Inhibitors of Nitric Oxide Production from the Flowers of Angelica furcijuga: Structures of Hyuganosides IV and V

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The methanolic extract from the flowers of Angelica furcijuga Kitagawa was found to inhibit nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages. From the methanolic extract, two new glycosides called hyuganosides IV and V, were isolated together with 28 known constituents. The structures of the new constituents were determined on the basis of chemical and physicochemical evidence. Furthermore, the inhibitory effects of 11 coumarin constituents on nitric oxide production were examined. Among them, 3′-angeloyl-cis-khellactone (IC50 = 82 μg/ml), (S)-(-)-oxypeucedanin (57 μg/ml), imperatorin (60 μg/ml), isoepoxypteryxin (53 μg/ml), and isopityxin (8.8 μg/ml) showed inhibitory activity.

Key words Angelica furcijuga; coumarin; nitric oxide production inhibitor; hyuganoside

Table 1. Inhibitory Effects of the MeOH Extract and EtOAc- and H2O-Soluble Fractions from the Flowers of Angelica furcijuga on NO Production in LPS-Activated Mouse Peritoneal Macrophages

<table>
<thead>
<tr>
<th>Inhibition (%)</th>
<th>IC50 (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>0 μg/ml</td>
<td>3 μg/ml</td>
</tr>
<tr>
<td>MeOH extract</td>
<td>0.0 ± 5.5</td>
</tr>
<tr>
<td>EtOAc-soluble fraction</td>
<td>0.0 ± 0.9</td>
</tr>
<tr>
<td>H2O-soluble fraction</td>
<td>0.0 ± 1.4</td>
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</table>

a) Each value represents the mean ± S.E.M. (n = 4). Significantly different from the control, b) p < 0.05, c) p < 0.01. d) Cytotoxic effects were observed.

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negative optical rotation (\([\alpha]_D^{25} = -5.5^\circ\) in MeOH). The molecular formula \(C_{20}H_{28}O_{11}\) was confirmed from the quasimolecular ion peaks at \(m/z\) 467 (M+Na)\(^{2}\) and 443 (M-H)\(^{2}\) in the positive- and negative-ion fast atom bombardment (FAB)-MS of \(1\) and with high-resolution FAB-MS. The IR spectrum of \(1\) showed absorption bands at 1719, 1638, 1478, and 1266 cm\(^{-1}\) ascribable to a carbonyl function and an aromatic ring, and strong absorption bands at 3410 and 1075 cm\(^{-1}\) suggestive of a glycosidic moiety. The UV spectrum of \(1\) showed an absorption maximum at 287 (\(\log e 3.60\)) nm. Acid hydrolysis of \(1\) with 1.0 M hydrochloric acid (HCl) liberated \(d\)-glucose, which was identified in HPLC analysis using an 

Inhibitory Effects on NO Production in LPS-Activated Mouse Peritoneal Macrophages

The inorganic free radical NO has been implicated in physiologic and pathologic processes, such as vasodilation, nonspecific host defense, ischemia-reperfusion injury, and chronic or acute inflammation. NO is produced by the oxidation of \(L\)-arginine by NO synthase (NOS). In the family of NOS, inducible NOS is specifically involved in the pathologic aspects with overproduction of NO and can be expressed in response to proinflammatory agents such as interleukin-1\(\beta\), tumor necrosis factor-\(\alpha\), and LPS in various cell types including macrophages, endothelial cells, and smooth muscle cells. As a part of our studies to characterize the bioactive components of natural medicines, we reported various NO production inhibitors, i.e., higher unsaturated fatty acids, poly-
acetylenes, Coumarins, Flavonoids, Stilbenes, Lignans, Sesquiterpenes, Diterpenes, Triterpenes, Diarylheptanoids, Cyclic Peptides, and Alkaloids. As a continuation of these studies, the effects of the coumarin constituents from the flowers and leaves of A. furcijuga on NO production from LPS-activated macrophages were examined, and the results are summarized in Table 2. The isolated constituents (I—3, 8—13) significantly inhibited the accumulation of nitrite, a product of NO, in the medium. Among them, three khellactone-type coumarins, 3’-angeloyl-cis-khellactone (3, IC_{50} = 82 \mu M), isoepeoxypteryxin (12, 53 \mu M), and isoptryxyn (13, 8.8 \mu M), and two furanocoumarins, (S)-(−)-oxypeucedanin (8, 57 \mu M) and imperatorin (9, 60 \mu M), showed stronger inhibitory effects without cytotoxic effects in the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

