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Carboxymethyl Flavonoids and A Monoterpene Glucoside from *Selaginella moellendorffii*

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A new dihydroflavone, 5-carboxymethyl-7,4'-dihydroxyflavonone (**1**), and its glucoside 5-carboxymethyl-7,4'-dihydroxyflavonone-7-*O*-β-D-glucopyranoside (**2**), and one new monoterpene glucoside, (4*Z*,6*E*)-2,7-dimethyl-8-hydroxyocta-4,6-dienoic acid 8-*O*-β-D-glucopyranoside (**3**), were isolated from the whole plants of *Selaginella moellendorffii*. Their structures were determined by spectroscopic methods and chemical transformation. Compound **2** was evaluated for the ability to enhance glucose consumption in normal and insulin-resistant L6 muscle cells induced by high concentrations of insulin and glucose. Glucose consumption in insulin-resistant cells (but not in normal cells) was increased 15.2 ± 3.3% ($p < 0.01$) by compound **2** at a concentration of 0.1 μM in the presence of insulin (1 nM).

Key words: Selaginellaceae, *Selaginella moellendorffii*, Flavonoids, Monoterpene glucoside, Insulin-resistance

INTRODUCTION

Selaginella moellendorffii Hieron. (Selaginellaceae), a perennial herb, is mainly distributed in the southern area of the Changjiang River in China and is used to treat jaundice, gonorrhea, bleeding, acute hepatitis and idiopathic thrombocytopenic purpura (ITP) in Chinese folk medicine (Sun et al., 1997; Shi et al., 2008; Zheng et al., 2008; Zhu et al., 2008). A lignanoside (Zheng et al., 2008), and flavonoids (Sun et al., 1997; Shi et al., 2008; Zhu et al., 2008; Cao et al., 2010) had been found in this plant. In our previous investigation, eight pyrrolidinoindoline alkaloids were found in this plant (Wang et al., 2009). In continuing studies on this plant, two new flavonoids, 5-carboxymethyl-7,4'-dihydroxyflavonone (**1**), 5-carboxymethyl-7,4'-dihydroxyflavonone-7-*O*-β-D-glucopyranoside (**2**), and one new monoterpene glucoside, (4*Z*,6*E*)-2,7-dimethyl-8-hydroxyocta-4,6-dienoic acid 8-*O*-β-D-glucopyranoside (**3**) were isolated from the whole plants of *S. moellendorffii*. The flavonoids enhanced glucose consumption of insulin-resistant cells in the presence and absence of insulin (Li et al., 2009). Therefore, compound **2** was evaluated for its ability to induce glucose utilization in normal and insulin-resistant L6 cells. The isolation and structure elucidation of these new compounds and bioassay results are reported.

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MATERIALS AND METHODS

General experimental procedures

Optical rotations were determined on a JASCO DIP-370 automatic digital polarimeter. UV spectra were recorded on a Shimadzu double-beam 210A spectrometer. IR spectra were recorded on a Bio-Rad FTS-

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135 infrared spectrophotometer. 1D- and 2D-NMR spectra were recorded on BRUKER AM-400 and DRX-500 spectrometers with TMS as internal standard. FAB-MS were measured on a VG Auto Spec-3000 mass spectrometer (glycerol as matrix in negative-ion mode). ESI-MS and HR-ESI-MS were measured on an API Qstar Pulsar 1 instrument. Column chromatography (CC) was performed over silica gel G (80-100 and 300-400 mesh), silica gel H (10-40 μm), and Sephadex LH-20 (40-70 μm; Amersham Pharmacia Biotech AB). TLC was conducted on precoated silica gel plates GF_{254} (Qingdao). HPLC separations were performed using an Agilent 1200 series pump equipped with a diode array detector and a semi-preparative Zorbax SB-C_{18} (5 μm, *f* 9.4 × 250 mm,) C-18 column. Trifluoroacetic acid (TFA) was from Sinopharm Chemical Reagent Co. Ltd.

