# A New Iridoid Glycoside with Nerve Growth Factor-Potentiating Activity, Gelsemiol 6′-*trans*-Caffeoyl-1-glucoside, from *Verbena littoralis*

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**A new iridoid glycoside, gelsemiol 6′-*trans*-caffeoyl-1-glucoside (1), was isolated from *Verbena littoralis*, together with four known phenylethanoid glycosides, acteoside (2), 2′-acetylacteoside (3), jionoside (4), and isoverbascoside (5). Their structures were elucidated by spectral data. Compound 1 showed weak enhancement of nerve growth factor (NGF)-mediated neurite outgrowth from PC12D cells.**

**Key words** *Verbena littoralis*; iridoid glycoside; phenylethanoid glycoside; nerve growth factor (NGF)-potentiating activity; PC12D cells

*Verbena littoralis* H. B. K. (Verbenaceae) is a perennial medicinal plant native to Paraguay.[1] In the course of our continual investigations of bioactive substances from medicinal plants,[2—4] we found that the extract of *V. littoralis* exhibited significant activity in enhancing nerve growth factor (NGF)-mediated neurite outgrowth from PC12D cells. The EtOAc-soluble materials of the MeOH extract of the aerial parts of *V. littoralis* were repeatedly subjected to silica gel column and Sephadex LH-20 column chromatography, followed by reverse-phase semipreparative HPLC to yield gelsemiol 6′-*trans*-caffeoyl-1-glucoside (**1**), together with four known phenylethanoid glycosides, acteoside (**2**), 2′-acetylacteoside (**3**),[5] jionoside (**4**),[6] and isoverbascoside (**5**),[7] by monitoring the potentiation of NGF action in PC12D cells.

Compound **1** was obtained as colorless needle crystals. Its molecular formula $C_{25}H_{32}O_{12}$ was determined by high-resolution fast atom bombardment mass spectrometry (HR-FAB-MS) to be *m*/*z* 525.1921 ($[M+H]^+$, calcd. 525.1972) and 547.1876 ($[M+Na]^+$, calcd. 547.1791), which indicates 10 degrees of unsaturation. The IR spectrum of **1** indicated the presence of hydroxyl (3600—3300 $cm^{-1}$), lactone (1745 $cm^{-1}$), and ester carbonyl (1700 $cm^{-1}$) moieties. The $^1H$- and $^{13}C$-NMR spectroscopic data (Table 1) aided by the distortionless enhancement by polarization transfer (DEPT) and $^1H$-detected heteronuclear multiple quantum coherence (HMQC) experiments disclosed the presence of two ester carbonyls, three $sp^2$ quaternary carbons (two of which were oxygen bearing), 10 $sp^3$ methines (five of which were oxygen bearing), five $sp^2$ methines, four $sp^3$ methylenes (three of which were oxygen bearing), and one methyl group. The initial analysis of the NMR spectral data of **1** indicated that the molecule consisted of the moieties of a monoterpene iridoid, a monosaccharide, and a phenylpropene (Fig. 1). In the $^{13}C$-NMR and DEPT spectra of **1**, six carbons appeared at $\delta_C$ 104.61 (C-1′), 75.03 (C-2′), 77.99 (C-3′), 71.81 (C-4′), 75.50 (C-5′), and 64.56 (C-6′), suggesting the presence of a $\beta$-glucopyranosyl group which was confirmed by the detailed analysis of correlations in the $^1H$–$^1H$ correlation spectroscopy (COSY) (Fig. 2), HMQC, heteronuclear multiple bond connectivity (HMBC) (Fig. 2) and nuclear Overhauser effect spectroscopy (NOESY) (Fig. 2) spectra of **1**.

In the $^1H$-NMR spectrum of **1**, two pairs of double doublet signals ($J$=10.0, 4.0 Hz and $J$=10.5, 4.0 Hz) at $\delta$ 3.52 (H-1a) and 4.05 (H-1b), and 3.81 (H-3a) and 3.86 (H-3b) were assigned to the C-1 ($\delta_C$ 69.37) and C-3 ($\delta_C$ 63.12) positions based on the following HMBC correlations: H-1a with C-5 ($\delta_C$ 45.49), C-8 ($\delta_C$ 34.13), and C-9 ($\delta_C$ 50.30); H-1b with C-5; and H-3a (H-3b) with C-4 ($\delta_C$ 45.59), C-5, and C-11 ($\delta_C$ 181.66), respectively. The cyclopentane ring was determined by analyzing $^1H$–$^1H$ COSY and HMBC spectral data (Fig. 2). One doublet signal at $\delta$ 0.96 (3H, $J$=6.5 Hz, $H_3$-10) was assigned to the C-10 ($\delta_C$ 17.64) based on the HMBC correlations of H-7$\beta$ ($\delta$ 1.44)/C-10, H-8/C-10, $H_3$-10/C-7 ($\delta_C$ 42.71), $H_3$-10/C-8, and $H_3$-10/C-9. The presence of a five-membered lactone ring was deduced by the analysis of the downfield chemical shift signals of C-6 ($\delta_C$ 85.36) and H-6 ($\delta$ 4.91), which revealed that the methine (C-6) was linked to an acyloxy group, and this was confirmed by the HMBC correlations of H-6/C-11. Hence the iridoid substructure was demonstrated as shown in Fig. 1.

