Pergamon

PII: S0031-9422(96)00443-8

Phytochemistry, Vol. 44, No. 1, pp. 83–87, 1997

0031-9422/97 $17.00 + 0.00

FLAVONOIDS AND PHENYLETHANOIDS FROM HAIRY ROOT CULTURES OF *SCUTELLARIA BAICALENSIS*

YU ZHOU, MASAO HIROTANI,* TAKAFUMI YOSHIKAWA and TSUTOMU FURUYA†

School of Pharmaceutical Sciences, Kitasato University, 9–1 Shirokane 5 Chome, Minato-ku, Tokyo 108, Japan; †Department of Applied Science, Okayama University of Science, 1–1 Ridai-cho, Okayama 700, Japan

(*Received in revised form* 11 *June* 1996)

Key Word Index—*Scutellaria baicalensis*; Labiatae; hairy roots; *Agrobacterium rhizogenes*; polymerase chain reaction; 5,7,2′,6′-tetrahydroxyflavone 2′-*O*-β-D-glucopyranoside; flavonoids; phenylethanoids.

Abstract—We successfully established hairy root cultures of *Scutellaria baicalensis* by direct inoculation on sterile seedlings with *Agrobacterium rhizogenes* pRil5834 harbouring pBI121. Transformation was proved by direct detection of the inserted T-DNA by the polymerase chain reaction. To determine the optimal medium for growth of hairy roots, the effects of five basal media were investigated; growth was best in B5 liquid medium. A new flavone glucoside, 5,7,2′,6′-tetrahydroxyflavone 2′-*O*-β-D-glucopyranoside 15 known flavonoids and five known phenylethanoids were isolated from the hairy root cultures of *S. baicalensis* for the first time. Their structures were elucidated by various spectroscopic methods. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Dried roots of *Scutellaria baicalensis* are a very old and well known drug in traditional Chinese medicine for the treatment of bronchitis, hepatitis, diarrhoea and tumours [1, 2]. Recent papers reported that flavonoids from the roots of this species have an inhibitory effect against human immunodeficiency virus (HIV-1) [3], human T cell leukemia virus type I (HTLV-I) [4] and mouse skin tumour promotion [5]. In this context, the production of bioactive flavonoids by plant tissue and organ cultures is an important and promising aspect of modern biotechnology. Studies on calli of *S. baicalensis* have been reported [6, 7] but an *Agrobacterium* mediated transformant (hairy root) of *S. baicalensis* has not been established hitherto. Hairy root cultures are considered to offer better prospects for the commercial production of secondary metabolites than undifferentiated cell cultures [8–10]. Moreover, there are a few reports that hairy roots have been induced from species of Labiatae [11]; hairy root cultures have been used to produce flavonoids [12].

We report herein the establishment of hairy root cultures of *S. baicalensis* and transformation that was proved by PCR [13]. Growth of *S. baicalensis* hairy root cultures was best in B5 liquid medium. We also report the structure of a new flavone glucoside 5,7,2′,6′-tetrahydroxyflavone 2′-*O*-β-D-glucopyranoside (**1**) determined by UV, ^{1}H and ^{13}C NMR spectral evidence. Fifteen known flavanoids (**2–16**) and five known phenylethanoids (**17–21**) were also isolated.

* Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Five adventitious root clones of *S. baicalensis* were induced by direct inoculation on sterile seedlings. The most actively grown strain, S.b. pBI121, was isolated and used in our experiments. Growth of hairy roots of S.b. pBI121 was investigated in a time-course experiment by culturing five different basal media: Gamborg B5 [14], Murashige and Skoog (MS) [15], 1/2MS [15], Woody plant (WP) [16] and Nitsch and Nitsch (NN) [17] liquid media. Growth was best in B5 liquid medium (Fig. 1). On the other hand, in MS medium, growth was inhibited, even at half-strength. Thus, we used Gamborg B5 medium for our experiments.

