# Medicinal Flowers. XXVII.[1] New Flavanone and Chalcone Glycosides, Arenariumosides I, II, III, and IV, and Tumor Necrosis Factor-$\alpha$ 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-$\alpha$ (TNF-$\alpha$, 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*-$\beta$-D-glucopyranoside (7), apigenin 7-*O*-$\beta$-D-glucopyranoside (14), apigenin 7-*O*-gentiobioside (16), and apigenin 7,4′-di-*O*-$\beta$-D-glucopyranoside (17) significantly inhibited TNF-$\alpha$-induced cytotoxicity in L929 cells at 30 $\mu$M.**

**Key words** *Helichrysum arenarium*; arenariumoside; medicinal flower; tumor necrosis factor-$\alpha$ 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.[2] 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 $\alpha$-pyrones, flavonoids, chalcones, and phthalides were isolated from the flowers[3,4] and roots.[5,6] During the course of our characterization studies on medicinal flowers,[1,7—21] we found that the methanolic extract from the flowers of *H. arenarium* inhibited on tumor necrosis factor-$\alpha$ (TNF-$\alpha$)-induced cytotoxicity in L929 cells. TNF-$\alpha$ 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-$\alpha$ have been implicated in hepatic injury induced by hepatic toxins, ischemia/reperfusion, vital hepatitis, and alcohol.[22—24] Therefore, TNF-$\alpha$ 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-$\alpha$-induced cell death in L929 cells, a TNF-$\alpha$-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-$\alpha$-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-$\alpha$-induced cytotoxicity in L929 cells (inhibition: 58.5±3.3% at 100 $\mu$g/ml). The methanolic extract was partitioned into an EtOAc–$H_2O$ (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 ($H_2O$→MeOH) to give $H_2O$- 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 $\mu$g/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 (2*S*)-helichrysin[26] (**5**, 0.13%), (2*R*)-helichrysin[27] (**6**, 0.016%), naringenin 7-*O*-$\beta$-D-glucopyranoside[28] (**7**, 0.0053%), 5,7-di-*O*-$\beta$-D-glucopyranosyl (2*S*)-naringenin[29] (**8**, 0.0045%), 5,7-di-*O*-$\beta$-D-glucopyranosyl (2*R*)-naringenin[29] (**9**, 0.0097%), helicioside A[30] (**10**, 0.0015%), (2*R*,3*R*)-dihydrokaempferol 7-*O*-$\beta$-D-glucopyranoside[31] (**11**, 0.010%), chalconaringenin 2′-*O*-$\beta$-D-glucopyranoside[32] (**12**, 0.013%), chalconaringenin 2′,4′-di-*O*-$\beta$-D-glucopyranoside[32] (**13**, 0.0060%), apigenin 7-*O*-$\beta$-D-glucopyra-

