Acetylated Flavonoid Glycosides Potentiating NGF Action from *Scoparia dulcis*

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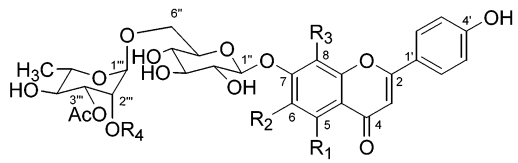
Three new acetylated flavonoid glycosides, 5,6,4′-trihydroxyflavone 7-*O*-α-L-2,3-di-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (**1**), apigenin 7-*O*-α-L-3-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (**2**), and apigenin 7-*O*-α-L-2,3-di-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (**3**), were isolated from *Scoparia dulcis* together with the known compound eugenyl *β*-D-glucopyranoside (**4**). Their structures were elucidated by spectroscopic analyses. Compounds **2** and **3** showed an enhancing activity of nerve growth factor-mediated neurite outgrowth in PC12D cells.

Scoparia dulcis L. (Scrophulariaceae) is a widespread tropical herbaceous medicinal plant, which has been used widely as a traditional folk medicine for its antipyretic and analgesic properties and for its use in treating bronchitis and gastric disorders in South America.[1] Previous investigations on *S. dulcis* have resulted in the isolation of diterpenes, flavonoids,[2,3] triterpenes,[4] and catecholamines.[1] However, there is no report on the isolation of acetylated flavonoid glycosides from this plant. In the course of our search for natural products that possess nerve growth factor (NGF)-potentiating activity or neurotrophic activity from medicinal plants, we have screened 20 Paraguayan plants for this activity in PC12D cells and found that the methanol extract of *S. dulcis* exhibited a significant activity enhancing NGF's action. Three new acetylated flavonoid glycosides, 5,6,4′-trihydroxyflavone 7-*O*-α-L-2,3-di-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (**1**), apigenin 7-*O*-α-L-3-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (**2**), and apigenin 7-*O*-α-L-2,3-di-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (**3**), were isolated from the aerial parts of *S. dulcis* by bioassay-guided fractionation together with a known compound, eugenyl *β*-D-glucopyranoside (**4**). Here we report the isolation and structure elucidation of compounds **1**−**3** and the biological activity of these compounds as enhancers of NGF action.

The ethyl acetate-soluble portion of the methanol extract of the aerial parts of *S. dulcis* was subjected to silica gel column chromatography (EtOAc−MeOH) to give seven fractions, I−VII. The active fraction IV was further resolved by a series of chromatographic separations including silica gel, Sephadex LH-20, and reversed-phase semipreparative HPLC (ODS column), to yield **1**−**4**.

The molecular formula of **1** was determined to be $C_{31}H_{34}O_{17}$ by HRFABMS [*m*/*z* 679.1852, $(M + H)^+$, Δ −2.0 mmu]. The IR spectrum indicated the presence of hydroxyl (3350 cm^{-1}), α,*β*-unsaturated carbonyl (1737 cm^{-1}), and aromatic rings (1606, 1560, 1488 cm^{-1}). The UV spectrum exhibited maximum absorptions at 333, 284, and 215 nm. Examination of the ^1H and ^{13}C NMR spectral data of **1** (Table 1) indicated that the molecule consisted of a flavone, two sugars, and two acetyl moieties.

