



# A stilbene and dihydrochalcones with radical scavenging activities from *Loiseleuria procumbens*

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## Abstract

A new dihydrochalcone, 6''-acetylphloridzosid, was isolated from the whole plant of *Loiseleuria procumbens* (L.) Desv. and identified as 2'-O-(6''-O-acetylglucopyranosyl)-4,4',6'-trihydroxydihydrochalcone by spectroscopic methods. In addition, one stilbene and three other dihydrochalcones were identified as (*E*)-piceid, phloretin (2',4,4',6'-tetrahydroxydihydrochalcone), phloridzosid (2'-O-glucopyranosyl-4,4',6'-trihydroxydihydrochalcone) and asebotin (2'-O-glucopyranosyl-4'-methoxy-4,6'-dihydroxydihydrochalcone), respectively. Some of these compounds showed scavenging properties towards the 2,2-diphenyl-1-picrylhydrazyl radical and antioxidant properties in a test with lysozyme. © 2000 Elsevier Science Ltd. All rights reserved.

**Keywords:** *Loiseleuria procumbens*; Ericaceae; Radical scavenging activity; Stilbene; Dihydrochalcone; *E*-piceid; Phloretol; Phloridzosid; Asebotin; 6''-Acetylphloridzosid

## 1. Introduction

In the course of our search for new antioxidants from higher plants, particular attention has been given to plants growing at high altitude (>2000 m). Such species have to face more intense UV radiation and may respond to the resulting oxidative stress by producing a greater diversity of antioxidant molecules. We detected in the methanolic whole plant extract of *Loiseleuria procumbens* (L.) Desv. (Ericaceae) compounds which reduced the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical in a TLC autographic assay (Cuendet et al., 1997; Takao et al., 1994). Analysis by HPLC-UV and TLC (Godin reagent) (Godin, 1954) showed the presence of dihydrochalcones or flavanones.

*L. procumbens* is a small bush growing in the subalpine to alpine regions of Europe and North America. Previous work on the plant had led to the isolation of

phloretic acid, phloridzosid, phloretol and (hydroxy-4-phenyl)-3-propionic acid (Serve et al., 1985, 1986).

## 2. Results and discussion

The methanolic extract of the whole plant was fractionated by a combination of CC on silicagel, medium-pressure liquid chromatography (MPLC) and gel filtration on Sephadex LH-20 to provide compounds 1–5 (see Section 3).

Compound 1 was identified as (*E*)-piceid (3-*O*-glucopyranosyl-5,4'-dihydroxystilbene) by comparison of its spectroscopic data (<sup>1</sup>H- and <sup>13</sup>C-NMR, UV, FAB-MS) with literature values (Waffo Teguo et al., 1996).

The reaction of compounds 2–5 with Godin reagent gave an orange color. The <sup>1</sup>H NMR spectra of these compounds revealed signals for two CH<sub>2</sub> groups, typical for a dihydrochalcone. By reference to the literature (Hufford and Oguntimein, 1980), it was possible to identify compounds 2–4 as phloretin, phloridzosid and asebotin, respectively.

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Compound **5**, named 6''-acetylphloridzoid, was obtained as a white microcrystalline powder. In its positive ion D/CI mass spectrum, a peak for the ammonium adduct  $[M + NH_4]^+$  was observed at  $m/z$  496. The pseudomolecular ion signal  $[M + H]^+$  was at  $m/z$  479. Other fragments at  $m/z$  292  $[(M + NH_4) - 204]^+$  and 275  $[(M + H) - 204]^+$  were attributed to the aglycone. The difference of 204 amu could be due to an acetylated hexosyl group (162 amu + 42 amu). The molecular formula  $C_{23}H_{26}O_{11}$  was deduced from the combined D/CI-MS,  $^{13}C$  and DEPT NMR data. The dihydrochalcone skeleton was confirmed by the signals for the H- $\beta$  protons in the  $^1H$  NMR spectrum ( $\delta$  2.88, 2H,  $t$ ,  $J = 7.8$  Hz) and the C- $\alpha$  and C- $\beta$  carbons in the  $^{13}C$  NMR spectrum ( $\delta$  46.83 and 30.83). All signals obtained for compound **5** were identical to those of **3**, except for the downfield shift of H-6'' and the upfield shift of C-5'' in the  $^1H$  and  $^{13}C$  NMR spectra. These data showed an esterification on the hydroxyl group at C-6''. Two supplementary signals appeared in the  $^{13}C$  NMR spectrum at  $\delta$  172.80 and 20.64, corresponding to a carbonyl- and a  $CH_3$ -group, respectively; in addition, a singlet at  $\delta$  2.03 corresponding to an acetyl group appeared in the  $^1H$  NMR spectrum. This signal showed the presence of an acetyl group at C-6''.