Recently, Wang et al. reported that imperatorin (9) isolated from the roots of Saponshnikovia divaricata inhibited NO production in the macrophage-like cell line RAW264.7. In agreement with the previous report, 9 substantially inhibited NO production in LPS-activated mouse peritoneal macrophages. In our previous study, we reported that the acyl groups at the 3’- and 4’-positions of khellactone-type coumarins (e.g., hyuganins, anomalin, etc.) were essential to inhibit LPS-induced NO production in mouse peritoneal macrophages.
However, the effects of the 3'- or 4'-monoacyl derivative have not been clarified. In the present study, 3'-angeloyl-cis-khellactone (3, 82 μm) with the 3'-angeloyl group showed stronger activity than laserpitin (4, >100 μm) with the 4'-angeloyl group, but both compounds (3, 4) showed less activity than anomalin (3.4 μm) with angeloyl groups at the 3'- and 4'-positions. These findings suggest that the 3'-acyl group contributes to the activity more than the 4'-acyl group and provide evidence that both the 3'- and 4'-acyl groups of khellactone-type coumarins are essential for the potent activity.

With regard to the activity of furanocoumarins (5–10), imperatorin (9, 60 μm) with the 5-O-prenyl group showed substantial inhibitory activity and its activity was stronger than that of xanthotoxin (5, >100 μm), Isomperatorin (10) with the 5-O-angeloyl group also showed stronger activity, but cytotoxic effects were observed. Thus the prenyl ether moiety at the 5-O- or 8-O-position in furanocoumarins is suggested to enhance the inhibition of NO production. On the other hand, (S)-(−)-oxypeedeanin (8, 57 μm) with the oxidized prenyl group showed less activity than 10, but did not show cytotoxic effects, while (R)-(+) and (S)-(−)-oxypeedeanedin hydrate (6, 7) lacked activity.

In conclusion, two new glycosides, hyganosides IV (1) and V (2), were isolated from the flowers of A. furcijuga and their structures were determined on the basis of chemical and physicochemical evidence. Among the coumarin constituents, 3'-angeloyl-cis-khellactone (3), (S)-(−)-oxypeedeanedin (8), imperatorin (9), isoeoxypteryxin (12), and isopyseryxin (13) inhibited NO production in LPS-activated mouse peritoneal macrophages, and some structural requirements of khellactone-type coumarins and furanocoumarins for the activity were clarified.

Table 3. Inhibitory Effects of Constituents from A. furcijuga on NO Production in LPS-Activated Mouse Peritoneal Macrophages

<table>
<thead>
<tr>
<th>Constituent</th>
<th>IC50 (μM)</th>
<th>Inhibition (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μM</td>
<td>1 μM</td>
</tr>
<tr>
<td>3'-Angeloyl-cis-khellactone (3)</td>
<td>0.0 ± 6.3</td>
<td>−5.0 ± 2.1</td>
</tr>
<tr>
<td>Laserpitin (4)</td>
<td>0.0 ± 7.9</td>
<td>−5.0 ± 6.5</td>
</tr>
<tr>
<td>Xanthotoxin (5)</td>
<td>0.0 ± 2.2</td>
<td>16.9 ± 5.5</td>
</tr>
<tr>
<td>(R)-(+) Oxypeedeanid hydrate (6)</td>
<td>0.0 ± 4.9</td>
<td>2.0 ± 4.0</td>
</tr>
<tr>
<td>(S)-(−)-Oxypeedeanid hydrate (7)</td>
<td>0.0 ± 6.7</td>
<td>4.5 ± 7.0</td>
</tr>
<tr>
<td>(S)-(−)-Oxypeedeanid hydrate (8)</td>
<td>0.0 ± 7.8</td>
<td>−3.3 ± 13.2</td>
</tr>
<tr>
<td>Imperatorin (9)</td>
<td>0.0 ± 3.4</td>
<td>20.8 ± 6.2</td>
</tr>
<tr>
<td>Isoimperatorin (10)</td>
<td>0.0 ± 8.7</td>
<td>1.8 ± 10.6</td>
</tr>
<tr>
<td>Umbelliferone (11)</td>
<td>0.0 ± 8.2</td>
<td>−10.9 ± 13.0</td>
</tr>
<tr>
<td>Isoepteryxin (12)</td>
<td>0.0 ± 2.7</td>
<td>14.2 ± 7.4</td>
</tr>
<tr>
<td>Isopyseryxin (13)</td>
<td>0.0 ± 1.2</td>
<td>20.4 ± 2.1</td>
</tr>
</tbody>
</table>