Plant material

S. moellendorffii was collected from Jingxi County, Guangxi Zhuang Autonomous Region, P. R. China, in June 2008. The plant was identified by Dr. Guang-Wan Hu (Kunming Institute of Botany, CAS), and a voucher specimen (No. JX0801) was deposited at the Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany, CAS.

Extraction and isolation

The air-dried whole plants of *S. moellendorffii* (6.0 kg) were exhaustively extracted with MeOH (4 h, 3 h, and 3 h, respectively) at 70°C. The solvent was evaporated under reduced pressure to give a residue (780.0 g), which was partitioned into three fractions, $CHCl_3$ (A, 32.0 g), Me_2CO (B, 154.0 g), and MeOH (C, 350.0 g) by silica gel column chromatography.

Fr. B was subjected to chromatography on a silica gel column ($CHCl_3$-MeOH = 20:1, 10:1, 5:1, 3:1, 1:1, 0:1) to afford six fractions (B_1-B_6). Fr. B_1 (7.8 g) was rechromatographed by RP-18 column chromatography (MeOH-H_2O = 10:90 → 90:10), and then was subjected to a Sephadex LH-20 column (MeOH). The fraction was subjected to a prepared silica gel column. Fr. B_4 (16.1 g) was subjected to RP-18 column chromatography (CC; MeOH-H_2O = 10:90 → 90:10) to give subfractions. The 50% MeOH fraction was submitted to column chromatography on Sephadex LH-20 (MeOH), and then was separated by silica gel column chromatography to give **1** (17.0 mg) eluted with EtOAc-Me_2CO-HCOOH (30:10:0.4). Fr. B_5 was subjected to RP-18 column chromatography (MeOH-H_2O = 10:90 → 90:10) to give subfractions. The 50% MeOH fraction was submitted to chromatography on Sephadex LH-20 (MeOH) and silica gel (EtOAc-Me_2CO = 5:1; EtOAc-Me_2CO-HCOOH = 50:50:1) columns to afford **3** (26.3 mg). Fr. B_6 was submitted to RP-18 column chromatography (MeOH-H_2O = 20:80) and then was separated by hemi-preparative HPLC [MeOH-H_2O (containing 0.05% TFA) = 40:60] to yield **2** (43.0 mg).

5-Carboxymethyl-7,4'-dihydroxyflavonone (1)

A colorless amorphous solid: $[\alpha]_D^{24.4}$ 0 (*c* = 0.60, MeOH); UV (MeOH) λ_{max} (log ε) nm: 311 (3.53), 278 (3.86), 222 (4.09); IR (KBr) ν_{max} 3398, 1661, 1609, 1583, 1518, 1449, 1274, 1161, 1047, 836 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data (Table I); FAB-MS : *m/z* 313 $[M - H]^-$; HR-ESI-MS: *m/z* 313.0727 $[M - H]^-$ (calcd for $C_{17}H_{13}O_6$, 313.0712).

5-Carboxymethyl-7,4'-dihydroxyflavonone-7-*O*-β-D-glucopyranoside (2)

A white amorphous solid (MeOH): $[\alpha]_D^{24.5}$ −41.9 (*c* = 0.62, MeOH); UV (MeOH) λ_{max} (log ε) nm: 314 (3.46), 273 (3.88), 222 (4.14), 202 (4.04); IR (KBr) ν_{max} 3396, 1716, 1692, 1662, 1609, 1573, 1520, 1441, 1268, 1171, 1086, 839 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data (Table II); FAB-MS: *m/z* 475 $[M - H]^-$; HR-ESI-MS: *m/z* 475.1259 $[M - H]^-$ (calcd for $C_{23}H_{23}O_{11}$, 475.1240).

