Phytochemistry, Vol. 26, No. 12, pp. 3225–3227, 1987.
Printed in Great Britain.

0031-9422/87 $3.00+0.00

PHENOLIC GLYCOSIDE COMPOSITION OF LEAVES AND CALLUS CULTURES OF *DIGITALIS PURPUREA*

MASAMI MATSUMOTO, SATOMI KOGA, YUKIHIRO SHOYAMA and ITSUO NISHIOKA

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

(*Revised received* 1 *June* 1987)

Key Word Index—*Digitalis purpurea*; Scrophulariaceae; comparative study; callus culture; phenolic glycoside.

Abstract—Five phenolic glycosides were isolated from the leaves of *Digitalis purpurea*. Four of the glycosides were identified as desrhamnosyl acteoside, forsythiaside, purpureaside A and purpureaside B respectively and the structure of other one was elucidated as 3,4-dihydroxyphenethylalcohol-6-*O*-caffeoyl-β-D-glucoside. Four phenolic glycoside were isolated from the callus tissue of *D. purpurea* and identified as purpureaside A, purpureaside B, acteoside and purpureaside C respectively.

INTRODUCTION

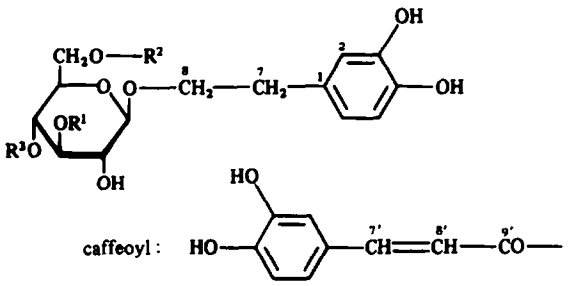
It is well-known that the leaves of *Digitalis spp.* contain cardiac glycosides, flavonoid glycosides [1] and anthraquinones [1, 2]. There are many reports of the use of tissue cultures of *Digitalis spp.* for clonal propagation [3], the preparation of protoplast-derived clones [4], and the production of cardiac glycosides [5] and anthraquinones [6]. In addition the biotransformation of cardiac glycosides and steroids by callus tissue has been investigated by many workers [7, 8]. This paper describes a comparative study of dihydroxyphenethylalcohol glycoside production by leaves and callus tissue of *D. purpurea* L.

RESULTS AND DISCUSSION

Isolation of phenolic glycosides from growing plant leaves

A methanol extract of leaves of *D. purpurea* afforded five phenolic glycosides (**1**–**5**). The FD-mass spectrum of compound **1** showed *m/z* 501 $[M+Na]^+$, 478 $[M]^+$ and 316 $[M-hexose]^+$ suggesting that it was a phenolic glycoside. Its ^1H NMR spectrum established the presence of a caffeoyl moiety and a 3,4-dihydroxyphenethylalcohol moiety and its ^{13}C NMR spectrum clearly showed the presence of a glucose moiety of which the C-6 hydroxyl group was linked with the caffeoyl group (shift of δ2.5 to lower field). From these results, **1** had to be 3,4-dihydroxyphenethylalcohol-6-*O*-caffeoyl-β-D-glucoside. This is the first time that **1** has been isolated from a natural source [9]*.

The ^1H NMR spectrum of compound **2** resembled that of **1** except in the aliphatic region corresponding to the glucose moiety. In the ^{13}C NMR spectrum of **2**, an acylation shift was observed for C-3 of the glucose moiety, confirming that the caffeoyl group was linked to the C-4 position of glucose. Finally, **2** was identified as desrhamnosyl acteoside by direct comparison with an authentic sample of this glycoside [10].



