Phytochemistry, Vol. 26, No. 4, pp. 983–986, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

PHENOLIC GLYCOSIDES FROM DISEASED ROOTS OF *REHMANNIA GLUTINOSA* VAR. *PURPUREA*

YUKIHIRO SHOYAMA, MASAMI MATSUMOTO and ITSUO NISHIOKA

Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan

(*Revised received* 16 *September* 1986)

Key Word Index—*Rehmannia glutinosa*; Scrophulariaceae; caffeoyl glycoside of 3,4-dihydroxyphenethyl alcohol; stress compound; antimicrobial activity.

Abstract—A new caffeoyl glycoside was isolated from the methanolic extract of the diseased root of *Rehmannia glutinosa* together with acteoside. The structure of the new phenol glycoside was elucidated as 3,4-dihydroxy-β-phenethyl-*O*-α-L-rhamnopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 6)-4-*O*-caffeoyl-β-D-glucopyranoside by spectral and chemical evidence. These compounds were detected as stress compounds in the roots of *Rehmannia glutinosa* following microbial infection. They inhibited the growth of the two *Pseudomonas* species.

INTRODUCTION

As part of a continuing study on medicinal plants in tissue culture, we described the clonal multiplication of *Rehmannia glutinosa* Libosch. var. *purpurea* Makino by shoot tip tissue culture [1]. The root of this Chinese drug plant contains many constituents [2–8]. However, there is still confusion about the relation between these constituents and the pharmacological activities of the drug, apart from the iridoid glycosides [3, 9] and the polysaccharide [10]. During screening experiments on fresh roots, it has been clearly shown that only the diseased roots contain phenolics. On the other hand, HPLC analysis indicates that catalpol, a major iridoid glycoside of fresh roots, disappears in diseased roots [11].

In this paper, we describe the structure determination of a new phenolic compound isolated from the methanolic extract of diseased roots in addition to acteoside and confirm that these are both stress compounds of this plant.

RESULTS AND DISCUSSION

Identification of two phenolics from diseased root of R. glutinosa

The methanolic extracts of diseased roots were partitioned with ethyl ether, ethyl acetate and *n*-butanol, successively. The *n*-butanol soluble fraction contained two major phenolics (R_f 0.76 and 0.29 in solvent 1; see Experimental) which were subjected to column chromatography on Sephadex LH-20 and MCI GEL CHP-20 P with stepwise-increasing concentration of methanol to give **1** and **2**.

1, $C_{29}H_{36}O_{15}$, was an amorphous substance, $[\alpha]_D^{20}$ −73.8°. Acid hydrolysis of **1** yielded glucose, rhamnose and 3,4-dihydroxyphenethyl alcohol. By alkaline hydrolysis caffeic acid was obtained. These components are in good agreement with ^{13}C NMR spectrum of acteoside [12]. Therefore, **1** was identified by the direct comparison with authentic acteoside (FD mass spectrum, ^{1}H NMR).

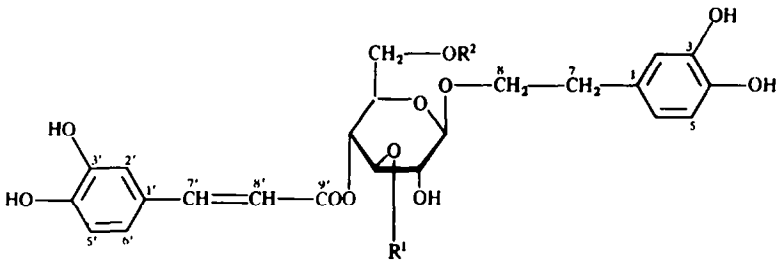
2, $C_{35}H_{46}O_{20}$, $[\alpha]_D^{20}$ −17.4°, was an amorphous substance. Upon acid hydrolysis of **2** with 2 N HCl, galactose, glucose, rhamnose and 3,4-dihydroxyphenethyl alcohol were detected by TLC. Alkaline hydrolysis of **2** afforded caffeic acid and the deacyl derivative. FD mass spectrum gave the M^+ of **2**, *m/z* 809 $[M + Na]^+$ confirming the molecular weight and associated major fragments at *m/z* 663 $[M + Na^+ - \text{rhamnosyl}]$ and 647 $[M + Na^+ - \text{hexose}]$, indicating that **2** is a 3,4-dihydroxyphenethyl alcohol triglycoside and that rhamnose is a terminal moiety.

