Acylated Flavonol Glycosides from the Flower of *Inula britannica*

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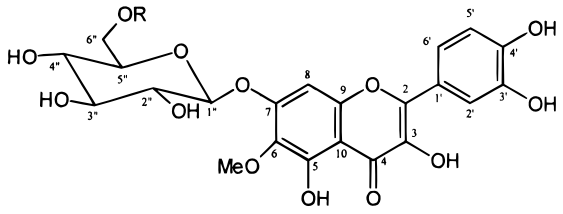
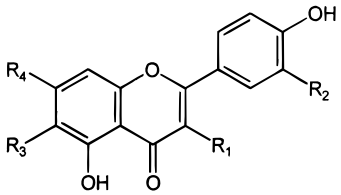
Three new acylated flavonol glycosides, patuletin 7-*O*-(6″-isobutyryl)glucoside (**1**), patuletin 7-*O*-[6″-(2-methylbutyryl)]glucoside (**2**), and patuletin 7-*O*-(6″-isovaleryl)glucoside (**3**), were isolated from the *n*-BuOH extract of *Inula britannica* flowers by bioassay-guided fractionation, together with other known flavonoids. The structures were elucidated by 1D and 2D NMR, FABMS, and other spectral analyses. The eight flavonoids, including new compounds (**1**−**3**), patulitrin (**7**), nepitrin (**8**), axillarin (**10**), patuletin (**11**), and luteolin (**12**), showed profound antioxidant activity in DPPH assay and cytochrome-*c* reduction assay using HL-60 cell culture system.

Inula britannica (Asteraceae) is a wild plant found in Eastern Asia, including Korea, Japan, and China. The flowers of *I. britannica* have been used for the treatment of digestive disorders, bronchitis, and inflammation in traditional medicine. The *n*-BuOH extract of *I. britannica* flowers showed antioxidant activity in preliminary DPPH assays. This prompted us to search for antioxidant compounds from this plant. Bioassay-guided fractionation of the *n*-BuOH extract led to the isolation of flavonoids **1**−**12**,



1 R = $COCH(CH_3)_2$
2 R = $COCH(CH_3)CH_2CH_3$
3 R = $COCH_2CH(CH_3)_2$



4 R_1 = OGlc, R_2 = H, R_3 = H, R_4 = OH
5 R_1 = OGlc, R_2 = OCH_3, R_3 = H, R_4 = OH
6 R_1 = H, R_2 = H, R_3 = OCH_3, R_4 = OGlc
7 R_1 = OH, R_2 = OH, R_3 = OCH_3, R_4 = OGlc
8 R_1 = H, R_2 = OH, R_3 = OCH_3, R_4 = OGlc
9 R_1 = OH, R_2 = H, R_3 = H, R_4 = OH
10 R_1 = OCH_3, R_2 = OH, R_3 = OCH_3, R_4 = OH
11 R_1 = OH, R_2 = OH, R_3 = OCH_3, R_4 = OH
12 R_1 = H, R_2 = OH, R_3 = H, R_4 = OH

including three new compounds (**1**−**3**): patuletin 7-*O*-(6″-isobutyryl)glucoside (**1**), patuletin 7-*O*-[6″-(2-methylbutyryl)]glucoside (**2**), and patuletin 7-*O*-(6″-isovaleryl)-glucoside (**3**), along with kaempferol 3-glucoside (**4**), isorhamnetin 3-glucoside (**5**), hispidulin 7-glucoside (**6**), patulitrin (**7**), nepitrin (**8**), kaempferol (**9**), axillarin (**10**), patuletin (**11**), and luteolin (**12**). In this paper, we report the isolation, structure elucidation, and biological activities of the compounds isolated from *I. britannica*.

