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Revision of Structures of Flavanoids from *Scutellaria indica* and Their Protein Tyrosine Phosphatase 1B Inhibitory Activity

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Abstract – The structures of flavonoids, 2(*S*)-5,7-dihydroxy-8,2'-dimethoxyflavanone (**1**), wogonin (**2**), 2(*S*)-5,7,2'-trihydroxy-8-methoxyflavanone (**3**), and 2(*S*)-5,2',5'-trihydroxy-7,8-dimethoxyflavanone (**4**), isolated from *Scutellaria indica* were revised on the basis of 2D NMR spectroscopy, including to gCOSY, gHSQC, and gHMBC. Compounds **1-4** were tested *in vitro* protein tyrosine phosphatase 1B (PTP1B) inhibitory activity. Compounds **2** and **4** exhibited weak PTP1B inhibitory activity with IC_{50} values of 208 and 337 μM, respectively.
Keywords – *Scutellaria indica*, flavonoid, revision of structure, PTP1B

Introduction

The whole herb of *Scutellaria indica* L. (Labiatae; perennial herb), known as "Han-xin-cao", is used for hemoptysis, hematemesis, anticancer, and other diseases in China, and distributed widely in Korea, Japan, China, Taiwan, and Indo-china (Miyaichi *et al.*, 1987). In the previous study on the bioactive compounds of *S. indica*, we isolated three flavanones and two flavones from the root of this plant and found cytotoxic activity against L1210, HL-60 and K562 tumor cells (Bae *et al.*, 1994). Flavonoids from this plant were assigned on the basis of the similarity of UV, IR, ^{1}H- and ^{13}C-NMR spectroscopic data to those their literature reports. During our systematic NMR study of flavonoids, we measures the ^{1}H- and ^{13}C-NMR spectra of 5,7-dihydroxy-8,2'-dimethoxyflavanone (**1**), wogonin (**2**), 5,7,2'-trihydroxy-8-methoxyflavanone (**3**), and 5,2',5'-trihydroxy-7,8-dimethoxyflavanone (**4**) from *S. indica*. The individual ^{1}H- and ^{13}C-NMR resonances of compounds **1-4** were assigned by using gradient (g) COSY, gHSQC, and gHMBC experiment. In addition, we describe protein tyrosine phosphatases 1B (PTP1B) inhibitory activity of compounds **1-4**.

Experimental

All melting points were determined on an Electrotherma SERIES IA9100 micro melting point apparatus and are uncorrected. IR and UV/VIS spectra were taken on Jasco IR Report-100 and Milton Roy Spectronics 3000 Array, respectively. Circular dichroic (CD) spectroscopy was obtained with Jasco J-600 spectropolarimeter. The NMR spectra were recorded on a Varian NMR System AS400 MHz, with chemical shifts being represented in ppm and tetramethylsilane used as an internal standard. Silica gel 60 (70-230 mesh, ASTM) was used for column chromatography. The spots were detected under UV radiation and by spraying with $FeCl_3$ solution, followed by heating.

Plant material – The roots of *S. indica* were collected during May, July, and August 1991 at Jin-do, Jeollanam-do Province, Korea, and dried at room temperature. A voucher specimen (No. 2589) was deposited at the herbarium of the Chungnam National University, Korea.

Isolation procedure – The dried and powdered roots of *S. indica* (505 g) were extracted with MeOH (5 × 2 L) by refluxing for 5 h to give 120 g solid extract. The residue (120 g) was diluted with water (1 L), and then partitioned successively with hexane (3 × 1 L), ether (3 × 1 L), EtOAc (3 × 1 L) and BuOH (3 × 1 L) to afford the hexane-(6 g), ether-(7.6 g), EtOAc-(17.1 g) and BuOH-soluble fractions (18.2 g), respectively. The ether-soluble fraction was chromatographed on a silica gel column. The column eluted using a stepwise gradient of hexane and EtOAc to give six fractions (Fr. 1-Fr. 6; 0.51 g, 0.42 g, 0.83 g, 0.58 g, 2.16 g, 2.96 g). Of six fractions, Fr. 2 was loaded onto a silica gel column, which was eluted with benzene : acetone (50 : 1, v/v) to give compound **1** (90 mg). Fr. 3 was recrystallized from EtOH to yield compound **2** (546 mg). The combined mixture, the mother liquor of Fr.