The ^{13}C NMR data of **2** indicated that the signals of glucose and rhamnose moieties of **2** were almost superimposable with those of acteoside with the exception of a lower shifted signal at 68.9 ppm (*ca* 6.9 ppm) which must be attributed to C-6 of glucose and that no low field shift in rhamnosyl and galactosyl moieties was observed (Table 1). Therefore, it is likely that galactose is linked at C-6 of glucose. The ^{1}H NMR spectrum of **2** closely resembled that of acteoside except that a new anomeric proton signal at 4.26 ppm (*d*, *J* = 9.5 Hz) due to galactose appeared.

Partial hydrolysis of **2** with 0.05 N HCl afforded three hydrolysates, $\mathbf{2}_a$, $\mathbf{2}_b$ and $\mathbf{2}_c$ $\mathbf{2}_a$ and $\mathbf{2}_c$ were directly identified with desrhamnosyl acteoside [13] and acteoside by comparisons with authentic samples (^{1}H NMR and ^{13}C NMR), respectively. These results reveal that **2** has the same partial structure as acteoside.

$\mathbf{2}_b$, $C_{29}H_{36}O_{16}$, was an amorphous substance; the FD mass spectrum gave the M^+ of $\mathbf{2}_b$ *m/z* 663 $[M + Na]^+$ confirming the molecular weight. The ^{13}C NMR spectrum of $\mathbf{2}_b$ resembled that of desrhamnosyl acteoside in the aliphatic region except a low field shift of C-6 of glucose and the addition of galactose signals (Table 1). These findings indicate that the structure of $\mathbf{2}_b$ is 3,4-dihydroxy-β-phenethyl-*O*-D-galactopyranosyl-(1 → 6)-4-*O*-caffeoyl-D-glucopyranoside.

Finally, the coupling constants of the anomeric proton signals (4.26 ppm, *d*, *J* = 7.5 Hz, 4.38 ppm, *d*, *J* = 7.5 Hz and 5.18 ppm, *br s*) in the ^{1}H NMR spectrum of **2** showed that glucose and galactose in **2** were β-linked and the rhamnose was α-linked. Thus **2**



1 R^1 = rha, R^2 = H (acteoside)
2 R^1 = rha, R^2 = gal
2b R^1 = H, R^2 = gal
2c R^1 = H, R^2 = H (desrhamnosylacteoside)

Table 1 ^{13}C NMR chemical shifts

		Desrhamnosyl acteoside*	Acteoside*	2*	2_b†
3,4-Dihydroxyphenetyl moiety	1	130.2	130.2	130.3	131.6
	2	116.4	116.4	116.4	116.4
	3	146.3	146.8	147.0	146.0
	4	145.4	145.3	145.4	144.6
	5	117.3	117.3	117.4	117.2
	6	120.3	120.3	120.5	121.3
	7	35.9	35.9	35.9	36.6
	8	71.2	71.1	71.1	72.4
Caffeate moiety	1′	126.5	126.7	126.8	127.7
	2′	115.5	115.6	115.7	115.2
	3′	146.5	147.4	147.4	146.8
	4′	150.1	151.0	150.8	149.8
	5′	116.5	116.6	116.6	116.5
	6′	122.2	122.2	122.6	123.1
	7′	147.1	148.3	148.6	147.8
	8′	114.5	114.5	114.7	114.7
	9′	167.2	167.0	167.2	168.7
Glucose	1	104.1	104.0	104.0	104.3
	2	74.9	76.0	75.5	75.9
	3	75.5	80.6	80.5	81.8
	4	72.3	70.1	70.1	75.2
	5	75.9	75.6	75.0	76.1
	6	62.0	62.0	68.9	69.3
Rhamnose	1		103.0	103.0	
	2		72.4	72.5	
	3		72.4	72.5	
	4		73.7	73.8	
	5		70.1	70.1	
	6		19.0	19.1	
Galactose	1			105.6	105.3
	2			72.5	72.6
	3			74.5	74.8
	4			70.1	70.3
	5			76.8	76.7
	6			62.1	62.5

