# Syringin 4-*O*-*β*-Glucoside, a New Phenylpropanoid Glycoside, and Costunolide, a Nitric Oxide Synthase Inhibitor, from the Stem Bark of *Magnolia sieboldii*

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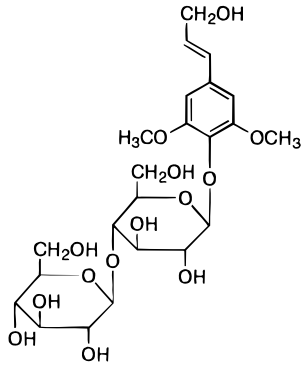
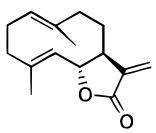
Syringin 4-*O*-*β*-glucoside (**1**), a new phenylpropanoid glycoside, and costunolide (**2**) were isolated from the stem bark of *Magnolia sieboldii*. The structures were determined by spectroscopic and chemical methods. Costunolide (**2**) exhibited strong nitric oxide synthase inhibitory activity in the endotoxin-activated murine macrophage, J774.1.

*Magnolia sieboldii* K. Koch (Magnoliaceae) is an important component of traditional Chinese medicine such as Magnoliae Cortex and Magnoliae Flos. A number of biologically active substances such as magnolol, honokiol,[1] magnoshinin, and magnosalin[2] have been isolated from plants of the Magnoliaceae. Therefore, several plants of this family (*M. fargesii*,[3] *M. officinalis*,[4] *M. denuata*,[5] *M. liliflora*[6]) have been studied thoroughly from the pharmacological point of view. Although the sesquiterpene lactones costunolide and 15-acetoxycostunolide[7] have been isolated from *M. sieboldii*, pharmacological studies and detailed phytochemical studies have not been reported. In a search for immunosuppressant agents, we studied the phytochemistry of this plant and isolated a new compound, syringin 4-*O*-*β*-glucoside (**1**), and seven known compounds. In this paper, we present the structure elucidation of syringin 4-*O*-*β*-glucoside (**1**) and the nitric oxide synthase inhibitory activity of costunolide (**2**) on the murine macrophage (J774.1) induced by endotoxin.




**1**




**2**

## Results and Discussion

The molecular composition of compound **1** ($C_{23}H_{34}O_{14}$) was established by negative ion HRFABMS. It exhibited a UV spectrum similar to that of syringinoside.[8] The IR spectrum of **1** showed OH (3422 $cm^{-1}$, broad), an aromatic double bond (1584, 1508, 1458 $cm^{-1}$), and a glycoside linkage (1100−1000 $cm^{-1}$). The $^1$H-NMR spectrum of **1** indicated the presence of *trans*-olefinic protons, doublet at $\delta$ 6.40 ($J$ = 16.1 Hz) and doublet-triplet at $\delta$ 6.33 ($J$ = 16.1, 4.4 Hz). The $^{13}$C-NMR spectrum suggested that the sugar moiety of **1** was cellobiose by comparison with $^{13}$C-NMR data of cellobiose. In addition, acid hydrolysis of **1** gave only D-glucose as a sugar moiety. Enzymatic hydrolysis of **1** using *β*-glucosidase produced the aglycon syringenin, which was identified by direct comparison with an authentic sample. On acetylation, **1** gave an octa-*O*-acetyl compound, showing eight acetyl methyl peaks and two anomeric protons at $\delta$ 5.20 (1H, d, $J$ = 7.8 Hz) and $\delta$ 4.50 (1H, d, $J$ = 8.2 Hz) in the $^1$H-NMR spectrum, indicating that there were two *β*-D-glucose moieties. The signals for two methoxyl groups at $\delta$ 3.77 and 56.4 in the $^1$H- and $^{13}$C-NMR spectra, respectively, showed that these methoxy groups were on equivalent positions in the phenyl ring and suggested that the sugar was linked to the aglycon moiety at C-4. The linkage between the two glucose moieties was observed to be a 1→4 linkage based on the $^1$H−$^{13}$C long-range COSY. Thus, compound **1** was determined to be syringin 4-*O*-*β*-glucoside. Syringin 4-*O*-*β*-glucoside (**1**) has not been reported previously from natural sources; however, Niwa et al.[8] reported syringinoside, a phenylpropanoid disaccharide with a glucose moiety linked to the C-6 position of syringin.