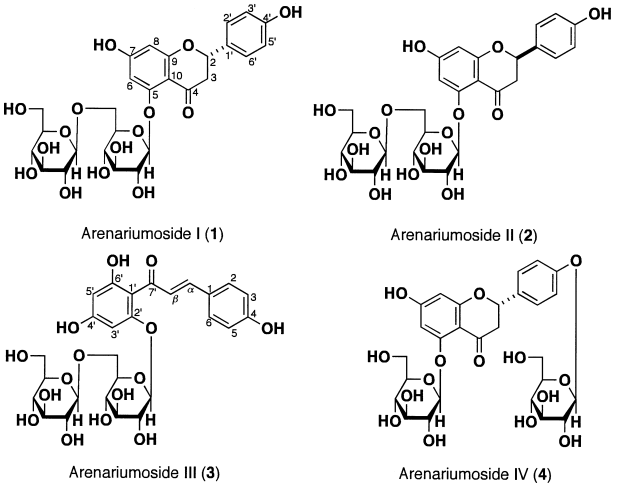


Chart 1

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noside[33] (**14**, 0.0025%), apigenien 7-*O*-*β*-D-glucopyranosiduronic acid methyl ester[34] (**15**, 0.0024%), apigenin 7-*O*-gentiobioside[35] (**16**, 0.0040%), apigenin 7,4′-di-*O*-*β*-D-glucopyranoside[36] (**17**, 0.019%), luteolin 7-*O*-*β*-D-glucopyranoside[37] (**18**, 0.0025%), luteolin 3′-*O*-*β*-D-glucopyranoside[37] (**19**, 0.0013%), scutellarein 7-*O*-gentiobioside[38] (**20**, 0.0017%), 6-hydroxyluteolin 7-*O*-*β*-D-glucopyranoside[39] (**21**, 0.020%), **22**[40] (0.0033%), kaempferol 3-*O*-*β*-D-glucopyranoside[37] (**23**, 0.58%), kaempferol 3-*O*-gentiobioside[41] (**24**, 0.0070%), kaempferol 3,7-di-*O*-*β*-D-glucopyranoside[42] (**25**, 0.0025%), kaempferol 3,4′-di-*O*-*β*-D-glucopyranoside[42] (**26**, 0.011%), **27**[43] (0.0040%), quercetin 3-*O*-*β*-D-glucopyranoside[37] (**28**, 0.037%), rutin[44] (**29**, 0.0032%), quercetin 3,3′-di-*O*-*β*-D-glucopyranoside[45] (**30**, 0.0015%), aureusidin 6-*O*-*β*-D-glucopyranoside[46,47] (**31**, 0.0025%), **32**[48] (0.0025%), tortoside B[49] (**33**, 0.0025%), 7-hydroxy-5-methoxyphthalide 7-*O*-*β*-D-glucopyranoside[50] (**34**, 0.19%), scopolin[51] (**35**, 0.017%), undulatoside A[52] (**36**, 0.0017%), **37**[53] (0.0023%), **38**[54] (0.018%), syringin[55] (**39**, 0.0037%), dihydrosyringin[56] (**40**, 0.0013%), **41**[57] (0.0029%), orcinol *β*-D-glucopyranoside[58] (**42**, 0.0035%), **43**[59] (0.0013%), **44**[60] (0.0012%), icariside $F_2$[61] (**45**, 0.0048%), **46**[62] (0.0045%), **47**[60] (0.0033%), icariside $D_1$[63] (**48**, 0.017%), **49**[64] (0.0090%), and adenosine[44] (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 ($[\alpha]_D^{26}$ −12.3° in MeOH). In the UV spectrum of **1**, absorption maxima were observed at 222 (log $\varepsilon$ 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, $C_{27}H_{32}O_{15}$, of **1** was determined by high resolution FAB-MS measurement. Acid hydrolysis of **1** with 1.0 M HCl liberated naringenin,[44] together with D-glucose, which was identified by HPLC using an optical rotation detector.[8,16,17,65] The $^1$H- and $^{13}$C-NMR (DMSO-$d_6$, Table 1) spectra of **1**, which were assigned by various NMR experiments,[66] showed signals assignable to a dihydropyrone moiety in flavanone structure by a characteristic ABX type coupling pattern {$\delta$ [2.65 (1H, dd, $J$=2.1, 16.5 Hz), 3.09 (1H, dd, $J$=12.4, 16.5 Hz), 3-$H_2$], 5.34 (1H, dd, $J$=2.0, 12.4 Hz, 2-H)}, a pair of *meta*-coupled aromatic protons [$\delta$ 6.10, 6.53 (1H each, both d, $J$=2.0 Hz, 8, 6-H)], and *ortho*-coupled $A_2B_2$ type aromatic protons [$\delta$ 6.80, 7.30 (2H each, both d, $J$=8.3 Hz, 3′,5′ and 2′,6′-H)] together with two glucopyranosyl parts [$\delta$ 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 glycopyranosyl 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 ($\delta_C$ 69.8) and between the *inner*-Glc-1-H and the 5-C ($\delta_C$ 161.5). The circular dichroic (CD) spectrum (MeOH) of **1** showed positive and negative maxima at 333 ($\Delta\varepsilon$=+4.95) and 302 ($\Delta\varepsilon$=−7.25) nm, which were similar to those of 5,7-di-*O*-*β*-D-glucopyranosyl (2*S*)-naringenin {**8**, CD (EtOH): $[\theta]_{333}$