(4*Z*,6*E*)-2,7-Dimethyl-8-hydroxyocta-4,6-dienoic acid 8-*O*-β-D-glucopyranoside (3)

A white amorphous solid ($CHCl_3$-MeOH): $[\alpha]_D^{26.2}$ −40.1 (*c* = 0.22, pyridine); UV (MeOH) λ_{max} (log ε) nm: 238 (3.71); IR (KBr) ν_{max} 3407, 1712, 1632, 1562, 1460, 1411, 1377, 1201, 1164, 1077, 1037 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 2.33 (br s, H-2), 2.31 (1H, br s, H-3a), 2.12 (1H, br s, H-3b), 5.60 (1H, m, H-4), 6.25 (1H, dd, *J* = 12.5, 10.5 Hz, H-5), 6.02 (1H, d, *J* = 10.5 Hz, H-6), 4.13 (1H, d, *J* = 12.7 Hz, H-8a), 3.92 (1H, d, *J* = 12.7 Hz, H-8b), 1.00 (3H, br s, H-9), 1.69 (3H, s, H-10), 4.08 (1H, d, *J* = 8.0 Hz, H-1'), 2.96 (1H, t, *J* = 8.0 Hz, H-2'), 3.03 (1H, m, H-3'), 3.02 (1H, m, H-4'), 3.10 (1H, m, H-5'), 3.64 (1H, br d, *J* = 11.5 Hz, H-6'a), 3.42 (1H, m, H-6'b); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 177.8 (C-1), 39.7 (C-2), 36.8 (C-3), 132.5 (C-4), 127.4 (C-5), 126.1 (C-6), 132.0 (C-7), 73.1 (C-8), 16.9 (C-9), 14.2 (C-10), 101.9 (C-1'), 73.5 (C-2'), 76.8 (C-3'), 70.1 (C-4'), 76.9 (C-5'), 60.1 (C-6'); FAB-MS: *m/z* 345 $[M - H]^-$; HR-ESI-MS: *m/z* 345.1548 $[M - H]^-$ (calcd for $C_{16}H_{25}O_8$, 345.1549).

Chemical conversion

Methyl esterification of compound 1

Compound **1** (11.0 mg) was dissolved in 5 mL Me_2CO and 0.5 mL Me_2SO_4 was added. The solution was stirred for 12 h at 50°C. The mixture was evaporated to dryness under vacuum, and the residue was dissolved in 30 mL H_2O and extracted with $CHCl_3$. The

$CHCl_3$-soluble extract was concentrated *in vacuo* to give a residue, which was chromatographed over a silica gel column ($CHCl_3$-Me_2CO = 50:1) to yield **1a** (3.6 mg).

Acid hydrolysis of compound 2

Compound **2** (10.0 mg) was subjected to acid hydrolysis in 2 mol/L HCl and heated at 90°C for 2 h. After cooling, the mixture was concentrated under vacuum to give a residue, which was submitted to silica gel column chromatography ($CHCl_3$-MeOH-HCOOH = 100:10:1; $CHCl_3$-MeOH-H_2O = 30:10:1) to afford compound **1** (3.2 mg) and D-glucose (3.2 mg) with an optical rotation value of $[\alpha]_D^{28.1}$ +14.3 (c = 0.22, H_2O).

Biological testing

L6 rat myoblasts (Cell Bank of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences) were maintained in DMEM supplemented with 10% FBS and differentiated into myotubes by exposure to DMEM supplemented with 2% FBS. Myotubes were cultured in 96-well plates and treated with 25 mM glucose and 100 nM insulin to induce insulin-resistant cells (Walker et al., 1989). Normal and resistant cells were exposed to compound **2** (0.01, 0.1, 1, and 10 μM) or rosiglitazone (5, 10, 20, and 40 μM) as a positive control for a period of 48 h, and then incubated for 15 min with or without insulin (1 nM). The consumption of glucose was assessed by measuring the concentration of glucose in the media in each well before and at 48 h of incubation, using the glucoseoxidase-peroxidase (GOD/POD) method (Yang et al., 2003), and calculating the decrease over 48 h. All data are expressed as mean ± S.E. from four to six experiments each performed in duplicate. They were analyzed for the significance of intergroup differences by student's t-tests. $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Compound **1** is a colorless solid having the molecular formula of $C_{17}H_{14}O_6$ by its HR-ESI-MS ($[M - H]^-$ at m/z 313.0727). The IR spectrum of **1** displayed the presence of hydroxy (3398 cm^{-1}), carbonyl (1661 cm^{-1}) and aromatic (1609, 1583, and 1518 cm^{-1}) groups. ^1H-NMR spectra of **1** (Table I) revealed signals for a *para*-disubstituted phenyl ring [δ_H 7.29 (2H, d, J = 8.2 Hz, H-2',6'), 6.81 (2H, d, J = 8.2 Hz, H-3',5')] and a 1,2,3,5-tetrasubstituted benzene ring [δ_H 6.33 (s, H-6) and 6.30 (s, H-8)]. Additionally, signals for one methylene [δ_C 46.2 (CH_2, C-3)] and one methine [δ_C 80.2 (CH, C-2)] groups were observed in the ^{13}C-NMR spectrum of **1**. In the HMBC spectrum of **1** (Fig. 1), correlations of H-2',6'/C-2 and H-2/C-2',6' were observed. Compound **1** might be a dihydroflavone according to the characteristics of its NMR data. Three carbon signals disappeared in the ^{13}C-NMR spectrum of **1** by analysis of its molecular formula of $C_{17}H_{14}O_6$. Therefore, the methyl ester derivative (**1a**) of compound **1** was prepared using Me_2SO_4.