Results and Discussion

The *n*-BuOH extract of *I. britannica* was chromatographed on Si gel using $CHCl_3$−MeOH (20:1) followed by stepwise addition of MeOH to afford 13 fractions. Each fraction was further subjected to Si gel, MCI gel, Sephadex LH-20 column chromatography, and reversed-phase HPLC to afford 12 flavonoids. Compound **1** was obtained as a yellow amorphous powder with $[\alpha]_D$ − 0.35° (*c* 1.00, MeOH). The UV spectrum of **1** in MeOH exhibited characteristic absorbance bands of flavonol at 258 and 372 nm. The band at 372 nm was shifted +60 nm by $AlCl_3$−HCl and +31 nm by NaOAc−H_3BO_3. These results suggested that compound **1** was a flavonol with a hydroxyl group at C-5 and an *ortho*-dihydroxyl group in the B ring.[1] Its IR spectrum indicated the presence of two carbonyl groups (1654 and 1719 cm^{-1}), which meant the flavonoid carbonyl and ester carbonyl groups. The molecular formula, $C_{26}H_{28}O_{14}$, was inferred from the $[M + 1]^+$ peak at *m*/*z* 565 in the FABMS, and it was supported by ^{13}C NMR and DEPT spectra, which showed 26 resonance lines consisting of three methyls, one methylene, 10 methines, and 12 quaternary carbons.

The ^1H and ^{13}C NMR spectra (Table 1) revealed that compound **1** was a flavonoid with glycosidic and acyl functionalities present. The ^1H NMR signals [δ 6.74 (1H, H-8, *s*), 6.89 (1H, H-5′, d, J = 8.5 Hz), 7.60 (1H, H-6′, d, J = 8.5 Hz), 7.74 (1H, H-2′, s), 3.87 (3H, OCH_3, s)] of **1** are consistent with a 3,5,6,7,3′,4′-hexahydroxyflavone derivative. Furthermore, it was suggested that compound **1** possessed one methoxyl group at C-6 and was substituted with one glucosyl moiety [one anomeric proton at δ 5.08 (1H, d, J = 6.6 Hz)] at C-7 when compared with ^{13}C NMR data of patuletin as a reference flavonoid.[2] In addition, ^1H NMR spectrum showed an isobutyrate at δ 2.54 (1H, m, J = 6.8, 7.1 Hz), 0.92 (3H, d, J = 7.1 Hz), 1.01 (3H, d, J = 6.8 Hz) as an acyl moiety, and it was confirmed by a base peak at *m*/*z* 71 derived from −$COCH(CH_3)_2$ in the mass spectrum. Lower field shifts of H-6″ (δ 4.17, 4.44) and C-6″ (δ 65.8) signals indicated the bonding site of the acyl function.[3] Unequivocal information could be obtained by 2D NMR (^1H−^1H COSY, ^{13}C−^1H COSY, and HMBC) spectra. The important connectivities between an acyl carbonyl group (C-1‴) and the two protons of H-6″ were observed in the HMBC (Table 1, Figure 1), which revealed

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Table 1. NMR Spectral Data of Patuletin 7-*O*-(6″-isobutyryl)glucoside (**1**), Patuletin 7-*O*-[6″-(2-methylbutyryl)]glucoside (**2**), and Patuletin 7-*O*-(6″-isovaleryl)glucoside (**3**)[a]

	1			**2**	**3**
carbon	δ_C	δ_H	HMBC	δ_C	δ_C
2	149.8			150.0	149.9
3	138.2			138.2	138.3
4	178.3			178.5	178.5
5	153.8			153.9	154.0
6	134.2			134.3	134.3
7	158.2			158.2	158.2
8	96.0	6.74 (s)	C-4, 6, 7, 9, 10	96.2	96.1
9	153.8			154.0	153.9
10	107.5			107.6	107.5
1′	124.7			124.7	124.7
2′	117.1	7.74 (s)	C-4′, 6′	117.0	117.1
3′	147.1			147.2	147.2
4′	149.8			149.9	149.9
5′	117.0	6.89 (d, 8.5)	C-1′, 3′	117.0	117.1
6′	122.6	7.60 (d, 8.5)	C-2′, 4′	122.6	122.7
OCH_3	62.3	3.87 (s)		62.3	62.3
1″	102.4	5.08 (d, 6.6)	C-3″	102.4	102.3
2″	75.5	3.56 (br s)		75.5	75.4
3″	78.6	3.56 (br s)		78.7	78.7
4″	72.5	3.34 (m)	C-3″	72.6	72.7
5″	76.6	3.75 (t, 7.6, 8.3)		76.5	76.4
6″a	65.8	4.44 (t, 7.1, 11.2)	C-1‴	65.8	65.6
6″b	65.8	4.17 (d, 11.7)	C-5″, 1‴	65.8	65.6
1‴	179.4			179.0	175.4
2‴	35.9	2.54 (m, 6.8, 7.1)	C-1‴, 3‴ (4‴)	43.0	44.9
3‴	20.1	1.01 (d, 6.8)	C-1‴, 2‴, 4‴	28.5	27.5
4‴	20.1	0.92 (d, 7.1)	C-1‴, 2‴, 3‴	12.4	23.2
5‴				17.8	23.2