1 R^1 = H, R^2 = caffeoyl, R^3 = H
2 R^1 = H, R^2 = H, R^3 = caffeoyl: desrhamnosyl acteoside
3 R^1 = H, R^2 = rha, R^3 = caffeoyl: forsythiaside
4 R^1 = glc, R^2 = H, R^3 = caffeoyl: purpureaside A
5 R^1 = glc, R^2 = rha, R^3 = caffeoyl: purpureaside B
6 R^1 = rha, R^2 = H, R^3 = caffeoyl: acteoside
7 R^1 = rha, R^2 = gal, R^3 = caffeoyl: purpureaside C

Compound **3** on acid hydrolysis yielded glucose and rhamnose, and on alkaline hydrolysis gave caffeic acid. Its ^{13}C NMR spectrum was in good agreement with that of forsythiaside [11]. Therefore, **3** was identified by direct comparison with authentic forsythiaside (^1H NMR, FD-MS).

The ^{13}C NMR spectrum of compound **4** contained a typical lower field shifted signal for a glucose residue linked to C-3 of an inner glucose unit. The FD-mass spectrum of **4** indicated that it contained two hexose molecules. Finally, **4** was identified by direct comparison with authentic 3,4-dihydroxyphenethylalcohol-3-*O*-β-D-glucopyranosyl-4-*O*-caffeoyl-β-D-glucoside (purpureaside A) [12].

Upon partial hydrolysis of compound **5** with 0.01 M HCl, **2**, **4** and forsythiaside [11] were detected by HPLC. Since it seemed likely that the C-3 and C-6 hydroxyl

*After publication, compound **1** was isolated from *Prunus grayana*: Shimomura, H., Sashida, Y. and Adachi, T. (1987) *Phytochemistry* **26**, 249.

groups on the inner glucose of **5** were linked to glucose and rhamnose, respectively, **5** was identified by direct comparison of its ^{13}CNMR spectrum with that of authentic 3,4-dihydroxyphenethylalcohol-3-*O*-β-D-glucopyranosyl-6-*O*-α-L-rhamnopyranosyl-4-*O*-caffeoyl-β-D-glucoside(purpureaside B) [12].

Formation of phenolic glycosides in tissue culture of D. purpurea

Various organs of *D. purpurea* were investigated for callus induction on media supplemented with different combinations of 2,4-D and BAP. The results showed that the most suitable organs were leaf and stem segments of seedling and that Murashige–Skoog medium [13] containing 1 ppm 2,4-D was the best medium for callus induction. When the callus was cultured in the same medium under continuous irradiation for six weeks, it grew rapidly and became greenish, soft and friable. Therefore, subculture of callus tissue was done in the same medium under the same condition every six weeks.

A methanol extract of the fresh callus tissue was partitioned with organic solvents and then repeatedly chromatographed on Sephadex LH-20 and MCIGEL CHP-20P to give four phenolic glycosides (**4**–**7**).

Compounds **4**–**6** were identified by direct comparisons of their physical and spectral data with those of authentic purpureaside A, purpureaside B and acetoside (verbascoside) [14], respectively.

Compound **7** was obtained as an amorphous powder. Upon acid hydrolysis with 1 M HCl, it give rhamnose, glucose and galactose (detected by TLC). In order to confirm the sugar linkage, **7** was partially hydrolized with 0.05 M HCl to give acetoside and 3,4-dihydroxyphenethylalcohol-6-*O*-β-D-galactopyranosyl-4-*O*-caffeoyl-β-D-glucose [12] which was identified by HPLC. Finally, **7** was identified by comparison of its ^{13}CNMR, ^{1}HNMR and FD-mass spectra with those of 3,4-dihydroxyphenethylalcohol-3-*O*-α-L-rhamnopyranosyl-6-*O*-β-D-galactopyranosyl-4-*O*-caffeoyl-β-D-glucoside(purpureaside C) [12].

