Medicinal Flowers. XXVII.1) New Flavanone and Chalcone Glycosides, Arenariumosides I, II, III, and IV, and Tumor Necrosis Factor-α Inhibitors from Everlasting, Flowers of Helichrysum arenarium

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The methanolic extract from the flowers of Helichrysum arenarium L. MOENCH was found to show inhibitory effect on tumor necrosis factor-α (TNF-α, 1 ng/ml)-induced cytotoxicity in L929 cells. From the methanolic extract, 50 constituents including four new flavanone and chalcone glycosides named arenariumosides I (1), II (2), III (3), and IV (4) were isolated. The stereostructures of 1—4 were elucidated on the basis of chemical and physicochemical evidence. Among the constituents, naringenin 7-O-β-D-glucopyranoside (7), apigenin 7-O-β-D-glucopyranoside (14), apigenin 7-O-gentiobioside (16), and apigenin 7,4′-di-O-β-D-glucopyranoside (17) significantly inhibited TNF-α-induced cytotoxicity in L929 cells at 30 μM.

Key words Helichrysum arenarium; arenariumoside; medicinal flower; tumor necrosis factor-α inhibitory activity; Asteraceae; everlasting

The Asteraceae plant, Helichrysum arenarium L. MOENCH, is wildly distributed in northern, southern, and central regions of Europe. The flowers of H. arenarium (Everlasting in English) have been used as choleretic, hepatoprotective, and detoxication agents in European folk medicine.25 The essential oil from the flowers of Helichrysum species, Immortelle, have been used for a cosmetic agent. As the chemical constituents from this herbal medicine, several pyrones, flavonoids, chalcones, and phthalides were isolated from the flowers3,4 and roots.5,6 During the course of our characterization studies on medicinal flowers,17–21 we found that the methanolic extract from the flowers of H. arenarium inhibited on tumor necrosis factor-α (TNF-α)-induced cytotoxicity in L929 cells. TNF-α is known to mediate a number of forms of organ injury through its induction of cellular apoptosis. In the case of liver, the biological effects of TNF-α have been implicated in hepatic injury induced by hepatic toxins, ischemia/reperfusion, vital hepatitis, and alcohol.22–24 Therefore, TNF-α is considered to be an important target in research to discover anti-inflammatory and hepatoprotective agents. On the basis of above-mentioned concept, we investigated protective constituents from naturally occurring products on TNF-α-induced cell death in L929 cells, a TNF-α-sensitive cell line.25 From the methanolic extract, four new flavanone and chalcone glycosides named arenariumosides I (1), II (2), III (3), and IV (4) were isolated together with 46 known compounds [(5—49) and adenosine]. This paper deals with the isolation and structure elucidation of four new glycosides (1—4) as well as effects of the principal constituents on TNF-α-induced cell death in L929 cells.