The ^1H NMR spectrum showed two one-proton singlets at *δ* 6.61 (H-3) and 6.89 (H-8) and a pair of two-proton doublet signals (*J* = 7.8 Hz) at *δ* 7.81 (H-2′, 6′) and 6.92 (H-3′, 5′). The ^{13}C NMR spectrum disclosed the existence of nine sp^2 quaternary carbons (C-2, C-4, C-5, C-6, C-7, C-9, C-10, C-1′, and C-4′), including one carbonyl (C-4) and six oxygen-bearing ones (C-2, C-5, C-6, C-7, C-9, and C-4′), and six sp^2 methine carbons (C-3, C-8, C-2′, C-3′, C-5′, and C-6′) for the flavone aglycone. The LRFABMS revealed a fragment peak at *m*/*z* 287 $[C_{15}H_{11}O_6]^+$, and EIMS showed a peak at *m*/*z* 286 $[C_{15}H_{10}O_6]^+$. On the basis of the above analyses of spectral data the flavone aglycone was suggested as 5,6,7,4′-tetrahydroxyflavone. In the ^1H NMR spectrum, the *β*-D-glucopyranosyl moiety signals were found at *δ* 5.10 (H-1″), 3.59 (H-2″), 3.53 (H-3″), 3.45 (H-4″), 3.75 (H-5″), 4.05 (H-6″a), and 3.77 (H-6″b). Moreover, the α-L-rhamnopyranosyl moiety signals appeared at *δ* 4.74 (H-1‴), 5.18 (H-2‴), 5.03 (H-3‴), 3.41 (H-4‴), 3.78 (H-5‴), and 1.77 (H_3-6‴). The ^{13}C NMR peaks of the glucose and rhamnose units were found at δ_C 102.7 (C-1″), 75.4 (C-2″), 78.1 (C-3″), 72.1 (C-4″), 77.9 (C-5″), and 68.0 (C-6″) and δ_C 99.9 (C-1‴), 71.9 (C-2‴), 73.9 (C-3‴), 72.1 (C-4‴), 71.0 (C-5‴), and 18.6 (C-6‴) by DEPT and HMQC experiments, respectively. The anomeric configuration of the rhamnopyranosyl was identified as the α configuration by the coupling constants $J_{H-1‴,H-2‴}$ = 1.8 Hz and $J_{H-1‴,C-1‴}$ = 173.5 Hz.[5] The ^1H NMR spectrum also revealed two three-proton singlets at *δ* 1.94 (2‴-$OCOCH_3$) and 1.78 (3‴-$OCOCH_3$), and these methyl groups were assigned to the two acetyl moieties, which were connected to the C-2‴ and C-3‴ sites of the rhamnose unit through an oxygen atom



	R_1	R_2	R_3	R_4
1	OH	OH	H	Ac
2	OH	H	H	H
3	OH	H	H	Ac

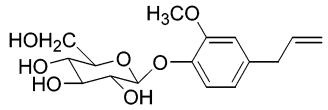


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Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1**−**3** (CD_3OD, ^{1}H NMR 600 MHz, ^{13}C NMR 150 MHz)