[a] ^{13}C NMR (75 MHz; δ in ppm), ^1H NMR (300 MHz; δ in ppm, multiplicity, J in Hz), and HMBC in 300 MHz.

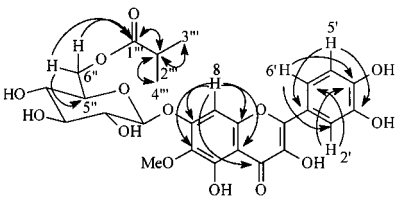

Figure 1. HMBC correlations of compound **1**.

the location of the acyl function at C-6″. These spectral data established **1** to be patuletin 7-*O*-(6″-isobutyryl)glucoside.

Compound **2** was a yellow, amorphous powder with $[\alpha]_D$ + 0.22° (*c* 1.00, MeOH). The UV and IR spectra of **2** were very similar to **1**, but its ^1H NMR spectrum showed different acyl signals compared with **1**. The molecular formula, $C_{27}H_{30}O_{14}$, was inferred from the $[M + 1]^+$ peak at *m*/*z* 579 in the FABMS, and it was supported by ^{13}C NMR and DEPT spectroscopy, which showed 27 carbon signals consisting of three methyls, two methylenes, 10 methines, and 12 quaternary carbons. The nature of the acyl function [δ 2.37 (1H, m), 1.47 (1H, m), 1.24 (1H, m), 0.62 (3H, t, J = 7.6 Hz), 1.00 (3H, d, J = 6.8 Hz)] was easily determined as a 2-methylbutyrate by a $^1H-^1H$ COSY spectrum. The structure of compound **2** is therefore characterized as patuletin 7-*O*-[6″-(2-methylbutyryl)glucoside].

The molecular formula of compound **3** was confirmed as $C_{27}H_{30}O_{14}$ from the FABMS. NMR and other spectroscopic data were nearly identical with those of **2**, the only difference was that an isovalerate ester group [δ 2.12 (1H, m), 1.89 (1H, m), 0.67 (3H, d, J = 6.6 Hz), and 0.63 (3H, d, J = 6.6 Hz) in the ^1H NMR spectrum] appeared instead of the 2-methylbutyrate of **2**. In conclusion, the structure of the novel compound **3** was determined to be patuletin 7-*O*-(6″-isovaleryl) glucoside.

Table 2. Antioxidant Activities of *n*-BuOH Fractions and Compounds from *I. britannica*

	DPPH assay (IC_{50})[a]		cytochrome-*c* reduction assay (IC_{50})[a]	
compound	μg/mL	μM	μg/mL	μM
1	14.3	25.3	10.6	18.8
2	23.5	40.6	11.2	19.4
3	14.3	24.7	11.7	20.2
4	>100		>50	
5	>100		>50	
6	>100		>50	
7	10.3	20.8	8.1	16.4
8	12.2	25.5	24.3	50.8
9	74.4	260.1	10.0	34.9
10	12.4	35.8	2.9	8.3
11	9.0	27.1	10.1	30.5
12	7.2	25.2	12.1	25.5
gallic acid	3.7	21.8	3.2	18.8

[a] Results are expressed as IC_{50} values (μg/mL and μM). Data for active fractions were mean of triplicates. Gallic acid used for positive control.

