7.5 Hz), 5.02 (*br s*)] in the ^{1}H NMR spectrum of **4** exhibited that the two glucoses in **4** were β-linked and rhamnose was α-linked, the structure of **4** could be assigned as 3,4-dihydroxy-β-phenethyl-*O*-β-D-glucopyranosyl-(1 → 3)-*O*-α-L-rhamnopyranosyl-(1 → 6)-4-caffeoyl-β-D-glucopyranoside.

The water soluble fraction of the callus was then acetylated in the usual manner and the product separated

by silica gel column chromatography. After repeated chromatography, **5b** was obtained as an amorphous powder. The EI-MS of **5b** gave the $[M-H]^+$ at m/z 375 confirming the molecular weight. In the ^1H NMR spectrum four acetyl groups and an ethyl group were observed. Alkaline hydrolysis of **5b** with methanolic sodium bicarbonate gave deacetylated compound **5a** which was identified as ethyl-β-D-glucose by means of the ^{13}C NMR spectrum [15].

The antibiotic activity of **3**, desrhamnosyl acteoside [13] and forsythiaside was tested using various bacteria. A significant antibiotic activity of **3** was observed against *Pseudomonas cepacia* and *P. maltophilia* at concentrations of 0.2–0.5 mg/disk. Forsythiaside and desrhamnosyl acteoside also showed antibiotic activity which was somewhat weaker than **3**, in the range of 0.2–0.5 mg/disk (Table 2). This is the first isolation of phenolic glycosides from callus tissue of *R. glutinosa*. Moreover, it is notable that acteoside, a phytoalexin of root of *R. glutinosa*, is formed in the callus tissue of *R. glutinosa* as a major phenolic.

EXPERIMENTAL

^1H NMR spectra were measured at 100 MHz and chemical shifts are given in δ (ppm) with TMS as internal standard. ^{13}C NMR spectra were taken on ppm scale with TMS as internal standard. HPLC analysis was run on a Toyosoda liquid chromatograph HLC-803 D with a Toyosoda absorbance detector UV-8 model 11. A column (4 mm × 300 mm) packed with Nucleosil 5C-18 was operated under the following conditions; column temp: room temp.; solvent: 35% MeCN; flow rate: 0.5 ml/min; detect: 325 nm. Methanolic $FeCl_3$ soln, UV and 10% H_2SO_4 were used for detection. CC was carried out with Sephadex LH-20 (25–100 μm) and MCI GEL CHP-20P. TLC was developed with solvents 1 and 2. Solvent 1: *n*-BuOH–HOAc–H_2O (4:1:5); solvent 2: EtOAc–MeOH–H_2O (7:3:0.2).

Callus culture of R. glutinosa *leaf and isolation of compounds.* Leaf segment cultured *in vitro* of *R. glutinosa* [5] were cultured on MS medium containing 2,4-D or NAA with or without BAP at 25 ± 1° under continuous light for 4 weeks. Subculture of callus was done every 4 weeks under the same conditions with those of callus induction. Accumulated fresh callus tissue (300 g) were homogenized with MeOH and stored overnight. After filtration, the solvent was evaporated and concd. The concd aq. soln was partitioned with Et_2O, EtOAc and *n*-BuOH, successively. The *n*-BuOH extract (1.4 g) was separated by CC on Sephadex LH-20 and MCI GEL CHP-20P using MeOH–H_2O and Me_2CO–H_2O mixtures to give compounds **1** (137.1 mg), **2** (99.5 mg), **3** (32.6 mg) and **4** (57.1 mg).

The aq. layer (3.6 g) described above was passed through Seppak (Waters Co. Ltd) and washed with H_2O and then 50% MeOH. The 50% MeOH eluate was separated by CC on silica gel using $CHCl_3$–MeOH–H_2O (6:4:1) to give Fr. 1–5. Fr. 1 was acetylated with Ac_2O–C_5H_5N and the crude acetate was purified by CC on silica gel eluting C_6H_6–Me_2CO (1:1) to give compound **5b** (13.8 mg). Compound **5b** was refluxed in MeOH containing $NaHCO_3$ to give compound **5a** (6.5 mg).

Compound **1**. An amorphous substance; $[\alpha]_D^{20}$ −6.5° (*c* 1.0; MeOH); FD-MS *m/z*: 647 $[M+Na]^+$, 625 $[M+H]^+$; it was directly identified with authentic acteoside by comparison with ^1H NMR and ^{13}C NMR spectra.

Compound **2**. An amorphous substance; $[\alpha]_D^{19}$ −17.8° (*c* 1.0; MeOH); FD-MS *m/z*: 647 $[M+Na]^+$; UV λ_{max}^{MeOH} nm (log ε): 220 (4.29), 246 (4.00), 290 (4.14), 330 (4.26); IR ν_{max}^{KBr} cm^{-1}: 3380 (OH),

Table 2. Antibacterial activity*

Compound	2	3	desrhamnosyl acteoside	2	3	desrhamnosyl acteoside	2	3	desrhamnosyl acteoside	2	3	desrhamnosyl acteoside
Concentration (mg/disk)		0.5			0.3			0.2			0.1	
Micro-organism												
Pseudomonas cepacia IID1340	273	468	398	174	425	181	74	384	106	33	33	33
P. maltophilia IID1275	65	345	29	20	153	84	9	105	4	4	33	4
Escherichia coli C6005	5	0	0	0	0	0	0	0	0	0	0	0

*Area of inhibition = inhibition area − disk area (mm^2).