This work constitutes the first report of the isolation of 3,4-dihydroxyphenethylalcohol glycosides from the leaves and callus culture of *D. purpurea*. Although the most usual sugar to be linked to the C-3 hydroxyl group of glucose is rhamnose or in one case, xylose (conandroside) [15], the leaves and callus tissues of *D. purpurea* contained 3-β-D-glucopyranosyl glucosides. It seems, therefore, that the 3-β-D-glucopyranosyl glucoside linkage may be a common feature in cell cultures of members of the Scrophulariaceae. The C-4 hydroxyl group of glucose is usually linked to a caffeoyl group. However, that of **1** is free and the caffeoyl group is attached to the C-6 hydroxyl group of glucose. Previously, 6-rhamnopyranosyl(forsythiaside) [11], 6-apiofuranosyl(forsythoside B) [16] and 6-glucopyranosyl(echinacoside) [17] glucosides have been isolated. The isolation of 6-galactopyranosyl glucoside (purpureaside C) provides a novel example.

B. E. Ellis reported on the formation of large amounts of acetoside (verbascoside) in suspension cultures of *Syringa vulgaris*, but the product pattern was simple [18]. By contrast, the callus of *D. purpurea* biosynthesized a range of products which was different from that of leaves. It has been suggested that these derivatives may act as a resistant component or protectant against attack by fungi or viruses [19–21]. In addition, we have isolated these derivatives as stress compounds from diseased root and callus culture of *Rehmannia glutinosa* var. *purpurea* (Scrophulariaceae) [22]. From these results, it seems that these derivatives may play an important part in pathogen resistance in *Digitalis spp.*

EXPEREIMENTAL

Mps: uncorr; ^{1}H NMR: 100 MHz, TMS as internal standard; detection: methanolic $FeCl_3$ soln, UV and 10% H_2SO_4; CC: Sephadex LH-20 and MCI GEL CHP-20P; TLC: Solv. 1: *n*-BuOH–HOAc–H_2O (4:1:5), solv. 2: EtOAc–MeOH–H_2O (7:3:0.2). HPLC: Nucleosil 5C-18 column (4 × 300 mm), column temp = room temp, 35% MeCN (0.5 ml/min), detection at 325 nm.

Isolation of **1**–**5** from D. purpurea *leaves.* Fresh leaves (240 g) were homogenized and extracted (× 3) with MeOH at room temp. After filtration, the solvent was evapd and the concd aq. soln partitioned with Et_2O, EtOAc and *n*-BuOH, successively. The *n*-BuOH extract (2.07 g) was repeatedly chromatographed on Sephadex LH-20 using H_2O–MeOH, H_2O and H_2O–Me_2CO to give **1** (24 mg), **2** (148 mg), **3** (77 mg), **4** (41 mg) and **5** (34 mg).

Compound **1**. Amorphous powder; $[\alpha]_D^{24}$ −32.3° (MeOH; *c* 1.3); FDMS *m/z*: 501, 478, 316; UV λ_{max}^{MeOH} nm (log ε): 215 (4.25), 248 (4.00), 291 (4.13), 332 (4.16); IR ν_{max}^{KBr} cm^{-1}: 3380 (OH), 1690 (CO), 1622, 1605 (C=C); ^{1}H NMR (DMSO-d_6): δ2.70 (2H, *t*, *J* = 8 Hz, 7-H), 4.20 (1H, *d*, *J* = 7.5 Hz, glc-1-H), 6.20 (1H, *d*, *J* = 16 Hz, 8′-H), 7.60 (1H, *d*, *J* = 16 Hz, 7′-H); ^{13}CNMR (DMSO-d_6): δ35.1 (C-7), 63.5 (glc-6), 70.1 (glc-4), 73.2 (glc-2), 73.7 (glc-5), 76.4 (glc-3), 102.9 (glc-1), 113.7 (C-8′), 114.7 (C-2′), 115.4 (C-5), 116.2 (C-2), 119.6 (C-6), 121.3 (C-6′), 125.4 (C-1′), 129.3 (C-1), 143.4 (C-4), 144.9 (C-3), 145.1 (C-3′), 145.5 (C-7′), 148.4 (C-4′), 166.4 (C-9′).

