Sterols isolated from seeds of *Panax ginseng* and their antiinflammatory activities

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**ABSTRACT**

**Background:** *Panax ginseng* C. A. Meyer, a perennial herb from the Araliaceae family, is a commonly used medicinal plant. Many studies have been conducted on the biologically active constituents of whole parts of *P. ginseng* (i.e., roots, leaves, flower buds, and fruits). However, the seeds of *P. ginseng* have not been intensively investigated. A new sterol glucoside, 3-O-β-d-glucopyranosyl-5,22,24-stigmastatrienol (1), and a known sterol, 5,22-stigmastadienol (2), were isolated from seeds of *P. ginseng* and were evaluated for their inhibitory activities on tumor necrosis factor (TNF)α-induced nuclear factor (NF)-κB and inducible nitric oxide synthase (iNOS) transcription in transfected HepG2 cells. The present work deals with the isolation, identification, and antiinflammatory activities of the two compounds. **Materials and Methods:** The compounds were isolated by a combination of silica gel and YMC R-18 column chromatography, and their structures were identified by analysis of spectroscopic data (1D, 2D-NMR, and MS). The antiinflammatory activities of the isolated compounds 1 and 2 were evaluated by luciferase reporter gene assays. **Results:** Two sterols have been isolated from the seeds of *P. ginseng*. Compound 1 is a previously unreported glucosidyl sterol. Compounds 1 and 2 both inhibited NFκB-luciferase activity, with IC₅₀ values of 8.1 and 4.8µM, respectively. They also inhibited iNOS-luciferase activity in TNFα-induced HepG2 cells, with IC₅₀ values of 2.2 and 2.9µM, respectively. **Conclusion:** The two isolated sterols have inhibitory effects on inflammation-related factors in HepG2 cells, as determined by luciferase reporter gene assays. Thus, seeds of *P. ginseng* are worthy of consideration for the development and research of antiinflammatory agents.

**Key words:** Antiinflammatory activity, *Panax ginseng*, seeds, 3-O-β-d-glucopyranosyl-5,22,24-stigmastatrienol

**INTRODUCTION**

Nuclear factor κB (NFκB) comprises a group of transcription factors known to regulate inflammatory responses and can be activated by the expression of inflammatory cytokines, such as tumor necrosis factor (TNF) or interleukin-1 (IL-1), and the presence of oxidant-free radicals. It is required for inducible nitric oxide synthase (iNOS) induction and is implicated in the induction of the human iNOS gene. Additionally, inhibitors of NF-κB activation minimally decrease iNOS expression. 

Ginseng (*Panax ginseng* C. A. Meyer), a traditional herbal medicine, has been used as a tonic for the treatment of various diseases. The entire ginseng plant, including leaves, flower buds, and berries, has been comprehensively studied, and many dammarane-type triterpenes have been characterized as principal components. However, the seeds of *P. ginseng* have not been investigated intensively. In our search for antiinflammatory components from the seeds of *P. ginseng*, a new sterol glucoside, 3-O-β-d-glucopyranosyl-5,22,24-stigmastatrienol (1), and a known sterol, 5,22-stigmastadienol (2), were isolated from seeds of *P. ginseng* using a combination of silica gel and YMC RP-18 column chromatography [Figure 1]. The structures were established on the basis of nuclear magnetic resonance (NMR), electrospray ionization mass spectrometry (ESI-MS), and a comparison with reports in the literature. Compounds 1 and 2 were evaluated for their inhibitory activities on TNF-α-induced NF-κB and iNOS transcription in transfected HepG2 cells.
MATERIALS AND METHODS

General
Optical rotations were measured with a DIP-360 digital polarimeter (Jasco, Easton, MD, USA). NMR spectra were obtained at room temperature on Bruker DRX 400 NMR spectrometers with tetramethylsilane (TMS) as internal standard. ESI-MS spectra were recorded on a Model 1100 LC-MSD Trap spectrometer (Agilent, Santa Clara, CA). Column chromatography was carried out silica gel (70-230 and 230-400 mesh, Merck, Darmstadt, Germany), and HP-20 Diaion (Mitsubishi Chemical, Tokyo, Japan). Analytical thin-layer chromatography (TLC) was performed on Kieselgel 60F254or RP-18F254S (Merck, Darmstadt, Germany, and YMC RP-18 resins (30-50 μm, Fuji Silysia Chemical Ltd., Aichi, Japan), and HP-20 Diaion (Mitsubishi Chemical, Tokyo, Japan). Analytical thin-layer chromatography (TLC) was performed on Kieselgel 60F254 or RP-18F254S (Merck, Darmstadt, Germany) glass plates and spots were visualized by spraying with 10% aqueous H2SO4 solution, followed by heating.

