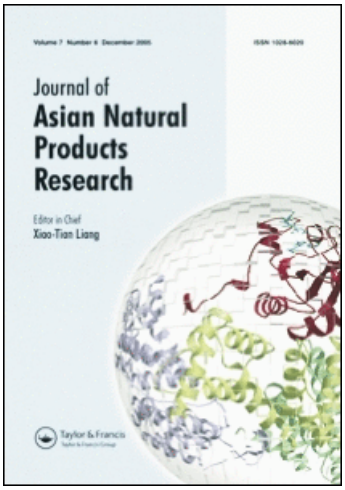

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Four new biflavonoids from *Selaginella uncinata* and their anti-anoxic effect

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Four new biflavonoids from *Selaginella uncinata* and their anti-anoxic effect

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The 60% ethanolic extract of *Selaginella uncinata* (Desv.) Spring possessed potent anti-anoxic effect in the anoxic PC12 cell assay. A phytochemical study of its EtOAc-soluble part led to the isolation of four new and three known biflavonoids. Their structures were established on the basis of physico-chemical properties and spectroscopic analysis. The absolute configurations of the new compounds were determined with the aid of circular dichroism (CD) spectroscopy. Compounds **4** and **5** showed potent anti-anoxic effect in the anoxic PC12 cell assay.

Keywords: *Selaginella uncinata* (Desv.) Spring; biflavonoids; anti-anoxic effect; uncinatabiflavones A–D

1. Introduction

Hypoxia is a common environmental stress that influences signaling pathways and cell functions. Several cell types, including neuroendocrine chromaffin cells, have evolved to sense oxygen levels and initiated specific adaptive responses to hypoxia. PC12, a rat pheochromocytoma cell line derived from a tumor of adrenal medulla chromaffin tissue, is an oxygen-sensitive cell type that provides a useful system to study the effects of hypoxia [1]. In our searching for anti-anoxic compounds, the 60% ethanolic extract of *Selaginella uncinata* (Desv.) Spring possessed potent anti-anoxic effect in the anoxic PC12 cell assay.

S. uncinata (Desv.) Spring is widely distributed in the southwest part of China. It has been used as a herbal medicine for the treatment of jaundice, dysentery, edema, and beriberoid diseases [2]. Recent research of this plant led to the isolation of several biflavonoids, chromone glycosides, and phenolic constituents [3,4].

In our present investigation, the EtOAc-soluble part from the 60% ethanolic extract of *S. uncinata* showed the strongest anti-anoxic effect (Figure 1). Four new and three known biflavonoids were isolated from the EtOAc-soluble part. Their structures were established on the basis of physico-chemical properties and spectroscopic analysis. Compounds **4** and **5** showed potent anti-anoxic effect in the anoxic PC12 cell assay.

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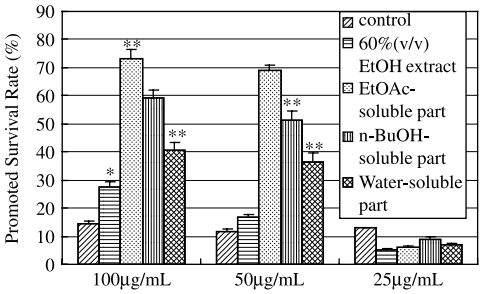
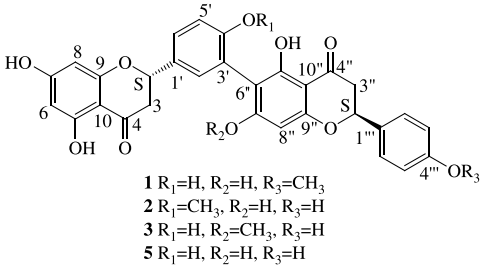
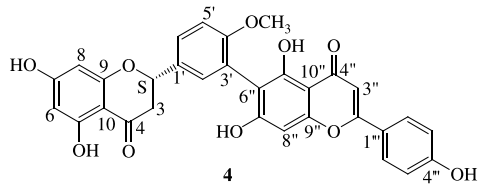


Figure 1. The anti-anoxic effect of 60% (v/v) EtOH extract, EtOAc-, *n*-BuOH-, and H_2O-soluble parts in the anoxic PC12 cell assay. Dates were expressed as mean ± SD ($n = 3$). Statistical significance was determined by Student's *t*-test. $*p < 0.05$, $**p < 0.01$ as compared with the control.