	1		**2**		**3**	
position	^{13}C	^{1}H (J = Hz)	^{13}C	^{1}H (J = Hz)	^{13}C	^{1}H (J = Hz)
2	167.9 s		167.7 s		167.7 s	
3	104.3 d	6.61, 1H, s	104.9 d	6.63, 1H, s	104.9 d	6.65, 1H, s
4	185.3 s		184.9 s		184.4 s	
5	148.8 s		163.9 s		163.8 s	
6	132.6 s		102.1 d	6.47, 1H, d, J = 1.8	102.5 d	6.49, 1H, d, J = 1.8
7	153.4 s		165.4 s		165.5 s	
8	96.0 d	6.89, 1H, s	96.6 d	6.75, 1H, d, J = 1.8	96.7 d	6.47, 1H, d, J = 1.8
9	152.3 s		159.9 s		159.9 s	
10	108.3 s		107.8 s		108.0 s	
1′	124.4 s		123.7 s		123.8 s	
2′	130.5 d	7.81, 1H, d, J = 7.8	130.5 d	7.85, 1H, d, J = 8.4	130.5 d	7.88, 1H, d, J = 9.0
3′	117.8 d	6.92, 1H, d, J = 7.8	118.0 d	6.91, 1H, d, J = 8.4	118.0 d	6.93, 1H, d, J = 9.0
4′	163.5 s		163.9 s		163.9 s	
5′	117.8 d	6.92, 1H, d, J = 7.8	118.0 d	6.91, 1H, d, J = 8.4	118.0 d	6.93, 1H, d, J = 9.0
6′	130.5 d	7.81, 1H, d, J = 7.8	130.5 d	7.85, 1H, d, J = 8.4	130.5 d	7.88, 1H, d, J = 9.0
1″	102.7 d	5.10, 1H, d, J = 7.8	102.3 d	5.06, 1H, d, J = 7.2	102.2 d	5.08, 1H, d, J = 7.2
2″	75.4 d	3.59, 1H, dd, J = 9.0, 7.8	75.6 d	3.46, 1H, m	75.5 d	3.45, 1H, m
3″	78.1 d	3.53, 1H, t, J = 9.0	78.6 d	3.48, 1H, m	78.6 d	3.49, 1H, m
4″	72.1 d	3.45, 1H, t, J = 9.0	72.2 d	3.38, 1H, t, J = 9.0	72.2 d	3.41, 1H, t, J = 9.0
5″	77.9 d	3.75, 1H, dd, J = 9.0, 6.0	78.0 d	3.71, 1H, dd, J = 9.0, 6.0	78.0 d	3.73, 1H, m
6″	68.0 t	4.05, 1H, d, J = 9.6	68.2 t	4.02, 1H, d, J = 9.6	68.2 t	4.04, 1H, d, J = 9.6
		3.77, 1H, dd, J = 9.6, 6.0		3.65, 1H, d, J = 9.6, 6.0		3.69, 1H, d, J = 9.6, 6.0
1‴	99.9 d	4.74, 1H, d, *J = 1.8*	102.6 d	4.69, 1H, d, J = 1.8	102.6 d	4.71, 1H, d, J = 1.8
2‴	71.9 d	5.18, 1H, dd, J = 3.6, *1.8*	70.7 d	3.99, 1H, dd, J = 3.6, 1.8	70.7 d	4.01, 1H, dd, J = 3.0, 1.8
3‴	73.9 d	5.03, 1H, dd, J = 9.6, 3.6	76.6 d	3.47, 1H, dd, J = 9.6, 3.6	76.6 d	4.95, 1H, dd, J = 10.0, 3.0
4‴	72.1 d	3.41, 1H, t, J = 9.6	72.1 d	3.48, 1H, t, J = 9.6	72.2 d	3.50, 1H, t, J = 10.0
5‴	71.0 d	3.78, 1H, dd, J = 9.6, 6.0	70.7 d	3.73, 1H, m	70.8 d	3.70, 1H, m
6‴	18.6 q	1.77, 3H, d, J = 6.0	18.7 q	1.16, 3H, J = 6.0	18.7 t	1.18, 3H, d, J = 6.6
2‴-O*CO*CH$_3$	172.2 s				173.1 s	
2‴-OCO*CH$_3$*	21.3 q	1.94, 3H, s			21.5 q	2.01, 3H, s
3‴-O*CO*CH$_3$	172.8 s		173.3 s		173.3 s	
3‴-OCO*CH$_3$*	21.5 q	1.78, 3H, s	21.7 q	1.92, 3H, s	21.8 q	1.94, 3H, s

by the HMBC correlations of H-2‴/2‴-OCOCH$_3$ and H-3‴/3‴-OCOCH$_3$ (Figure S1), respectively.

Finally, the connection of these substructures was completed by the HMBC spectrum of **1**. The α-L-2,3-di-*O*-acetylrhamnopyranosyl unit was linked to C-6″ of the *β*-D-glucopyranosyl moiety by the ^3J interactions of H-6″a/C-1‴, H-6″b/C-1‴, and H-1‴/C-6″. Furthermore, the sugar chain was connected to the C-7 position of the flavone aglycone through an oxygen atom by the HMBC correlation of H-1″/C-7 (Figure S1). Thus, compound **1** was determined to be 5,6,4′-trihydroxyflavone 7-*O*-α-L-2,3-di-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside.

The structure of **2** was evaluated by comparing its spectral data with those of **1**. Compound **2** had a molecular formula of $C_{29}H_{32}O_{15}$ by HRFABMS [*m*/*z* 643.1668, $(M + Na)^+$, Δ +3.1 mmu]. For the aglycone moiety, the differences between **2** and **1** occurred at ring A. The ^{1}H NMR spectrum of **2** showed the typical pattern of apigenin: three one-proton signals at *δ* 6.63 (H-3), 6.47 (H-6), and 6.75 (H-8) and two two-proton doublet signals (J = 8.4 Hz) at *δ* 7.85 (H-2′, 6′) and 6.91 (H-3′, 5′). For the glycosyl moiety, the acetyl resonances occurred at δ_C 173.3 (3‴-OCOCH$_3$) and 21.7 (3‴-OCOCH$_3$) in the ^{13}C NMR spectrum and at *δ* 1.92 (3‴-OCOCH$_3$) in the ^{1}H NMR spectrum. Full analyses of the ^{1}H and ^{13}C NMR spectral data of **2**, supported by the ^{1}H−^{1}H COSY, DEPT, HMQC, and HMBC experiments, permitted assignment of all proton and carbon resonances (Table 1). Consequently, compound **2** was determined to be apigenin 7-*O*-α-L-3-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside.