In order to prove transformation, direct confirmation of transferred DNA was tried using the polymerase chain reaction (PCR) amplification method using three different parts in T-DNA as the primers. To determine the insertion of TL-DNA or TR-DNA of the Ri plasmid, PCR was performed using primers which can detect either TL-DNA rol A and B [18] or the rol B^{TR} gene [19] and the agropine synthase gene [20]. PCR amplification analysis of DNAs from transformed hairy roots gave the expected 1541 bp, 1672 bp and 816 bp bands with the primer combination rol A-1 and rol B-2 (lane 2), AGS-2 and AGS-1 (lane 5), and TR-1 and TR-2 (lane 8) same bands (lane 1, 4 and 7) that pLJ1 and pLJ85 as positive controls yielded (Fig. 2). PLJ1

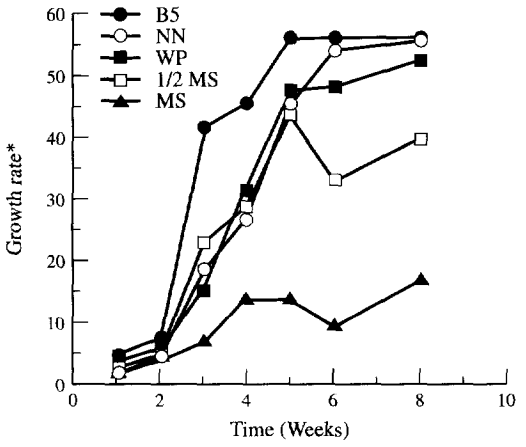


Fig. 1. Growth of *Scutellaria baicalensis* hairy roots (S.b. pBI121 strain) cultured in five basal liquid media. *Expressed as g fresh weight of harvest per inoculum.

and pLJ85 are cosmid clones containing entire TL-DNA and TR-DNA, respectively, of pRiHRI [21]. On the contrary, no visible band was obtained from untransformed callus (lane 3, 6 and 9). These results show that the hairy root strain S.b. pBI121 contained both the TL-DNA and TR-DNA region of the Ri plasmid.

After five weeks culture, S.b. pBI121 strain hairy roots were dried at 60° and extracted with ethanol (3 hr × 5) and then with water (3 hr × 1). The ethanol extract was partitioned into ether–water and *n*-butanol–water solvent systems, successively. By a combination of silica gel column chromatography and HPLC, six flavone glycosides (**1–6**) and five phenylethanoids (**17–21**) were isolated from the *n*-butanol extract and seven flavones (**7–13**) from the ether extract. The water extract was subjected to Diaion HP20 column chromatography and further separation was achieved by HPLC; three flavone glucuronides (**14–16**) were isolated. Among these, 20 were identified as the known compounds, viscidulin III-2′- *O*-β-D-glucoside (**2**) [22], 5,2′,6′-trihydroxy-7,8-dimethoxyflavone 2′-*O*-β-D-glucopyranoside (**3**) [23], 5,2′,6′-trihydroxi-6,7-dimethoxy-flavone 2′-*O*-β-D-glucopyranoside (**4**) [24], 6-*C*-α-L-arabinopyranosyl-8-*C*-β-D-glucopyranosylchrysin (**5**) [25], 6-*C*-β-D-glucopyranosil-8-*C*-α-L-arabinopyranosylchrysin (**6**) [25], chrysin (**7**) [26], baicalein (**8**) [27], wogonin (**9**) [27], skullcapflavone I (**10**) [28], rivularin (**11**) [29], 5,2′-dihydroxy-6,7,8-trimethoxyflavone (**12**) [30], skullcapflavone II (**13**) [31], baicalin (**14**) [27], 5,2′-dihydroxy-7,8,6′trimethoxyflavone 2′-*O*-β-D-glucuronopyranoside (**15**) [32], wogonoside (**16**) [33] martynoside (**17**) [34], leucosceptoside A (**18**) [35], 2-(3-hydroxy-4-methoxyphenylethyl)1-*O*-α-L-rhamnopyranosyl-(1→3)-β-D-(4-*O*-feruloyl)-glucopyranoside (**19**) [36], acteoside (**20**) [37], 4-hydroxy-β-phenylethyl-β-D-glucopyranoside (**21**) [37], by analysis of their mass, ^{1}H and ^{13}C NMR spectral data.