The *trans*-caffeoyl substructure was elucidated by analyzing the remaining signals of $^{13}C$- and $^1H$-NMR spectra data of **1** (Table 1) aided by the $^1H$–$^1H$ COSY and HMBC spectral data (Fig. 2).

The connections of three substructures were determined based on the HMBC spectrum. The C-1′ carbon atom of the $\beta$-glucopyranosyl group was attached to the C-1 position of the iridoid aglycon through an oxygen atom based on the obvious correlations from H-1a (H-1b) to C-1′ and from H-1′ to C-1 in the HMBC spectrum. The carbonyl C-1″ of the *trans*-caffeoyl group was linked to the C-6′ of the $\beta$-glucopyranosyl group through an oxygen atom because the HMBC spectrum revealed the correlations of H-6′a (H-6′b)/C-1″. Thus the planar structure of gelsemiol 6′-*trans*-caffeoyl-1-glucoside was elucidated to be **1**.

The NOESY spectrum of **1** allowed many of the stereochemical features of compound **1** to be assigned. The presence of cross-peaks of H-5/H-6, H-6/H-7$\beta$, H-7$\beta$/$H_3$-10, H-9/H-5, and H-9/$H_3$-10 indicated that H-5, H-6, H-9, and 8-$CH_3$ were oriented on the same side of the cyclopentane in a $\beta$-position. H-4 and H-8 were in an $\alpha$-position on the basis of the correlations of H-4/H-8 and H-7$\alpha$/H-8. For the conformation of the sugar moiety, the NOESY correlations among

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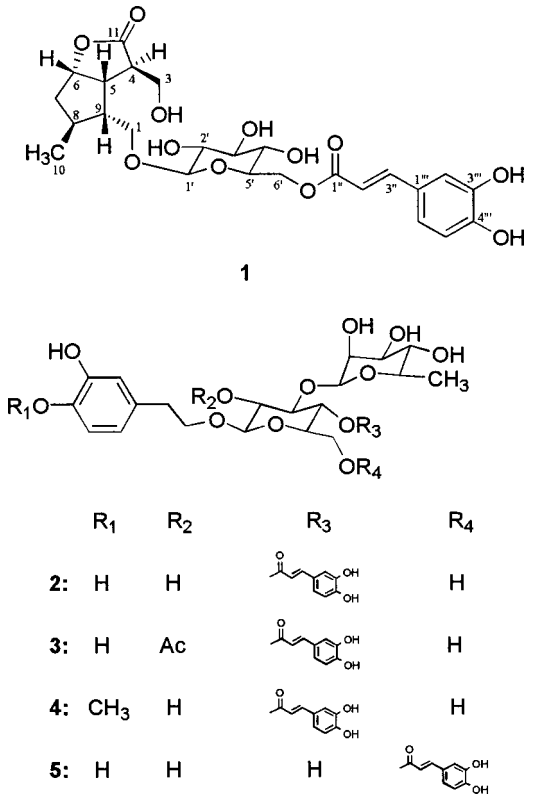


Fig. 1. Chemical Structures of **1**—**5**

Table 1. NMR Spectral Data of **1** ($CD_3OD$, $^1$H-NMR 500 MHz, $^{13}$C-NMR 125 MHz)