2. Results and discussion

The EtOAc-soluble part was subjected to silica gel, Sephadex LH-20, and ODS column chromatography, and finally purified by preparative reverse-phase HPLC to afford seven compounds (Figure 2). All the compounds showed brick red when reacted with Mg/HCl, and deep blue when reacted with aqueous $FeCl_3$ solution, which indicated that they were flavonoids.





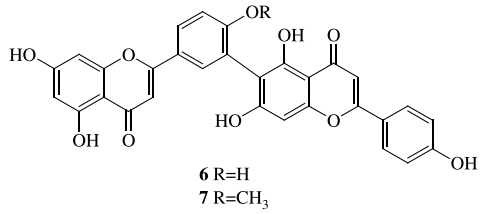


Figure 2. Chemical structures of compounds **1**–**7**.

Compound **1** was obtained as an amorphous yellow powder. Its UV spectrum showed absorption maxima at λ_{max} (nm) 327 (3.88) and 288 (log ε 4.48). Its IR spectrum showed the presence of hydroxyl group (3357 cm^{-1}), conjugated carbonyl (1636 cm^{-1}), and aromatic rings (1504 and 1454 cm^{-1}). The molecular formula was established as $C_{31}H_{24}O_{10}$ by HRESIMS at *m/z* 579.1240. The ^1H NMR spectrum (Table 1) showed the presence of AMX coupling system signals at δ 7.46 (1H, dd, $J = 8.6$, 2.0 Hz, H-6′), 7.21 (1H, d, $J = 2.0$ Hz, H-2′), and 7.07 (1H, d, $J = 8.6$ Hz, H-5′), AA′XX′ coupling system signals at δ 7.35 (2H, d, $J = 8.7$ Hz, H-2‴, 6‴) and 6.82 (2H, d, $J = 8.7$ Hz, H-3‴, 5‴), and two meta-coupled proton signals at δ 5.91 (1H, d, $J = 2.2$ Hz, H-8) and 5.89 (1H, d, $J = 2.2$ Hz, H-6). Six double doublets at δ 5.52 (1H, dd, $J = 12.7$, 2.9 Hz, H-2), 3.33 (1H, dd, $J = 17.1$, 12.7 Hz, H-3α), 2.73 (1H, dd, $J = 17.1$, 2.9 Hz, H-3β), 5.48 (1H, dd, $J = 12.7$, 2.9 Hz, H-2″), 3.23 (1H, dd, $J = 17.1$, 12.7 Hz, H-3″α), and 2.69 (1H, dd, $J = 17.1$, 2.9 Hz, H-3″β) were also revealed by the ^1H NMR spectrum, indicating the presence of two flavanone subunits. The ^{13}C NMR spectrum (Table 2) showed signals for 31 carbons, including two carbonyl groups at δ 196.5 and 196.3; 14 quaternary sp^2 carbons at δ 166.9, 164.6, 163.5, 163.0, 161.7, 160.9, 157.9, 157.8, 129.9, 129.0, 122.0, 106.2, 101.8, and 101.5; 10 tertiary sp^2 carbons at δ 131.1, 128.4 (× 2), 127.4, 115.2 (× 2), 111.2, 95.9, 95.1, and 94.6; and five sp^3 carbon atoms at δ 78.5, 78.4, 55.6, and 42.0 (× 2). All the chemical shifts of carbons connected with protons were confirmed using the HSQC experiment. All the ^1H and ^{13}C NMR spectral data mentioned above indicated that compound **1** was a biflavonoid consisting of two flavanone units. The two subunits, designated I and II (Figure 3), were determined according to

Table 1. ^{1}H NMR spectral data (400 MHz, DMSO-d_6) of compounds **1**–**4**[a].

