Antioxidative Phenylethanoid and Phenolic Glycosides from *Picrorhiza scrophulariiflora*

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One new phenylenthanoid glycoside, scroside D (2), was isolated from the roots of *Picrorhiza scrophulariiflora* (Scrophulariaceae), together with nine known phenylethanoid and phenolic glycosides: 2-(3,4-dihydroxyphenyl)-ethyl-*O*-*β*-D-glucopyranoside (1), 2-(3-hydroxy-4-methoxyphenyl)-ethyl-*O*-*β*-D-glucopyranosyl (1→3)-*β*-D-glucopyranoside (3), scroside B (4), hemiphroside A (5), plantainoside D (6), scroside A (7), androsin (8), piceoside (9), and 6-*O*-feruloyl-*β*-D-glucopyranoside (10). The structures of these compounds were elucidated using spectroscopic methods. The antioxidative activities of these isolated compounds were evaluated based on their scavenging effects on hydroxyl radicals and superoxide anion radicals, respectively. Compounds 1, 2, and 6 showed potent antioxidative effects as those of ascorbic acid and the structure–activity relationship is discussed.

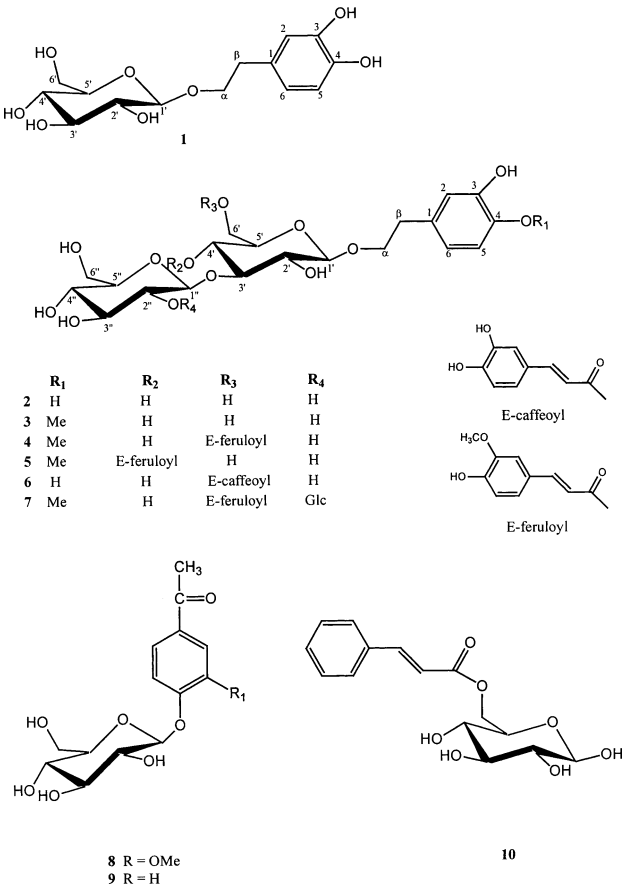
Key words *Picrorhiza scrophulariiflora*; Scrophulariaceae; phenylethanoid glycoside; phenolic glycoside; antioxidative activity

The plant *Picrorhiza scrophulariiflora* PENNELL (Scrophulariaceae) grows in high-altitude regions (above 4400 m) in southeast Tibet and northwest Yunnan province in China. The root of this plant is used in traditional Chinese medicine for the treatment of damp-heat dysentery, jaundice, and steaming of bone.[1] *Picrorhiza kurrooa*, the other unique species of the same genus, is also widely utilized in India for the treatment of jaundice, indigestion, common fever, acute viral hepatitis, and bronchial asthma.[2] The hepatoprotective action of *P. kurrooa* is not fully understood but may be attributed to inhibition of the generation of oxygen anions and scavenging of free radicals.[3] Picroside I and kutkoside, iridoid glycosides obtained from *P. kurrooa*, were reported to be scavengers of superoxide anion radicals.[4] Iridoid glycosides, triterpenoids, phenolic glycosides, and phenylethanoid glycosides have been isolated from the roots of *P. scrophulariiflora*.[5—7]

In our screening program searching for antioxidative natural substances from traditional Chinese medicine, the *n*-BuOH portion from the ethanol extract of *P. scrophulariiflora* showed potent antioxidative activity evaluated based on its scavenging effects on hydroxyl radicals and superoxide anion radicals, with the concentrations exhibiting 50% inhibition (IC_{50}) being 2.82 and 1.98 mg/ml, respectively. Further bioassay-guided analysis led to the isolation of 10 phenylethanoid and phenolic glycosides from the active *n*-BuOH fraction from *P. scrophulariiflora*. In this paper, we describe the isolation and structure elucidation of a new phenylethanoid glycoside, scroside D (**2**), along with nine known glycosides obtained from this plant. The antioxidative activities of these 10 glycosides were evaluated based on their scavenging effects on hydroxyl radicals produced by H_2O_2/Fe^{2+}, and superoxide anion radicals produced by xathine/xanthine oxidase systems.