Final proof of structure **5** was obtained by alkaline hydrolysis which afforded phloridzoid. With the use of HPLC and TLC co-chromatography, this derivative was found to be compound **3**. Thus, compound **5** is 6''-acetylphloridzoid.

Since the methanolic extract of *L. procumbens* showed activity in the DPPH TLC assay, the radical scavenging properties of compounds **1–5** were evaluated against this radical in a spectrometric assay (Lamaison et al., 1991). Quercetin and BHT (2,6-di(*tert*-butyl)-4-methylphenol) were used as reference compounds. The activities of **1** and **2** were comparable to that of BHT, while the other compounds did not significantly reduce the free radical (Table 1).

The antioxidant activities of compounds **1–5** were

Table 1

Percentage of DPPH<sup>a</sup> radical reduction by compounds **1** and **2** at different concentrations. (measurement at 517 nm, determination after 30 min)

Concentration ( $\mu M$ )	Quercetin	BHT <sup>b</sup>	<b>1</b>	<b>2</b>
80.0	96.6	41.3	54.2	33.9
40.0	95.8	29.5	33.7	26.0
20.0	84.3	14.9	17.9	18.8
10.0	43.8	8.1	9.5	12.4
5.0	21.8	3.3	4.7	7.6
2.5	11.0	3.3	2.4	4.4

<sup>a</sup> DPPH = 2,2-diphenyl-1-picrylhydrazyl.

<sup>b</sup> BHT = 2,6-di(*tert*-butyl)-4-methylphenol.

also evaluated in a test with lysozyme by a capillary electrophoretic method (Salvi et al., 1999). Trolox (6-hydroxy-2,5,7,8-tetraethylchroman-2-carboxylic acid) and chlorogenic acid were used as reference compounds. Compound **1** presented the highest activity, but remained less active than trolox and chlorogenic acid. Compounds **4** and **5** showed modest activity and **2** and **3** had a pro-oxidant effect (Table 2).

### 3. Experimental

#### 3.1. General

TLC: Silica gel 60 F<sub>254</sub> sheets (Merck);  $CHCl_3$ –MeOH–H<sub>2</sub>O (65:35:5). Open column chromatography (CC): Sephadex LH-20 (Pharmacia) and silica gel (63–200  $\mu m$ , Merck). Medium-pressure liquid chromatography (MPLC): home-packed LiChroprep-RP-18 column (15–25  $\mu m$ , 460  $\times$  36 mm i.d., Merck). Anal. HPLC: Hewlett Packard 1050 instrument equipped with a photodiode array detector; Nucleosil RP-18 column (7  $\mu m$ ; 250  $\times$  4 mm i.d., Macherey-Nagel); MeCN–H<sub>2</sub>O (5:95  $\rightarrow$  50:50 in 30 min, 0.05%  $CF_3COOH$ , 1 ml/min). UV: Shimadzu UV-160A and Perkin-Elmer Lambda-3 spectrophotometers. Mp: Mettler FP-80/82 hot-stage apparatus; uncorrected.  $[\alpha]_D^{20}$ : Perkin-Elmer 241 polarimeter.  $^1H$  and  $^{13}C$  NMR: Varian Inova 500 spectrometer;  $\delta$  in ppm rel. to TMS;  $J$  in Hz; carbon multiplicities from DEPT experiments. D/CI-MS: Finnigan-MAT TSQ-700 triple-stage quadrupole instrument. High-performance capillary electrophoresis: Beckman P/ACE Capillary Electrophoresis. Capillary: 50  $\mu m$  i.d. fused silica capillary, 47 cm in length (40 cm effective length up to the detector). Prior to filling a new capillary with running buffer, it was hydrated by high pressure rinse (20 psi), a 10 min rinse with 1 M HCl was followed by a 3 min