| Position | $\delta_H$ [a)] | $J$ (in Hz) | $\delta_C$ [a)] |
|---|---|---|---|
| 1a | 3.52 | dd, 10.0, 4.0 | 69.37 (t) |
| b | 4.05 | dd, 10.0, 4.0 | |
| 3a | 3.81 | dd, 10.5, 4.0 | 63.12 (t) |
| b | 3.86 | dd, 10.5, 4.0 | |
| 4 | 2.87 | dt, 8.0, 4.0 | 45.59 (d) |
| 5 | 3.14 | m | 45.49 (d) |
| 6 | 4.91 | dd, 7.0, 6.0 | 85.36 (d) |
| 7$\alpha$ | 2.04 | dd, 14.0, 6.0 | 42.71 (t) |
| $\beta$ | 1.44 | ddd, 14.0, 12.0, 6.0 | |
| 8 | 1.76 | m | 34.13 (d) |
| 9 | 1.91 | m | 50.30 (d) |
| 10 | 0.96 | d, 6.5 | 17.64 (q) |
| 11 | | | 181.66 (s) |
| 1′ | 4.27 | d, 8.0 | 104.61 (d) |
| 2′ | 3.19 | t, 8.0 | 75.03 (d) |
| 3′ | 3.51 | dd, 9.5, 8.0 | 77.99 (d) |
| 4′ | 3.34 | t, 9.5 | 71.81 (d) |
| 5′ | 3.52 | ddd, 9.5, 6.0, 2.5 | 75.50 (d) |
| 6′a | 4.38 | dd, 12 .0, 6.0 | 64.56 (t) |
| b | 4.47 | dd, 12.0, 2.5 | |
| 1″ | | | 169.08 (s) |
| 2″ | 6.28 | d, 16.0 | 114.90 (d) |
| 3″ | 7.57 | d, 16.0 | 147.25 (d) |
| 1‴ | | | 127.66 (s) |
| 2‴ | 7.04 | d, 2.0 | 115.17 (d) |
| 3‴ | | | 146.86 (s) |
| 4‴ | | | 149.69 (s) |
| 5‴ | 6.77 | d, 8.0 | 116.53 (d) |
| 6‴ | 6.94 | dd, 8.0, 2.0 | 123.05 (d) |

*a*) $^1$H- and $^{13}$C-NMR signals were assigned by $^1$H–$^1$H COSY, long range $^1$H–$^1$H COSY, DEPT, HMQC, and HMBC experiments.

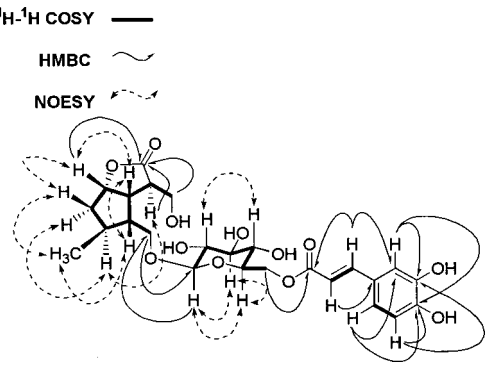


Fig. 2. Selected Correlations of the HMBC, NOESY and $^1$H–$^1$H COSY Spectra of **1**

the protons of the $\beta$-glucopyranosyl moiety and the coupling constants (see Table 1, in particular, $J_{1',2'}$=8.0 Hz) indicated a $^1C_4$ chair conformation of the $\beta$-glucopyranose moiety. The absolute stereostructure of the sugar was determined to be the D-form by the HPLC analysis of a thiazolidine derivative which was obtained by condensing the aqueous acidic hydrolysate of **1** with L-cysteine methyl ester hydrochloride.[8] The iridoid moiety of **1** is related to gelsemiol, which had been isolated from *V. littoralis*[2] and *Gelsemium sempervirens*,[9] and has the absolute configuration depicted in Fig. 1.

From the MeOH extract of *V. littoralis*, four known phenylethanoid glycosides, acteoside (**2**) 2′-acetylacteoside (**3**), jionoside (**4**), and isoverbascoside (**5**) were also isolated and identified by comparison of their spectra data with those in the literature.[5—7] The propensity of **1**—**5** to enhance the effects on the NGF to stimulate neurite outgrowth was assessed using methodology previously reported.[2] In control experiments, the populations of neurite-bearing cells were 20.5% and 100% following incubation with NGF 2 ng/ml and 30 ng/ml, respectively. The NGF (2 ng/ml)-induced increase in the proportion of the neurite-bearing cells in PC12D cells was enhanced 10% by **1** (30 $\mu$M). However, **2**—**5** (3—30 $\mu$M) were not found to have NGF-potentiating activity.

## Experimental

**General Procedures** The melting points were determined on a Yanaco micro-melting point apparatus and were uncorrected. UV spectra were recorded on a Hitachi U-2000 spectrophotometer. IR spectra were recorded on an IR-408 spectrometer. One-dimensional (1D) and 2D NMR spectra were recorded in $CD_3OD$ on JEOL GX-500 (500 MHz for $^1$H, 125 MHz for $^{13}$C) and JEOL ECP-600 (600 MHz for $^1$H, 150 MHz for $^{13}$C) spectrometers. FAB-MS and HR-FAB-MS were recorded on JMS AX500 and JMS DX303 spectrometers.

