A New Flavone *C*-Glycoside and Antiplatelet and Vasorelaxing Flavones from *Gentiana arisanensis*

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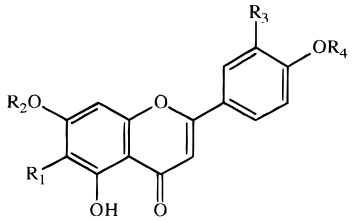
A new flavone *C*-glycoside, isovitexin 6″-*O*-glucoside (**1**), and three known flavonoids, quercetin, isovitexin, and luteolin-7-*O*-*β*-D-glucoside, have been further isolated from the whole plant of *Gentiana arisanensis* Hayata. The new compound was characterized by spectral methods and chemical reactions. The antiplatelet effects of isovitexin 6″-*O*-glucoside (**1**), isoorientin (**2**), **2** peracetate (**3**), isovitexin (**4**), luteolin 7-*O*-*β*-D-glucoside (**5**), luteolin (**6**), isoorientin 6″-*O*-glucoside (**7**), and **7** peracetate (**8**) were studied using washed rabbit platelets. Of the compounds tested, **6** showed potent antiplatelet effects on arachidonic acid (AA)-induced platelet aggregation (IC_{50} = 43.5 μM). The effect of **2**, **5**, and **6** on the contraction of rat thoracic aorta was also studied. Compound **6** depressed markedly the contraction induced by Ca^{2+} (1.9 mM) in high-K^+ (80 mM) medium, with an IC_{50} of about 156 μM and also inhibited noradrenaline (3 μM)-induced phasic and tonic contractions, with an IC_{50} of about 68 and 72 μM, respectively.

Recently we demonstrated that quercetin and kaempferol derivatives and apigenin inhibited the aggregation of rabbit platelets caused by various inducers.[1−3] In our search for antiplatelet compounds from Formosan Gentianaceous plants, we previously reported the isolation of two known compounds, isoorientin (**2**) and isoorientin 6″-*O*-glucoside (**7**), and a new flavone *C*-glycoside, isoorientin 6″-*O*-caffeate from *Gentiana arisanensis* Hayata.[4] In a continuation of this research a new flavone *C*-glycoside, isovitexin 6″-*O*-glucoside (**1**) and three known flavonoids, quercetin, isovitexin (**4**), and luteolin-7-*O*-*β*-D-glucoside (**5**) were further isolated from this plant. The structural characterization of **1** and the antiplatelet and vasorelaxing activities of the isolates are reported herein.



1: R_1=Glc(6→1)Glc, R_2=R_3=R_4=H
2: R_1=Glc, R_2=R_4=H, R_3=OH
4: R_1=Glc, R_2=R_3=R_4=H
5: R_1=R_4=H, R_2=Glc, R_3=OH,
6: R_1=R_2=R_4=H, R_3=OH
7: R_1=Glc(6→1)Glc, R_2=R_4=H, R_3=OH

Compound **1** showed UV absorption similar to that of isovitexin (**4**).[5] Its IR spectrum exhibited absorption bands at 3500 (OH) and 1640 (CO). The ^{1}H-NMR spectrum of **1** indicated the presence of two glucosyl anomeric proton signals at δ 4.85 (d, J = 7.5 Hz, H-1‴) and 5.79 (d, J = 10 Hz, H-1″), two one proton singlets at δ 6.70 (H-8) and 6.84 (H-3), and two pairs of 2H doublets at δ 7.20 (J = 8.4 Hz, H-3′ and H-5′) and 7.85 (J = 8.4 Hz, H-2′ and H-6′). Based on the above evidence, and the presence of bathochromic shifts induced by $AlCl_3$ and NaOAc and the absence of bathochromic shifts induced by NaOAc−H_3BO_3 in the UV spectrum, **1** was concluded to be an isovitexin *O*-glucoside. Acid hydrolysis of **1** afforded glucose, as detected by TLC, and isovitexin (**4**), identified by comparison of spectral data with those of authentic sample.

The ^{13}C-NMR spectra of **1** and **4** (Table 1) were assigned by ^{1}H-decoupled spectra, DEPT pulse sequence, and comparison of chemical shifts with those of isovitexin and isoorientin 6″-*O*-glucoside reported in literature.[4,6] The chemical shift values of glucosyl C-6″ and C-5″ of **1** indicated a downfield and upfield shift, respectively, compared to those of corresponding data for **4** (Table 1). Therefore **1** was characterized as isovitexin 6″-*O*-glucoside (1).