Position	1	2	3	4
2	5.52 (1H, dd, J = 12.7, 2.9 Hz)	5.42 (1H, dd, J = 13.4, 2.8 Hz)	5.49 (1H, dd, J = 12.6, 2.9 Hz)	5.53 (1H, dd, J = 12.8, 2.7 Hz)
3	3.33 (1H, dd, J = 17.1, 12.7 Hz)	3.15 (1H, dd, J = 17.4, 13.4 Hz)	3.35 (1H, dd, J = 17.0, 12.6 Hz)	3.18 (1H, dd, J = 17.0, 12.8 Hz)
	2.73 (1H, dd, J = 17.1, 2.9 Hz)	2.78 (1H, dd, J = 17.4, 2.8 Hz)	2.71 (1H, dd, J = 17.0, 2.9 Hz)	2.74 (1H, dd, J = 17.0, 2.7 Hz)
6	5.89 (1H, d, J = 2.2 Hz)	5.88 (2H, brs)	5.87 (2H, brs)	5.87 (1H, d, J = 2.0 Hz)
8	5.91 (1H, d, J = 2.2 Hz)			5.90 (1H, d, J = 2.0 Hz)
2′	7.21 (1H, d, J = 2.0 Hz)	7.26 (1H, d, J = 2.2 Hz)	7.39 (1H, d, J = 2.2 Hz)	7.27 (1H, d, J = 2.2 Hz)
5′	7.07 (1H, d, J = 8.6 Hz)	7.00 (1H, d, J = 8.4 Hz)	6.87 (1H, d, J = 8.5 Hz)	7.12 (1H, d, J = 8.6 Hz)
6′	7.46 (1H, dd, J = 8.6, 2.0 Hz)	7.35 (1H, dd, J = 8.4, 2.2 Hz)	7.43 (1H, dd, J = 8.5, 2.2 Hz)	7.50 (1H, dd, J = 8.6, 2.2 Hz)
2″	5.48 (1H, dd, J = 12.7, 2.9 Hz)	5.48 (1H, dd, J = 13.1, 2.6 Hz)	5.55 (1H, dd, J = 13.2, 2.7 Hz)	
3″	3.23 (1H, dd, J = 17.1, 12.7 Hz)	3.25 (1H, dd, J = 16.8, 13.1 Hz)	3.25 (1H, dd, J = 17.0, 13.2 Hz)	6.80 (1H, s)
	2.69 (1H, dd, J = 17.1, 2.9 Hz)	2.60 (1H, dd, J = 16.8, 2.6 Hz)	2.75 (1H, dd, J = 17.0, 2.7 Hz)	
6″				
8″	6.05 (1H, s)	6.04 (1H, s)	6.41 (1H, s)	6.63 (1H, s)
2‴/6‴	7.35 (2H, d, J = 8.7 Hz)	7.15 (2H, d, J = 8.6 Hz)	7.36 (2H, d, J = 8.7 Hz)	7.95 (2H, d, J = 8.8 Hz)
3‴/5‴	6.82 (2H, d, J = 8.7 Hz)	6.70 (2H, d, J = 8.6 Hz)	6.82 (2H, d, J = 8.7 Hz)	6.95 (2H, d, J = 8.8 Hz)
OH-5	12.16 (1H, brs)			12.15 (1H, brs)
OH-5″	12.38 (1H, brs)			13.18 (1H, brs)
OMe-4′		3.70 (3H, s)		3.72 (3H, s)
OMe-4‴	3.78 (3H, s)			
OMe-7″			3.78 (3H, s)	

[a]Multiplicity and coupling constant (J in Hertz) assigned in parentheses; brs, broad singlet; d, doublet; dd, double doublet; s, singlet.

Table 2. ^{13}C NMR spectral data (100 MHz, DMSO-d_6) of compounds **1**–**4**.