Table 2

Antioxidant activity of compounds **1–5** against lysozyme studied by capillary electrophoresis

Compounds	IC <sub>50</sub> ( $\mu M$ )
<b>1</b>	60 $\pm$ 14.0
<b>2</b>	Pro-oxidant effect
<b>3</b>	22 $\pm$ 5.2 <sup>a</sup>
<b>4</b>	190 $\pm$ 24.0
<b>5</b>	100 $\pm$ 17.0
Trolox <sup>b</sup>	31 $\pm$ 8.7
Chlorogenic acid	10 $\pm$ 2.3

<sup>a</sup> Extrapolated value from measures of 1–50  $\mu M$  solutions, because of the pro-oxidant effect observed at 100  $\mu M$ .

<sup>b</sup> Trolox = 6-hydroxy-2,5,7,8-tetraethylchroman-2-carboxylic acid. IC<sub>50</sub> represents the concentration inhibiting oxidation by 50%. Values are expressed as mean  $\pm$  SD for duplicate experiments.

Table 3

<sup>1</sup>H NMR spectral data of compounds **3** and **5** (measured in CD<sub>3</sub>OD)<sup>a</sup>

Assignment	<b>3</b>	<b>5</b>
H-2	7.06 (1H, <i>d</i> , <i>J</i> = 8.3)	7.07 (1H, <i>d</i> , <i>J</i> = 8.3)
H-3	6.69 (1H, <i>d</i> , <i>J</i> = 8.3)	6.70 (1H, <i>d</i> , <i>J</i> = 8.8)
H-5	6.69 (1H, <i>d</i> , <i>J</i> = 8.3)	6.70 (1H, <i>d</i> , <i>J</i> = 8.8)
H-6	7.06 (1H, <i>d</i> , <i>J</i> = 8.3)	7.07 (1H, <i>d</i> , <i>J</i> = 8.3)
H- $\alpha$		
H- $\beta$	2.87 (2H, <i>t</i> , <i>J</i> = 7.6)	2.88 (2H, <i>t</i> , <i>J</i> = 7.8)
H-3'	6.18 (1H, <i>d</i> , <i>J</i> = 2.4)	6.15 (1H, <i>d</i> , <i>J</i> = 2.0)
H-5'	5.97 (1H, <i>d</i> , <i>J</i> = 2.4)	6.00 (1H, <i>d</i> , <i>J</i> = 2.0)
H-1''	5.05 (1H, <i>d</i> , <i>J</i> = 7.3)	5.05 (1H, <i>d</i> , <i>J</i> = 7.3)
H-2''		
H-3''		
	3.35–3.52 (5H, unresolved, H-2''–H-5'' and H- $\alpha$ )	3.37–3.50 (4H, unresolved, H-2''–H-4'' and H- $\alpha$ )
H-4''		
H-5''		
Ha-6''	3.91 (1H, <i>dd</i> , <i>J</i> = 12.4, 1.9)	3.65 (1H, <i>ddd</i> , <i>J</i> = 10.0, 6.6, 2.1)
Hb-6''	3.72 (1H, <i>dd</i> , <i>J</i> = 12.4, 5.4)	4.40 (1H, <i>dd</i> , <i>J</i> = 12.0, 2.1)
Ac-CH <sub>3</sub>	–	4.27 (1H, <i>dd</i> , <i>J</i> = 12.0, 6.6)
		2.03 (3H, <i>s</i> )

<sup>a</sup> Proton coupling constant (*J* in Hz), signal multiplicities and integrations are in parentheses.

rinse with H<sub>2</sub>O, a 10 min rinse with 1% NaOH, a 3 min rinse with H<sub>2</sub>O, and finally a 10 min rinse with the running buffer (phosphate buffer 50 mM pH 3.0). The running buffer was filtered through a 2  $\mu$ m filter (Titan syringe filter, Infochroma, Zug, Switzerland). The temperature of the capillary during electrophoretic analysis was kept constant at 30°C by a liquid thermostating system.