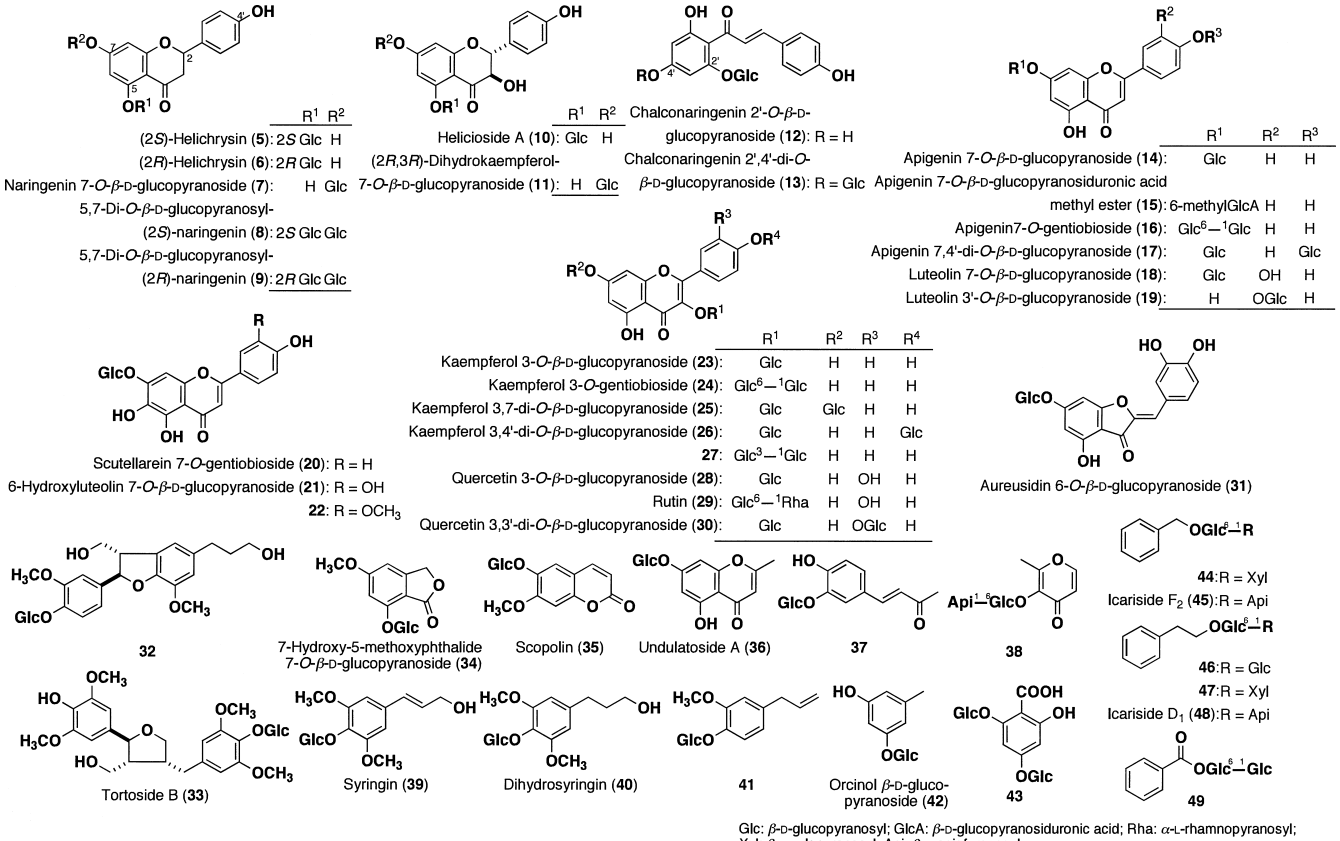


Chart 2

Table 1. $^{1}$H- and $^{13}$C-NMR Data of Arenariumosides I (**1**), II (**2**), and IV (**4**)

| Position | **1** | | **2** | | **4** | |
|---|---|---|---|---|---|---|
| | $\delta_H$ ($J$ Hz) | $\delta_C$ | $\delta_H$ ($J$ Hz) | $\delta_C$ | $\delta_H$ ($J$ Hz) | $\delta_C$ |
| 2 | 5.34 (dd, 2.0, 12.4) | 80.4 | 5.32 (dd, 1.6, 12.4) | 80.3 | 5.46 (dd, 2.7, 13.7) | 77.8 |
| 3 | 2.65 (dd, 2.1, 16.5) | 46.4 | 2.69 (dd, 1.6, 17.2) | 46.4 | 2.68 (dd, 2.7, 16.4) | 44.2 |
| | 3.09 (dd, 12.4, 16.5) | | 2.97 (dd, 12.4, 17.2) | | 3.06 (dd, 13.7, 16.4) | |
| 4 | | 193.1 | | 193.0 | | 189.8 |
| 5 | | 161.5 | | 162.2 | | 160.6 |
| 6 | 6.53 (d, 2.0) | 100.1 | 6.62 (d, 1.4) | 100.3 | 6.41 (d, 1.4) | 98.8 |
| 7 | | 166.8 | | 166.9 | | 164.8 |
| 8 | 6.10 (d, 2.0) | 99.5 | 6.13 (d, 1.4) | 99.5 | 6.11 (d, 1.4) | 97.6 |
| 9 | | 166.1 | | 166.6 | | 164.0 |
| 10 | | 106.8 | | 107.1 | | 105.4 |
| 1′ | | 131.1 | | 131.0 | | 131.9 |
| 2′,6′ | 7.30 (2H, d, 8.3) | 128.2 | 7.30 (2H, d, 8.2) | 129.0 | 7.43 (2H, d, 8.9) | 127.9 |
| 3′,5′ | 6.80 (2H, d, 8.3) | 115.1 | 6.80 (2H, d, 8.2) | 116.5 | 7.06 (2H, d, 8.9) | 116.1 |
| 4′ | | 159.0 | | 159.0 | | 157.4 |
| | (5-*O*-*Glc*$^{6}$—$^{1}$Glc) | | (5-*O*-*Glc*$^{6}$—$^{1}$Glc) | | (5-*O*-*Glc*) | |
| 1 | 4.80 (d, 7.6) | 103.8 | 4.76 (d, 7.6) | 104.7 | 4.71 (d, 7.6) | 103.3 |
| 2 | 3.25 (m) | 74.6 | 3.36 (m) | 74.7 | 3.27 (m) | 73.4 |
| 3 | 3.65 (m) | 77.3 | 3.69 (m) | 77.1 | 3.30 (m) | 76.5 |
| 4 | 3.41 (m) | 71.4 | 3.42 (m) | 71.4 | 3.17 (m) | 69.6 |
| 5 | 3.67 (m) | 77.8 | 3.67 (m) | 77.8 | 3.30 (m) | 77.5 |
| 6 | 3.86 (m) | 69.8 | 3.87 (m) | 69.8 | 3.74 (m) | 60.6 |
| | 4.18 (dd, 2.0, 11.6) | | 4.20 (br d, *ca.* 12) | | 3.50 (m) | |
| | (5-*O*-Glc$^{6}$—$^{1}$*Glc*) | | (5-*O*-Glc$^{6}$—$^{1}$*Glc*) | | (4′-*O*-*Glc*) | |
| 1 | 4.42 (d, 7.2) | 104.8 | 4.47 (d, 7.6) | 104.8 | 4.89 (d, 7.6) | 100.2 |
| 2 | 3.48 (m) | 75.0 | 3.48 (m) | 75.4 | 3.27 (m) | 73.1 |
| 3 | 3.67 (m) | 78.0 | 3.67 (m) | 78.0 | 3.27 (m) | 75.5 |
| 4 | 3.55 (m) | 71.6 | 3.55 (m) | 71.7 | 3.17 (m) | 69.5 |
| 5 | 3.67 (m) | 78.0 | 3.67 (m) | 78.0 | 3.30 (m) | 77.0 |
| 6 | 3.67 (m) | 62.7 | 3.69 (m) | 62.7 | 3.50 (m) | 60.6 |
| | 3.86 (m) | | 3.87 (m) | | 3.74 (m) | |

Measured in DMSO-$d_6$.