Position	**1**	**2**	**3**	**4**
2	78.4	77.7	77.6	78.4
3	42.0	41.7	41.5	42.0
4	196.5	196.3	195.4	196.3
5	163.5	163.1	163.1	163.5
6	95.9	95.0	95.6	95.9
7	166.9	166.7	166.9	166.8
8	95.1	94.7	94.7	95.1
9	163.0	162.5	162.3	163.0
10	101.8	101.2	101.1	101.8
1′	129.9	129.4	131.4	130.0
2′	131.1	130.7	127.6	130.9
3′	122.0	121.5	122.6	121.8
4′	157.8	157.5	157.6	157.8
5′	111.2	110.3	113.9	111.3
6′	127.4	126.6	127.8	127.7
2″	78.5	78.0	78.6	163.7
3″	42.0	42.0	41.5	102.9
4″	196.3	195.7	197.0	181.9
5″	160.9	159.4	153.2	158.9
6″	106.2	105.2	102.1	109.4
7″	164.6	164.4	159.9	162.0
8″	94.6	95.3	91.9	93.4
9″	161.7	162.0	159.6	156.3
10″	101.5	101.2	101.1	103.5
1‴	129.0	128.5	128.1	121.3
2‴	128.4	127.5	128.0	128.5
3‴	115.2	114.6	114.8	116.1
4‴	157.9	157.0	157.4	161.2
5‴	115.2	114.6	114.8	116.1
6‴	128.4	127.5	128.0	128.5
OMe-4′		55.0		55.6
OMe-4‴	55.6			
OMe-7″			56.1	

the correlations of H-6′/C-4′, C-2′; H-2′/C-4′, C-2; H-2/C-4, C-2′, C-1′; H-3α/C-4, C-2; H-3β/C-4; H-2‴, 6‴/C-4‴, C-2″; H-3‴, 5‴/C-4‴, C-1‴; H-2″/C-4″, C-2‴; H-3″α/C-4″, C-2″; H-3″β/C-4″ in the HMBC spectrum. The methoxyl group was linked at C-4‴ in the subunit I, for its protons correlated with C-4‴ in the HMBC spectrum.

A further examination of the HMBC spectrum of **1** clearly showed the correlations of OH-5″/C-6″, C-10″; H-2′/C-6″; H-8″/C-7″, C-9″, C-6″, C-10″, which indicated that the interlinkage position of the subunits I and II was between C-3′ and C-6″. The ^1H and ^{13}C NMR signal assignments were achieved by

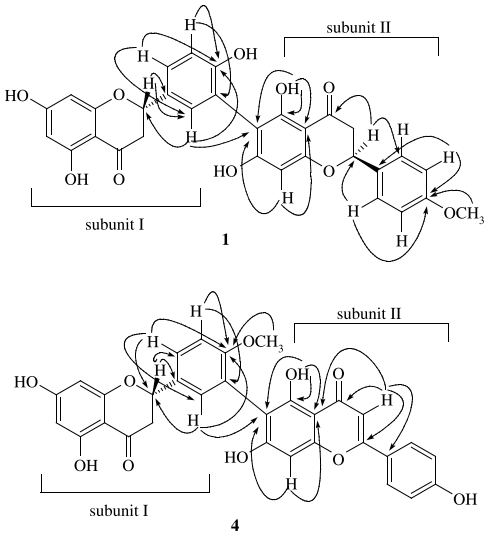


Figure 3. Key HMBC of compounds **1** and **4**.

the combination of ^1H–^1H COSY, HSQC, and HMBC spectral elucidation, and comparison with the literature values of tetrahydrorobustaflavone [5].

The CD spectrum of **1** showed a positive Cotton effect at 327 nm ($[\Delta\varepsilon]_{327\,nm}$ +6.82) and a negative Cotton effect at 288 nm ($[\Delta\varepsilon]_{288\,nm}$ −15.15). Therefore, both C-2 and C-2″ were assigned the *S* configurations in accordance with the literature values of compound (2*S*, 2″*S*)-tetrahydroamentoflavone [6]. Thus, compound **1** is a new compound and elucidated as (2*S*, 2″*S*)-2,3,2″,3″-tetrahydrorobustaflavone-4‴-methyl ether, named as uncinatabiflavone A.