Acid hydrolysis of **1**. Compound **1** (5 mg) in 2M HCl was refluxed for 2 hr. The reaction mixture was passed through Amberlite IRA-400 and the eluate subjected to PC using *n*-BuOH–C_5H_5N–H_2O (6:4:3) to detect glucose (R_f 0.39).

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

Compound **2**. Amorphous substance; $[\alpha]_D^{24}$ −19.8° (MeOH; *c* 1.1); FDMS *m/z*: 501 $[M+Na]^+$, 478 $[M]^+$, 316 $[M-glc]^+$; UV λ_{max}^{MeOH} nm (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 (DMSO-d_6): δ2.78 (2H, *t*, *J* = 8 Hz, 7-H), 4.30 (1H, *d*, *J* = 7.5 Hz, glc-1-H), 6.22 (1H, *d*, *J* = 16 Hz, 8′-H), 7.60 (1H, *d*, *J* = 16 Hz, 7′-H); ^{13}CNMR (C_5H_5N-d_5): δ104.1 (glc-1), 74.9 (glc-2), 75.5 (glc-3), 72.3 (glc-4), 75.9 (glc-5), 62.0 (glc-6).

Compound **3**. Amorphous substance; $[\alpha]_D^{19}$ −17.8° (MeOH; *c* 1.0); UV λ_{max}^{MeOH} nm (log ε): 220 (4.29), 246 (4.00), 290 (4.14), 330 (4.26); ^{1}H NMR (DMSO-d_6): δ1.08 (3H, *d*, *J* = 6 Hz, rha-1-H), 2.77 (2H, *t*, *J* = 8 Hz, 7-H), 3.86 (2H, *t*, *J* = 8 Hz, 8-H), 4.32 (1H, *d*, *J* = 7.5 Hz, glc-1-H), 4.70 (1H, *br s*, rha-1-H), 6.28 (1H, *d*, *J* = 16 Hz, 8′-H), 7.49 (1H, *d*, *J* = 16 Hz, 7′-H); ^{13}CNMR (C_5H_5N-d_5): δ104.5 (glc-1), 75.0 (glc-2), 75.7 (glc-3), 71.4 (glc-4), 74.6 (glc-5), 67.5 (glc-6), 102.4 (rha-1), 72.5 (rha-2), 72.4 (rha-3), 73.8 (rha-4), 69.8 (rha-5), 18.5 (rha-6).

Compound **4**. Amorphous powder; $[\alpha]_D^{19}$ −54.3° (MeOH; *c* 0.8); negative FABMS *m/z*: 639 $[M-H]^-$, 477 $[M-H-glc]^-$; UV λ_{max}^{MeOH} nm (log ε): 218 (4.11), 247 (3.83), 291 (3.94), 333 (4.08); IR λ_{max}^{KBr} cm^{-1}: 3380 (OH), 1690 (CO), 1623, 1605 (C=C); ^{13}CNMR (C_5H_5N-d_5): δ103.9 (glc-1), 76.2 (glc-2), 84.8 (glc-3), 70.5 (glc-4), 74.6 (glc-5), 62.0 (glc-6), 106.6 (glc-1′), 76.2 (glc-2′), 78.0 (glc-3′), 71.4 (glc-4′), 78.2 (glc-5′), 62.5 (glc-6′).