Chromatography of the $CHCl_3$-soluble fraction afforded (−)-germacrene A and (+)-*β*-elemene,[9] which were identified by comparison of spectral data with literature values. Costunolide (**2**) was isolated, and its $^{13}$C-NMR signals were assigned completely, for the first time, by 2D NMR.[10,11] Other compounds, *β*-sitosterol and its *β*-D-glucoside, were identified by comparing spectral data with those of authentic samples. Syringin was isolated from the EtOAc-soluble fraction. A glycoside from the *n*-BuOH-soluble fraction was identified as echinacoside by acid hydrolysis and comparison of $^{13}$C-NMR values with the literature.[12]

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**Table 1.** Nitric Oxide Synthase Inhibitory Activity Against LPS-Induced Macrophage (J774.1) Cells by Constituents Isolated from the Stem Bark of *Magnolia sieboldii*[a]

| group | concentration ($\mu$g/mL) | nitrite concentration ($\mu$M) | inhibition (%)[b] |
|---|---|---|---|
| normal | | 1.62 ± 0.72 | |
| control | | 25.02 ± 1.11 | 0.0 |
| magnolioside (**1**) | 100 | 19.44 ± 0.53[c] | 22.3 |
| costunolide (**2**) | 1 | 12.44 ± 0.38[d] | 50.3 |
| costunolide (**2**) | 100 | 1.05 ± 0.48[d] | 95.8 |
| costunolide (**2**) | 100 | 1.34 ± 0.46[d] | 94.6 |
| syringin | 100 | 21.93 ± 0.36 | 12.3 |
| echinacoside | 100 | 22.43 ± 0.32 | 10.4 |

[a] Results are expressed as mean ± SD, $n = 4$. [b] Inhibition is expressed as % comparing with the endotoxin treated control. [c] Significantly different from the control, $*p < 0.05$. [d] Significantly different from the control, $p < 0.001$.

The nitric oxide (NO) radical functions efficiently as a mediator, a messenger, or a regulator of cell function in a number of physiological systems and pathophysiological states.[13] Therefore, it is considered that the development of NO synthase inhibitors are important. NO produced by activated macrophages has been shown to be involved in tumor necrosis factor (TNF)-induced shock, hypotension, and vasodilation.[13,14] In addition, NO produced by macrophages is a highly reactive free radical that has another important action as a mediator of inflammatory responses.[14] In the present study, we examined the NO synthase inhibitory activity of eight compounds from *M. sieboldii* in murine macrophage cell lines (J774.1). The NO synthase inhibitory activity on J774.1 was measured in terms of nitrite formation compared with that of an endotoxin-induced control.[15] As shown in Table 1, compound **2** inhibited NO production by endotoxin-activated J774.1 cells (1 $\mu$g/mL) in contrast to the mild inhibitory activity of **1**. Syringin and echinacoside both showed mild inhibitory activity at 100 $\mu$g/mL, while other compounds, germacranolides, dihydrocostunolide, and arctiopicrin showed no significant inhibitory activity up to 500 $\mu$g/mL under similar conditions (no data shown). Compound **2** showed significant inhibitory activity even at a concentration of 1 $\mu$g/mL, and cell viability was observed to be 92% by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at this concentration.[16] These results suggest that **2** is a very potent inhibitory agent against NO production and also indicate that it could be a strong immunosuppressive agent.

## Experimental Section

**General Experimental Procedures.** Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-360 digital polarimeter at 25 °C. IR spectra were recorded on a Hitachi 260-01 spectrometer in KBr disks. UV spectra were obtained on a Shimadzu UV-2200 UV−VIS spectrophotometer. EIMS (ionization voltage, 70 eV) and FABMS were measured with a JEOL JMS DX-300 spectrometer. $^1$H- and $^{13}$C-NMR spectra were taken on a JEOL JNM-GX 400 spectrometer with TMS as an internal standard. 2D NMR spectra ($^1$H−$^1$H COSY, $^1$H−$^{13}$C COSY, $^1$H−$^{13}$C long-range COSY) were measured by the use of JEOL standard pulse sequences.