Compound **2** was obtained as an amorphous yellow powder. Its UV spectrum showed absorption maxima at λ_{max} (nm) 325 (3.84) and 289 (4.66). Its IR spectrum showed the presence of hydroxyl group (3356 cm^{-1}), conjugated carbonyl (1636 cm^{-1}), and aromatic rings (1508 and 1458 cm^{-1}). The molecular formula was established as $C_{31}H_{24}O_{10}$ by HRESIMS at *m/z* 579.1241, which indicated that **2** was an isomer of compound **1**. The ^1H and ^{13}C NMR spectra of compound **2** were quite similar to those of **1**. The structure of **2** was fully established

by ^{1}H–^{1}H COSY, HSQC, and HMBC spectral analysis. The methoxyl group at δ 3.70 (3H, s) showed a correlation with C-4′ in the HMBC spectrum, which indicated that the methoxyl group was located at C-4′. It was further confirmed by the correlation between H-5′ and OMe-4′ in the NOESY spectrum. The CD spectrum of **2** showed a positive Cotton effect at 325 nm ($[\Delta\varepsilon]_{325\,nm}$ +4.85) and a negative Cotton effect at 288 nm ($[\Delta\varepsilon]_{288\,nm}$ −8.33), suggesting that both C-2 and C-2″ were *S* configurations. Compound **2** was determined as (2*S*, 2″*S*)-2,3,2″,3″-tetrahydrorobustaflavone-4′-methyl ether, which is a new compound and named as uncinatabiflavone B.

Compound **3** was obtained as an amorphous yellow powder. Its UV spectrum showed absorption maxima at λ_{max} (nm) 328 (3.88) and 288 (4.70). Its IR spectrum showed the presence of hydroxyl group (3356 cm^{-1}), conjugated carbonyl (1643 cm^{-1}), and aromatic rings (1508 and 1450 cm^{-1}). The CD spectrum of **3** showed a positive Cotton effect at 328 nm ($[\Delta\varepsilon]_{328\,nm}$ +4.24) and a negative Cotton effect at 288 nm ($[\Delta\varepsilon]_{288\,nm}$ −5.45), suggesting that both C-2 and C-2″ were *S* configurations. Thus, compound **3**, which is a new compound, was deduced as (2*S*, 2″*S*)-2,3,2″,3″-tetrahydrorobustaflavone-7″-methyl ether and named as uncinatabiflavone C.

Compound **4** was obtained as a pale yellow crystal, m.p. 225–227°C. Its UV spectrum showed absorption maxima at λ_{max} (nm) 334 (4.57) and 286 (4.66). Its IR spectrum showed the presence of hydroxyl (3387 cm^{-1}), conjugated carbonyl (1642 cm^{-1}), and aromatic rings (1612, 1570, and 1454 cm^{-1}). The molecular formula was established as $C_{31}H_{24}O_{10}$ by HRESIMS at *m/z* 577.1124. The ^{1}H NMR spectrum (Table 1) showed the presence of AMX coupling signals at δ 7.50 (1H, dd, J = 8.6, 2.2 Hz, H-6′), 7.27 (1H, d, J = 2.2 Hz, H-2′), and 7.12 (1H, d, J = 8.6 Hz, H-5′), AA′XX′ coupling signals at δ 7.95 (2H, d, J = 8.8 Hz, H-2‴, 6‴) and 6.95 (2H, d, J = 8.8 Hz, H-3‴, 5‴), and two meta-coupled proton signals at δ 5.90 (1H, d, J = 2.0 Hz, H-8) and 5.87 (1H, d, J = 2.0 Hz, H-6). A one-proton singlet at δ 6.80 (H-3″) and three double doublets at δ 5.53 (1H, dd, J = 12.8, 2.7 Hz, H-2), 3.18 (1H, dd, J = 17.0, 12.8 Hz, H-3α), and 2.74 (1H, dd, J = 17.0, 2.7 Hz, H-3β) were also revealed by the ^{1}H NMR spectrum, indicating the presence of a flavanone and a flavone unit. The ^{13}C NMR spectrum (Table 2) showed 31 carbon signals, including two carbonyl groups at δ 196.3 and 181.9; 15 quaternary sp^2 carbon signals at δ 166.8, 163.7, 163.5, 163.0, 162.0, 161.2, 158.9, 157.8, 156.3, 130.0, 121.8, 121.3, 109.4, 103.5, and 101.8; 11 tertiary sp^2 carbons at δ 130.9, 128.5 (×2), 127.7, 116.1 (×2), 111.3, 102.9, 95.9, 95.1, and 93.4; three sp^3 carbon atoms at δ 78.4, 55.6, and 42.0. All the chemical shifts of carbons connected with protons were confirmed using the HSQC experiment. Based on the ^{1}H and ^{13}C NMR spectral data analysis, the skeleton structure of **4** was deduced to be a biflavonoid consisted of a flavone unit and a flavanone unit. The two subunits, designated I and II (Figure 3), were also established by the correlations of H-6′/C-4′, C-2′, C-2; H-2′/C-4′, C-6′, C-2; H-2/C-1′, C-6′; H-3α/C-4, C-2; H-3β/C-4; H-2‴, 6‴/C-4‴, C-2″; H-3″/C-4″, C-2″, C-1‴, C-10″ in the HMBC spectrum. The methoxyl group was assigned at the position of C-4′ in the subunit I by its HMBC correlation with C-4′.