The ^{13}C-NMR spectrum of compound **1a** (Table I) exhibited 20 signals of carbon for two aromatic, and two carbonyl, one methine, two methylene, and three methoxy groups. According to the HMBC spectrum of **1a** (Fig. 1), it was elucidated as 5-(2-*O*-methyl)-carboxymethyl-7,4'-dimethoxyflavonone. Thus, compound **1**

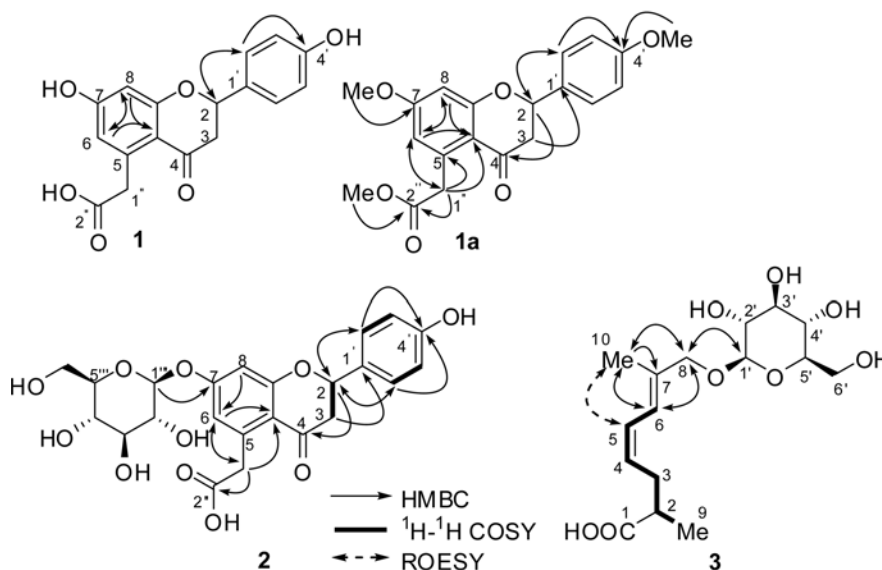

Fig. 1. Key 2D NMR correlations of compounds **1**-**3** and **1a**

Table I. ^{1}H-NMR and ^{13}C-NMR data (δ in ppm, J in Hz) of **1** and **1a**

Position	**1**[a]		**1a**[b]	
	δ_H	δ_C	δ_H	δ_C
2	5.31 (d, J = 12.6)	80.2	5.40 (dd, J = 13.7, 2.7)	79.1
3	3.00 (m) 2.62 (br d, J = 16.1)	46.2	3.06 (dd, J = 16.6, 13.7) 2.62 (dd, J = 16.6, 2.7)	45.0
4		not observed		191.8
5		142.1		138.5
6	6.33 (s)	115.6	6.40 (d, J = 2.2)	114.3
7		166.7		164.6
8	6.30 (s)	103.3	6.47 (d, J = 2.2)	100.5
9		165.1		165.0
10		113.7		113.1
1'		131.4		130.7
2'/6'	7.29 (2H, d, J = 8.4)	129.0	7.39 (2H, d, J = 8.8)	127.7
3'/5'	6.81 (2H, d, J = 8.4)	116.3	6.95 (2H, d, J = 8.8)	114.1
4'		158.9		159.9
1''	3.95 (2H, br s)	not observed	4.02 (d, J = 16.6) 3.94 (d, J = 16.6)	41.1
2''		not observed		171.7
7-*O*-CH$_3$			3.82 (3H, s)	55.5
4'-*O*-CH$_3$			3.83 (3H, s)	55.3
2''-*O*-CH$_3$			3.73 (3H, s)	51.8