a) Each value represents the mean ± S.E.M. (n=4). Significantly different from the control, b) p<0.05, c) p<0.01. d) Cytotoxic effects were observed.
4-2 (300 mg) was purified by HPLC [MeOH–H2O (35 : 65, v/v)] to give seven fractions [fr. 4-1 (0.4 g), fr. 4-2 (1.1 g), fr. 4-3 (1.1 g), fr. 4-4 (2.5 g), fr. 4-5 (0.4 g)]. Fraction 5 (0 : 100, v/v) was purified by HPLC [MeOH–H2O (70 : 30, v/v)] to give praeroside II (28 mg), fr. 2-10 (83 mg). Fraction 2-5 (300 mg) was purified by HPLC [MeOH–H2O (50 : 50, v/v)] to give apiosylskimmin (22 mg), fr. 2-6 (775 mg), fr. 2-7 (1546 mg), fr. 2-8 (916 mg), fr. 2-9 (807 mg), fr. 2-10 (83 mg). Fraction 2-5 (300 mg) was purified by HPLC [MeOH–H2O (50 : 50, v/v)] to give praeurosides II (28 mg), fr. 2-10 (83 mg). Fraction 2-5 (300 mg) was purified by HPLC [MeOH–H2O (50 : 50, v/v)] to give praeurosides II (28 mg), fr. 2-10 (83 mg).

**Hydrolysates IV (1):** A white powder; [ε]230 = −5.5° (c = 1.00, MeOH).


**Hydrolysates V (2):** A white powder; [ε]230 = −43.1° (c = 1.20, MeOH).

High-resolution positive-ion FAB-MS: Calcd for C14H18O5 Na+: 245.1580. Found: 245.1586. UV [MeOH, λmax (log ε)]: 291 (3.70). IR (KBr): 3453, 1709, 1655, 1082 cm−1.

**Acid Hydrolysis of 1 and 2** A solution of 1 or 2 (2.0 mg each) in 1.0 M HCl (0.1 ml) was heated under reflux for 1 h. After cooling, the reaction mixture was extracted with EtOAc (0.1 ml). The H2O layer was analyzed by HPLC under the following conditions: detection: optical rotation, column: Kaseisorb LC NH2-60 5 250 x 4.6 mm i.d., 5 μm (Tokyo Kasei Kogyo Co., Ltd.), mobile phase: CH3CN–H2O (3 : 1, v/v), flow rate: 0.8 ml/min, injection volume: 10 μl, column temperature: room temperature. Identification of r-glucose present in the H2O layer was carried out by comparison of its retention time and optical rotation with that of authentic sample.

**Enzymatic Hydrolysis of 2** A solution of 2 (5.8 mg, 0.014 mmol) in 0.2 M acetic buffer (pH 4.4, 2.0 ml) was treated with β-glucosidase (15.0 mg, Sigma) and the solution was stirred at 38°C for 24 h. After treatment of the reaction mixture with EtOH, the mixture was evaporated to dryness under reduced pressure and the residue was purified by reverse-phase silica gel column chromatography [MeOH (H2O–MeOH)] to give 2a (3.1 mg, 91%).


**Bioassay** NO Production from LPS-Stimulated Macrophages

Inhibitory effects on NO production by mouse macrophages were evaluated using the method reported previously. Peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice by washing with 6 ml of ice-cold phosphate-buffered saline (PBS), and cells (5 x 10^7 cells/well) were suspended in 200 μl of RPMI 1640 supplemented with 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), and preincubated in 96-well microplates at 37°C in 5% CO2 in air for 1 h. Nonadherent cells were removed by washing the cells with PBS, and the adherent cells were cultured in fresh medium containing LPS (10 μg/ml) and test compound (1—100 μm) for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent. Cytotoxicity was determined using the MTT colorimetric assay. Briefly, after 20-h incubation with test compounds, MTT (10 μl, 5 mg/ml in PBS) solution was added to the wells. After 4 h culture, the medium was removed, and isopropyl alcohol containing 0.04% HCl was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm (reference, 655 nm). When the optical density of the sample-treated group was reduced to less than 80% of that in the vehicle-treated group, the test compound was considered to exhibit cytotoxic effects.

References and Notes

15) These known compounds were identified by comparison of their
authentic samples.
27 The 1H- and 13C-NMR spectra of 1, 2 and 2a were assigned with the aid of distortionless enhancement by polarization transfer (DEPT) and homo- and heterocorrelation spectroscopy (1H–1H, 13C–1H COSY), and HMBC experiments.