+23652 (positive maximum), $[\theta]_{288}$ −30669 (negative maximum)}.[29] Thus, the absolute stereostructure of the 2-position in **1** was elucidated to be *S* orientation.[67] On the basis of the above-mentioned evidence, the structure of arenariumoside I was determined to be (2*S*)-naringenin 5-*O*-*β*-D-glucopyranosyl(1→6)-*β*-D-glucopyranoside (**1**).

Arenariumoside II (**2**), $C_{27}H_{32}O_{15}$, was also observed as a pale yellow powder with negative optical rotation ($[\alpha]_D^{26}$ −64.5° in MeOH). Acid hydrolysis of **2** with 1.0 M HCl liberated naringenin together with D-glucose. The proton and carbon signals in the $^{1}$H- and $^{13}$C-NMR (DMSO-$d_6$, Table 1) spectra[66] of **2** were superimposable on those of **1** {**2**: $\delta$ [2.69 (1H, dd, $J$=1.6, 17.2 Hz), 2.97 (1H, dd, $J$=12.4, 17.2 Hz), 3-$H_2$], 4.47 (1H, d, $J$=7.6 Hz, *terminal*-Glc-1-H), 4.76 (1H, d, $J$=7.6 Hz, *inner*-Glc-1-H), 5.32 (1H, dd, $J$=1.6, 12.4 Hz, 2-H), 6.13, 6.62 (1H each, both d, $J$=1.4 Hz, 8, 6-H)}, 6.80, 7.30 (2H each, both d, $J$=8.2 Hz, 3′,5′ and 2′,6′-H)}. The connectivities of two *β*-D-glucopyranosyl moieties in **2** were clarified by HMBC experiment, in which long-range correlations were observed between the *terminal*-Glc-1-H and the *inner*-Glc-6-C ($\delta_C$ 69.8) and between the *inner*-Glc-1-H and the 5-C ($\delta_C$ 162.2). The CD spectrum (MeOH) of **2** showed negative and positive maxima at 333 ($\Delta\varepsilon$=−1.23) and 286 ($\Delta\varepsilon$=+2.74) nm, which were similar to those of 5,7-di-*O*-*β*-D-glucopyranosyl (2*R*)-naringenin {**9**, CD (EtOH): $[\theta]_{328}$ −2269 (negative maximum), $[\theta]_{284}$ +8707 (positive maximum)}.[29] Thus, the absolute stereostructure of the 2-position in **2** was elucidated to be *R* orientation.[67] Consequently, arenariumoside II was determined to be (2*R*)-naringenin 5-*O*-*β*-D-glucopyranosyl(1→6)-*β*-D-glucopyranoside (**2**).

Arenariumoside III (**3**) was isolated as a pale yellow powder with negative optical rotation ($[\alpha]_D^{25}$ −62.1° in MeOH). In the positive-ion FAB-MS of **3**, the quasimolecular ion peak was observed at *m*/*z* 619 $(M+Na)^+$ and the molecular formula $C_{27}H_{32}O_{15}$ was determined by high resolution FAB-MS measurement, which was the same as those of **1** and **2**. The IR spectrum of **3** showed absorption bands at 3569, 1687, 1655, 1509, 1458, and 1075 $cm^{-1}$ ascribable to hydroxyl, *α*,*β*-unsaturated carbonyl, and ether functions and aromatic ring. The UV spectrum of **3** indicated the presence of the chalcone moiety from the characteristic absorption maximum at 349 (log $\varepsilon$ 4.15) nm. Acid hydrolysis of **3** with 1.0 M HCl liberated naringenin together with D-glucose, which was identified by HPLC analysis. The $^{1}$H- and $^{13}$C-NMR (DMSO-$d_6$, Table 2) spectra[66] of **3** showed signals assignable to a pair of *meta*-coupled aromatic protons [$\delta$ 5.97, 6.25 (1H each, both br s, 5′, 3′-H)], *ortho*-coupled $A_2B_2$ type aromatic protons [$\delta$ 6.82, 7.64 (2H each, both d, $J$=8.2 Hz, 3,5 and 2,6-H)], and a pair of *trans*-olefinic protons [$\delta$ 7.64, 7.97 (1H each, both d, $J$=15.1 Hz, *α*, *β*-H)] together with two glucopyranosyl parts [$\delta$ 4.22 (1H, d, $J$=7.5 Hz, *inner*-Glc-1-H), 5.05 (1H, d, $J$=7.6 Hz, *terminal*-Glc-1-H)]. Finally, the connectivities of the two *β*-D-glucopyranosyl moieties in **3** were elucidated on the basis of HMBC experiment, which showed long-range correlations were observed between the *terminal*-Glc-1-H and the *inner*-Glc-6-C ($\delta_C$ 68.0)