The antiplatelet effects of **1**, **2**, **2** peracetate (**3**), **4**, **5**, aglycon of **5** (**6**), **7**, and **7** peracetate (**8**) were studied using procedures to evaluate the aggregation of washed rabbit platelets induced by thrombin (0.1 U/mL), arachidonic acid (AA) (100 μM), collagen (10 μg/mL), and platelet-activating factor (PAF) (2 ng/mL), and the results are shown in Table 2. The *C*- or *O*-glycosylflavones **1**, **2**, **4**, and **5** did not show potent antiplatelet effects on platelet aggregation induced by various inducers. The *O*-glycosylation or acetylation of *C*-glycosylflavone also did not enhance the antiplatelet effects on platelet aggregation induced by various inducers. Compound **6** (aglycon of **2**, **5**, and **7**) exhibited potent antiplatelet effects on AA- and collagen-induced platelet aggregation and significant antiplatelet effects on thrombin- and PAF-induced platelet aggregation. Its inhibitory effects on collagen-, thrombin-, and PAF-induced platelet aggregation were marked above 300 μM but insignificant below 300 μM. Compound **6** inhibited AA-induced platelet aggregation in a concentration-

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Table 1. ^{13}C-NMR Chemical Shifts of **1** and **4**[a]

carbon	**1** (pyridine-d_5)	**4** (pyridine-d_5)
2	164.2	164.3
3	103.8	103.9
4	182.8	182.9
5	157.5	157.5
6	109.8	110.1
7	162.6	165.0
8	94.9	94.7
9	161.9	162.1
10	105.3	104.8
1′	122.9	122.2
2′	128.8	128.9
3′	116.8	116.8
4′	161.9	162.7
5′	116.8	116.8
6′	128.8	128.9
1″	75.8	75.6
2″	72.8	72.9
3″	80.4	80.6
4″	71.7	71.9
5″	81.3	83.0
6″	70.0	62.7
1‴	105.3	
2‴	75.0	
3‴	78.4	
4‴	71.4	
5‴	78.5	
6‴	62.5	

[a] The number of protons directly attached to each carbon was verified with DEPT pulse sequence.

Table 2. Effects of **1**−**8** and Aspirin on Platelet Aggregation Induced by Thrombin, Arachidonic Acid (AA), Collagen, and Platelet-Activating Factor (PAF) in Washed Rabbit Platelets[a]

	aggregation (%)			
agent (μM)	thrombin (0.1U/mL)	AA (100 μM)	collagen (10 μg/mL)	PAF (2 ng/mL)
control	92.8 ± 0.5 (3)	93.0 ± 0.5 (4)	90.2 ± 1.7 (3)	90.3 ± 1.6 (4)
1 (300)	87.4 ± 0.7 (3)	84.9 ± 0.1 (3)	85.6 ± 0.1 (3)	84.6 ± 2.9 (3)
2 (300)	85.5 ± 2.0 (3)	66.9 ± 6.9 (3)[c]	60.8 ± 8.6 (3)[c]	77.1 ± 4.9 (3)[d]
3 (300)	79.0 ± 4.4 (3)[d]	72.4 ± 3.6 (3)[b]	68.6 ± 1.9 (3)[b]	74.7 ± 5.0 (3)[d]
4 (300)	86.5 ± 0.8 (3)[d]	85.1 ± 1.1 (3)	84.7 ± 0.3 (3)	83.9 ± 3.3 (3)
5 (300)	87.0 ± 2.6 (3)	80.2 ± 1.9 (4)[c]	80.5 ± 1.3 (3)[b]	71.9 ± 5.7 (4)[d]
6 (300)	84.7 ± 1.4 (3)[c]	0.0 ± 0.0 (5)[b]	2.8 ± 2.3 (3)[b]	53.0 ± 6.2 (4)[b]
6 (150)		0.0 ± 0.0 (5)[b]		
6 (60)		20.5 ± 13.6 (5)[b]		
6 (30)		53.0 ± 10.1 (5)[c]		
6 (15)		79.2 ± 3.6 (5)		
7 (300)	85.7 ± 1.9 (3)	83.3 ± 1.0 (3)	85.4 ± 0.3 (3)	83.5 ± 4.6 (3)
8 (100)		79.9 ± 1.9 (3)[c]	80.5 ± 0.9 (3)[c]	
aspirin (50)	91.9 ± 2.5 (3)	0.0 ± 0.0 (3)[b]	85.4 ± 3.9 (3)	90.5 ± 1.2 (3)