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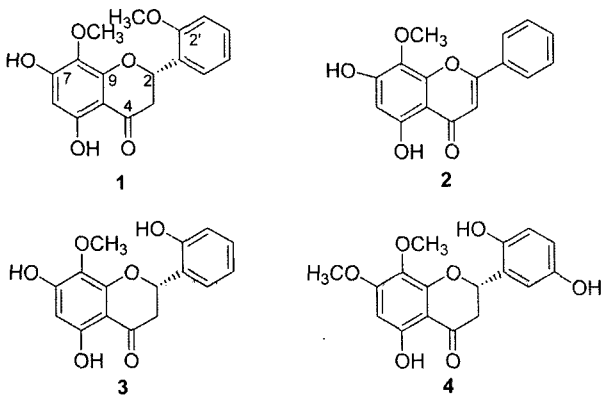

Fig. 1. Structures of compounds **1-4** from *S. indica*.

3 and Fr. 4, was chromatographed on a silica gel column with benzene : acetone (20 : 1 → 5 : 1, v/v) to yield compounds **3** (27 mg) and **4** (55 mg).

5,7-Dihydroxy-8,2'-dimethoxyflavanone (1) – pale yellowish white needles; mp 208-212 °C; IR ν_{max} cm^{-1}: 3200, 1635, 1600. UV (MeOH) λ_{max} nm (log ε): 291 (4.23), 342 (3.66); (MeOH + $AlCl_3$) 316 (4.37), 406 (3.62); (MeOH + $AlCl_3$ + HCl) 313 (4.32), 394 (3.63); (MeOH + NaOMe) 278 (3.60), 330 (4.41); (MeOH + NaOAc): 329 (4.43). ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2. CD (*c* 0.00004, MeOH) $[\theta]^{20}$ (nm): +7850 (311) (positive maximum), –42536 (287) (negative maximum).

Wogonin (2) – yellow needles; mp 203 °C; IR ν_{max} cm^{-1}: 3350, 1650, 1610, 1580. UV (MeOH) λ_{max} nm (log ε): 277 (4.52); (MeOH + $AlCl_3$) 295 (4.47), 333 (4.05), 406 (3.80); (MeOH + $AlCl_3$ + HCl) 295 (4.45), 333 (3.98), 411 (3.75); (MeOH + NaOMe) 286 (4.50), 385 (3.92); (MeOH + NaOAc): 381 (3.92). ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2.

5,7,2'-Trihydroxy-8-methoxyflavanone (3) – white needles; mp 197-202 °C; IR ν_{max} cm^{-1}: 3470, 1638, 1610. UV (MeOH) λ_{max} nm (log ε): 290 (4.28), 342 (3.69); (MeOH + $AlCl_3$) 315 (4.40), 402 (3.67); (MeOH + $AlCl_3$ + HCl) 312 (4.34), 398 (3.64); (MeOH + NaOMe) 330 (4.44); (MeOH + NaOAc): 329 (4.43). ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2. CD (*c* 0.000037, MeOH) $[\theta]^{20}$ (nm): +7378 (311) (positive maximum), –38432 (287) (negative maximum).

5,2',5'-Trihydroxy-7,8-dimethoxyflavanone (4) – white needles; mp 192-194 °C; IR ν_{max} cm^{-1}: 3380, 1630, 1605, 1590. UV (MeOH) λ_{max} nm (log ε): 290 (3.95), 346 (3.40); (MeOH + $AlCl_3$) 312 (4.37), 406 (3.40); (MeOH + $AlCl_3$ + HCl) 312 (4.04), 402 (3.36); (MeOH + NaOMe) 286 (3.81), 411 (3.56). ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2. CD (*c* 0.00004, MeOH) $[\theta]^{20}$ (nm): +7850 (311) (positive maximum), –42536 (287) (negative maximum).