Some acylated glycoside flavonols at sugar hydroxyl groups via ester linkage have been identified as plant constituents. Most acyl groups are phenolic acids[4] such as *p*-coumaric, caffeic, or gallic acid, but aliphatic moieties, such as isobutyrate, 2-methylbutyrate,[5] and isovalerate described here, are rare. Therefore, it is meaningful to report compounds **1**−**3** as aliphatic acyl flavonols from natural sources for the first time. Compounds **4**−**12** were identified by comparing their physical and spectral data with the literature values: kaempferol 3-glucoside (**4**),[6] isorhamnetin 3-glucoside (**5**),[7] hispidulin 7-glucoside (**6**),[8] patulitrin (**7**),[9] nepitrin (**8**),[10] kaempferol (**9**),[6] axillarin (**10**),[11] patuletin (**11**),[12] and luteolin (**12**).[6]

For the screening and evaluation of antioxidant activity of pure compounds and/or plant extracts, DPPH and cytochrome-*c* reduction assays were adopted. Compounds **1**−**3**, **7**, **8**, and **10**−**12** exhibited inhibitory activity as strong as the positive control against TPA-induced free radical formation in a HL-60 cell culture system and showed free radical scavenging activity in the DPPH assay (Table 2). Three new acylated flavonoids (IC_{50} of **1**−**3**, 10.6, 11.2, 11.7 μg/mL, respectively) showed similar activity with parent compounds, patuletin (IC_{50}, 10.1 μg/mL) and patulitrin (IC_{50}, 8.1 μg/mL). Generally, the presence of hydroxyl substituents on the flavonoid nucleus enhanced activity, whereas substitution by methoxyl groups diminished antioxidant activity. Substitution patterns on the B-ring especially affected antioxidant potencies of the flavonoids.[16] The di-OH substitution at 3′ and 4′ of the B-ring is particularly important to the oxygen radical absorbing activity of a flavonoid.[17] These trends are consistent with inactive flavonoids (**4**−**6** and **9**) that possess monohydroxyl in the B-ring.

Experimental Section

General Experimental Procedures. Melting points were determined with a DuPont 910 differential scanning calorimeter. UV spectra were recorded in MeOH using a Shimadzu UV-1601 PC spectrophotometer. IR spectra were recorded in KBr using a Perkin−Elmer 1710 spectrometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL-GSX 300 spectrometer (300 MHz). Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. Column chromatography was performed over Si gel 60 (230−400 mesh, Merck), MCI gel (CHP 20P 75−100 μm, Mitsubishi Chemical Co., Japan), or Sephadex LH-20 (25−100 μm, Pharmacia).

Plant Materials. The flowers of *I. britannica* were purchased from Korea Export and Import Federation of Drugs,

Seoul, Korea, in 1994, and identified by Dr. Dae Suk Han, an emeritus professor, College of Pharmacy, Seoul National University. A voucher specimen (SNUP-089) has been deposited in the Herbarium of the College of Pharmacy, Seoul National University in Korea.