*In pyridine-d_5.
†In CD_3OD.

is 3,4-dihydroxy-β-phenethyl-*O*-α-L-rhamnopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 6)-4-*O*-caffeoyl-β-D-glucopyranoside.

Accumulation of **1** *and* **2** *during fungal infection*

Slices of fresh root of *R. glutinosa* in Murashige–Skoog medium [14] supplemented with sucrose were inoculated

separately with *Aspergillus fumigatus*, *Fusarium moniliforme*, *Candida albicans* and *Saccharomyces cerevisiae*. Inoculation with fungi resulted in a general brownish surface appearance of the root slices after 24 hr. **1** and **2** were uniformly absent from the control (H_2O) inoculation. However, the methanol extraction of infected root slices afforded **1** and **2**, identified by TLC and IR in comparison with authentic samples, respectively. This result shows that **1** and **2** are stress compounds of the root of *R. glutinosa*.

Previously, we showed that the callus tissue of *R. glutinosa* produced acteoside and three related glycosides [15]. Moreover, from the finding that catalpol, a major iridoid glycoside, disappeared in the diseased root, it is apparent that the secondary metabolism is altered by infection [11]. Recently, Kitagawa *et al.* pointed out that the crude market drug of *R. glutinosa* contains acteoside and suggested that it was formed during drug processing [9]. These findings strongly support the idea that **1** and **2** are stress compounds of the root.

Antimicrobial activity

It is well known that acteoside and the related phenolic glycosides have antibiotic activity [15, 16]. To investigate the activity of **2** with acteoside and desrhamnosyl acteoside, they were tested against various bacteria and fungi (Table 2). Acteoside was particularly active against *Pseudomonas cepacia* and *P. maltiphilia*; concentrations of 0.3 mg/disc produced clear zones of inhibition. A weak activity was found against *E. coli* and *Saccharomyces cerevisiae*. **2** showed the same activity as **1**.

Since a major disease of *R. glutinosa* roots is due to infection with *Pseudomonas chicolli* [17], it appears that **1** and **2** may be considered as phytoalexins.

EXPERIMENTAL

All mps were uncorr. ^{1}H NMR spectra were measured at 100 MHz and chemical shifts are given on a ppm scale with TMS as internal standard. 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-20 P. TLC was developed with solv. 1 and solv. 2. Solv. 1: *n*-BuOH–HOAc–H_2O (4:1:5), solv. 2: EtOAc–MeOH–H_2O (7:3:0.2).

Isolation of **1** *and* **2**. Dried diseased roots of *R. glutinosa* (515 g) were extracted with MeOH (10 l.) 3 times at room temp. The extracts (139 g) were partitioned between Et_2O, EtOAc and *n*-BuOH, successively. The EtOAc extracts (2.6 g) and the *n*-BuOH extracts (52 g) were repeatedly chromatographed on Sephadex LH-20 using H_2O–MeOH and H_2O–Me_2CO to give **1** (2.29 g). The latter fractions of *n*-BuOH extracts and H_2O soluble layer (100 g) were repeatedly chromatographed on Sephadex LH-20 and MCI gel CHP-20 P using H_2O–MeOH to give **2** (1.23 g).