The flowers of H. arenarium (cultivated in Poland) were extracted with methanol to give a methanolic extract (19.8% from the dried flowers). The methanolic extract was found to inhibit TNF-α-induced cytotoxicity in L929 cells (inhibition: 58.5±3.3% at 100 μg/ml). The methanolic extract was partitioned into an EtOAc-H₂O (1:1, v/v) mixture to furnish an EtOAc-soluble fraction (7.6%) and an aqueous phase. The aqueous phase was subjected to Diaion HP-20 column chromatography (H₂O → MeOH) to give H₂O- and MeOH-eluted fractions (8.6% and 3.2%, respectively). Since the MeOH-eluted fraction showed the inhibitory activity (56.7±11.1% at 100 μg/ml), it was subjected to normal- and reversed-phase column chromatographies, and finally HPLC to give arenariumosides I (1, 0.0045% from the dried flowers), II (2, 0.0059%), III (3, 0.0046%), and IV (4, 0.0034%) together with (25)-helichrysin26 (5, 0.13%), (2R)-helichrysin27 (6, 0.016%), naringenin 7-O-β-D-glucopyranoside28 (7, 0.0053%), 5,7-di-O-β-D-glucopyranosyl (2S)-naringenin29 (8, 0.0045%), 5,7-di-O-β-D-glucopyranosyl (2R)-naringenin29 (9, 0.0097%), heliciside A30 (10, 0.0015%), (2R,3R)-dihydrokaempferol 7-O-β-D-glucopyranoside31 (11, 0.010%), chalconaringenin 2′-O-β-D-glucopyranoside32 (12, 0.013%), chalconaringenin 2′,4′-di-O-β-D-glucopyranoside33 (13, 0.0060%), apigenin 7-O-β-D-glucopyra-
noside33) (14, 0.0025%), apigenin 7-O-β-D-glucopyranosyliduronic acid methyl ester41 (15, 0.0024%), apigenin 7-O-gentiobioside36 (16, 0.0040%), apigenin 7,4’-di-O-β-D-glucopyranoside35 (17, 0.019%), luteolin 7-O-β-D-glucopyranoside37 (18, 0.0025%), luteolin 3’-O-β-D-glucopyranoside39 (19, 0.0013%), scutellarein 7-O-gentiobioside38 (20, 0.0017%), 6-hydroxyluteolin 7-O-β-D-glucopyranoside39 (21, 0.020%), 2240 (0.0033%), kaempferol 3-O-β-D-glucopyranoside35 (23, 0.58%), kaempferol 3-O-gentiobioside43 (24, 0.0070%), kaempferol 3,7-di-O-β-D-glucopyranoside42 (25, 0.0025%), kaempferol 3,4’-di-O-β-D-glucopyranoside42 (26, 0.011%), 2740 (0.0040%), quercetin 3-O-β-D-glucopyranoside38 (28, 0.037%), rutin44 (29, 0.0032%), quercetin 3,3’-di-O-β-D-glucopyranoside45 (30, 0.0015%), aureusin 6-O-β-D-glucopyranoside46,47 (31, 0.0025%), 3248 (0.0025%), tortoside B49 (33, 0.0025%), 7-hydroxy-5-methoxyphthalide 7-O-β-D-glucopyranoside50 (34, 0.19%), scopolin51 (35, 0.017%), undulatoside A52 (36, 0.0017%), 3753 (0.0023%), 3854 (0.018%), syringin55 (39, 0.0037%), dihydroxyringin56 (40, 0.0013%), 4157 (0.0029%), orcinol β-D-glucopyranoside58 (42, 0.0035%), 4359 (0.0013%), 4460 (0.0012%), 4561 (0.0048%), 4662 (0.0045%), 4763 (0.0033%), icariside D50 (48, 0.0179), 4954 (0.00909), and adenosin64 (0.0055%).

Structures of Arenariumosides I (1), II (2), III (3), and IV (4) Arenariumoside I (1) was isolated as a pale yellow powder with negative optical rotation ([α]D = −12.3° in MeOH). In the UV spectrum of 1, absorption maxima were observed at 222 (log ε 4.67) and 284 (4.46) nm, suggestive of the flavanone structure.65 The IR spectrum of 1 showed absorption bands at 1655, 1638, 1509, and 1458 cm−1 assignable to chelated carbonyl function and aromatic ring and strong absorption bands at 3569 and 1071 cm−1 suggestive of a glycoside moiety. The positive-ion fast atom bombardment (FAB)-MS of 1 showed a quasimolecular ion peak at m/z 619 (M+Na)⁺. The molecular formula, C27H35O15, of 1 was determined by high resolution FAB-MS measurement. Acid hydrolysis of 1 with 1.0 M HCl liberated naringenin,66 together with d-glucose, which was identified by HPLC using an optical rotation detector.8,16,17,65 The 1H- and 13C-NMR (DMSO-d6) spectrum of 1 (Table 1) spectra of 1, which were assigned by various NMR experiments,66 showed signals assignable to a dihydroxypropane moiety in flavanone structure by a characteristic ABX type coupling pattern {δ [2.65 (1H, dd, J = 2.1, 16.5 Hz), 3.09 (1H, dd, J = 12.4, 16.5 Hz), 3.54 (1H, dd, J = 2.0, 12.4 Hz, 2-H)], a pair of meta-coupled aromatic protons [δ 6.10, 6.53 (1H each, both d, J = 2.0 Hz, 8, 6-H)], and ortho-coupled A-B, type aromatic protons [δ 6.80, 7.30 (2H each, both d, J = 8.3 Hz, 3’,5’ and 2’,6’-H)] together with two glucopyranosyl parts [δ 4.42 (1H, d, J = 7.2 Hz, terminal-Glc-1-H), 4.80 (1H, d, J = 7.6 Hz, inner-Glc-1-H)]. The connectivities of glucopyranosyl parts were determined by a heteronuclear multiple-bond correlations (HMBC) experiment on 1. Namely, long-range correlations were observed between the terminal-Glc-1-H and the inner-Glc-6-C (δc 69.8) and between the inner-Glc-1-H and the 5-C (δc 161.5). The circular dichroic (CD) spectrum (MeOH) of 1 showed positive and negative maxima at 333 (Δε = +4.95) and 302 (Δε = −7.25) nm, which were similar to those of 5,7-di-O-β-D-glucopyranosyl (2S)-naringenin 8, CD (EtOH): [θ]333
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<th>Position</th>
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<th>$\delta_{\text{H}}$ (J Hz)</th>
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<td>3.50 (m)</td>
<td>100.2</td>
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Table 1. $^1$H- and $^{13}$C-NMR Data of Arenariumosides I (1), II (2), and IV (4)