**Plant Material.** Stem bark of *Magnolia sieboldii* K. Koch was collected in August 1994, on Chiak Mountain, Kangwon Province, Korea, and the plant was identified by Prof. S. C. Lim (Department of Botanical Resources, Sangji University, Wonju, Korea). A voucher specimen is deposited in the herbarium of Life Science and Natural Resources, Sangji University, Wonju, Korea.

**Extraction and Isolation.** Dried stem bark (7.2 kg) of *M. sieboldii* was pulverized and extracted three times with MeOH under reflux. The MeOH extract was filtered and evaporated on a rotatory evaporator under reduced pressure to obtain a viscous mass (540 g) of MeOH extract. This material was suspended in $H_2O$ and then partitioned with $CHCl_3$, EtOAc, and *n*-BuOH to give a $CHCl_3$-soluble fraction (214 g), an EtOAc-soluble fraction (26 g), and an *n*-BuOH-soluble fraction (53 g).

A part of the $CHCl_3$-soluble fraction (20 g) was subjected to column chromatography on Si gel (Wakogel C 200, Wako Pure Chemicals, Osaka, Japan). The column was eluted with *n*-hexane−EtOAc (3:1), and four fractions were collected. Fraction 1 (85 mg) was rechromatographed on 10% $AgNO_3$-impregnated Si gel column chromatography. Initial elution with *n*-hexane and then with an *n*-hexane−EtOAc gradient yielded two sesquiterpenes, (−)-germacrene (15.5 mg) and (+)-$\beta$-elemene (21.1 mg). Fraction 2 was subjected to preparative TLC (Si gel) with *n*-hexane−EtOAc (10:1) to give costunolide (**2**) (122.0 mg). Fraction 3 was rechromatographed on Si gel column chromatography eluting with *n*-hexane−EtOAc (5:1) and finally purified by crystallization with $CHCl_3$−MeOH (1:1) to give $\beta$-sitosterol (400.5 mg). Purification of subfraction 4 was achieved by crystallization from $CHCl_3$−MeOH (1:1) to yield $\beta$-sitosterol $\beta$-D-glucoside (56.2 mg). The EtOAc-soluble fraction (20 g) was subjected to Si gel column chromatography with the solvent $CHCl_3$−MeOH−$H_2O$ (7:3:1) to afford fractions 5 and 6. Recrystallization of fractions 5 and 6 with MeOH yielded syringin (133.2 mg) and syringin 4-*O*-$\beta$-glucoside (**1**) (205.6 mg), respectively. Repeated Sephadex LH-20 column chromatography of the *n*-BuOH-soluble fraction yielded echinacoside (82.5 mg).