1; an amorphous powder; mp 128–133°; $[\alpha]_D^{20}$ −73.8° (MeOH, c = 1.0); FD-MS m/z: 647 $[M+Na]^+$, 625 $[M+H]^+$, 479 $[M+H-146]^+$ (found: C, 54.57; H, 6.11; $C_{29}H_{36}O_{15}\cdot H_2O$ requires: C, 54.20; H, 5.96%); UV λ_{max}^{MeOH} nm (log ε): 221 (4.29), 245 (4.02), 290 (4.15), 330 (4.27); IR ν_{max}^{KBr} cm^{-1}: 3380 (OH), 1690 (CO), 1625, 1605 (C=C); ^{1}H NMR (CD_3OD) ppm: 1.01 (3H, *d*, J = 6 Hz), 2.76 (2H, *t*, J = 8 Hz), 3.88 (1H, *d*, J = 8 Hz), 4.36 (1H, *d*, J = 7.5 Hz), 5.20 (1H, *br s*), 6.20 (1H, *d*, J = 16 Hz), 7.60 (1H, *d*, J = 16 Hz).

2; an amorphous powder; $[\alpha]_D^{20}$ −17.4° (MeOH, c = 1.0); FD-MS m/z: 809 $[M+Na]^+$, 663 $[M+Na-146]^+$, 647 $[M+Na-162]^+$ (found: C, 52.33; H, 6.22; $C_{35}H_{46}O_{20}\cdot H_2O$ requires: C, 52.23; H, 6.01%); UV λ_{max}^{MeOH} nm (log ε): 332 (3.90), 290 (3.74), 245 (3.67), 230 (3.72); IR ν_{max}^{KBr} cm^{-1}: 3360 (OH), 1690 (CO), 1625, 1600 (C=C); ^{1}H NMR (CD_3OD) ppm: 1.08 (3H, *d*, J = 6 Hz, rha-6-H), 2.79 (2H, *t*, J = 8 Hz, α-H), 3.88 (1H, *d*, J = 8 Hz, β-H), 4.26 (1H, *d*, J = 7.5 Hz, gal-1-H), 4.38 (1H, *d*, J = 7.5 Hz, glc-1-H), 5.18 (1H, *br s*, rha-1-H), 6.28 (1H, *d*, J = 16 Hz, 8′-H), 7.60 (1H, *d*, J = 16 Hz, 7′-H); ^{13}C NMR see Table 1.

Acid hydrolysis of **2**. **2** (5 mg) was dissolved in 1N HCl. The mixture was refluxed for 2 hr. The reactant was passed through Amberlite IRA-400 and the eluate was conducted on PC in solvent: *n*-BuOH–C_5H_5N–H_2O (6:4:3) to detect glucose (R_f 0.37), rhamnose (R_f 0.67) and galactose (R_f 0.33).

Alkaline hydrolysis of **2**. **2** (5 mg) was dissolved in 1N NaOH. 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 extracts were subjected to TLC to detect caffeic acid.

Partial hydrolysis of **2**. **2** (30 mg) was dissolved in 0.05 N HCl and heated at 95° for 1 hr. After evaporation the residue was chromatographed on Sephadex LH-20 using H_2O–MeOH to give $\mathbf{2}_a$ (4 mg), $\mathbf{2}_b$ (2.4 mg) and $\mathbf{2}_c$ (9.8 mg). $\mathbf{2}_a$ was identified with acteoside by comparison of ^{1}H NMR and ^{13}C NMR with an authentic sample. $\mathbf{2}_b$; an amorphous powder; $[\alpha]_D^{20}$ −11.3° (MeOH, c = 0.3); FD-MS m/z: 663 $[M+Na]^+$; UV λ_{max}^{MeOH} nm (log ε): 218 (4.25), 247 (3.93), 291 (4.13), 330 (4.20); IR ν_{max}^{KBr} cm^{-1}: 3375 (OH), 1690 (CO), 1620, 1610 (C=C); ^{1}H NMR (CD_3OD) ppm: 2.78 (2H, *t*, J = 8 Hz, α-H), 4.24 (1H, *d*, J = 7.5 Hz, gal-1-H), 4.37 (1H, *d*, J = 7.5 Hz, glc-1-H), 6.29 (1H, *d*, J = 16 Hz, 8′-H), 7.60 (1H, *d*, J = 16 Hz, 7′-H). $\mathbf{2}_c$ (desrhamnosyl acteoside); an amorphous powder; $[\alpha]_D^{24}$ −59.1° (MeOH, c = 0.25); FD-MS m/z: 501 $[M+Na]^+$, 478 $[M]^+$, 316 $[M-162]^+$; UV λ_{max}^{MeOH} (log ε): 218 (4.24), 247 (3.96), 290 (4.08), 333 (4.21); IR ν_{max}^{KBr} cm^{-1}: 3380 (OH), 1690 (CO), 1625, 1600 (C=C); ^{1}H NMR (CD_3OD) ppm: 2.80 (2H, *t*, J = 8 Hz, α-H), 4.38 (1H, *d*, J = 7.5 Hz, glc-1-