Assay method of PTP1B inhibitory activity – PTP1B

Table 2. ^{13}C-NMR (100 MHz) spectroscopic data of compounds **1-4**

carbon	1[a]	2[a]	3[a]	4[a]
2	74.6	163.7	74.9	75.0
3	41.7	105.7	41.6	42.0
4	197.1	182.7	197.1	197.8
5	159.2	158.1	159.3	159.6
6	96.6	99.8	96.6	93.6
7	160.6	150.3	160.9	161.7
8	129.1	128.5	129.2	129.8
9	155.1	156.9	155.2	154.4
10	102.4	104.4	102.3	102.9
1'	127.0	131.5	125.5	126.1
2'	156.8	127.0	155.0	147.1
3'	112.0	129.9	116.1	116.9
4'	130.5	132.8	130.1	116.4
5'	121.2	129.9	119.8	150.6
6'	127.4	127.0	127.5	113.7
OCH_3	56.3 (C-2') 61.1 (C-8)	61.7 (C-8)	61.0 (C-8)	57.0 (C-7) 61.1 (C-8)

[a]DMSO-d_6

Table 1. ^{1}H-NMR (400 MHz) spectroscopic data of compounds **1-4**

H	1[a]	2[a]	3[a]	4[a]
2	5.71 dd (2.8, 13.0)		5.69 dd (3.0, 13.2)	5.63 dd (2.8, 12.8)
$3_{equ.}$ $3_{axi.}$	2.69 dd (2.8, 17.2) 3.16 dd (13.0, 17.2)	6.94 s	2.71 dd (3.0, 17.2) 3.18 dd (13.2, 17.2)	2.75 dd (2.8, 17.2) 3.11 dd (12.8, 17.2)
6	5.93 s	6.29 s	5.93 s	6.26 s
2'		8.04 m		
3'	7.05 d (7.6)	7.58 m	6.87 m	6.66 d (8.4)
4'	7.34 td (1.2, 7.6)	7.58 m	7.18 td (1.2, 7.6)	6.57 dd (1.2, 8.4)
5'	7.00 t (7.6)	7.58 m	6.85 m	
6'	7.51 dd (1.2, 7.6)	8.04 m	7.43 dd (1.2, 7.6)	6.85 d (1.2)
OCH_3	3.58 s (C-8) 3.77 s (C-2')	3.83 s (C-8)	3.60 s (C-8)	3.62 s (C-8) 3.82 s (C-7)

[a]DMSO-d_6. δ values in ppm and coupling constant (in parentheses) in Hz

(human, recombinant) was purchased from BIOMOL® International LP (USA) and the enzyme activity was measured using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate (Na *et al.*, 2006). To each 96-well (final volume : 200 μL) were added 2 mM *p*-NPP and PTP1B (0.05-0.1 μg) in a buffer containing 50 mM citrate (PH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT) with or without test compounds. Following incubation at 37 °C for 30 min, the reaction was terminated with 10 M NaOH. The amount of produced *p*-nitro phenol was estimated by measuring the absorbance at 405 nm. The nonenzymatic hydrolysis of 2 mM *p*-NPP was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme (Lin *et al.*, 1992).

Results and Discussion

Repeated column chromatography of the ether-soluble fraction of the MeOH extraction from the roots of *S. indica* led to the isolation of three flavanones (**1**, **3**, **4**) and one flavone (**2**).