**Syringin 4-*O*-$\beta$-glucoside (1):** colorless needles (MeOH); mp 228−230 °C; $[\alpha]_D$ −76.7° (*c* 0.3, MeOH); UV $\lambda_{max}$ (MeOH) (log $\epsilon$) 221.6 (4.20), 265.8 (3.90); IR $\nu_{max}$ (KBr) 3422 (broad, OH), 2909 (CH), 1635 (olefin), 1584, 1508, 1458, 1419 (C=C), 1339, 1243, 1133, 1100−1000 (glycoside), 635 cm$^{-1}$; $^1$H NMR (DMSO-$d_6$) $\delta$ 6.72 (2H, s, H-2, 6), 6.40 (1H, d, $J$ = 16.1 Hz, H-7), 6.33 (1H, dt, $J$ = 16.1, 4.4 Hz, H-8), 5.25 (1H, d, $J$ = 4.6 Hz, 2″-OH), 5.11(1H, d, $J$ = 4.6 Hz, 2′-OH), 5.05 (1H, d, $J$ = 6.0 Hz, 3″-OH), 5.02 (1H, d, $J$ = 5.0 Hz, 4″-OH), 4.95 (1H, d, $J$ = 7.3 Hz, H-1′), 4.88 (1H, d, $J$ = 4.8 Hz, $C_9$-OH), 4.73 (1H, br s, 3′-OH), 4.68 (1H, t, $J$ = 4.2 Hz, 6″-OH), 4.45 (1H, t, $J$ = 5.4 Hz, 6′-OH), 4.29 (1H, d, $J$ = 7.9 Hz, H-1″), 4.11 (2H, br t, $J$ = 4.4 Hz, H-9), 3.77 (6H, s, $OCH_3 \times 2$), 3.71 (1H, m, $H_b$-6″), 3.60 (2H, m, $H_{a,b}$-6′), 3.43 (1H, m, $H_a$-6″), 3.43 (1H, m, H-4′), 3.40 (1H, m, H-3′), 3.29 (1H, m, H-2′), 3.22 (1H, m, H-5′), 3.20 (1H, m, H-5″), 3.17 (1H, m, H-3″), 3.07 (1H, m, H-4″), 3.02 (1H, m, H-2″); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 152.8 (C-5), 152.8 (C-3), 132.9 (C-1), 130.3 (C-8), 133.7 (C-4), 128.5 (C-7), 104.4 (C-6), 104.4 (C-2), 61.6 (C-9), 56.4 (OMe), Glu 102.3 (C-1′), 80.3 (C-4′), 75.1 (C-5′), 74.9 (C-3′), 73.4 (C-2′), 60.5 (C-6′), terminal Glu 103.2 (C-1″), 76.9 (C-5″), 76.5 (C-3″), 73.9 (C-2″), 70.1 (C-4″), 61.1 (C-6″); positive ion FABMS *m*/*z*

535 $[M + 1]^+$; negative ion HRFABMS *m/z* 533.1859 (calcd for $C_{23}H_{33}O_{14}$, 533.1871).

**Acetylation of 1.** A mixture of compound **1** (30 mg), $Ac_2O$ (1 mL), and pyridine (2 mL) was left to stand at room temperature for 12 h. After decomposition of the excess reagent with cold $H_2O$, the precipitate from the reaction mixture was washed with $H_2O$ and evaporated *in vacuo* to obtain syringin 4-*O*-*β*-glucoside octaacetate (31 mg): mp 228 °C; $[\alpha]_D$ −60.0°, IR $\nu_{max}$ (KBr) 2938 (C−H), 1737 (CO), 1636 (olefin), 1585, 1506, 1457 (aromatic C=C), 1420, 1372, 1338, 1223 (acetyl), 1043 (glycoside), 604 $cm^{-1}$; $^1$H-NMR ($CDCl_3$) $\delta$ 7.06 (2H, s, H-2, 6), 6.54 (1H, d, $J$ = 15.7 Hz, H-7), 6.18 (1H, dt, $J$ = 15.7, 6.4 Hz, H-8), 5.20 (1H, d, $J$ = 7.3 Hz, H-1″), 4.50 (1H, d, $J$ = 7.9 Hz, H-1′), 3.79 (6H, s, 3, 5-OMe), 2.08, 2.07, 2.05, 2.01, 2.00, 1.99, 1.96 (Ac of sugar × 7), 1.55 ($C_9$-OAc).

**Acid Hydrolysis of 1.** Compound **1** was hydrolyzed in 5% $H_2SO_4$ in MeOH−$H_2O$ (1:1) under reflux for 3 h. After neutralization with $NH_4OH$ followed by extraction with $CHCl_3$, the aqueous layer was evaporated *in vacuo* to give a residue. The resulting residue was applied to a TLC plate and developed with EtOAc−MeOH−$H_2O$−AcOH (13:6:3:3). The $R_f$ value of the product was identical to that of D-glucose shown by co-TLC.

**Enzymatic Hydrolysis of 1.** A solution of **1** (25 mg) in a mixture of EtOH (5 mL) and 0.2 M $K_2HPO_4$−0.1 M citric acid buffer (pH 4.0) (10 mL) was treated with *β*-glucosidase, and the mixture was kept for 24 h at 37 °C with gentle stirring. The reaction product mixture was extracted with EtOAc. The EtOAc extract was washed with brine, dried, and concentrated. The residue was separated by preparative TLC with $CHCl_3$−MeOH−$H_2O$ (25:8:5, lower layer) to give syringenin (sinapyl alcohol). Syringenin was identified by comparison of mixed mp and co-TLC with an authentic sample.