A further examination of the HMBC spectrum of **4** clearly showed the following correlations of OH-5″/C-6″, C-10″; H-2′/C-6″; H-8″/C-7″, C-9″, C-6″, C-10″, which unambiguously confirmed that the interlinkage position of the subunits I and II was at C-3′ and C-6″. The ^{1}H and ^{13}C NMR signal assignments were achieved by the combination of ^{1}H–^{1}H COSY, HSQC, and HMBC spectral elucidation, and comparison with the literature values of caesalflavone [7].

The CD spectrum of **4** showed a positive Cotton effect at 334 nm ($[\Delta\varepsilon]_{334\,nm}$ +6.06) and a negative Cotton effect at 288 nm ($[\Delta\varepsilon]_{288\,nm}$ −6.97). Therefore, C-2 was assigned the *S* configuration in accordance with the literature values of (2*S*)-2,3-dihydroamentoflavone [6]. Compound **4** is determined to be a new compound: (2*S*)-2,3-dihydrorobustaflavone-4′-methyl ether and named as uncinatabiflavone D.

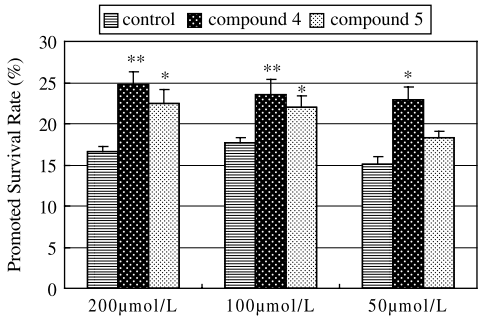


Figure 4. The anti-anoxic effect of compounds **4** and **5** in the anoxic PC12 cell assay. Dates were expressed as mean ± SD ($n = 3$). Statistical significance was determined by Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with the control.

Along with the four new biflavonoids mentioned above, three known biflavonoids (2*S*, 2″*S*)-tetrahydrorobustaflavone (**5**) [8], robustaflavone (**6**) [9], and robustaflavone 4′-methyl ether (**7**) [10] were also isolated.

The anti-anoxic activity of compounds **4** and **5** was evaluated by the anoxic PC12 cell assay (Figure 4). Compound **4** displayed a potent anti-anoxic effect, and compound **5** showed a moderate effect.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a YANACO apparatus (uncorrected). The IR spectra were recorded on a SHIMADZU FTIR8900 spectrophotometer using KBr disks, and the UV spectra were obtained on a SHIMADZU UV2401PC spectrophotometer. The optical rotations were taken on a JASCO P-1020 digital polarimeter, and the CD spectra were recorded on a JASCO 810 spectropolarimeter. The 1D- and 2D-NMR spectra were obtained on a Bruker AV-400 spectrometer, with TMS as an internal standard. The mass spectra were determined on a Bruker Esquire 2000 spectrometer, and HRESIMS were acquired using a Micromass Q-TOF mass spectrometer. HPLC were recorded on an Agilent 1100 Series high-performance liquid chromatograph. Silica gel (Qing Dao Hai Yang Chemical Group Co., Qing Dao, China), ODS-A120-S150 purchased from YMC Co., Ltd (made in Komatsu, Japan), and Sephadex LH-20 purchased from Amersham Biosciences (made in Uppsala, Sweden) were used for column chromatography.