Plant material
The seeds of P. ginseng were collected in Geumsan province, which is well-known for ginseng cultivation in Korea, in August 2009, and were taxonomically identified by one of us (Young Ho Kim). Voucher specimens (CNU09105) have been deposited at the College of Pharmacy, Chungnam National University.

Extraction and Isolation
The powdered seeds of P. ginseng (4.0 kg) were extracted in MeOH three times (5.0 L × 3, 50°C) and the combined extracts were concentrated in vacuo to dryness. The MeOH residue (202.0 g) was suspended in H2O (0.8 L), then partitioned with ethyl acetate (EtOAc, 0.8 L × 3), and the EtOAc-soluble fraction (83.7 g) was subjected to a silica gel column eluted with a gradient of EtOAc in n-hexane (100% n-hexane, 10, 20, 25, 50, and 100% EtOAc; v/v) to give seven fractions (Fr. 1 ~ Fr. 7). Fr. 5 (5.1 g) was purified by YMC RP-18 column with MeOH: acetone: H2O (15:10:5, v/v/v) to obtain compound 2 [11.5 mg, 0.005% (w/w) of MeOH extract]. The water layer was subjected to a Diaion HP-20 column eluted with a gradient of MeOH in H2O (0, 25, 50, 75, and 100% MeOH; v/v) to give six fractions, F1~F6. 5 (6.6 g) was purified on silica gel columns and eluted with CHCl3:MeOH (7:0.1, v/v) to obtain 1 [37.0 mg, 0.018% (w/w)].

3-O-β-d-Glucopyranosyl-5,22,24-stigmastatrienol (1)
White amorphous powder; mp 280–300°C; ESI-MS: m/z 573.4 [M + H]+; IR (KBr): 3481, 2933, 1463, 1379, 1164, 1075, 1024/cm; 1H-NMR (400 MHz, C5D5N): δ 0.67 (3H, s, CH3-18), 0.93 (3H, s, CH3-19), 1.03 (3H, d, J = 6.6 Hz, CH3-27), 1.04 (3H, d, J = 6.6 Hz, CH3-26), 1.10 (3H, d, J = 6.0 Hz, CH3-21), 1.64 (3H, d, J = 6.6 Hz, CH3-29), 4.07 (1H, m, H-3), 5.09 (1H, d, J = 15.0 Hz, H-23), 5.24 (1H, dd, J = 6.0, 15.0 Hz, H-22), 5.26 (1H, q, J = 6.6 Hz, H-28), 5.36 (1H, m, H-6), glucose unit: 5.07 (1H, d, J = 7.8 Hz, H-1′), 3.97 (1H, m, H-2′), 4.31 (1H, m, H-3′), 4.30 (1H, m, H-4′), 3.94 (1H, m, H-5′), 4.58 (1H, d, J = 12.0 Hz, H-6a′), 4.43 (1H, m, H-6b′); 13C-NMR (100 MHz, C5D5N): δ 146.1 (C-24), 141.2 (C-5), 139.2 (C-22), 129.8 (C-23), 122.2 (C-6), 117.4 (C-28), 102.8 (C-1′), 78.9 (C-5′), 78.8 (C-3′), 78.3 (C-3), 75.6 (C-2′), 71.9 (C-4′), 63.0 (C-6′), 56.4 (C-14), 56.3 (C-17), 50.5 (C-9′), 42.5 (C-13), 40.1 (C-20), 39.5 (C-4′), 37.1 (C-1), 36.6 (C-10), 32.4 (C-8), 32.2 (C-7), 30.4 (C-12), 29.6 (C-2′), 28.5 (C-25), 26.5 (C-16), 24.7 (C-15), 21.3 (C-11), 19.4 (C-19), 21.2 (C-26), 21.1 (C-27), 19.2 (C-21), 12.7 (C-29), 12.1 (C-18).

5,22-Stigmastadienol (2)
White amorphous powder; mp 164–165°C; ESI-MS: m/z 411.2 [M–H]+; This compound exhibited comparable spectroscopic data (1H- and 13C-NMR) to published values.[9]

Luciferase assay
NFkB-Luc and iNOS-Luc plasmids were kindly provided by Dr. Kyoon E. Kim (Chungnam National University, Daejeon, Korea). According to the manufacturer’s protocol, all cells were transfected with optimized amount of DNA plasmids (NFkB-Luc and iNOS-Luc plasmids) using Promega luciferase assay kits (Promega, Madison, WI). HepG2 cells were seeded at 1.5 × 104 cells/well in a 12-well plate and grown for 24 h prior to transfection. The transfected Hep-G2 cells were pretreated for 1 h with either vehicle (dimethyl sulfoxide (DMSO)) and a positive control, followed by 1 h of treatment with 10 ng/mL TNFα. Unstimulated Hep-G2 cells were used as a negative control. Luciferase activity was assayed using an LB 953 Autolumat (EG&G Berthold) as described previously.[10] The data were presented as a mean ± SD of three independent experiments, performed in triplicate.