**Costunolide (2):** colorless needles, mp 105−106 °C; $[\alpha]_D$ +117° (*c* 0.20 , $CHCl_3$); UV $\lambda_{max}$ (KBr) (log $\epsilon$) end absorbance 210 (4.05) nm; IR $\nu_{max}$ ($CHCl_3$) $\delta$ 1765, 1667, 1285, 1136, 995, 930 $cm^{-1}$; $^1$H-NMR ($CDCl_3$) $\delta$ 6.18 (1H, d, $J$ = 3.6 Hz, $H_a$-13), 5.52 (1H, d, $J$ = 3.6 Hz, $H_b$-13), 4.83 (1H, dd, $J$ = 10.1, 4.3 Hz, $H_\beta$-6), 4.71 (1H, d, $J$ = 10.1 Hz, H-5), 4.54 (1H, t, $J$ = 8.9 Hz, H-1), 2.55 (1H, m, H-7), 2.41 (2H, m, H-3), 2.31−2.21 (2H, m, H-2), 2.21−1.96 (4H, m, H-8, 9), 1.68 (3H, s, 15-$CH_3$), 1.40 (3H, s, 14-$CH_3$); $^{13}$C-NMR ($CDCl_3$) $\delta$ 169.56 (C-12), 140.51 (C-4), 139.66 (C-11), 136.35 (C-10), 126.91 (C-5), 126.57 (C-1), 118.74 (C-13), 81.13 (C-6), 49.68 (C-7), 40.36 (C-9), 38.81 (C-3), 27.40 (C-8), 25.60 (C-2), 15.49 (C-15), 15.43 (C-14); EIMS *m/z* 232 $[M]^+$.

**Cell Culture.** The murine monocyte macrophage cell line, J774.1, was obtained from the Japan Cancer Research Bank (JCRB, Tokyo Japan). The J774.1 cells were maintained continuously in 75-$cm^2$ plastic culture flasks (Falcon) in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with penicillin (100 Units/mL), streptomycin (100 $\mu$g/mL), and 10% fetal calf serum. Cells were harvested by gentle scraping and passaged every 3−6 days by dilution of a suspension of the cells 1:10 in fresh medium.

**Measurement of Nitrite Production.** NO production in J774.1 cells was assayed by measuring the accumulation of nitrite in the culture medium by the Griess reaction. The J774.1 cells were removed from culture flasks by vigorous pipetting and centrifuged, then resuspended in the medium to a concentration of $2 \times 10^5$ cells/mL. Cells were plated in 24-well culture plates (Falcon) and allowed to adhere for 24 h. Thereafter, the medium was replaced with fresh medium or medium containing endotoxin and/or the presence of sample and was incubated at 37 °C in 5% $CO_2$ in air for up to 48 h. Briefly, 300 $\mu$L of culture supernatant from control cells or cells stimulated with endotoxin were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% $H_3PO_4$) at room temperature for 10 min. Absorbency was read at 560 nm using an ELISA analyzer (Model ETY-96, Toyo Sokki Co., Ltd., Kanagawa). The nitrite concentration in the medium was determined with sodium nitrite used as a standard. Data are expressed as the total $\mu$M nitrite production by $2 \times 10^5$ cells for 48 h, as indicated in the figures. Data are expressed as the mean ± S.D. Statistical significance of the data was evaluated using the Students *t*-test.

**Cell Viability.** Cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetazolium bromide (MTT, Sigma)-based colorimetric assay. Cells ($5 \times 10^3$ cells/well) in 96-well plates were exposed to drug samples at 37 °C in 5% $CO_2$ in air for up to 48 h. After completion of the drug treatment, 10 $\mu$L of MTT (5 mg/mL) solution was added to each well, followed by 100 $\mu$L of complete medium. After incubation for 4 h at 37 °C, MTT solution in the medium was removed. The incorporated formazan crystals in viable cells were solubilized with 100 $\mu$L of DMSO. The absorbency of each well was then read at 540 nm using an ELISA analyzer.

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