Compound **5**. Amorphous substance; $[\alpha]_D^{27}$ −16.0° (MeOH; *c* 1.0); negative FABMS *m/z*: 785 $[M-H]^-$, 623 $[M-H-glc]^-$; UV λ_{max}^{MeOH} nm (logε): 218 (4.28), 247 (3.94), 290 (4.07), 3.30 (4.20); ^{1}H NMR (DMSO-d_6): δ 1.06 (3H, *d*, *J* = 6 Hz, rha-6-H), 2.76 (2H, *t*, *J* = 8 Hz, 7-H), 3.88 (2H, *t*, *J* = 8 Hz, 8-H), 4.40 (1H × 2, *d*, *J* = 7.5 Hz, glc-1-H and glc-1′-H), 4.73 (1H, *t*, *J* = 9.5 Hz, glc-4-H), 5.02 (1H, *br s*, rha-1-H), 6.28 (1H, *d*, *J* = 16 Hz, 8′-H), 7.45 (1H, *d*, *J* = 16 Hz, 7′-H); ^{13}C NMR (C_5H_5N-d_5): δ 104.0 (glc-1), 76.2 (glc-2), 84.4 (glc-3), 70.4 (glc-4), 74.5 (glc-5), 67.3 (glc-6), 102.5 (rha-1), 72.7 (rha-2), 72.1 (rha-3), 73.9 (rha-4), 69.9 (rha-5), 18.6 (rha-6), 106.5 (glc-1′), 76.2 (glc-2′), 78.3 (glc-3′), 71.5 (glc-4′), 78.1 (glc-5′), 62.7 (glc-6′).

Partial hydrolysis of **5**. Compound **5** (5 mg) was dissolved in 0.05 M HCl (1 ml) and the mixture heated at 95° for 1.5 hr. The reactant was removed *in vacuo* and the residue subjected to TLC and HPLC. Three peaks were identified which run with authentic **2**, **3** and **4**, respectively.

Callus culture of D. purpurea. Leaf and stem segments of seedling (1-week-old) were cultured on MS medium containing 2,4-D (1 ppm) at 25 ± 1° under continuous light for 6 weeks. Subculture of callus was done every 6 weeks under the same conditions as those used for callus induction.

Isolation of **4–7** *from* D. purpurea *callus*. Accumulated fresh callus tissue (1.3 kg) was homogenized with MeOH and stored overnight. After filtration, the solvent was evapd and the concd aq. soln partitioned with Et_2O, EtOAc and *n*-BuOH, successively. The *n*-BuOH extract (6.67 g) was separated by CC on Sephadex LH-20 using MeOH–H_2O and Me_2CO–H_2O, after which MCI GEL CHP-20P using MeOH–H_2O gave **4** (4.9 mg), **5** (11.2 mg), **6** (36.2 mg) and **7** (72.4 mg). Compounds **4–6** were directly identified with authentic purpureaside A, purpureaside B and acetoside (^{1}H NMR and ^{13}C NMR).

Compound **7**. Amorpous powder; $[\alpha]_D^{27}$ −16.3° (MeOH; *c* 1.0); negative FABMS *m/z*: 785 $[M-H]^-$, 623 $[M-H-$galactosyl$]^-$; UV λ_{max}^{MeOH} nm (logε): 230 (3.72), 245 (3.67), 290 (3.74), 332 (3.90); IR ν_{max}^{KBr} cm^{-1}: 3360 (OH), 1690 (CO), 1625, 1600 (C=C); ^{1}H NMR (CD_3OD): δ 1.08 (3H, *d*, *J* = 6 Hz, rha-6-H), 2.79 (2H, *t*, *J* = 8 Hz, C-7-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, C-8′-H), 7.60 (1H, *d*, *J* = 16 Hz, C-7′-H); ^{13}C NMR (C_5H_5N-d_5): δ 19.1 (rha-6), 35.9 (C-7), 62.1 (gal-6), 68.9 (glc-6), 70.0 (glc-4, rha-5), 70.3 (gal-4), 71.3 (C-8), 72.5 (rha-2,3, gal-2), 73.8 (rha-4), 74.5 (gal-3), 75.0 (glc-2), 75.5 (glc-5), 76.8 (gal-5), 80.6 (glc-3), 103.0 (rha-1), 104.0 (glc-1), 105.5 (gal-1), 114.4 (C-2′), 115.8 (C-8′), 116.5 (C-2, C-5′), 117.5 (C-5), 120.5 (C-6), 122.7 (C-6′), 126.8 (C-1′), 130.4 (C-1), 145.4 (C-4), 147.0 (C-3), 147.5 (C-3′), 148.5 (C-7′), 150.7 (C-4′), 167.3 (C-9′).