### 3.2. Plant material

Whole plant of *L. procumbens* (L.) Desv. was collected in Col du Sanetsch (altitude 2250 m), Switzerland, in July 1996. A voucher specimen (No. 96162) is deposited at the Institut de Pharmacognosie et Phytochimie, University of Lausanne, Switzerland.

### 3.3. Extraction and isolation

The dried whole plant (630 g) was ground and extracted at room temperature successively with CH<sub>2</sub>Cl<sub>2</sub> (3  $\times$  2000 ml) and MeOH (3  $\times$  2000 ml) to yield 64 and 165 g of extract, respectively. A portion (70 g) of the MeOH extract was subjected to CC on silica gel eluted with CHCl<sub>3</sub>–MeOH (9:1–1:1) to provide 13 fractions (1–13). Fraction 4 was purified by gel filtration on Sephadex LH-20 CHCl<sub>3</sub>–MeOH (1:1) to yield phloretin (**2**; 15 mg). Fractions 7 and 10 were separated by MPLC (RP-18, MeOH–H<sub>2</sub>O 25:75–1:1, flow rate 4 ml/min) affording fractions 7a–f and 10a–p, respectively. Fractions 7c, 10g, 10i and 10n contained pure **5** (950 mg), (*E*)-piceid (**1**; 440 mg), phloridzoid (**3**; 790 mg) and asebotin (**4**; 21 mg), respectively.

### 3.4. Reduction of 2,2-diphenyl-1-picrylhydrazyl (2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl; DPPH) radical

30  $\mu$ l of a solution containing the compound to be tested and 200  $\mu$ l of MeOH were added to 50  $\mu$ l of a 0.02% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percent of activity was calculated.

### 3.5. Antioxidant activity against lysozyme

Lysozyme (1 mg/ml) with antioxidants in various concentrations were incubated for 20 min, at 40  $\pm$  0.1°, in phosphate buffer (10 mM, pH 7.4  $\pm$  0.1). The antioxidants were dissolved in MeOH and a small volume (5  $\mu$ l) was added to the protein solution, so as to limit the amount of organic solvent in the assay (total volume 0.5 ml) to 1% (v/v). The oxidation was initiated by the addition of AAPH (2,2'-azobis(2-amidinopropane)-dihydrochloride) dissolved in phosphate buffer.

Protein oxidations were performed in the presence of 10 mM AAPH with or without antioxidants at 40  $\pm$  0.1° during 60 min, with continuous shaking, under air atmosphere.

Immediately after incubation, the samples were placed in ice for 10 s in order to sufficiently decrease the temperature to stop the free radical generation from AAPH. 200  $\mu$ l of the oxidation samples were mixed with the internal standard to obtain the following final concentrations, lysozyme 0.4 mg/ml with 0.3 mg/ml  $\alpha$ -chymotrypsinogen A as internal standard.

Samples were placed on the inlet tray of the P/ACE

Table 4

$^{13}\text{C}$  NMR spectral data of compounds **3** and **5** (measured in  $\text{CD}_3\text{OD}$ )