Results and Discussion

The ethanol extract of the plant was suspended in water and then successively extracted with petroleum ether (60—90 °C), EtOAc, and *n*-BuOH. The *n*-BuOH portion was chromatographed repeatedly on silica gel, C_{18}, and Sephadex LH-20 to afford compounds **1**—**10**. The other nine known glycosides were subsequently identified as 2-(3,4-dihydroxyphenyl)-ethyl-*O*-*β*-D-glucopyranoside (**1**),[8] 2-(3-hydroxy-4-methoxyphenyl)-ethyl-*O*-*β*-D-glucopyranosyl (1→3)-*β*-D-glucopyranoside (**3**),[9] scroside B (**4**),[7] hemiphroside A (**5**),[9] plantainoside D (**6**),[10] scroside A (**7**),[7] androsin (**8**),[6] piceoside (**9**),[11] and 6-*O*-feruloyl-*β*-D-glucopyranoside (**10**),[12] by comparison of spectral data with the data reported in the lit-



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erature. The purity of these compounds was confirmed by TLC and HPLC (purity >90% for all compounds).

Scroside D (**2**) was obtained as white amorphous powder, which darkened on exposure to air, especially in solution. The high resolution electrospray ionization mass spectrometry (HR-ESI-MS) of **2** showed a quasimolecular ion $[M+NH_4]^+$ at *m/z* 496.2006, consistent with the molecular formula of $C_{20}H_{30}O_{13}$. The ^{13}C-NMR spectrum of **1** displayed 20 signals, of which eight were assigned to the aglycone moiety, including one aromatic ring and one hydroxyethyl group; the remaining 12 signals corresponded to two hexose sugar residues. The ^{1}H-NMR spectrum of **1** displayed an ABX system assigned to a 1,3,4-trisubstituted aromatic ring [δ 6.69 (1H, d, J=2.0 Hz, H-2), 6.66 (1H, d, J=8.0 Hz, H-5), 6.55 (1H, dd, J=2.0, 8.0 Hz, H-6)], an A_2B_2 system assigned to a hydroxyethyl group [δ 3.86 (1H, m, H-α_a), 4.02 (1H, m, H-α_b), 2.78 (2H, m, H_2-β)], and two anomeric signals [δ 4.34 (1H, d, J=7.9 Hz, H-1′ of inner Glc), 4.54 (1H, d, J=7.8 Hz, H-1″ of terminal Glc)]. These data suggest that **2** should be a phenylethanoid glycoside. Acid hydrolysis of **2** afforded only D-glucose, compound **1**, and 3,4-dihydroxyphenylethyl alcohol, which were identified on TLC with authentic samples. The sugar was identified by direct comparison with authentic sample using HPLC and optical rotation measurement. Furthermore, the NMR data of **2** led to the assumption that the sugar portion of the molecule was composed of two β-D-glucose residues. Compared with the ^{13}C-NMR data of **1**, C-3′ of the inner glucose of **2** was shifted downfield by +10.2 ppm, and C-2′ and C-4′ were each shifted upfield by −0.7 and −1.6 ppm, respectively, suggesting that the terminal glucose residue is glycosylated at the C-3′ position of the inner glucose. Furthermore, the HMBC spectrum of **2** supported the above observation (Fig. 1). Hence compound **1** was established to be 2-(3,4-dihydroxyphenyl)-ethyl-*O*-β-D-glucopyranosyl (1→3)-β-D-glycopyranoside.

The antioxidative activities of compounds **1**—**10** were estimated based on their scavenging effects on hydroxyl radicals produced by H_2O_2/Fe^{2+} and superoxide anion radicals produced by xathine/xanthine oxidase systems. The results of these assays are given in Table 1. In the scavenging assay of hydroxyl radicals, compounds **1**, **2**, and **6** showed potent scavenging effects with IC_{50} values of 55.9, 48.7, and 39.3 μM, respectively, comparable with that of the well-known antioxidant ascorbic acid with an IC_{50} value of 51.8 μM. In the scavenging assay of superoxide anion radicals, compounds **1**, **2**, and **6** also exhibited significant scavenging effects with IC_{50} values of 86.5, 84.5, and 74.8 μM, comparable with that of ascorbic acid with an IC_{50} value of 86.2 μM. Phenolic glycosides **8**, **9**, and **10**, which have no free phenolic group in their structure, exhibited no or little scavenging effects on either hydroxyl radicals or superoxide anion radicals. The above experimental results suggest that the free phenolic group, especially the catechol group, in molecular structures plays a key role in their antioxidative effects. The number of sugar chains or the E-feruloyl group substituted at the C-4′ or 6′ position of glucose might have no obvious influence on their antioxidative activities. These results also suggest that the antioxidative activities of *P. scrophulariiflora* are partly attributed to these phenylethanoid glycosides.