Table 2. Antibacterial activity of phenolic glycosides*

Compound	1	2	1	2	1	2	1	2	1	2
Concentration (mg/disc)	1		0.5		0.3		0.2		0.1	
Micro-organism										
Pseudomonas cepacia II D 1340	408	—	264	308	151	209	28	26	0	9
P. maltiphilia II D 1275	402	—	233	241	104	153	0	33	0	10
P. aeruginosa II D 1042	28	—	13	0	0	0	0	0	0	0
P. fluorescens II D 5115	11	—	0	0	0	0	0	0	0	0
Escherichia coli C 6005	5	—	3	2	0	2	0	0	0	0
Saccharomyces cerevisiae IAM 4512	151	—	±	0	0	0	0	0	0	0

*Area of inhibition [inhibition area − disc area (mm^2)].

H), 6.29 (1H, *d*, J = 16 Hz, 8'-H), 7.60 (1H, *d*, J = 16 Hz, 7'-H).

Accumulation of **1** *and* **2** *during fungal infection.* Fresh roots (100 g) of *R. glutinosa* were surface sterilized with 2.5% NaOCl for 25 min and then 70% EtOH for 1 min and washed with sterile H_2O twice. After slicing (2 mm in thickness), 1/4 of the slices were incubated with individual fungi as depicted in Table 2 at 25° for 24 hr. The slices were washed with H_2O and then homogenized with MeOH. The MeOH extracts were partitioned with Et_2O and *n*-BuOH. Individual *n*-BuOH extracts were subjected to TLC and then collected and chromatographed on Sephadex LH-20 using H_2O–MeOH and H_2O–Me_2CO to give **1** (17.8 mg) and **2** (5.6 mg) which were identified by TLC and IR with authentic samples.

Antibiotic assay. Escherichia coli C 600S was obtained from Dr. T. Miki, Faculty of Pharmaceutical Sciences of this University. *Streptococcus pyogenes* II D 690, *Streptococcus pyogenes* II D 698, *Staphylococus aureus* II D 671, II D 980, *Pseudomonas aeruginosa* II D 1042, *P. maltophilia* II D 1275, *P. fluorescens* II D 5115, *P. cepacia* II D 1340 and *Bacillus subtilis* II D 864 were obtained from Institute of Medical Science, University of Tokyo, *Aspergillus fumigatus* IAM 3006, *Saccharomyces cerevisiae* IAM 4512 and *Candida albicans* IAM 4966 were obtained from Institute of Applied Microbiology, University of Tokyo. *Fusarium moniliforme* was obtained from the Research Center for Medical Mycology, Faculty of Medicine, Teikyo University. A paper-disc method was carried out following the method of [18] with modification. The antibiotic assay discs (6 mm diameter) were loaded with a prescribed amount of **1** and **2** in absolute EtOH as shown in Table 2. A control disc was treated with EtOH alone in each plate. After evaporation of EtOH completely the discs were arranged in Petri dishes (6 cm diameter) containing broth medium and agar (0.8%) (Nissui Co. Ltd.) seeded with the test bacterium. Individual bacterial strain was cultured at 30° for 24 hr in nutrient broth. A drop of the culture (approximately 10^6 cells) was added to the agar surface and spread out uniformely with a glass rod. Plates were incubated at 30° for 2 days and diameters of inhibition zones were measured. Antibacterial activity was calculated as inhibition area (mm^2) − disc area (mm^2) and shown in Table 2.