[a]Recorded in CD$_3$OD (^{1}H-NMR/^{13}C-NMR at 400/100 MHz); [b]Recorded in CDCl$_3$ (^{1}H-NMR/^{13}C-NMR at 400/100 MHz).

Table II. ^{1}H-NMR and ^{13}C-NMR data (δ in ppm, J in Hz) of **2**

Position	**2a**[a]		**2b**[a]	
	δ_H	δ_C	δ_H	δ_C
2	5.40 (dd, J = 13.0, 3.0)	78.57	5.39 (dd, J = 13.0, 3.0)	78.55
3	3.12 (m) 2.60 (d, J = 15.0)	44.3	3.12 (m) 2.60 (d, J = 15.0)	44.3
4		191.8		191.7
5		139.2		139.2
6	6.55 (s)	115.0	6.55 (s)	115.0
7		161.8		161.7
8	6.60 (br. s)	102.5	6.60 (br. s)	102.5
9		164.1		164.0
10		113.8		113.8
1'		129.1		129.0
2'/6'	7.33 (2H, d, J = 8.2)	128.5	7.33 (2H, d, J = 8.2)	128.4
3'/5'	6.78 (2H, d, J = 8.2)	115.1	6.78 (2H, d, J = 8.2)	115.0
4'		157.8		157.8
1''	3.90 (d, J = 16.2) 3.84 (d, J = 16.2)	40.6	3.90 (d, J = 16.2) 3.84 (d, J = 16.2)	40.6
2''		171.9		171.9
1'''	5.00 (d, J = 7.5)	99.5	4.98 (d, J = 7.5)	99.4
2'''	3.20 (m)	73.1	3.20 (m)	73.1
3'''	3.14 (m)	76.3	3.14 (m)	76.3
4'''	3.14 (m)	69.4	3.14 (m)	69.4
5'''	3.36 (m)	76.99	3.36 (m)	76.97
6'''	3.64 (br d, J = 11.5) 3.43 (m)	60.5	3.64 (br d, J = 11.5) 3.43 (m)	60.5

[a]Recorded in DMSO-d_6 (^{1}H-NMR/^{13}C-NMR at 500/100 MHz); The data in a same line of **2a** and **2b** were interchangeable.

was identified as 5-carboxymethyl-7,4'-dihydroxyflavonone. Because the optical rotation value of compound **1** is zero, it might be a racemate.

Compound **2** was obtained as a pair of compounds, **2a** and **2b**. Its molecular formula was established to be $C_{23}H_{24}O_{11}$ by HR-ESI-MS ($[M - H]^-$ at m/z 475.1259). The IR spectrum indicated the presence of hydroxy (3396 cm^{-1}), carbonyl (1716 and 1692 cm^{-1}), and aromatic (1609, 1573, and 1520 cm^{-1}) groups. The ratio of **2a** and **2b** was about 1:1 by hydrogen integration in the ^{1}H-NMR spectrum of **2**. Comparison of NMR data of **2** (Table II) with those of **1** and **1a** suggested that the former is a β-glucoside of **1**. Acid hydrolysis of **2** yielded compound **1** and D-glucose identified by comparison with an authentic sample on TLC. The glucosyl group was located at C-7 by the HMBC correlation from H-1''' to C-7. According to the above analysis, compound **2** was elucidated as 5-carboxymethyl-7,4'-dihydroxyflavonone-7-*O*-β-D-glucopyranoside. Because there is only a chiral carbon (C-2) in the aglycone of compound **2,** compounds **2a** and **2b** might be a pair of C-2 isomers.