Table 2. $^{1}$H- and $^{13}$C-NMR Data of Arenariumoside III (**3**)

| Position | **3** | |
|---|---|---|
| | $\delta_H$ ($J$ Hz) | $\delta_C$ |
| 1 | | 125.9 |
| 2,6 | 7.64 (2H, d, 8.2) | 130.7 |
| 3,5 | 6.82 (2H, d, 8.2) | 115.8 |
| 4 | | 159.8 |
| $\alpha$ | 7.64 (d, 15.1) | 143.0 |
| $\beta$ | 7.97 (d, 15.1) | 124.0 |
| 1′ | | 105.3 |
| 2′ | | 160.1 |
| 3′ | 6.25 (br s) | 94.5 |
| 4′ | | 164.3 |
| 5′ | 5.97 (br s) | 96.9 |
| 6′ | | 166.0 |
| 7′ | | 192.1 |
| | (2′-*O*-*Glc*$^{6}$—$^{1}$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 |
| | 4.02 (br d, *ca.* 12) | |
| | (2′-*O*-Glc$^{6}$—$^{1}$*Glc*) | |
| 1 | 5.05 (d, 7.6) | 100.3 |
| 2 | 3.45 (m) | 73.5 |
| 3 | 3.10 (m) | 76.3 |
| 4 | 3.26 (m) | 69.7 |
| 5 | 3.58 (m) | 76.7 |
| 6 | 3.45 (m) | 60.8 |
| | 3.63 (m) | |

Measured in DMSO-$d_6$.

and between the *inner*-Glc-1-H and the 2′-C ($\delta_C$ 160.1). Thus, the structure of arenariumoside III was constructed as chalconaringenin 2′-*O*-$\beta$-D-glucopyranosyl(1→6)-$\beta$-D-glucopyranoside (**3**).

Arenariumoside IV (**4**) was also isolated as a pale yellow powder with negative optical rotation ($[\alpha]_D^{27}$ −58.7° in MeOH). The UV and IR spectra of **4** indicated the presence of a flavanone glycoside structure and the molecular formula, $C_{27}H_{32}O_{15}$, 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 D-glucose. The proton and carbon signals in the $^{1}$H- and $^{13}$C-NMR (DMSO-$d_6$, Table 1) spectra[66] of **4** showed signals due to a neringenin moiety {$\delta$ [2.68 (1H, dd, $J$=2.7, 16.4 Hz), 3.06 (1H, dd, $J$=13.7, 16.4 Hz), 3-$H_2$], 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 [$\delta$ 4.71 (1H, d, $J$=7.6 Hz, 5-*O*-Glc-1-H), 4.89 (1H, d, $J$=7.6 Hz, 4′-*O*-Glc-1-H)]. The connectivities of two $\beta$-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 ($\delta_C$ 160.6) and between the 4′-*O*-Glc-1-H and the 4′-C ($\delta_C$ 157.4). In addition, the CD spectrum (MeOH) of **4** showed positive and negative maxima at 331 ($\Delta\varepsilon$=+3.82) and 298 ($\Delta\varepsilon$=−7.39) nm, which were similar to those of **1**. Thus, the structure of are-

Table 3. Effects of Constituents from the Flowers of *H. arenarium* on TNF-$\alpha$-Induced Cytotoxicity in L929 Cells