Carbon no.	<b>3</b>	<b>5</b>
1	133.84	133.81
2	130.31	130.32
3	116.03	116.05
4	156.14	156.23
5	116.03	116.05
6	130.31	130.32
$\alpha$	46.83	46.83
$\beta$	30.73	30.83
CO	206.48	206.49
1'	106.73	106.87
2'	167.18	167.31
3'	95.40	95.82
4'	165.77	165.82
5'	98.33	98.44
6'	162.15	162.03
1''	101.90	101.92
2''	74.62	74.60
3''	78.24	78.21
4''	70.98	71.31
5''	78.36	75.48
6''	62.34	64.44
Ac-CO	—	172.80
Ac-CH <sub>3</sub>	—	20.64

instrument and introduced into the capillary by a low pressure (0.5 psi) injection for 5 s, followed by a running buffer injection for 1 s. During the capillary zone electrophoresis analyses, a constant voltage of 15 kV was applied. The detection was carried out by UV/Vis spectrophotometry at 200 nm. Between runs, the capillary was washed with 1 M HCl, followed by reconditioning with running buffer during 5 min. Each sample analysis was done in duplicate or triplicate.

The percentages of inhibition were calculated by the ratio (peak height of the sample protein/peak height of the internal standard protein) in reference to the controls, i.e. sample with maximal oxidation and with no oxidation (Salvi et al., 1999).

### 3.6. Alkaline hydrolysis of **1**

1 mg of **1** in KOH 0.5 N was left at room temperature for 15 h; the reaction mixture was acidified to pH 5 and then partitioned with *n*-BuOH in which phloridzoid was detected by HPLC and TLC.

### 3.7. 6''-Acetylphloridzoid (**5**)

Microcrystalline white powder.  $[\alpha]_{\text{D}}^{20} -37^\circ$  (MeOH, *c*0.5). Mp 103–107°C. UV  $\lambda_{\text{max}}$  (MeOH) nm (log  $\epsilon$ ): 223 (4.30), 284 (4.20). D/CI-MS *m/z*: 496  $[\text{M} + \text{NH}_4]^+$ , 479  $[\text{M} + \text{H}]^+$ , 292  $[(\text{M} + \text{NH}_4)-6''\text{-O-acetylglucosyl}]^+$ , 274  $[\text{M}-6''\text{-O-acetylglucosyl}]^+$ .  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR: Tables 3 and 4.

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### References

- Cuendet, M., Hostettmann, K., Potterat, O., 1997. Iridoid glucosides with free radical scavenging properties from *Fagraea blumei*. Helvetica Chimica Acta 80, 1144–1152.
- Godin, P., 1954. A new reagent for paper chromatography of polyols and cetoses. Nature 174, 134.
- Hufford, C.D., Oguntimein, B.O., 1980. Dihydrochalcones from *Uvaria angolensis*. Phytochemistry 19, 2036–2038.
- Lamaison, J.L., Petitjean-Freyet, C., Carnat, A., 1991. Lamiacées médicinales à propriétés antioxydantes, sources potentielles d'acide rosmarinique. Pharmaceutica Acta Helveticae 66, 185–188.
- Salvi, A., Carrupt, P.-A., Testa, B., 1999. Development of a capillary electrophoresis method to monitor protein oxidation and antioxidant protection. Helvetica Chimica Acta 82, 870–878.
- Serve, L., Combaut, G., Piovetti, L., 1985. Isolement, identification et dosage de l'acide (hydroxy-4-phényl)-3-propionique par chromatographie liquide haute-performance des composés phénoliques de *Loiseleuria procumbens* Desf. (Ericaceae). Journal of Chromatography 322, 257–260.
- Serve, L., Combaut, G., Piovetti, L., 1986. Dosage par chromatographie liquide haute-performance des phloridzoides, phlorétol et acid phlorétique de *Loiseleuria procumbens* (L.) Desv. (Ericaceae). Journal of Chromatography 355, 318–327.
- Takao, T., Kitatani, F., Watanabe, N., Yagi, A., Sakata, K., 1994. A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. Bioscience, Biotechnology and Biochemistry 58, 1780–1783.
- Waffo Teguo, P., Decendit, A., Krisa, S., Deffieux, G., Vercauteren, J., Mérillon, J.-M., 1996. The accumulation of stilbene glycosides in *Vitis vinifera* cell suspension cultures. Journal of Natural Products 59, 1189–1191.