Acid hydrolysis of **7**. Compound **7** (5 mg) was refluxed in 2M HCl for 2 hr. The reaction mixt. was passed through Amberlite IRA-400 and the eluate subjected to PC using *n*-BuOH–C_5H_5N–H_2O (6:4:3) to detect rhamnose (R_f 0.70), glucose (R_f 0.40) and galactose (R_f 0.35).

Partial hydrolysis of **7**. Compound **7** (5 mg) was dissolved in 0.05 M HCl (1 ml) and the mixture was heated at 95° for 1.5 hr. The reaction mixture was evapd *in vacuo* and the residue was subjected to TLC (solv 1) and HPLC. Three peaks were identified with authentic **2** (R_f 0.92; R_t 10.0 min), acetoside (R_f 0.67; R_t 9.0 min) and 3,4-dihydroxyphenethylalcohol-6-*O*-*β*-D-galactopyranosyl-4-*O*-caffeoyl-*β*-D-glucoside (R_f 0.53) [12], respectively.

Acknowledgements—We wish to thank Mr Y. Tanaka, Miss Y. Soeda, Mr R. Isobe and the members of the Central Analysis Room of this University for ^{1}H NMR, ^{13}C NMR, FDMS and microanalysis.

REFERENCES

1. Paris, R. (1954) *Compt. Rend.* **238**, 932.
2. Paris, R. (1940) *Compt. Rend.* **133**, 46.
3. Erdei, I., Kiss, Z. and Maliga, P. (1981) *Plant Cell Reports* **1**, 34.
4. Diettrich, B., Neumann, D. and Luckner, M. (1980) *Planta Med.* **38**, 275.
5. Kartuig, T., Russheim, U. and Maunz, B. (1976) *Planta Med.* **29**, 275.
6. Furuya, T. and Kojima, H. (1971) *Phytochemistry* **10**, 1.
7. Furuya, T., Kawaguchi, K. and Hirotani, M. (1973) *Phytochemistry* **12**, 1621.
8. Alfermann, A. W., Boy, H. M., Doller, P. C., Hagedorn, W., Heins, M., Wahl, J. and Reinhard, E. (1977) in *Plant Tissue Culture and its Bio-technological Application* (Barz, W., Reinhard, E. and Zenk, M. H., eds) p. 125. Springer, Berlin.
9. Matsumoto, M., Koga, S., Shoyama, Y. and Nishioka, I. (1986) Abstract Papers of the 106th Annual Meeting of the Pharmaceutical Society of Japan, p. 238.
10. 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.
11. Nishibe, S., Okabe, K., Tsukamoto, H., Sakushima, A. and Hisada, S. (1982) *Chem. Pharm. Bull.* **30**, 458.
12. Shoyama, Y., Matsumoto, M. and Nishioka, I. (1986) *Phytochemistry* **5**, 1633.
13. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.
14. Birkofer, L., Kaiser, C. and Thomas, U. (1968) *Z. Naturforsh B 23*, 1059.
15. Nonaka, G. and Nishioka, I. (1977) *Phytochemistry* **16**, 1265.
16. Endo, K., Takahashi, K., Abe, T. and Hikino, H. (1982) *Heterocycles* **19**, 761.
17. Beeker, H. and Hsieh, W. C., Wylde, R., Laffite, C. and Andary, C. (1982) *Z. Naturforsh.* **37C**, 351.
18. Ellis, B. E. (1983) *Phytochemistry* **22**, 1941.
19. Legrand, M., Fritig, B. and Hirth, L. (1976) *Phytochemistry* **15**, 1353.
20. Thiel, K. D., Helbig, B., Klochig, R., Wutzler, P., Sprossig, M. and Schweizer, H. (1981) *Pharmazie* **36**, 50.
21. Pierpoint, W. S., Ireland, R. J. and Carpenter, J. M. (1977) *Phytochemistry* **16**, 29.
22. Shoyama, Y., Matsumoto, M. and Nishioka, I. (1987) *Phytochemistry* **26**, 983.