[a] Washed rabbit platelets were preincubated with various agents, aspirin, or DMSO (0.5%, control) at 37 °C for 3 min, and the inducer was then added. Percentages of aggregation are presented as mean ± s.e.m. (n). [b] $p < 0.001$. [c] $p \leq 0.01$. [d] $p < 0.05$ as compared with control values.

dependent manner, and the IC_{50} value was about 43.5 μM with minimal effect at 15 μM and maximal effect at 150 μM. Apigenin (aglycon of **1** and **4**) (100 μg/mL) almost completely abolished the aggregation induced by AA, markedly inhibited those by ADP and PAF, and did not significantly affect those by thrombin and ionophore A 23187.[3] It is clearly indicated the *C*- or *O*-glycosylation of flavone luteolin (**6**) or apigenin did not enhance the antiplatelet effects.

Aspirin, a cyclooxygenase inhibitor, was used in this study as a positive control. It was found (Table 2) that aspirin (50 μM) inhibited completely the platelet aggregation induced by AA but not that induced by collagen, thrombin, or PAF. But **6** (300 μM) produces substantial inhibition of PAF and almost complete inhibition of collagen. Further experiments are needed to elucidate the mechanism of action.

Table 3. Effects of **2**, **5**, and **6** on High K^+- and Ca^{2+}-induced and Noradrenaline-induced Contraction of Rat Thoracic Aorta[a]

compound (μM)	K^+ (80 mM) + Ca^{2+} (1.9 mM)	noradrenaline (3 μM) phasic	noradrenaline (3 μM) tonic
control	100 ± 7.2	100 ± 4.1	100 ± 5.3
2 (150)	106 ± 6.0	91.7 ± 3.7	86.6 ± 6.2
5 (150)	93.4 ± 1.5	84.5 ± 0.8	88.2 ± 1.7
6 (300)	28.6 ± 0[b]	14.9 ± 3.1[b]	22.9 ± 4.7[b]
(90)	49.9 ± 0.9[c]	29.3 ± 11.3[c]	32.6 ± 4.7[b]
(30)	69.5 ± 3.2	64.7 ± 13.1	52.3 ± 4.6[c]
(9)		92.8 ± 0.7	99.2 ± 15.9

[a] Rat aorta was preincubated with **2**, **5**, and **6** or DMSO (0.1%, control) at 37 °C for 15 min; then high K^+ (80 mM) and Ca^{2+} (1.9 mM) or noradrenaline (3 μM) was added. Percentages of the contraction were calculated and presented as mean ± s.e.m. (n = 3). [b] $p < 0.001$. [c] $p < 0.01$ as compared with the respective control values.

In the rat thoracic aorta, **6** inhibited markedly the contraction induced by Ca^{2+} (1.9 mM) in high-K^+ (80 mM) medium in a concentration-dependent manner, with an IC_{50} of about 156 μM (Table 3). Compound **6** also inhibited the noradrenaline (3 μM)-induced phasic and tonic contraction in a concentration-dependent manner (Table 3), with IC_{50} of about 68 μM and 72 μM, respectively. These dual activities indicate that luteolin (**6**) may be developed as antithrombotic agent.

Experimental Section

General Experimental Procedures. Melting points are uncorrected. UV spectra were obtained on a JASCO UV-vis spectrophotometer; ^{1}H- and ^{13}C-NMR (400 MHz) spectra were recorded on Varian Unity-400 spectrometer; IR spectra were recorded on a Hitachi Model 260-30 spectrometer; and MS were obtained on a JMS-HX 100 mass spectrometer.

Plant Material. The fresh whole plants of *G. arisanensis* (1.5 kg) were collected at Yu Shieh, Chiayi Hsieh, Taiwan, during April 1994, and a voucher specimen deposited in the author's laboratory.