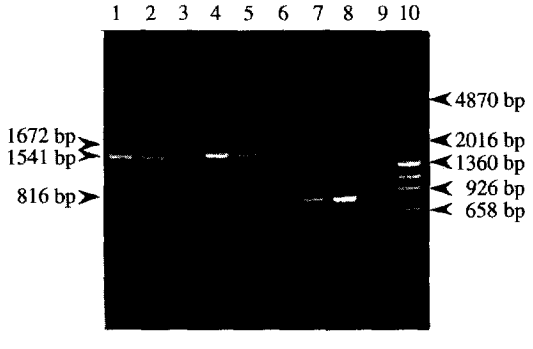


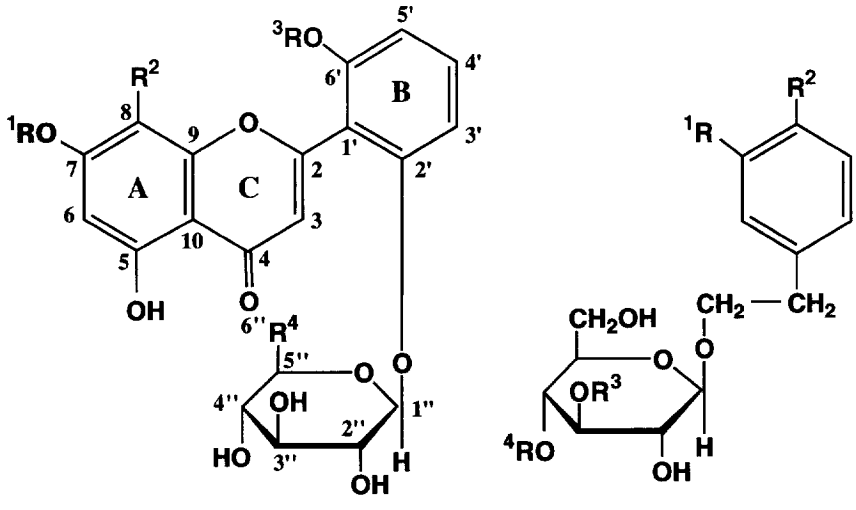
Fig. 2. PCR amplifications from DNA of adventitious root and callus of *Scutellaria baicalensis*. 1, pLJ1 positive control (TL-DNA); 2, S.b. pBI121; 3, S.b. callus; 4, pLJ85 positive control (TR-DNA); 5, S.b. pBI121; 6, S.b. callus; 7, pLJ85 positive control (TR-DNA); 8, S.b. pBI121; 9, S.b. callus; 10, pHY marker DNA.

Compound **1**, obtained as a pale yellow amorphous powder, exhibited a $[M+H]^+$ peak at *m/z* 449.1081 ($C_{21}H_{21}O_{11}$) in its high resolution positive FAB mass spectrum and showed the presence of a hydroxyl group (3440 cm^{-1}) and a conjugated carbonyl group (1680 cm^{-1}) in its IR spectrum. The ^{13}C NMR spectrum showed signals arising from one flavone (15 carbons, C-2 to C-10 and C-1′ to C-6′) and a hexose (δ 60.8, 69.7, 73.3, 76.8, 77.2 and 100.5), whose chemical shift values were in good agreement with those of the β-D-glucose (Table 1). The UV spectrum with shift reagents suggested that **1** was a 5,7-dihydroxyflavone derivative [38]. The ^{1}H NMR spectrum (Tables 2) showed a chelated hydroxyl (δ 12.90), a non-coupled olefinic (δ 6.23), *meta*-coupled aromatic (δ 6.18 and 6.30) and ABC-type aromatic (δ 6.61, 6.70 and 7.25) proton signals. This signal pattern suggested that **1** had a 5(OH),7(OH)2′,6′-substituted flavone structure. This suggestion was supported by data from the HMBC spectrum (Table 3). In the ^{1}H NMR spectrum the coupling constant ($J = 8$ Hz) of the anomeric proton signal at δ 4.87 indicated the β-configuration of the glucopyranoside moiety. The position of attachment of the glucose in **1** was decided by a HMBC experiment. The anomeric proton signal at δ 4.87 (H-1″) showed long-range correlation with the carbon at 156.3 (C-2′), indicating that the glucose moiety is linked to the C-2′ hydroxyl group of B-ring (Table 3). From the combined spectral data, **1** was concluded to be 5,7,2′,6′-tetrahydroxyflavone 2′-*O*-β-D-glucopyranoside.