1700 (CO), 1628, 1600 (C=C); ^{1}H NMR (DMSO-d_6): δ1.08 (3H, *d*, *J* = 6 Hz, rha H-1), 2.77 (2H, *t*, *J* = 8 Hz, H-7), 3.86 (2H, *t*, *J* = 8 Hz, H-8), 4.32 (1H, *d*, *J* = 7.5 Hz, glc H-1), 4.70 (1H, *br s*, rha H-1), 6.28 (1H, *d*, *J* = 16 Hz, H-8'), 7.49 (1H, *d*, *J* = 16 Hz, H-7'); ^{13}C NMR: see Table 1; identified with forsythiaside by comparison with data in refs [10, 11].

Compound **3**. An amorphous powder; $[\alpha]_D^{19}$ −18.1° (*c* 0.3; MeOH); FD-MS *m/z*: 679 $[M+K]^+$, 663 $[M+Na]^+$. (Found: C, 52.86; H, 5.80. $C_{29}H_{36}O_{16}\cdot H_2O$ requires: C; 52.88; H, 5.82%.) UV λ_{max}^{MeOH} nm (log ε): 218 (4.11), 247 (3.83), 291 (3.94), 333 (4.08); IR ν_{max}^{KBr} cm^{-1}: 3380 (OH), 1690 (CO), 1623, 1605 (C=C); ^{1}H NMR (DMSO-d_6): δ4.22 (1H, *d*, *J* = 8 Hz, glc H-1'), 4.34 (1H, *d*, *J* = 8 Hz, glc H-1), 6.30 (1H, *d*, *J* = 16 Hz, H-8'), 7.50 (1H, *d*, *J* = 16 Hz, H-7'); ^{13}C NMR: see Table 1.

Acid hydrolysis of **3**. Compound **3** (5 mg) was dissolved in 1 N HCl and the mixture was refluxed for 2 hr. The reactant was passed through Amberlite IRA-400. The eluate was conducted on PC using *n*-BuOH–C_5H_5N–H_2O (6:4:3) to detect glucose (R_f 0.37).

Alkaline hydrolysis of **3**. Compound **3** (5 mg) was dissolved in 1 N NaOH and the mixture was heated at 50° under N_2 for 1 hr. The reactant was passed through Amberlite IR-120. The eluate was extracted with Et_2O. The Et_2O extract was subjected to TLC to detect caffeic acid.

Compound **4**. An amorphous powder; $[\alpha]_D^{19}$ −14° (*c* 1.0; MeOH); FD-MS *m/z*: 809 $[M+Na]^+$. (Found: C, 51.80; H, 6.10. $C_{35}H_{46}O_{20}\cdot\frac{1}{2}H_2O$ requires: C, 51.66; H, 6.07%.) UV λ_{max}^{MeOH} nm (log ε): 218 (4.28), 247 (3.94), 290 (4.07), 3.30 (4.20); IR ν_{max}^{KBr} cm^{-1}: 3380 (OH), 1690 (CO), 1625, 1600 (C=C); ^{1}H NMR (DMSO-d_6): δ1.06 (3H, *d*, *J* = 6 Hz, rha H-6), 2.76 (2H, *t*, *J* = 8 Hz, H-7), 3.88 (2H, *t*, *J* = 8 Hz, H-8), 4.40 (1H × 2, *d*, *J* = 7.5 Hz, glc H-1 and H-1'), 4.73 (1H, *t*, *J* = 9.5 Hz, glc H-4), 5.02 (1H, *br s*, rha H-1), 6.28 (1H, *d*, *J* = 16 Hz, H-8'), 7.45 (1H, *d*, *J* = 16 Hz, H-7'); ^{13}C NMR: see Table 1.

Acid hydrolysis of **4**. Compound **4** (5 mg) in 1 N HCl was refluxed for 2 hr. The reactant was passed through Amberlite IRA-400 and the eluate was conducted on PC using *n*-BuOH–C_5H_5N–H_2O (6:4:3) to detect rhamnose (R_f 0.67) and glucose (R_f 0.37).

Alkaline hydrolysis of **4**. Compound **4** (5 mg) in 1 N NaOH was heated at 50° under N_2 for 1 hr. The reactant was passed through Amberlite IR-120 and the eluate was extracted with Et_2O. The Et_2O extract was subjected to TLC to detect caffeic acid.

Partial hydrolysis of **4**. Compound **4** (5 mg) was dissolved in 0.05 N HCl (1 ml) and the mixture was heated at 95° for 1 hr. The reactant was evaporated *in vacuo* and the residue was subjected to TLC and HPLC. Three peaks, 4_a, 4_b and 4_c were identified with authentic compound **3** (R_t 7.5 min), forsythiaside (R_t 8.0 min) and desrhamnosyl acteoside (R_t 10.0 min), respectively.

Compound **5b**. An amorphous powder; $[\alpha]_D^{20}$ +14.8° (*c* 1.1; $CHCl_3$); EI-MS *m/z*: 375 $[M-H]^+$, 331 $[M-Ac-2H]^+$, 303 $[M-Ac-Et-H]^+$; ^{1}H NMR ($CDCl_3$): δ1.18 (3H, *m*, Me), 2.16 (12H, Ac × 4), 3.40–4.00 (3H, *m*, glc H-5, $-OCH_2-$), 4.10 (1H, *dd*, *J* = 12, 2 Hz, glc H-6), 4.30 (1H, *dd*, *J* = 12, 4 Hz, glc H-6), 4.48 (1H, *d*, *J* = 8 Hz, anomeric H), 4.80–5.40 (3H, *m*, glc H-2, 3, 4).

Compound **5a**. An amorphous powder; ^{13}C NMR (CD_3OD): δ15.0 (Me), 58.0 ($-OCH_2$), 61.6 (C-6), 72.5 (C-4), 74.4 (C-2), 75.8 (C-5), 76.9 (C-3), 104.6 (C-1).

Antibiotic assay was investigated by previously reported methods [6] and the results are given in Table 2.

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