| | Inhibition (%) | | | | | |
|---|---|---|---|---|---|---|
| TNF-$\alpha$ (1 ng/ml) | − | + | + | + | + | + |
| Conc. ($\mu$M) | 0 | 0 | 3 | 10 | 30 | 100 |
| Arenariumoside III (**3**) | 100.0±1.4** | 0.0±4.1 | −1.5±0.4 | 0.0±2.8 | 1.9±2.8 | −2.9±2.8 |
| (2*R*)-Helichrysin (**6**) | 100.0±14.4** | 0.0±3.1 | −1.5±4.6 | −1.8±7.6 | −2.2±5.2 | −17.8±1.9 |
| Naringenin 7-*O*-Glc (**7**) | 100.0±4.3** | 0.0±3.1 | 7.6±7.3 | 12.0±3.4 | 24.7±6.9** | 39.1±2.2** |
| **8** | 100.0±4.0** | 0.0±1.1 | 1.6±6.5 | 2.5±2.8 | 0.0±1.8 | −2.1±1.2 |
| **9** | 100.0±5.2** | 0.0±5.2 | −2.4±3.7 | −2.9±3.2 | −0.4±1.5 | 0.0±5.8 |
| Helicioside A (**10**) | 100.0±2.9** | 0.0±4.7 | −5.3±3.9 | 6.9±4.9 | 5.6±2.2 | 6.0±5.0 |
| (2*R*,3*R*)-Dihydrokaempferol 7-*O*-Glc (**11**) | 100.0±8.1** | 0.0±2.5 | 2.4±1.1 | 7.6±1.4 | 11.8±2.0 | 23.4±0.4* |
| Chalconaringenin 2′-*O*-Glc (**12**) | 100.0±4.9** | 0.0±2.5 | −0.9±1.9 | 1.9±1.9 | 1.1±1.2 | 1.4±0.9 |
| Chalconaringenin 2′,4′-di-*O*-Glc (**13**) | 100.0±3.7** | 0.0±2.5 | −1.3±1.4 | −2.2±1.9 | −2.2±0.6 | −4.3±0.5 |
| Apigenin 7-*O*-Glc (**14**) | 100.0±12.0** | 0.0±7.2 | 12.1±8.0 | 34.8±2.6* | 40.7±7.9** | 31.1±5.4** |
| Apigenin 7-*O*-gentiobiosaide (**16**) | 100.0±4.0** | 0.0±3.2 | 9.1±2.7 | 10.3±4.0 | 23.9±4.4** | 40.1±1.0** |
| Apigenin 7,4′-di-*O*-Glc (**17**) | 100.0±3.3** | 0.0±0.9 | 7.9±0.5* | 17.4±1.3** | 20.8±0.8** | 15.4±1.5** |
| 6-Hydroxyluteolin 7-*O*-Glc (**21**) | 100.0±3.3** | 0.0±7.9 | 5.5±3.8 | 6.7±3.1 | 4.4±1.3 | −3.2±3.7 |
| Kaempferol 3-*O*-Glc (**23**) | 100.0±8.1** | 0.0±4.8 | −1.6±5.3 | 5.4±4.5 | 4.1±3.0 | 9.2±2.6 |
| Kaempferol 3-*O*-gentiobioside (**24**) | 100.0±23.1** | 0.0±9.2 | 6.7±12.7 | 13.3±6.7 | 14.5±6.8 | 35.3±11.7 |
| Kaempferol 3,7-di-*O*-Glc (**25**) | 100.0±4.3** | 0.0±1.5 | −5.0±1.8 | −5.2±1.1 | −7.5±1.1 | −7.9±1.0 |
| Kaempferol 3,4′-di-*O*-Glc (**26**) | 100.0±9.7** | 0.0±7.3 | −2.9±11.5 | 8.8±10.0 | −11.7±1.5 | 4.5±4.7 |
| **27** | 100.0±5.2** | 0.0±2.4 | 4.3±1.6 | 3.1±1.5 | 5.1±4.4 | 21.4±2.4** |
| Quercetin 3-*O*-Glc (**28**) | 100.0±2.4** | 0.0±2.0 | −2.9±1.8 | 0.6±1.5 | −0.7±3.7 | 5.3±1.8 |
| Rutin (**29**) | 100.0±11.8** | 0.0±4.2 | −13.8±5.5 | −20.3±5.1 | −28.5±7.8 | −40.0±14.7 |
| Quercetin 3,3′-di-*O*-Glc (**30**) | 100.0±3.5** | 0.0±1.6 | −1.1±1.3 | 3.1±5.4 | 3.3±1.6 | 0.4±1.3 |
| Aureusidin 6-*O*-Glc (**31**) | 100.0±5.0** | 0.0±3.0 | 5.7±4.7 | 11.1±2.8 | 15.1±1.5 | 28.2±1.1** |
| 7-Hydroxy-5-methoxyphthalide 7-*O*-Glc (**34**) | 100.0±1.4** | 0.0±1.4 | −1.3±2.5 | −0.3±3.5 | 3.0±1.2 | 1.0±1.1 |
| Scopolin (**35**) | 100.0±1.1** | 0.0±2.1 | −3.3±1.2 | 1.9±1.3 | 0.8±1.2 | 0.1±0.8 |
| Undulatoside A (**36**) | 100.0±9.3** | 0.0±1.7 | −0.3±5.3 | 3.6±3.3 | −2.4±3.7 | −3.0±2.4 |
| **37** | 100.0±2.9** | 0.0±2.7 | −1.2±3.8 | −0.3±3.6 | −9.2±2.2 | −27.4±1.1 |
| Syringin (**39**) | 100.0±11.2** | 0.0±4.6 | −4.7±4.3 | −8.3±2.4 | −14.2±1.9 | −14.4±1.4 |
| Orcinol Glc (**42**) | 100.0±8.5** | 0.0±1.7 | 1.9±2.8 | −1.7±1.4 | 1.4±3.0 | −3.5±1.4 |
| Icariside $F_2$ (**45**) | 100.0±12.4** | 0.0±4.7 | 6.6±4.7 | 2.5±5.2* | −2.8±5.3 | −8.5±3.9 |
| **46** | 100.0±9.9** | 0.0±2.4 | 14.5±5.7 | −2.5±8.2 | 7.5±7.9 | 6.0±2.2 |
| **47** | 100.0±6.1** | 0.0±6.7 | 3.2±3.0 | 0.7±5.0 | 5.3±2.3 | 2.5±3.5 |
| Icariside $D_1$ (**48**) | 100.0±6.0** | 0.0±4.6 | −3.4±1.1 | −0.9±1.9 | −3.4±1.9 | −5.2±2.3 |
| **49** | 100.0±4.9** | 0.0±4.5 | −0.8±2.0 | −2.9±0.7 | 1.3±2.6 | −4.4±2.7 |
| Piperine[19] | 100.0±2.6** | 0.0±1.3 | 5.5±1.6* | 5.3±1.4* | 10.6±0.9** | 41.8±1.4** |
| Silybin[19] | 100.0±3.6** | 0.0±2.6 | 5.3±2.8 | 22.0±3.8** | 48.0±4.1** | 50.8±3.9** |

Each value represents the mean±S.E.M. ($n$=4). Significantly different from the control, * $p$<0.05, ** $p$<0.01.

nariumoside IV was determined as (2*S*)-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 those 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,[68—70] 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 rotations, Horiba SEPA-300 digital polarimeter ($l$=5 cm); CD spectra, JASCO J-720WI spectrometer; UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; $^1$H-NMR spectra, JEOL JNM-ECA600 (600 MHz), JNM-LA500 (500 MHz), and EX-270 (270 MHz) spectrometers; $^{13}$C-NMR spectra, JEOL JNM-ECA600 (150 MHz), JNM-LA500 (125 MHz), and EX-270 (68 MHz) spectrometers with tetramethylsilane as an internal standard; and HPLC detector, Shimadzu RID-6A refractive index and SPD-10A*vp* UV–VIS detectors. HPLC column, Cosmosil 5$C_{18}$-MS-II (Nacalai Tesque Inc., 250×4.6 mm i.d.) and (250×20 mm i.d.) columns were used for analytical and preparative purposes, respectively.