**Measurements:**
- Carbon signals in the $^1$H- and $^{13}$C-NMR (DMSO-d$_6$).
- Recorded at 400 MHz for $^1$H and 100.6 MHz for $^{13}$C.

**Assignments:**
- Data are reported in ppm (δ) with coupling constants (J Hz).

**Signatures:**
- Assignments were corroborated by HMBC and NOESY experiments.

**Notes:**
- The assignments were made based on the characteristic absorption maxima at 349 (log e 1.45 nm) of the chalcone moiety from the characteristic absorption maxima at 306 and 298 nm.

**Structure:**
- Arenariumoside II (2), $C_{27}$H$_{32}$O$_{15}$, was also observed as a pale yellow powder with negative optical rotation ([α]$_{D}^{25}$ = −62.1° in MeOH).
- Arenariumoside III (3) was isolated as a pale yellow powder with negative optical rotation ([α]$_{D}^{25}$ = −64.5° in MeOH).
- The absolute stereostructure of the 2-position in 1 was elucidated to be S orientation. Consequently, arenariumoside II was determined to be (2R)-naringenin 5-$O$-β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl (2).
and between the inner-Glc-1-H and the 2′-C (δC 160.1). Thus, the structure of arenariumoside III was constructed as chalconaringenin 2′-O-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside (3).

Arenariumoside IV (4) was also isolated as a pale yellow powder with negative optical rotation ([α]D 58.7° in MeOH). The UV and IR spectra of 4 indicated the presence of a flavanone glycoside structure and the molecular formula, C37H33O16, was determined by a quasimolecular ion peak in the positive-ion FAB-MS and the high resolution FAB-MS measurements. The acid hydrolysis of 4 liberated naringenin together with α-glucose. The proton and carbon signals in the 1H- and 13C-NMR (DMSO-d6, Table 1) spectra of 4 showed signals due to a naringenin moiety (δ [2.68 (1H, dd, J = 2.7, 16.4 Hz), 3.06 (1H, dd, J = 13.7, 16.4 Hz), 3-H, 5.46 (1H, dd, J = 2.7, 13.7 Hz, 2-H), 6.11, 6.41 (1H each, both d, J = 1.4 Hz, 8, 6-H), and 7.06, 7.43 (2H each, both d, J = 8.9 Hz, 3′, 5′ and 2′, 6′-H)) together with two glucopyranosyl parts (δ 4.71 (1H, d, J = 7.6 Hz, 5-O-Glc-I-H), 4.89 (1H, d, J = 7.6 Hz, 4′-O-Glc-1-H)). The connectivities of two β-D-glucopyranosyl moieties in 4 were clarified by HMBC experiment, in which long-range correlations were observed between the 5-O-Glc-1-H and the 5-C (δC 160.6) and between the 4′-O-Glc-I-H and the 4-C (δC 157.4). In addition, the CD spectrum (MeOH) of 4 showed positive and negative maxima at 331 (Δε = +3.82) and 298 (Δε = −7.39) nm, which were similar to those of 1. Thus, the structure of are-

Table 2. 1H- and 13C-NMR Data of Arenariumoside III (3)

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(2′-O-β-D-Glucose-5′-Glc)

1  4.22 (d, 7.5) 103.2
2  3.45 (m) 73.5
3  3.63 (m) 76.0
4  3.00 (m) 69.9
5  3.58 (m) 76.6
6  3.63 (m) 68.0
7  4.02 (br d, α = 12) 100.3

Table 3. Effects of Constituents from the Flowers of H. arenarium on TNF-α-Induced Cytotoxicity in L929 Cells

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<th>Concentration (μM)</th>
<th>Inhibition (%)</th>
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<tr>
<td>100</td>
<td>100.0 ± 1.4**</td>
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</table>

Each value represents the mean ± S.E.M. (n = 4). Significantly different from the control, *p < 0.05, **p < 0.01.
narioside IV was determined as (2S)-naringenin 5,4'-di-O-β-D-glucopyranoside (4).