**Plant Material** The aerial parts of *V. littoralis* H. B. K. 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, São Paulo, Brazil). A voucher specimen (No. 68531) is deposited in Tohoku University Graduate School of Pharmaceutical Sciences (Sendai, Japan).

**Extraction and Isolation** The aerial parts of *V. littoralis* (5 kg) were extracted with MeOH (15 l) three times. The MeOH extract (250 g) was partitioned between EtOAc and $H_2O$ (1 : 1). Part (20 g) of the EtOAc-soluble fraction (90 g) was subjected to a silica gel column (8×80 cm, EtOAc/MeOH, 100 : 0→0 : 100) to give 10 fractions, fr. 1 (111 mg), fr. 2 (1106 mg), fr. 3 (457 mg), fr. 4 (6170 mg), fr. 5 (4397 mg), fr. 6 (1592 mg), fr. 7 (100 mg), fr. 8 (5021 mg), fr. 9 (1338 mg), and fr. 10 (423 mg). Further purification of fr. 10 was achieved on a Sephadex LH-20 column (5×120 cm, MeOH) followed by reverse-phase semipreparative HPLC (YMC-AM 324, ODS, i.d. 300×10 mm, 33% MeOH in $H_2O$, 1 ml/min) to afford **1** (23.2 mg),

jionoside (**4**, 14.2 mg, $[\alpha]_D^{27}$ −75.1°, $c$=0.20, MeOH), and isoverbascoside (**5**, 12.9 mg, $[\alpha]_D^{27}$ −72.3°, $c$=0.23, MeOH), from fr. 8 to yield acteoside (**2**, 79.3 mg, $[\alpha]_D^{27}$ −77.4°, $c$=0.31, MeOH) and 2′-acetylacteoside (**3**, 3.5 mg, $[\alpha]_D^{27}$ −65.2°, $c$=0.10, MeOH) by monitoring the potentiation of the NGF action in PC12D cells.

Gelsemiol 6′-*trans*-Caffeoyl-1-glucoside (**1**): Colorless needle crystals (MeOH), mp 122—124 °C. $[\alpha]_D^{27}$ +3.4° ($c$=0.23, MeOH). UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 217 (4.4), 244 (3.5), 329 (4.8). IR (neat) $cm^{-1}$: 3600—3300, 1745, 1700, 1635, 1600, 1520, 1450. $^1H$- and $^{13}C$-NMR data (Table 1). FAB-MS *m*/*z*: 547, 525, 363, 325, 201, 163, 115, 93. HR-FAB-MS *m*/*z*: 525.1921 $[M+H]^+$ (Calcd for $C_{25}H_{33}O_{12}$: 525.1972), 547.1876 $[M+Na]^+$ (Calcd for $C_{25}H_{32}O_{12}Na$: 547.1791).

**HPLC Analysis of Sugar Component in 1** A solution of **1** (1.5 mg) in 4 N aqueous HCl (2.0 ml) was heated under reflux for 7 h. The reaction mixture was neutralized with 1 N NaOH and concentrated under reduced pressure. The residue was dissolved in pyridine (1 ml) and treated with L-cysteine methyl ester hydrochloride (4.5 mg) at 60 °C for 1 h. The resulting thiazolidine derivatives were analyzed by HPLC. HPLC conditions: detection, UV (220 nm) and RI; column, Hibar LiChrosorb $NH_2$ (4.6×250 mm); mobile phase, 95% MeOH (in $H_2O$); flow rate 1 ml/min. $t_R$: methyl 2-(D-glucopentahydroxypentyl)-thiazolidine-4*R*-carboxylate=19.7 and 26.5 min. Each $t_R$ was identical with that of an authentic sample.

**Bioassay Procedure** PC12D cells were dissociated by incubation with 1 mM ethylene glycol-bis-(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid in phosphate-buffered saline for 30 min and then were seeded in 24-well culture plates ($2\times10^4$ 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 control and 2 ng/ml for test samples), 1% fetal calf serum, 2% horse serum, and various concentrations of test compounds (3, 10, 30 $\mu$M). All test compound stock solutions were prepared at 100 mM 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 neurite-bearing cells. The ratio of the neurite-bearing cells to total cells (with at least 100 cells examined/viewing area; three viewing areas/well; six wells/sample) was determined and expressed as a percentage. The data were analyzed using the Student *t*-test.

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