Extraction and Isolation. The fresh whole plants (1.5 kg) were extracted as previously reported.[4] The EtOAc extract was chromatographed on Si gel. Elution with $CHCl_3$−MeOH (1:2) yielded quercetin (20 mg). Elution with $CHCl_3$−MeOH−H_2O (5:1:0.5) and EtOAc−MeOH−H_2O (9:1:1) yielded a brown mixture of eluates. The eluates were further chromatographed on Sephadex LH-20. Elution with MeOH yielded **2** (40 mg) and 4 (30 mg). The *n*-BuOH extract was also chromatographed on Si gel eluted with EtOAc−MeOH−H_2O (4:1:1). After rechromatography on Sephadex LH-20 with MeOH, the 50% MeOH eluates gave **5** (55 mg), isoorientin 6″-caffeoate (15 mg), **7** (70 mg), and **1** (20 mg). The compounds quercetin, **4**, and **5** were identified by UV, IR, NMR and MS and compared the spectral data with those of authentic samples.[7−9]

Isovitexin 6″-*O*-glucoside (1): yellowish powder (MeOH), mp 214−216 °C; UV (MeOH) λ_{max} 260, 325, (MeOH−$AlCl_3$) 250, 300 (sh), 350, 400 (sh), (MeOH−NaOAc) 255, 335, 400 (sh), (MeOH−NaOAc−H_3BO_3) unchanged; IR (KBr) ν_{max} (3500 (OH), 1640 (CO); ^{1}H-NMR (pyridine-d_5, 400 MHz), see text; ^{13}C-NMR (pyridine-d_5, 400 MHz), see Table 1; FABMS (*m*-nitrobenzyl alcohol) (positive mode) *m/z*: no molecular ion peak, 413 (M − *O*-glucose − H) (1), 286 (1) 270 (M − *C*-glucose +

H), 176 (69), 165 (15), 154 (50), 136 (64), 133 (93), 121 (16), 118 (13), 93 (26), 77 (100) (6).

Preparation of Isoorientin Peracetate (3), Luteolin (6), and isoorientin 6″-*O*-glucoside Peracetate (8). Compounds **2** (1) and **7** were acetylated with anhydrous pyridine and Ac_2O, and **5** was hydrolyzed with concd HCl by the usual method to yield **3**, **6**, and **8**. Compounds **3**, **6**, and **8** were identified by spectral methods, and we compared the spectral data to those of authentic samples.[4,8]

Platelet Aggregation Assays. Washed rabbit platelets were obtained from ethylene diamine tetraacetic acid (EDTA)-anticoagulated platelet-rich plasma (PRP) according to procedures described previously.[10] Platelet numbers were counted by a Coulter Counter (Model ZM) and adjusted to 4.5×10^8 platelets/mL. The platelet pellets were suspended in Tyrode's solution containing (mM): 136.8 NaCl, 2.8 KCl, 11.9 $NaHCO_3$, 2.1 $MgCl_2$, 0.33 NaH_2PO_4, 1.0 $CaCl_2$, and 11.2 glucose with 0.35% bovine serum albumin. All glassware was siliconized. Four minutes before addition of the aggregation inducer, the platelet suspension was stirred at 1200 rev min^{-1}. Aggregation was measured by the turbidimetric method.[11] The absorbance of the platelet suspension was taken as 0% aggregation, and the absorbance of platelet-poor plasma or platelet-free Tyrode's solution was 100% aggregation. Aggregation was measured by a Lumi-aggregometer (Chrono-Log Co.) connected to dual channel recorders. Compounds were dissolved in DMSO. In order to eliminate the effect of solvent on platelet aggregation, the final concentration of DMSO was fixed at 0.5%. Collagen (type 1, bovine Achilles tendon), obtained from Sigma Chemical Co. (St. Louis, MO), was homogenized in 25 mM of HOAc and stored at −70 °C at a concentration of 1 mg/mL. PAF(1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), also purchased from Sigma, was dissolved in $CHCl_3$ and diluted into 0.1% bovine serum albumin−saline solution immediately prior to use. AA, ADP, bovine serum albumin, noradenaline, and EDTA (disodium salt) were also purchased from Sigma. Thrombin (bovine), was obtained from Park Davis Co. (Detroit MI) and dissolved in 50% glycerol to give a stock solution of 100 NIH units/mL.

Aortic Contraction. Wistar rats of either sex, 250−300 g, were killed by a flow to the head. The thoracic aorta was isolated and excess fat and connective tissue were removed. Vessels were cut into rings of about 5 mm in length, mounted in organ baths containing 5 mL of Krebs' solution, maintained at 37 °C, and bubbled with a 95% O_2−5% CO_2 mixture. Two stainless-steel hooks were inserted into the aortic lumen; one was fixed, while the other was connected to a transducer. Aortas were equilibrated in the medium for 90 min with three changes of Krebs' solution and maintained under an optimal tension of 1 g before specific experimental protocols were initiated; contractions were recorded isometrically via a force displacement transducer connected to a Gould polygraph (Model 2400). The final concentration of DMSO was fixed at 0.1%.

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