Compound **1** was obtained as a pale yellowish white needle, mp 208-212 °C. The ultraviolet (UV) spectrum exhibited absorption bands at 291 and 342 nm due to A and B rings of the flavonoids. The bathochromic shift induced by an addition of $AlCl_3$ or NaOAc, suggested the presence of free hydroxyl groups at C-5 and C-7, respectively (Min *et al.*, 2001). The ^{1}H-NMR spectrum of **1** showed an ABX-type split signals at δ 2.69 (1H, dd, J = 2.8, 17.2 Hz, H-$3_{equ.}$), 3.16 (1H, dd, J = 13.0, 17.2 Hz, H-$3_{axi.}$) and 5.71 (1H, dd, J = 2.8, 13.0 Hz, H-2), which were assignable to a flavanone moiety (Shen *et al.*, 2006) (Table 1). This observation was further supported by the ^{13}C-NMR spectral assignment (an oxygenated methane carbon at δ 74.6, a methylene carbon at δ 41.7, and a carbonyl carbon at δ 197.1) coupled to DEPT, gHSQC, and gHMBC experiments (Table 2). Furthermore, one singlet signal in the ^{1}H-NMR spectrum was observed at δ 5.93, which correlated with an oxygenated aromatic carbon at δ_C 129.1 (C-8) and a quaternary carbon at δ_C 102.4 (C-10) in the gHMBC spectrum, assignable to H-6 of A-ring of the flavanone unit, compared with that of sophoraflavone G isolated form *Sophora moorcroftiang* (Shirataki *et al.*, 1988). Four aromatic proton signals at δ 7.00 (1H, t, J = 7.6 Hz, H-5'), 7.05 (1H, d, J = 7.6 Hz, H-3'), 7.34 (1H, td, J = 1.2, 7.6 Hz, H-4') and 7.51 (1H, dd, J = 1.2, 7.6 Hz, H-6') indicated the presence of a 2'-substituted phenyl group in ring B. This was further confirmed by the gHMBC spectrum, which showed correlation peaks between at δ_H 7.51 (H-6') and δ_C 74.6 (C-2), and between at δ_H 7.34 (H-4') and δ_C 156.8 (C-2'). In addition, the ^{1}H-NMR spectrum showed two methoxy groups at δ_H 3.58 and 3.77, which correlated with aromatic carbons at δ_C 129.1 (C-8) and 156.8 (C-2') in the gHMBC spectrum, respectively. The stereochemistry of C-2 of compound **1** was deduced from the circular dichroism (CD) spectrum. It is known that 2(*S*)-configuration of flavanone showed a positive Cotton effect due to n-p* transition (~ 330 nm) and a negative Cotton effect due to p-p* transition (270 ~ 290 nm) in the CD spectrum (Gaffield, 1970). The CD spectrum of compound **1** showed positive and negative Cotton effect at 311 and 287 nm, respectively, indicated that the asymmetric carbon at C-2 was concluded to be *S*. Thus, compound **1** was characterized as 2(*S*)-5,7-dihydroxy-8,2'-dimethoxyflavanone (Tomimory *et al.*, 1984).

Compound **2** was isolated as a yellow needle, mp 203 °C. The UV absorption bands at 277 nm and bathochromic shifts by addition of $AlCl_3$ and NaOAc, indicated the presence of free hydroxyl groups at C-5 and C-7, respectively. It exhibited two aromatic singlet signals at δ 6.29 and 6.94 (Table 1), which were assignable to C-6 and C-3 protons on flavone moiety in the ^{1}H-NMR spectrum, compared with that of scutellaprostin A from *Scutellaria prostrate* (Kikuchi *et al.*, 1991). The flavone moiety was further supported by the ^{13}C-NMR spectral data to contain 15 resonance peaks, including two olefinic carbons at δ 105.7 and 163.7, a carbonyl carbon at δ 182.7, four oxygenated aromatic carbons at δ 128.5, 150.3, 156.9 and 158.1, and eight aromatic carbons (Table 2). In addition, five aromatic proton signals at δ 7.58 (3H, m, H-3',4',5') and 8.04 (2H, m, H-2',6') supported the presence of an unsubstituted phenyl group in ring B on the flavone moiety. This was further confirmed by the gHMBC spectrum, which showed correlation peaks between at δ_H 8.04 (H-2',6') and δ_C 163.7 (C-2), and between at δ_H 7.58 (H-3',4',5') and δ_C 131.5 (C-1'). Furthermore, the ^{1}H-NMR spectrum showed a methoxy signal at δ_H 3.83, which correlated with aromatic carbons at δ_C 128.5 (C-8) in the gHMBC spectrum. Therefore, compound **2** was determined to be wogonin (5,7-dihydroxy-8-methoxyflavone) (Tomimory *et al.*, 1985).

Compound **3** was obtained as a white needle, mp 197-202 °C. The UV spectrum exhibited absorption bands at 290 and 342 nm due to A and B rings of the flavonoids. The bathochromic shift induced by an addition of $AlCl_3$ or NaOAc, suggested the presence of free hydroxyl groups at C-5 and C-7, respectively. Inspection of ^{1}H- and ^{13}C-NMR spectral data of compound **3** revealed the presence of the same structural moiety as in compound **1**. It exhibited