Of the 21 compounds identified, **1** was isolated from hairy root cultures of *S. baicalensis* for the first time as a new compound. Although compound **15** in *S. rivularis*, compounds **18** and **20** in *S. rivularis* and *S. prostrata*, compound **19** in *Rehmannia glutinosa* and **21** in *Syringa vulgaris* have been reported already [21–36], these compounds were isolated from *S. baicalensis* for the first time.

EXPERIMENTAL

Mps: uncorr. IR: KBr discs. ^{1}H and ^{13}C NMR: 400 and 100 MHz, respectively, in DMSO-d_6. CC: silica gel (C-200).



Flavonoid

	R^1	R^2	R^3	R^4
1*	H	H	H	CH_2OH
15	Me	OMe	Me	CO_2H

*: new compound

Phenylethanoid

	R^1	R^2	R^3	R^4
18	OH	OH	rhamnosyl	feruloyl
19	OH	OMe	rhamnosyl	caffeoyl
20	OH	OH	rhamnosyl	caffeoyl
21	H	OH	H	H

Fig. 3. Structures of compounds isolated from *Scutellaria baicalensis* hairy roots.

Plant material. Sterile plants of *S. baicalensis* Georgi were germinated from seeds surface-sterilized with 10% (w/v) chlorinated lime soln for 20 min followed by washing × 3 with sterile H_2O. Subsequently, they were cultured on MS medium [15] at 25° in the dark.

Bacterial strain. Agrobacterium rhizogenes pRil5834/pBI121 (chimeric CaMV 35S-GUS gene in pBI121 the mini-Ti plasmid containing *gus* and *neo*) [39] was used in this expt. It was grown on YEB-B agar (YEB agar containing 50 mg l^{-1} rifampicin and 25 mg l^{-1} kanamycin) [40], and maintained by subculturing onto the same medium at 24° in the dark.

Induction of hairy roots. Hairy roots of *S. baicalensis* were induced by direct inoculation with *A. rhizogenes* pRil5834/pBI121 on sterile seedlings 3 weeks after germination. The bacteria in the generated adventitious root tips were eliminated on MS agar medium containing 2.5 mg l^{-1} IBA and 80 mg l^{-1} claforan at first, then to 500 mg l^{-1} claforan followed by 5 mg l^{-1} IBA

Table 1. ^{13}C NMR spectral data of compound **1** (100 MHz, δ, DMSO-d_6)

C	**1**
2	161.3
3	112.3
4	181.9
5	161.6
6	98.7
7	164.2
8	94.0
9	158.4
10	104.1
1′	110.3
2′	156.3
3′	105.6
4′	132.2
5′	109.6
6′	156.5
Glucose	
1″	100.5
2″	73.3
3″	76.8
4″	67.7
5″	77.2
6″	60.8

Table 2. ^1H NMR spectral data of compound **1** (400 MHz, δ, DMSO-d_6)

H	**1**	
3	6.23	1H, *s*
6	6.18	1H, *d*(2)
8	6.30	1H, *d*(2)
3′	6.70	1H, *d*(8)
4′	7.25	1H, *dd*(8, 8)
5′	6.61	1H, *d*(8)
Glucose		
1″	4.87	1H, *d*(8)
2″	3.04	1H, *dd*(9, 8)
3″	3.19	1H, *dd*(9, 9)
4″	3.08	1H, *dd*(9, 9)
5″	3.27	*br*
6″	3.40	*br d* (12)
	3.66	*br d* (12)
OH	12.90	1H, *s*
	10.10	1H, *s*

Coupling constants (Hz) in parentheses.