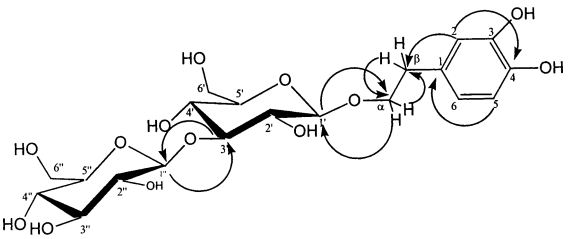

Fig. 1. The Key HMBC Correlations of **2**

Table 1. Hydroxyl Radical and Superoxide Anion Radical Scavenging Activities of Compounds **1**—**10**

Compound	IC_{50} (μM)	
	Hydroxyl radical	Superoxide anion
1	55.9	86.5
2	48.7	84.5
3	112.5	195.0
4	94.9	233.0
5	110.5	208.5
6	39.3	74.8
7	98.0	167.7
8	>400	>400
9	>400	>400
10	>400	>400
Ascorbic acid	51.8	86.2

Experimental

General Melting points were determined on an X-4 melting point apparatus (Beijing, China) and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Nicolet Impact 410 FT-IR instrument. UV spectra were recorded using a Shimadzu UV-2501 spectrophotometer. NMR spectra were obtained on a Bruker AV 500 Avance spectrometer with CD_3OD as solvent. ESI-MS was performed on a HP 1100 HPLC/EST instrument. HR-ESI-MS was obtained on an Applied Biosystems Mariner 5140 spectrometer. Column chromatography was carried out using octodecyl silica gel (ODS) (C_{18}, 40—63 μm, Merck), with Sephadex LH-20 (Pharmacia) as a stationary phase. TLC was performed on precoated silica gel 60 F_{254} or RP-18 F_{254} plates (Merck) and visualized under UV light and by spraying with vanillin reagent followed by heating. HPLC was performed on a Waters 600E pump equipped with a refractive index detector.

Plant Material The roots of *P. scrophulariiflora* were collected in Zhong-Dian County (altitude 4400 m), Yunnan province, People's Republic of China, in October 1999, and authenticated by Dr. Ming-Jian Qin of the China Pharmaceutical University. A voucher specimen (no. 991006) was deposited in the herbarium of China Pharmaceutical University, Nanjing.

Extraction and Isolation The air-dried and powdered roots of *P. scrophulariiflora* (1.0 kg) were extracted with 95% EtOH (3×3 l) under reflux. The EtOH extract was suspended in water and then successively extracted with petroleum ether (60—90 °C), EtOAc, and *n*-BuOH. The *n*-BuOH extract was subjected to silica gel (200—300 mesh) column chromatography using $CHCl_3$–CH_3OH (90 : 10→60 : 40) as eluents to yield five fractions, based on silica gel TLC ($CHCl_3$–CH_3OH–H_2O, 6.5 : 3.5 : 1, lower layer). Each fraction containing phenylethanoid and phenol glycosides was further chromatographed over silica gel eluted with $CHCl_3$–MeOH (90 : 10 or 80 : 20), Sephadex LH-20 eluted with MeOH–H_2O (60 : 40→100 : 0), and C_{18} low-pressure column eluted with MeOH–H_2O, respectively, to afford glycosides **1** (60 mg), **2** (200 mg), **3** (120 mg), **4** (25 mg), **5** (25 mg), **6** (25 mg), **7** (60 mg), **8** (200 mg), **9** (40 mg), and **10** (50 mg).

Acid Hydrolysis of 2 A solution of compound **2** (25 mg) in 2 M HCl–MeOH (1 : 1, 5 ml) was refluxed at 90 °C for 2 h. After being diluted with H_2O and neutralized with Ag_2CO_3, the solution was extracted with EtOAc. The EtOAc layer was evaporated and chromatographed on a silica gel column eluted with $CHCl_3$–MeOH (88 : 12) to yield the aglycone 3,4-dihydroxyphenylethyl alcohol, and compound **1**, which were identified by TLC in comparison with authentic samples. 3,4-Dihydroxyphenylethyl alco-

hol: *Rf* 0.84; **1**: *Rf* 0.25, lower layer of $CHCl_3$–MeOH–H_2O (6.5 : 3.5 : 1). The H_2O layer was concentrated and passed through an Alltech C_{18} SPE cartridge and then separated repeatedly by HPLC [LichroCART NH_2 column (5 μm, 4.6×250 mm); mobile phase, MeCN–H_2O (75 : 25); flow rate, 1.0 ml/min; detection, refractive index (RI)] to afford D-glucose (7.5 mg, t_R, 9.5 min; $[\alpha]_D^{20}$ +52.2°).[13]