3.2 Plant material

Herbs of *S. uncinata* were collected in Guangxi Province, China, in August 2004, and were identified by Professor Sun Qi-shi (Shenyang Pharmaceutical University, Shenyang, China). A voucher specimen (No. Y01156SU) is deposited in the Department of Natural Products Chemistry, Shenyang Pharmaceutical University.

3.3 Extraction and isolation

The air-dried whole herbs (4.2 kg) of *S. uncinata* were refluxed with 60% (v/v) EtOH and the extract was concentrated under vacuum to afford a viscous residue (856 g). The residue was dissolved in water and partitioned successively with EtOAc and water-saturated *n*-BuOH to give three parts, EtOAc-(160 g), *n*-BuOH-(90.4 g), and H_2O (600 g)-soluble parts. The EtOAc-soluble part was separated by column chromatography over silica gel (200–300 mesh) with a $CHCl_3$–MeOH gradient system to yield 15 fractions. Fraction 7 (18 g), eluted with $CHCl_3$–MeOH (95:5), was separated on a Sephadex LH-20 column ($CHCl_3$–MeOH, 1:1), an ODS column (MeOH–H_2O, 7:3), preparative reverse-phase HPLC (Shimadzu, 20 × 250 mm, MeOH–H_2O–HAc, 65:35:0.1, flow rate 10 ml/min), and then by crystallization to give compound **1** (50 mg). Fraction 6 (8.5 g), eluted with $CHCl_3$–MeOH (97:3), was further separated on a silica gel (200–300 mesh) column (cyclohexane–acetone, 6:4), a Sephadex LH-20 column ($CHCl_3$–MeOH, 9:1), an ODS column (MeOH–H_2O, 7:3), and then passed through preparative reverse-phase HPLC (Shimadzu, 20 × 250 mm, MeOH–H_2O–HAc, 65:35:0.1, flow rate 10 ml/min) to give compounds **2**

(33 mg) and **3** (45 mg). Fraction 8 (19.2 g), eluted with $CHCl_3$–MeOH (9:1), was separated on a Sephadex LH-20 column ($CHCl_3$–MeOH, 1:1), an ODS column (MeOH–H_2O, 7:3), preparative reverse-phase HPLC (Shimadzu, 20 × 250 mm, MeOH–H_2O–HAc, 65:35:0.1, flow rate 10 ml/min), and then by crystallization to give compounds **4** (30 mg) and **5** (30 mg). Compounds **6** (50 mg) and **7** (250 mg) were obtained from fraction 15 (500 mg) by preparative reverse-phase HPLC (Shimadzu, 20 × 250 mm, MeOH–H_2O–HAc, 70:30:0.1, flow rate 10 ml/min).

*3.3.1 (2S, 2″S)-2,3,2″,3″-Tetrahydrorobustaflavone-4‴-methyl ether (**1**)*

An amorphous yellow powder; $[\alpha]_D^{28.4}$ −3.80 (*c* 1.0, DMSO); UV λ_{max} (MeOH) ($\log_e \varepsilon$) nm: 327 (3.88), 288 (4.47), 223 (4.66), 202 (4.75); IR ν_{max} (KBr) cm^{-1}: 3357, 1636, 1504, 1454, 1339, 1254, 1246, 1157, 1088, 829; ^1H NMR (400 MHz, DMSO-d_6): Table 1; ^{13}C NMR (100 MHz, DMSO-d_6): Table 2; ESIMS: 579 $[M + Na]^+$, 555$[M\text{-}H]^-$; HRESIMS *m/z*: 579.1240 $[M + Na]^+$ (calcd for $C_{31}H_{24}O_{10}Na$, 579.1267); CD (MeOH): $[\Delta\varepsilon]_{327\,nm}$ +6.82, $[\Delta\varepsilon]_{288\,nm}$ −15.15.