Table 3. $^{1}H-^{13}C$ long-range correlation by HMBC of compound **1**

H	Correlated C
4.87 (H-1″)	156.3 (C-2′)
6.18 (H-6)	161.6 (C-5), 164.2 (C-7), 94.0 (C-8), 104.1 (C-10)
6.23 (H-3)	104.1 (C-10), 181.9 (C-4), 161.3 (C-2), 110.3 (C-1′)
6.30 (H-8)	164.2 (C-7), 98.7 (C-6), 104.1 (C-10), 158.4 (C-9)
6.61 (H-5′)	110.3 (C-1′), 105.6 (C-3′), 156.5 (C-6′)
6.70 (H-3′)	110.3 (C-1′), 156.3 (C-2′), 109.6 (C-5′)
7.25 (H-4′)	156.3 (C-2′), 156.5 (C-6′)
12.90 (HO-C-5)	161.6 (C-5), 98.7 (C-6), 104.1 (C-10)

and 100 mg l^{-1} claforan. Finally, adventitious roots were cultured on YEB agar medium to confirm elimination of bacteria. Five axenic adventitious root clones were isolated. Axenic adventitious roots were subcultured in hormone-free B5 liquid medium [14] every 5 weeks.

Culture conditions. To find the optimal medium for growth, S.b. pBI121 strain hairy roots were cultured in 100 ml flasks containing 50 ml hormone-free B5 [14], MS [15], 1/2MS [15], WP [16] and NN [17] liquid media. Growth of hairy roots was investigated. Mass culture of hairy roots was carried out using 500 ml Erlenmeyer flasks containing 250 ml Gamborg B5 medium containing 3% sucrose. All cultures of this expt were performed on a rotary shaker at 63 r.p.m. at 25° in the dark.

Amplification of transformed DNA by PCR. Hairy root and untransformed callus tissue (50 mg) of *S. baicalensis* were homogenized with 400 μl extraction buffer (20 mM Tris–HCl pH 7.5; 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Solns were centrifuged at 15 000 r.p.m. for 5 min at room temp; 300 μl of *iso*-PrOH was added to the supernatants. The mixed solns were incubated at room temp. for 2 min, then centrifuged at 15 000 r.p.m. for 5 min at room temp. Pellets were washed with 1 ml of 70% EtOH and then dried under vacuum. Dried pellets were resuspended in 100 μl TE buffer (10 mM Tris–HCl, pH 7.5; 0.1 mM EDTA pH 8.0) and 5 μl of the soln was used for 100 μl PCR reaction mixt.

The amplification reaction mixt. contained 10 mM Tris–HCl, pH 8.3 at 25°, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs, 20 pM oligonucleotide primers and 2.5 U Taq polymerase in 100 μl. PCR reactions were performed in a HOEI Thermocycler Model TC-100 using the following profile, 30 cycles of 94° for 1 min, 55° for 2 min and 72° for 3 min, plus an extension step at 72° for 7 min. Amplified DNAs were analyzed by EtBr staining after 1.2% agarose gel electrophoresis.

Six oligonucleotides, rol A-1, TR-1, AGS-2, rol B-2, TR-2 and AGS-1 were designed to identify the insertion of TL-DNA or TR-DNA of the Ri-plasmid into the plant genome by PCR amplification. Rol A-1 (5′-ATGGAATTAGCCGGACTAAACG-3′) or TR-1 (5′-GGCCCAAAAAAACATTCCCACC-3′) and AGS-2 (5′-AATCGTTCAGAGAGCGTCCGAAGTT-3′) correspond to the *N*-terminal-coding region of the TL-DNA rol A gene [18] or the TR-DNA rol B^{TR} gene [19] and the agropine synthase gene [20], respectively. Also, Rol B-2 (5′-ATGGATCCCAAATTGCTATTCC-3′) or TR-2 (5′-CTAAGCGCTCGTCCGTGTCTCC-3′) and AGS-1 (5′-CGGAAATTGTGGCTCGTTG-TGGAC-3′) correspond to the complimentary sequence of the *C*-terminal-coding region of the TL-DNA rol B gene or the rol B^{TR} and the agropine synthase gene, respectively.