The molecular formula of compound **3** was established as $C_{16}H_{26}O_8$ by HR-ESI-MS, indicating 4 degrees of unsaturation. The IR spectrum indicated the presence of hydroxy (3407 cm^{-1}) and carbonyl (1712 cm^{-1}) groups, and conjugated double bonds (1632 and 1562 cm^{-1}). The NMR spectra of **3** showed the existence of a β-glucosyl moiety [δ_H 4.08 (J = 8.0 Hz, H-1'); δ_C 101.9 (C-1'), 73.5 (C-2'), 76.8 (C-3'), 70.1 (C-4'), 76.9 (C-5'), and 61.1 (C-6')]. The remaining 10 carbon signals for two double bonds, and one carboxyl, two methyl, two methylene, and one methine groups were observed in the ^{13}C-NMR spectrum of **3**. Because all of the 4 degrees of unsaturation were accounted for, compound **3** was deduced to be an open-chain monoterpene glucoside.

A connection of C-9-C-2 to C-6 was confirmed by ^{1}H-^{1}H COSY spectra of **3**. The HMBC correlations from H_3-10 to C-6, C-7 and C-8, and from H-1' to C-8 indicated that all fragments were linked together as shown in Fig. 1 expect for C-1. Because C-2 is a methine group, C-1 must attach to this position. The C-4-C-5 double bonds were deduced as *Z*-configuration according to the coupling constant between H-4 and H-5 ($J_{4,5}$ = 12.5 Hz), while the C-6-C-7 double bonds are *E*-configuration by the ROESY correlations (Fig. 1) between H-5 and H_3-10. Therefore, compound **3** was identified as (4*Z*,6*E*)-2,7-dimethyl-8-hydroxyocta-4,6-dienoic acid 8-*O*-β-D-glucopyranoside.

Compound **2** was evaluated for the ability to enhance glucose consumption in normal and insulin-resistant L6 muscle cells induced by high concentrations of insulin and glucose. As shown in Table III, a 37.2~39.4% decrease in basal glucose consumption was observed in the insulin-resistant L6 muscle cells after treatment with high concentrations of insulin and glucose. Compound **3** significantly enhanced glucose consumption in insulin-resistant cells, with an increase of 15.2 ± 3.4% at the glucose concentrations of 0.1 and 0.01 μM in the presence of insulin (1 nM), but not in the absent of insulin. Moreover, no changes in glucose consumption were observed in the normal cells treated with compound **3** (data not shown), which suggested that compound **2** enhances the sensitivity of resistant cells in response to insulin and decreases insulin resistance.

Table III. Effects of compound **2** on glucose consumption in insulin-resistant L6 muscle cells

Groups	Concentration (M)	Glucose consumption (mM)	
		- Insulin	+ Insulin (1 nM)
Control	0.1% DMSO	6.85 ± 0.89	6.39 ± 0.23
Control + Insulin			6.88 ± 0.42
Model Control		4.14 ± 0.66**	3.67 ± 0.34**
Model + Insulin			3.94 ± 0.27
Positive Control (Rosiglitazone)	5×10^{-6}	3.66 ± 0.08	3.35 ± 0.45
	1×10^{-5}	4.42 ± 0.60	4.17 ± 0.33
	2×10^{-5}	4.39 ± 0.40	4.43 ± 0.37▲
	4×10^{-5}	4.15 ± 0.34	5.08 ± 0.73▲
Compound **3**	1×10^{-8}	4.04 ± 0.20	4.46 ± 0.38▲
	1×10^{-7}	4.21 ± 0.21	4.54 ± 0.30▲▲
	1×10^{-6}	4.27 ± 0.04	3.78 ± 0.77
	1×10^{-5}	4.08 ± 0.33	3.56 ± 0.93

$\bar{x} \pm s$, $n = 4$, $^{**}p < 0.01$, compared with control group, $^▲p < 0.05$, $^{▲▲}p < 0.01$, compared with model control group (t-test).

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