*3.3.2 (2S, 2″S)-2,3,2″,3″-Tetrahydrorobustaflavone-4′-methyl ether (**2**)*

An amorphous yellow powder; $[\alpha]_D^{28.0}$ −5.00 (*c* 1.0, DMSO); UV λ_{max} (MeOH) ($\log_e \varepsilon$) nm: 325 (3.84), 289 (4.66), 203 (4.91); IR ν_{max} (KBr) cm^{-1}: 3356, 3256, 1636, 1508, 1458, 1342, 1254, 1157, 1084, 829; ^1H NMR (400 MHz, DMSO-d_6): Table 1; ^{13}C NMR (100 MHz, DMSO-d_6): Table 2; ESIMS *m/z*: 579 $[M + Na]^+$, 555 $[M\text{-}H]^-$; HRESIMS *m/z*: 579.1241 $[M + Na]^+$ (calcd for $C_{31}H_{24}O_{10}Na$, 579.1267); CD (MeOH): $[\Delta\varepsilon]_{325\,nm}$ +4.85, $[\Delta\varepsilon]_{288\,nm}$ −8.33.

*3.3.3 (2S, 2″S)-2,3,2″,3″-Tetrahydrorobustaflavone-7″-methyl ether (**3**)*

An amorphous yellow powder; $[\alpha]_D^{28.8}$ −7.50 (*c* 1.0, DMSO); UV λ_{max} (MeOH) ($\log_e \varepsilon$) nm: 328 (3.88), 288 (4.70), 229 (4.87), 211 (4.90), 204 (4.90); IR ν_{max} (KBr) cm^{-1}: 3356, 1643, 1508, 1450, 1342, 1288, 1223, 1157, 1084, 1065, 833; ^1H NMR (400 MHz, DMSO-d_6): Table 1; ^{13}C NMR (100 MHz, DMSO-d_6): Table 2. ESIMS *m/z*: 579 $[M + Na]^+$, 555$[M\text{-}H]^-$; HRESIMS *m/z*: 579.1252 $[M + Na]^+$ (calcd for $C_{31}H_{24}O_{10}Na$, 579.1267); CD (MeOH): $[\Delta\varepsilon]_{328\,nm}$ +4.24, $[\Delta\varepsilon]_{288\,nm}$ −5.45.

*3.3.4 (2S)-2,3-Dihydrorobustaflavone-4′-methyl ether (**4**)*

A pale yellow crystal; m.p. 225–227°C; $[\alpha]_D^{29}$ +5.26 (*c* 1.0, DMSO); UV λ_{max} (MeOH) ($\log_e \varepsilon$) nm: 334 (4.57), 286 (4.66), 214 (4.88); IR ν_{max} (KBr) cm^{-1}: 3386, 1651, 1612, 1570, 1454, 1358, 1281, 1246, 1165, 1092, 837, 814; ^1H NMR (400 MHz, DMSO-d_6): Table 1; ^{13}C NMR (100 MHz, DMSO-d_6): Table 2; ESIMS *m/z*: 577 $[M + Na]^+$, 553$[M\text{-}H]^-$; HRESIMS *m/z*: 577.1124 $[M + Na]^+$ (calcd for $C_{31}H_{22}O_{10}$ Na, 577.1111); CD (MeOH): $[\Delta\varepsilon]_{334\,nm}$ +6.06, $[\Delta\varepsilon]_{288\,nm}$ −6.97.

3.4 The anoxic PC12 cell assay

The PC12 cells were cultured for five generations before use. The cells were dispersed with a pipette and seeded in a 35 mm culture dish at a density of 2×10^5-cells/ml, 2 ml/per dish. The culture medium consisted of 85% DMEM (Gibco, Grand Island, NY, USA), 5% fetal bovine serum (Hyclone), 10% heat-inactivated (56°C for 30 min) horse serum (Hyclone, South Logan, UT, USA), and glutamine 0.10 g/l. The PC12 cells were incubated in a gas mixture of 90% air and 10% CO_2 atmosphere, at 37°C. Cultured for 8 days, the PC12 cells were divided into groups with three dishes per group. Ten percent of DMSO (DMSO:normal saline, 1:9) was added to the PC12 cells as a control. The samples in 10% DMSO were added to the PC12 cells, respectively. After 24 h, the cells were moved into a sealed container with 90% N_2 and 10% CO_2 gas for 12 h. The cell viability was assessed by trypan blue exclusion. The viable cells were counted under inverted phase contrast microscope

(400 ×) in 10 visual fields randomly [11]. The data were presented as mean ± SD ($n = 10$) visual fields and statistics were performed by Student's *t*-test.

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