Acknowledgements—The authors would like to thank Dr. T. Miki of this University for the provision of *E. coli* and useful discussion. The authors are also grateful to Mr. K. Takemori of this University for valuable suggestions and helpful comments. Thanks are due to Mr. Y. Tanaka, Miss Y. Soeda, Mr. R. Isobe and the members of the Central Analysis Room of this University for ^1H NMR, ^{13}C NMR, FD-MS and microanalysis.

REFERENCES

1. Shoyama, Y., Nagano, M. and Nishioka, I. (1983) *Planta Med.* **48**, 124.
2. Kitagawa, I., Nishimura, T., Furubayashi, A. and Yoshioka, I. (1971) *Yakugaku Zasshi* **91**, 593.
3. Oshio, H. and Inouye, H. (1982) *Phytochemistry* **21**, 133.
4. Tomoda, M., Kato, S. and Onuma, M. (1971) *Chem. Pharm. Bull.* **19**, 1455.
5. Tomoda, M., Tanaka, M. and Kondo, N. (1971) *Chem. Pharm. Bull.* **19**, 2411.
6. Kitagawa, I., Fukuda, Y., Taniyama, T. and Yoshikawa, M. (1986) *Chem. Pharm. Bull.* **34**, 1399.
7. Yoshikawa, M., Fukuda, Y., Taniyama, T. and Kitagawa, I. (1986) *Chem. Pharm. Bull.* **34**, 1403.
8. Yoshikawa, M., Fukuda, Y., Taniyama, T., Cha, B. C. and Kitagawa, I. (1986) *Chem. Pharm. Bull.* **34**, 2294.
9. Yoshikawa, M., Fukuda, Y., Taniyama, T., Cha, B. C., Kitagawa, I., Nakase, K., Kimura, I. and Kimura, M. (1986) The 6th symposium on the Development and Application of Naturally Occurring Drug Materials, Abstracts, p. 31.
10. Endo, K., Matsuura, A., Kanazawa, K., Onosato, A., Konno, C. and Hikino, H. (1984) Abstract Papers of the 104th Annual Meeting of the Pharmaceutical Society of Japan, p. 165.
11. Matsumoto, M., Shoyama, Y., Nishioka, I., and Shimomura, K. (1984) Abstract Papers of the 31st Annual Meeting of the Japan Society of Pharmacognosy, p. 63.
12. Birkofer, L., Kaiser, C. and Thomas, V. Z. (1968) *Z. Naturforsch.* 1051.
13. Shimomura, Y., Sashida, Y., Ogawa, K., Hara, R. and Miyamoto, K. (1982) Abstract Papers of the 29th Annual Meeting of the Japan Society of Pharmacognosy, p. 26.
14. Murashige, T. and Skoog, T. (1962) *Physiol. Plant.* **15**, 473.
15. Shoyama, Y., Matsumoto, M. and Nishioka, I. (1986) *Phytochemistry* **25**, 1633.
16. Nishibe, S., Okabe, K., Tsukamoto, H., Sakushima, A., Hisada, S., Baba, H. and Akisada, T. (1982) *Chem. Pharm. Bull.* **30**, 4548.
17. Nishi, K., Suyama, K. and Enjyoji, K. (1982) Abstract Papers of the 29th Annual Meeting of the Japan Society of Pharmacognosy, p. 49.
18. Gnanamanicham, S. S. and Smith, D. A. (1980) *Phytopathology* **70**, 894.