Extraction and Isolation. The dried flowers of *I. britannica* (7.8 kg) were extracted three times with 80% MeOH using an ultrasonic apparatus for 3 h. The MeOH extract (1083.1 g) was suspended in water and partitioned with *n*-hexane, $CHCl_3$, and *n*-BuOH, successively. The *n*-BuOH extract (322.0 g) was subjected to Si gel column chromatography eluting with $CHCl_3$−MeOH (20:1) followed by stepwise addition of MeOH to yield 13 fractions. Fraction 8 (34.1 g) was applied to MCI gel column chromatography (water→25% MeOH→50% MeOH →75% MeOH→100% MeOH) to yield five fractions. Fraction 8-5 was rechromatographed on MCI gel (50% MeOH→75% MeOH→100% MeOH), Sephadex LH-20 (MeOH), and preparative TLC (EtOAc−formic acid−acetic acid−water, 300:11:11: 27) and was purified by HPLC (RP_{18}, 4 μm, 270 nm, AcCN−1% acetic acid, 30:70; **1**: t_R = 16.18 min; **2**: t_R = 22.38 min; **3**: t_R = 23.80 min) to give **1** (24 mg), **2** (5 mg), and **3** (3 mg). Fraction 8-4 was subjected to MCI gel (50% MeOH→75% MeOH→100% MeOH), Si gel ($CHCl_3$−MeOH, 10:1), and Sephadex LH-20 (MeOH) chromatography and purified by HPLC (RP_{18}, 4 μm, 270 nm, AcCN−1% acetic acid, 20:80; **4**: t_R = 31.00 min; **5**: t_R = 34.24 min; **6**: t_R = 41.93 min) to give compounds **4** (12 mg), **5** (5 mg), and **6** (5 mg). Fraction 9 (20.0 g) was applied to MCI gel (H_2O→25% MeOH→50% MeOH →75% MeOH→100% MeOH) to yield five fractions. Fraction 9-3 was chromatographed on a Sephadex LH-20 (MeOH) to yield two fractions. Fraction 9-3-1 was purified by HPLC (RP_{18}, 10 μm, 270 nm, 62% MeOH) to yield compound **7** (70 mg, t_R = 11.46 min), and fraction 9-3-2 was purified by HPLC (RP_{18}, 10 μm, 270 nm, 60% MeOH) to yield compound **8** (60 mg, t_R = 13.88 min).

Fraction 4 (4.1 g) was applied to Si gel column chromatography ($CHCl_3$−MeOH, 30:1), vacuum column chromatography ($CHCl_3$−EtOAc−MeOH, 100:24:4), and purified by HPLC (RP_{18}, 10 μm, 254 nm, 80% MeOH, **9** (60 mg: t_R = 13.88 min, **10**: t_R = 26.46 min) to give **9** (60 mg) and **10** (60 mg). Fraction 6 (24.4 g) was chromatographed on Si gel eluting with $CHCl_3$−EtOAc−MeOH gradients (10:1:1 → 1:1:1) to yield 10 fractions. Fraction 6-5 was purified by Sephadex LH-20 column chromatography (MeOH) to yield compounds **11** (9 mg) and **12** (10 mg).

Patuletin 7-*O*-(6″-isobutyryl)glucoside (1): yellow powder; mp 147−149 °C $[\alpha]_D$ −0.35° (*c* 1.00, MeOH); UV (MeOH) λ_{max} nm 372, 258, (NaOH) 424, 341, 247, ($AlCl_3$) 439, 272, ($AlCl_3$/HCl) 432, 269, (NaOAc) 386, 260, (NaOAc/H_3BO_3) 403, 264; IR (KBr) ν_{max} 3421 (OH), 1719 (ester C═O), 1654 (α,β-unsaturated C═O), 1070 (C−O); ^{1}H NMR (300 MHz, CD_3OD), see Table 1; ^{13}C NMR (75 MHz, CD_3OD), see Table 1; FABMS (glycerol) *m*/*z* 565 $[M + H]^+$ (7), 333 [aglycon + H]$^+$ (4), 133 (100) EIMS 70 eV *m*/*z* 332 [aglycon]$^+$ (97), 314 [aglycon − 18]$^+$ (52), 289 [aglycon − 43]$^+$ (90), 71(100).