Compound **3** showed a pseudomolecular ion peak at *m*/*z* 663 $(M + H)^+$, and the molecular formula, $C_{31}H_{34}O_{16}$, was established by HRFABMS [*m*/*z* 663.1952, $(M + H)^+$, Δ +2.9 mmu]. The ^{1}H and ^{13}C NMR spectral data of **3** were comparable with those of **2**, except for the presence of an additional acetyl group at δ_C 173.1 (2‴-OCOCH$_3$) and 21.5 (2‴-OCOCH$_3$) in the ^{13}C NMR spectrum of **3** and at *δ* 2.01 (2‴-OCOCH$_3$) in the ^{1}H NMR spectrum of **3**. The acetyl group was linked to the C-2‴ position by the HMBC correlation of H-2‴/2‴-OCOCH$_3$. All protons and carbons of **3** could be assigned on the basis of the analyses of the 2D NMR spectral data of **3** including ^{1}H−^{1}H COSY, HMQC, and HMBC spectra. Thus, compound **3** was elucidated to be apigenin 7-*O*-α-L-2,3-di-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside.

The known compound **4** was identified as eugenyl *β*-D-glucopyranoside on the basis of comparing its spectral data with literature values.[6]

The propensity of **1**−**4** to enhance the effects of NGF to stimulate neurite outgrowth from PC12D cells was assessed using the methodology previously reported.[7] In control experiments, the percentages of neurite-bearing cells were 27.1% following incubation with 2 ng/mL NGF and 71.3% with 30 ng/mL NGF after 48 h, respectively. Compounds **2** and **3** (10, 30, and 100 *μ*mol) did not induce neurite outgrowth from PC12D cells in the absence of NGF, but **2** and **3** at a concentration of 100 *μ*mol markedly increased the NGF (2 ng/mL)-induced proportion of neurite-bearing cells by 16.1% and 14.9%, respectively. However, **1** and **4** at concentrations of 10−100 *μ*mol did not show NGF-potentiating activity on PC12D cells.

We successfully isolated the acetylated flavone glycosides **1**−**3** from *S. dulci* and demonstrated their NGF-potentiating activity for the first time. Compounds that can enhance the action of NGF to stimulate neurite outgrowth from PC12D cells may be useful in the treatment of neurological disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and human immunodeficiency virus associated dementia (HAD).[8,9]

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanaco micro-melting point apparatus and are uncorrected. Optical rotations were measured with a Horiba SEPA-300 polarimeter. The ultraviolet spectra were recorded on a Shimadzu UV-260 spectrophotometer, and the infrared spectra were obtained on a Shimadzu FTIR-8300 spectrophotometer. 1D and 2D NMR spectra were recorded in CD_3OD on a JEOL ECP-600 instrument. Chemical shifts were measured using a residual MeOH-d_4 multiplet (δ_H 3.30 and δ_C 49.0) as internal standard. EIMS and FABMS were recorded on JMS DX-303 and JMS AX-500 spectrometers.

Plant Material. The aerial parts of *S. dulcis* L. were provided by Seiwa Pharmaceuticals Co., Ltd. (Ibaragi, Japan), on April 20, 2000. The botanical identification was made by Mr. Tetsuo Nakasumi (Instituto de Pesquisas de Plantas Medicinais do Brasil, Sao Paulo, Brazil). A voucher specimen (No. 68534) is deposited in the Graduate School of Pharmaceutical Sciences, Tohoku University (Sendai, Japan).