an ABX-type split protons at δ 2.71 (1H, dd, J = 3.0, 17.2 Hz, H-$3_{equ.}$), 3.18 (1H, dd, J = 13.2, 17.2 Hz, H-$3_{axi.}$) and 5.69 (1H, dd, J = 3.0, 13.2 Hz, H-2), five aromatic signals (δ 5.93, s, H-6; δ 6.85, m, H-5'; 6.87, m, H-3'; δ 7.18, td, J = 1.2, 7.6 Hz, H-4'; δ 7.43, dd, J = 1.2, 7.6 Hz, H-6'), and a methoxy group at δ 3.60 in the ^{1}H-NMR spectrum (Table 1). The methoxy group (δ_H 3.60) correlated with a quaternary aromatic carbon at δ_C 129.2 (C-8) in the gHMBC spectrum. The C-2 stereochemistry of compound **3** was confirmed by the CD data in the same way as in the case of compound **1**. Therefore, the structure of compound **3** was established as 2(*S*)-5,7,2'-tryhydroxy-8-methoxyflavnone (Miyaichi *et al.*, 1987).

Compound **4** was isolated as a white needle, mp 192-194 °C. The UV spectrum exhibited absorption bands at 290 and 346 nm due to A and B rings of the flavonoids. The ^{1}H-NMR spectrum of compound **4** showed an ABX-type split signals at δ 2.75 (1H, dd, J = 2.8, 17.2 Hz, H-$3_{equ.}$), 3.11 (1H, dd, J = 12.8, 17.2 Hz, H-$3_{axi.}$) and 5.63 (1H, dd, J = 2.8, 12.8 Hz, H-2), which were assignable to a flavanone moiety, compared with that of compound **1**. One singlet signal in the ^{1}H-NMR spectrum was observed at δ 6.26, which correlated with an oxygenated aromatic carbon at δ_C 129.8 (C-8) and a quaternary carbon at δ_C 102.9 (C-10) in the gHMBC spectrum, assignable to H-6 of A-ring of the flavanone unit. In addition, three aromatic proton signals at δ 6.57 (1H, dd, J = 1.2, 8.4 Hz), 6.66 (1H, d, J = 8.4 Hz), and 6.85 (1H, d, J = 1.2 Hz) indicated the presence of a 1,2,5-trisubstituted phenolic ring, compared with that of 2(*S*)-5,7,2',5'-tetrahydroxy-6-methoxyflavanone isolated from *Scutellaria scandens* (Miyaichi *et al.*, 1988). This was further confirmed by the gHMBC spectrum, which showed correlation peaks between at δ_H 6.66 (H-3') and δ_C 150.6 (C-5'), between at δ_H 6.57 (H-4') and δ_C 147.1 (C-2'), and between at δ_H 6.85 (H-6') and δ_C 147.1 (C-2')/75.0 (C-2). In addition, the ^{1}H-NMR spectrum showed two methoxy groups at δ_H 3.62 and 3.82, which correlated with aromatic carbons at δ_C 129.8 (C-8) and 161.7 (C-7) in the gHMBC spectrum. The 2(*S*)-stereochemistry was determined by the same way as in the case of compound **1**. From these results, the structure of compound **4** was determined to be 2(*S*)-5,2',5'-trihydroxy-7,8-dimethoxyflavanone (Miyaichi *et al.*, 1987).

Compounds **1-4** were evaluated for their PTP1B inhibitory activity, and the results are presented in Table 3. Wogonin (**2**) and 2(*S*)-5,2',5'-trihydroxy-7,8-dimethoxy-flavanone (**4**) showed weak inhibitory activity with IC_{50} values of 208 and 337 μM, respectively. However, 2(*S*)-5,7-dihydroxy-8,2'-dimethoxyflavanone (**1**) and 2(*S*)-5,7,2'-tryhydroxy-8-methoxyflavnone (**3**) were inactive against PTP1B inhibitory activity. These results suggested that the flavonoid isolated from *S. indica* showed weak inhibitory activity against PTP1B, compared with that of RK-682 (IC_{50}, 4.5 μM) used as a positive control (Hamaguchi *et al.*, 1995).

Table 3. Inhibitory activity of the compounds **1-4** against PTP1B

compound	IC_{50} values (μM)[a]
2(*S*)-5,7-dihydroxy-8,2'-dimethoxyflavanone (**1**)	> 500
wogonin (**2**)	208
2(*S*)-5,7,2'-trihydroxy-8-methoxyflavanone (**3**)	> 500
2(*S*)-5,2',5'-trihydroxy-7,8-dimethoxyflavanone (**4**)	337
RK-682[b]	4.5

[a]The value represent the mean ± S.D. of three experiments.
[b]Used as positive control.

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