Effects of the Constituents on TNF-α-Induced Cytotoxicity in L929 Cells Next, we examined the effects of the constituents from H. arenarium on TNF-α-induced cytotoxicity in L929 cells. As shown in Table 3, naringenin 7-O-β-D-glucopyranoside (7), inhibition: 24.7 ± 6.9% at 30 μM, apigenin 7-O-β-D-glucopyranoside (14, 40.7 ± 7.9%), apigenin 7-O-gentiobioside (16, 23.9 ± 4.4%), and apigenin 7,4'-di-O-gentiobioside (17, 20.8 ± 0.8%), were found to show inhibitory activity. This evidence indicated that these constituents were found to decrease in the sensitivity of L929 cells to TNF-α. Many compounds, which inhibit cell death by production on TNF-α have been reported, but there are few reports about compounds which selectively reduce the sensitivity of L929 cells to TNF-α.

Experimental

The following instruments were used to obtain physical data: specific rotation (°D) and ultraviolet-visible (UV) spectra (λmax (log ε)) with a JASCO J-7201 spectrometer; infrared spectra (νmax) with a JASCO IR-400 spectrometer; 1H-NMR (400 MHz, DMSO-d6) and 13C-NMR (100 MHz, DMSO-d6) spectra were recorded on a JEOL ECX-600 spectrometer, and mass spectra (m/z) were measured using a JEOL JMS-SX102 mass spectrometer. The following experimental conditions were used for chromatography: ordinary phase column chromatography: Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., Aichi, Japan, 100—200 mesh); TLC, precoated TLC plates with Silica gel 60F254 (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 RP-18 WF 254S (Merck, 0.25 mm); and de-
turate was neutralized with Amberlite IRA-400 (OH− form) and then the resin was removed by filtration. After removal of the solvent from the filtrate under reduced pressure, the residue was separated by Sep-Pak C18 cartridge column (H2O → MeOH). The H2O-eluted fraction was subjected to HPLC analysis under the following conditions: HPLC column, Kasisorb LC NH2-60-5, 4.6 mm i.d. x 250 mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan); mobile phase, CH3CN-H2O (85:15, v/v); flow rate 0.8 ml/min]. Identification of α-glucose from 1 to 4 present in the H2O-eluted fraction was carried out by the mass spectrometry retention time and optical rotation with that of an authentic sample. t2: 13.9 min (positive optical rotation). On the other hand, the MeOH-eluted fraction was purified by HPLC [CH3CN–1% aqueous AcOH (40:60, v/v)] to furnish naringenin46 (2.0 mg, 73% from 1, 2.1 mg, 73% from 2, 1.3 mg, 72% from 3, and 1.3 mg, 72% from 4).

**Bioassay Method. Inhibitory Effect on TNF-α-Induced Cytotoxicity in L929 Cells** Inhibitory effect on TNF-α-induced cell death in L929 cells was assayed by the method described in our previous report.19 Briefly, a suspension of 2 × 104 cells [obtained from Dainippon Pharmaceutical (Osaka, Japan)] in 100 μl of minimum essential Eagle medium supplemented with 1% non-essential amino acid solution (Invitrogen), fetal calf serum (FCS, 10%), penicillin G (100 units/ml), and streptomycin (100 μg/ml) was incubated in a 96-well microplate. After 44 h of incubation in the medium containing TNF-α (1 ng/ml) with or without a test sample, the viability of the cells was assessed by the MTT colorimetric assay. Each test sample was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration was 0.5%).

**Statistics** Values were expressed as means ± S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett’s test was used for statistical analysis.

**Acknowledgements** O. M., T. M., and K. N. were supported by a Grant-in-Aid for Scientific Research from 'High-tech Research Center' Project for Private Universities: matching fund subsidy from The Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), 2007–2011 and also supported by a Grant-in-Aid for Scientific Research by Japan Society for the Promotion of Science (JSPS). M. Y., H. M., and S. N. were supported by the 21st COE Program, Academic Frontier Project, and a Grant-in-Aid for Scientific Research from MEXT.

**References and Notes**


66) The $^1$H- and $^{13}$C-NMR spectra of 1—4 were assigned with the aid of distortionless enhancement by polarization transfer (DEPT), double quantum filter correlation spectroscopy (DQF COSY), heteronuclear multiple quantum coherence (HMOC), and heteronuclear multiple bond connectivity (HMBC) experiments.