Scroside D (**2**): White amorphous powder, mp 240—242 °C (MeOH). $[\alpha]_D^{20}$: −42.6° (c=0.8, MeOH). UV λ_{max} (MeOH) nm (log ε): 204 (3.41), 282 (3.12). IR (KBr) cm^{-1}: 3422, 1610, 1619, 1523, 1445, 1383, 1283, 1156, 1115, 1077. ESI-MS *m/z*: 479 $[M+H]^+$ ($C_{20}H_{30}O_{13}$). HR-ESI-MS *m/z*: 496.2006 (Calcd for $[C_{20}H_{30}O_{13}+NH_4]^+$, 496.2025). Elemental analysis C: 49.88%, H: 6.28% (Calcd for $C_{20}H_{30}O_{13}$). ^{1}H-NMR (500 MHz, CD_3OD): δ: aglycone 6.69 (1H, d, J=2.0 Hz, H-2), 6.66 (1H, d, J=8.0 Hz, H-5), 6.55 (1H, dd, J=2.0, 8.0 Hz, H-6), 4.02 (1H, m, H-α_a), 3.70 (1H, m, H-α_b), 2.77 (2H, m, H_2-β); inner glucose 4.34 (1H, d, J=7.9 Hz, H-1′), 3.36 (1H, t, J=8.7 Hz, H-2′), 3.53 (1H, t, J=8.9 Hz, H-3′), 3.41 (1H, t, J=8.7 Hz, H-4′), 3.87 (1H, dd, J=4.2, 11.9 Hz, H-6′a), 3.63 (1H, dd, J=3.5, 12.0 Hz, H-6′b); terminal glucose 4.54 (1H, d, J=7.8 Hz, H-1″), 3.88 (1H, dd, J=4.2, 12.0 Hz, H-6″a), 3.70 (1H, dd, J=3.5, 12.0 Hz, H-6″b); ^{13}C-NMR (125 MHz, CD_3OD): δ: aglycone 131.5 (C-1), 116.3 (C-2), 146.1 (C-3), 144.7 (C-4), 117.1 (C-5), 121.3 (C-6), 72.1 (C-α), 36.6 (C-β); inner glucose 103.9 (C-1′), 74.5 (C-2′), 88.2 (C-3′), 70.1 (C-4′), 77.6 (C-5′), 62.7 (C-6′); terminal glucose 105.3 (C-1″), 75.5 (C-2″), 77.9 (C-3″), 71.6 (C-4″), 78.2 (C-5″), 62.7 (C-6″).

Hydroxyl Radical Scavenging Assay The hydroxyl radical scavenging assay was carried out by measuring the clearance of test compound for hydroxyl radicals generated from the Fe^{2+}/H_2O_2 system. 1,10-Phenanthroline (0.75 mM) and $FeSO_4$ (0.75 mM), both dissolved in phosphate-buffered saline (pH 7.4), were mixed thoroughly. Then distilled water, 0.1% H_2O_2, and various concentrations of samples were added. After coincubation at 37 °C for 1 h, the absorption value at 536 nm was measured. Ascorbic acid was used as a positive control. Reactions were carried out in triplicate. The scavenging rate of hydroxyl radicals was calculated according to the following equation: scavenging rate (%)=$(A_{ctl}-A_{sample})/(A_{ctl}-A_0)\times100$, where A_{sample} is the absorbance of the sample containing test compounds, A_{ctl} is the absorbance of the control, and A_0 is the absorbance of blank sample containing all reagents except the text compound.[14]

Superoxide Anion Radical Scavenging Assay The scavenging assay of superoxide anion radicals was performed according to the instructions in the commercial test kit purchased from Nanjing Jiancheng Bioengineering Company (Nanjing, China). In brief, the xanthine–xanthine oxidase system can induce the generation of superoxide anion radicals, which oxidize hydroxylamine to form nitrite. Nitrite was mixed with Griess reagent for 10 min at room temperature. Absorbance was determined at 540 nm after chromophores formed. The A_{540} value decreases if the sample has a direct scavenging effect on superoxide anion radicals. The scavenging rate of superoxide anion radicals was calculated according to the following equation: scavenging rate (%)=$(A_{ctl}-A_{sample})/A_{ctl}\times100$.[15]

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