Extraction and isolation. After 5 weeks culture, hairy roots were harvested with nylon mesh and dried at 60° for 1 week. Dried hairy roots (1 kg) were refluxed with EtOH (7, 5, 5, 5 and 5 l) × 5 and with H_2O (6 l) once for 3 hr each. The EtOH (207.63 g) and H_2O extracts (217.71 g) were obtained after removal of the EtOH under red. pres. and H_2O by freeze-drying. The EtOH extract (41.86 g) was dissolved in H_2O (900 ml) and shaken successively with Et_2O (900 ml × 5) and *n*-BuOH (900 ml × 5). Et_2OH and *n*-BuOH layers were evapd to dryness. The *n*-BuOH extract (6.17 g) was subjected to CC over silica gel (*ca* 350 g Wako gel C-200) and eluted with a $CHCl_3$–MeOH solvent system to yield 6 frs (A–F). Further purification of frs A–F was achieved by HPLC to give **3** (26.8 mg) R_t 2.8 min and **4** (19.2 mg) R_t 9.0 min from fr. A by HPLC-1, **17** (30 mg) R_t 9.2 min from fr. B by HPLC-2, **1** (5.9 mg), R_t 10 min, **2** (20 mg), R_t 16 min and **21** (22.3 mg), R_t 7 min from fr. C, **18** (63.8 mg), R_t 17.6 min and **19** (4.5 mg), R_t 22.2 min from fr. D, **20** (97.9 mg), R_t 11.8 min from fr. E, **5** (92.2 mg), R_t 14.4 min and **6** (67.4 mg), R_t 18.6 min from fr. F, by HPLC-3. The Et_2O extract (9.22 g) was subjected to CC over silica gel (*ca* 200 g Wako gel C-200) and eluted with *n*-hexane–EtOAc–HOAc (1000:140:1) to give 10 frs (I–X). Further purification of frs VI–VIII was performed by HPLC-4 to give **7** (11 mg), R_t 32 min, **8** (12 mg), R_t 19 min, **9** (175 mg), R_t 28 min, **10** (21 mg), R_t 35 min, **11** (10 mg), R_t 16 min, **12** (11 mg), R_t 42 min, and **13** (55 mg), R_t 20 min. The H_2O extract (70 g) was subjected to Diaion HP20 CC, washed with H_2O and then eluted with MeOH. The MeOH eluate was concd under red. pres. and the MeOH extract purified by HPLC-2 to give **14** (248.6 mg), R_t 15.1 min, **15** (6 mg), R_t 21 min, and **16** (71.8 mg), R_t 25.6 min.

5,7,2′,6′-Tetrahydroxyflavone 2′-O-β-D-glucopyranoside (**1**). Fr. C was purified by HPLC-1 and compound **1** was isolated from the fr. containing the peak at R_t 10 min, yield 5.9 mg. Pale yellow amorphous powder. FAB-MS *m/z*: 449 $[M+H]^+$. HR-FAB-MS *m/z*: $C_{21}H_{21}O_{21}$ (required 449.1084, $[M+H]^+$ at *m/z* 449.1081). IR ν_{max}^{KBr} cm^{-1}: 3440 (OH), 1680 (conjugated CO), 1590 (aromatic ring). UV λ_{max}^{MeOH} nm (log ε): 257 (4.02), 298 (3.75); + $AlCl_3$: 267 (4.03), 312 (3.75); + $AlCl_3$/HCl: 267 (4.03), 312 (3.75); 1 + NaOAc: 263 (4.04), 322 (3.68); + NaOAc/H_3BO_3: 257 (4.02), 298 (3.75). ^{13}C and ^{1}H NMR: Tables 1 and 2.

HPLC. Analysis and isolation of compounds were run on an instrument fitted with a detector and a differential refractometer. A column (19 mm × 150 mm) packed with μ-Bondasphere 5 μ C18-100 Å was used and the solvents were as follows (flow rate 6 ml min^{-1}): HPLC-1: MeOH–H_2O–HOAc (11:10:1), HPLC-2: MeOH–H_2O–HOAc (10:10:1), HPLC-3: MeOH–H_2O–HOAc (15:25:2), HPLC-4: MeOH–H_2O–HOAc (12:7:1).

Acknowledgements—We wish to thank Mr Hekai Rui for providing the seeds of *S. baicalensis* and Prof. Kazuki Saito for providing the bacteria. We are also grateful to the members of the Analytical Centre of this University for NMR and MS.

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