Cytotoxicity assay
An 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega Celltiter 96-Aqueous One Solution Assay) was used to analyze...
the effect of compounds on cell viability as described by the manufacturer (Promega).

**RESULTS AND DISCUSSION**

Compound 1 was obtained as a white amorphous powder, and HRESI results yield a molecular formula of C_{35}H_{57}O_{6}, based on the experimental [M+H]^+ peak at m/z 573.4183 (calculated 573.4149). The infrared (IR) spectrum showed the presence of a hydroxyl at 3481/cm. Based on $^1$H- and $^{13}$C-NMR data, 1 was identified as a sterol glucoside, containing a β-d-glucopyranosyl unit. The large coupling constant ($J=7.8$Hz) of the anomeric proton at δ 5.07 in the $^1$H-NMR spectrum of 1 indicates the β configuration. The deshielded oxymethine signal at δ 78.3 for C-3 indicates the presence of a 3-O-β-d-glucopyranosyl bond in 1.[3] The $^1$H-NMR spectrum of 1 showed six methyl groups at δ 0.67 (3H, s, CH$_3$-18), 0.93 (3H, s, CH$_3$-19), 1.03 (3H, d, $J=6.6$Hz, CH$_3$-27), 1.04 (3H, d, $J=6.6$Hz, CH$_3$-26), 1.10 (3H, d, $J=6.0$Hz, CH$_3$-21), and 1.64 (3H, d, $J=6.6$Hz, CH$_3$-29), as well as an oxygenated methine proton at δ 3.98 (1H, m, H-3), and three types of olefinic protons at δ 5.09 (1H, d, $J=15.0$Hz, H-23), 5.26 (1H, dd, $J=6.0$, 15.0Hz, H-22), 5.24 (1H, q, $J=6.6$Hz, H-28), and at δ 5.36 (1H, m, H-6), which are characteristic for a sterol with trans-double bond protonin the alkyl chain. As shown in Figure 2, key heteronuclear multiple-bond correlation spectroscopy (HMBC) correlations were observed between the following proton and carbon atoms: H-1′ and C-3; H-3 and C-2, and C-4; H-6 and C-4, and C-8; H-18 and C-13, and C-14; H-19 and C-5, and C-9; H-21 and C-20, and C-22; H-22 and C-17, C-20, and C-23; H-23 and C-20, and C-25; H-26 and C-24;H-27 and C-24; and H-29 and C-24, and C-28. Thus, compound 1 was determined to be 3-O-β-d-glucopyranosyl-5,22,24-stigmastatrienol, which has not been previously reported.

Compound 2 was obtained as a white powder and showed a molecular ion peak at m/z 411.2 [M–H] in the ESIMS spectrum. The $^1$H- and $^{13}$C-NMR spectra of 2 indicated a typical sterol structure containing 29 carbons. They showed six methyl groups (H-18, H-19, H-21, H-26, H-27, and H-29), two types of olefinic protons (H-22, H-23, and H-6), and an oxygenated methine proton (H-3). The configuration of the hydroxyl group at C-3 (δ 71.8) was determined to be α by comparison with previously reported chemical shift: α with an upfield-shifted C-3 at δ 72.7 and β with a downfield-shifted C-3 at δ 84.4.[3] From the above evidence and a comparison with the literature,[9] compound 2 was identified as 5,22-stigmastadienol.

To investigate the effect of the two sterols (compounds 1 and 2) from seeds of *P. ginseng* on TNFα-induced NFkB and iNOS, the nuclear transcription luciferase-reporter system was used. Compounds 1 and 2 inhibited NFkB activity with IC$_{50}$ values of 8.1 and 4.8μM, respectively, compared with an IC$_{50}$ value of 3.8μM for the positive control. Compounds 1 and 2 also inhibited iNOS activity with IC$_{50}$ values of 2.2 and 2.9μM, respectively, compared with an IC$_{50}$ value of 0.7μM for the positive control [Table 1]. To confirm the antiinflammatory activity of the tested compounds, cell viability was simultaneously determined using a colorimetric MTS assay (Promega). Neither compound 1 nor compound 2 exhibited a significant effect on cell viability at the concentration tested (data not shown). Thus, this study suggests that the compounds 1 and 2 are considered for the development and research of antiinflammatory agents.

**REFERENCES**


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**Table 1: Effects of compounds 1 and 2 on NF-kB and iNOS luciferase activities in TNFα-induced HepG2 cells**

<table>
<thead>
<tr>
<th>Compound</th>
<th>NFkB (μM)</th>
<th>iNOS (μM)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>8.1 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>4.8 ± 0.1</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>Impressive acid*</td>
<td>3.8 ± 0.2</td>
<td>–</td>
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</tbody>
</table>

*Positive control;**1-Pyrrolidinecarboxothioic acid ammonium salt"

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