The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., Aichi, Japan, 150—350 mesh); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., Aichi, Japan, 100—200 mesh); TLC, precoated TLC plates with Silica gel 60$F_{254}$ (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 $F_{254S}$ (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, precoated TLC plates with Silica gel RP-18 $WF_{254S}$ (Merck, 0.25 mm); and detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous $H_2SO_4$ followed by heating.

**Plant Material** The flower buds of *H. arenarium* cultivated in Poland were purchased *via* Tochimoto Tenkaido Co., Ltd., Osaka, Japan. The plant material was identified by one of authors (M. Y.). A voucher specimen of this plant is on file in our laboratory.

**Extraction and Isolation** The dried flowers of *H. arenarium* (3.0 kg) were extracted three times with MeOH under reflux for 3 h. Evaporation of the solvent under reduced pressure provided a methanolic extract (593.8 g, 19.8%). The methanolic extract (543.8 g) was partitioned between an EtOAc–$H_2O$ (1 : 1, v/v) mixture, and removal of the solvents *in vacuo* yielded an EtOAc-soluble fraction (210.0 g, 7.6%) and an aqueous phase. The aqueous phase was subjected to Diaion HP-20 column chromatography (3.0 kg, $H_2O$→MeOH) to give $H_2O$-eluted fraction (237.2 g, 8.6%) and MeOH-eluted fraction (88.6 g, 3.2%). The MeOH-eluted fraction (68.6 g) was subjected to normal-phase silica gel column chromatography [2.5 kg, $CHCl_3$–MeOH–$H_2O$ (20 : 3 : 1→10 : 3 : 1→7 : 3 : 1, lower layer→6 : 4 : 1, v/v/v)→MeOH] to give 12 fractions [Fr. 1 (0.85 g), Fr. 2 (1.20 g), Fr. 3 (0.90 g), Fr. 4 (1.80 g), Fr. 5 (6.40 g), Fr. 6 (11.00 g), Fr. 7 (5.40 g), Fr. 8 (4.00 g), Fr. 9 (7.10 g), Fr. 10 (5.80 g), Fr. 11 (6.10 g), and Fr. 12 (17.10 g)]. Fraction 4 (1.80 g) was subjected to reversed-phase silica gel column chromatography [70 g, MeOH–$H_2O$ (15 : 85→80 : 20, v/v)→MeOH] and HPLC [MeOH–$H_2O$ (30 : 70, v/v)] to give 7-hydroxy-5-methoxyphthalide 7-*O*-*β*-D-glucopyranoside (**34**, 476.8 mg, 0.12%). Fraction 5 (4.00 g) was purified by reversed-phase silica gel column chromatography [300 g, MeOH–$H_2O$ (15 : 85→80 : 20, v/v)→MeOH] and HPLC [MeOH–$H_2O$ (15 : 85—45 : 55, v/v)] to furnish (2*S*)-helichrysin (**5**, 320.0 mg, 0.069%), (2*R*)-helichrysin (**6**, 18.0 mg, 0.0045%), naringenin 7-*O*-*β*-D-glucopyranoside (**7**, 21.5 mg, 0.0053%), apigenin 7-*O*-*β*-D-glucopyranoside (**14**, 10.0 mg, 0.0025%), luteolin 7-*O*-*β*-D-glucopyranoside (**18**, 10.0 mg, 0.0025%), luteolin 3′-*O*-*β*-D-glucopyranoside (**19**, 5.3 mg, 0.0013%), kaempferol 3-*O*-*β*-D-glucopyranoside (**23**, 2.30 g, 0.58%), tortoside B (**33**, 10.0 mg, 0.0025%), 7-hydroxy-5-methoxyphthalide 7-*O*-*β*-D-glucopyranoside (**34**, 150.0 mg, 0.037%), scopolin (**35**, 68.0 mg, 0.017%), undulatoside A (**36**, 11.0 mg, 0.0017%), **37** (11.3 mg, 0.0023%), syringin (**39**, 15.0 mg, 0.0037%), dihydrosyringin (**40**, 7.0 mg, 0.0013%), and **41** (11.8 mg, 0.0029%). Fraction 7 (5.40 g) was subjected by reversed-phase silica gel column chromatography [300 g, MeOH–$H_2O$ (15 : 85→70 : 30, v/v)→MeOH] and HPLC [MeOH–$H_2O$ (10 : 90—40 : 60, v/v)] to furnish **5** (223.0 mg, 0.055%), **6** (17.0 mg, 0.0042%), chalconaringenin 2′-*O*-*β*-D-glucopyranoside (**12**, 305.5 mg, 0.076%), quercetin 3-*O*-*β*-D-glucopyranoside (**28**, 40.0 mg, 0.010%), **32** (10.0 mg, 0.0025%), oricinol *β*-D-glucopyranoside (**42**, 12.2 mg, 0.0035%), **47** (6.2 mg, 0.0015%), icariside $D_1$ (**48**, 90.0 mg, 0.017%), and adenosine (22.0 mg, 0.0055%). Fraction 8 (4.00 g) was subjected to reversed-phase silica gel column chromatography [300 g, MeOH–$H_2O$ (15 : 85→60 : 40, v/v)→MeOH] and HPLC [MeOH–$H_2O$ (18 : 82—40 : 60, v/v) or $CH_3CN$–$H_2O$ (8 : 92—11 : 89, v/v)] to give **5** (20.0 mg, 0.0050%), **6** (30.0 mg, 0.0075%), helicioside A (**10**, 6.0 mg, 0.0015%), (2*R*,3*R*)-dihydrokaempferol 7-*O*-*β*-D-glucopyranoside (**11**, 40.0 mg, 0.010%), **12** (25.0 mg, 0.0057%), **28** (70.0 mg, 0.018%), **44** (5.0 mg, 0.0012%), icariside $F_2$ (**45**, 19.0 mg, 0.0048%), and **47** (7.2 mg, 0.0018%). Fraction 9 (7.10 g) was subjected by reversed-phase silica gel column chromatography [300 g, MeOH–$H_2O$ (15 : 85→60 : 40, v/v)→MeOH] and HPLC [MeOH–$H_2O$ (9 : 92—35 : 65, v/v)] to furnish scutellarein 7-*O*-*β*-D-glucopyranoside (**20**, 7.0 mg, 0.0017%), **22** (13.0 mg, 0.0033%), **23** (13.0 mg, 0.0032%), **27** (17.0 mg, 0.0040%), **28** (13.3 mg, 0.0033%), aureusidin 6-*O*-*β*-D-glucopyranoside (**31**, 10.0 mg, 0.0025%), and **49** (38.0 mg, 0.0090%). Fraction 10 (5.10 g) was subjected to reversed-phase silica gel column chromatography [200 g, MeOH–$H_2O$ (15 : 85→60 : 40, v/v)→MeOH] and HPLC [MeOH–$H_2O$ (15 : 85—40 : 60, v/v), or MeOH–$CH_3CN$–$H_2O$ (12 : 8 : 80, v/v/v)] to give arenariumosides I (**1**, 18.0 mg, 0.0045%) and II (**2**, 15.4 mg, 0.0038%), **5** (4.0 mg, 0.0010%), apigenin 7-*O*-gentiobioside (**16**, 16.0 mg, 0.0040%), 6-hydroxyluteolin 7-*O*-*β*-D-glucopyranoside (**21**, 76.0 mg, 0.019%), **23** (4.8 mg, 0.0012%), kaempferol 3-*O*-gentiobioside (**24**, 28.0 mg, 0.0070%), rutin (**29**, 13.0 mg, 0.0032%), **38** (70.0 mg, 0.018%), and **46** (18.0 mg, 0.0045%). Fraction 11 (5.00 g) was subjected to reversed-phase silica gel column chromatography [300 g, MeOH–$H_2O$ (10 : 90→60 : 40, v/v)→MeOH] and HPLC [MeOH–$H_2O$ (10 : 90–45 : 55, v/v)] to give arenariumosides II (**2**, 8.5 mg, 0.0021%), III (**3**, 18.4 mg, 0.0046%), and IV (**4**, 13.9 mg, 0.0034%), 5,7-di-*O*-*β*-D-glucopyranosyl (2*S*)-naringenin (**8**, 18.0 mg, 0.0045%), 5,7-di-*O*-*β*-D-glucopyranosyl (2*R*)-naringenin (**9**, 39.0 mg, 0.0097%), chalconaringenin 2′,4′-di-*O*-*β*-D-glucopyranoside (**13**, 24.0 mg, 0.0060%), apigenin 7-*O*-*β*-D-glucopyranosiduronic acid methyl ester (**15**, 9.7 mg, 0.0024%), apigenin 7,4′-di-*O*-*β*-D-glucopyranoside (**17**, 7.4 mg, 0.019%), **21** (4.0 mg, 0.0010%), kaempferol 3,7-di-*O*-*β*-D-glucopyranoside (**25**, 10.0 mg, 0.0025%), kaempferol 3,4′-di-*O*-*β*-D-glucopyranoside (**26**, 44.7 mg, 0.011%), quercetin 3,3′-di-*O*-*β*-D-glucopyranoside (**30**, 6.0 mg, 0.0015%), and **43** (5.5 mg, 0.0013%).