Extraction and Isolation. The aerial parts of *S. dulcis* (1 kg) were extracted with MeOH (3 × 8 L), and the MeOH extract (205 g) was partitioned by EtOAc, *n*-BuOH, and H_2O. The EtOAc-soluble fraction (61 g) was subjected to silica gel column chromatography (EtOAc−MeOH) to give fractions I−VII. The most active fraction, IV (1.8 g), was separated by a series of bioassay-directed chromatographic separations, employing Sephadex LH-20 and silica gel column chromatographic steps and culminating in a reversed-phase semipreparative HPLC on a YMC-AM 324 column (ODS, 30 × 1 cm i.d. stainless column, 43% MeOH in H_2O, 1 mL/min) to give compounds **1** (10.2 mg) and **4** (14.6 mg). By a similar procedure and final purification with a semipreparative HPLC column (YMC-AM 324, ODS, 30 × 1 cm i.d. stainless column, 46% MeOH in H_2O, 1 mL/min) separation of fraction IV afforded compounds **2** (1.9 mg) and **3** (2.1 mg).

5,6,4′-Trihydroxyflavone 7-*O*-α-L-2,3-di-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (1): yellow powder (MeOH); mp 168−171 °C; $[\alpha]_D^{27}$ −55.4° (*c* 0.9, MeOH); UV (MeOH) λ_{max} (log ϵ) 333 (4.42), 284 (3.80), 215 (4.81) nm; IR (film) ν_{max} 3373, 2932, 1737, 1606, 1560, 1488 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); EIMS *m*/*z* 303 (10), 286 (100), 257 (6), 231 (6), 168 (34); FABMS *m*/*z* 701 $[M + Na]^+$ (14), 679 $[M + H]^+$ (9), 307 (12), 287 (31), 176 (27), 154 (100), 136 (86); HRFABMS *m*/*z* 679.1852 (calcd for $C_{31}H_{35}O_{17}$, 679.1872).

Apigenin 7-*O*-α-L-3-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (2): yellow powder (MeOH); $[\alpha]_D^{27}$ −45.4° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 333 (4.10), 275 (3.35), 215 (4.40) nm; IR (film) ν_{max} 3370, 2932, 1740, 1603, 1555, 1480 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); FABMS *m*/*z* 643 $[M + Na]^+$ (7), 621 $[M + H]^+$ (2), 601 (3), 413 (17), 271 (17), 171 (20), 149 (31), 115 (100); HRFABMS *m*/*z* 643.1668 (calcd for $C_{29}H_{32}O_{15}Na$, 643.1637).

Apigenin 7-*O*-α-L-2,3-di-*O*-acetylrhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (3): yellow powder (MeOH); $[\alpha]_D^{27}$ −50.2° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 330 (4.20), 278 (3.45), 215 (4.15) nm; IR (film) ν_{max} 3375, 2928, 1740, 1603, 1555, 1460 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); FABMS *m*/*z* 663 $[M + H]^+$ (2), 647 (2), 413 (38), 391 (8), 279 (4), 149 (100); HRFABMS *m*/*z* 663.1952 (calcd for $C_{31}H_{35}O_{16}$, 663.1923).

Bioassay Procedure. PC12D cells were dissociated by incubation with 1 mmol of ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) in phosphate-buffered saline (PBS) for 30 min and then were seeded in 24-well culture plates (2×10^4 cells/well) coated with poly-L-lysine. After 24 h, the medium was changed to test medium containing various concentrations of NGF (30 ng/mL for positive control, 2 ng/mL for test samples and significant difference control), 1% fetal calf serum, 2% horse serum, and various concentrations of test compounds (10, 30, and 100 μmol). All stock solutions of the test compounds were prepared at 50 mmol in DMSO. After 48 h the cells were fixed with 2% glutaraldehyde at 37 °C for 1 h. The neurite outgrowth was assessed under a phase-contrast microscope. Neurite processes with a length equal to or greater than the diameter of the neuron cell body were scored as a neurite-bearing cell. The ratio of the neurite-bearing cells to total cells (with at least 100 cells examined/viewing area; 3 viewing areas/well; 6 wells/sample) was determined and expressed as a percentage. The data were analyzed by the Student *t*-test.

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Supporting Information Available: Selected HMBC correlations of **1** and spectra of **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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