Patuletin 7-*O*-[6″-(2-methylbutyryl)]glucoside (2): yellow powder; $[\alpha]_D$ + 0.22° (*c* 1.00, MeOH); UV (MeOH) λ_{max} 368, 256, (NaOH) 447, 356, ($AlCl_3$) 441, 271, ($AlCl_3$/HCl) 427, 268, (NaOAc) 399, 263, (NaOAc/H_3BO_3) 401, 320 nm; IR (KBr) ν_{max} 3421 (OH), 2915 (CH), 1718 (ester C═O), 1653 (α,β-unsaturated C═O), 1560, 1477, 1277, 1201, 1070 (C−O); ^{1}H NMR (300 MHz, CD_3OD) δ 6.82 (1H, s, H-8), 7.75 (1H, s, H-2′), 6.85 (1H, d, *J* = 8.5, H-5′), 7.63 (1H, d, *J* = 8.5, H-6′), 3.89 (3H, s, OCH_3), 5.11 (1H, d, *J* = 6.6, H-1″), 3.55 (2H, m, H-2″, -3″), 3.34 (1H, m, H-4″), 3.79 (1H, dd, *J* = 7.6, 8.3, H-5″), 4.45 (1H, d, *J* = 11.2, H-6″a), 4.17 (1H, dd, *J* = 7.8, 11.7, H-6″b), 2.37 (1H, m, *J* = 6.4, 7.1, H-2‴), 1.47 (1H, m, H-3‴a), 1.24 (1H, m, H-3‴b), 0.62 (3H, t, *J* = 7.6, H-4‴), 1.00 (3H, d, *J* = 6.8, H-5‴); ^{13}C NMR (75 MHz, CD_3OD), see Table 1; FABMS (glycerol) *m*/*z* 579 $[M + H]^+$ (3), 133 (100); EIMS 70 eV *m*/*z* 332 [aglycon]$^+$ (97), 314 [aglycon − 18]$^+$ (52), 289 [aglycon − 43]$^+$ (90), 71(100).

Patuletin 7-*O*-(6″-isovaleryl)glucoside (3): yellow powder; $[\alpha]_D$ + 0.24° (*c* 1.00, MeOH); UV (MeOH) λ_{max} 374, 259, (NaOH) 423, 361, ($AlCl_3$) 441, 272, ($AlCl_3$/HCl) 432, 269, (NaOAc) 386, 266, (NaOAc/H_3BO_3) 395, 264 nm; IR (KBr) ν_{max} 3422 (OH), 1718 (ester C═O), 1653 (α,β-unsaturated C═O), 1598, 1560, 1479, 1278, 1201, 1072 (C−O); ^{1}H NMR (300 MHz, CD_3OD) δ 6.80 (1H, s, H-8), 7.76 (1H, s, H-2′), 6.89 (1H, d, *J* = 8.3, H-5′), 7.66 (1H, d, *J* = 8.3, H-6′), 3.88 (3H, s, OCH_3), 5.10 (1H, d, *J* = 7.1, H-1″), 3.57 (2H, m, H-2″, -3″), 3.31 (1H, m, H-4″), 3.79 (1H, dd, *J* = 7.6, 8.3, H-5″), 4.42 (1H, d, *J* = 11.0, H-6″a), 4.22 (1H, dd, *J* = 7.8, 11.7, H-6″b), 2.12 (1H, m, H-2‴), 1.89 (1H, m, H-3‴), 0.67 (3H, d, *J* = 6.6, H-4‴), 0.63 (3H, d, *J* = 6.6, H-5‴); ^{13}C NMR (75 MHz, CD_3OD), see Table 1; FABMS (glycerol) *m*/*z* 579 $[M + H]^+$ (22), 333 [aglycon + H]$^+$ (13), 133 (100); EIMS 70 eV *m*/*z* 332 [aglycon]$^+$ (97), 314 [aglycon − 18]$^+$ (52), 289 [aglycon − 43]$^+$ (90), 71 (100).

Antioxidant Assay. DPPH assay was performed essentially according to the modified method of Kirby and Schmidt:[18] 95 μL of 3.2 × 10^{-4} M of DPPH solution in absolute EtOH and 5 μL of sample solution in DMSO were mixed in a 96-well plate. The optical density was measured at 515 nm after incubation of the plate for 1 h at 37 °C. The DPPH control contained no sample but was otherwise identical. The cytochrome-*c* reduction assay was performed according to Sharma et al.[19] HL-60 Cells were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 1% penicillin−streptomycin at 37 °C in humidified atmosphere at 5% CO_2 in air. Differentiation was induced by 7-day treatment with 1.3% DMSO, and the cells were cultured in a 96-well plate (1 × 10^6 cells per well) in HBSS. After the addition of TPA (8 μM) to induce free radical formation, cytochrome *c* (160 μM) and samples were added. The cells were incubated for 1 h at 37 °C, and antioxidant activity was determined by monitoring absorbance at 550 nm. The same reaction mixture, without the HL-60 cells, was used as a blank control.

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