Arenariumoside I (**1**): A pale yellow powder, $[\alpha]_D^{26}$ −12.3° ($c$=0.90, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{27}H_{32}O_{15}Na$ $(M+Na)^+$ 619.1639; Found 619.1646. CD (nm, $\Delta\varepsilon$, MeOH): 302 (−7.25), 333 (+4.95). UV [$\lambda_{max}$ (log $\varepsilon$), MeOH]: 222 (4.67), 284 (4.46) nm. IR (KBr, cm$^{-1}$): 3569, 1655, 1638, 1509, 1458, 1071. $^1$H-NMR (500 MHz, DMSO-$d_6$) $\delta$: given in Table 1. $^{13}$C-NMR (125 MHz, DMSO-$d_6$) $\delta_C$: given in Table 1. Positive-ion FAB-MS $m/z$: 619 $(M+Na)^+$.

Arenariumoside II (**2**): A pale yellow powder, $[\alpha]_D^{26}$ −64.5° ($c$=0.20, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{27}H_{32}O_{15}Na$ $(M+Na)^+$ 619.1639; Found 619.1644. CD (nm, $\Delta\varepsilon$, MeOH): 286 (+2.74), 333 (−1.23). UV [$\lambda_{max}$ (log $\varepsilon$), MeOH]: 223 (4.69), 282 (4.45) nm. IR (KBr, cm$^{-1}$): 3568, 1655, 1638, 1509, 1458, 1071. $^1$H-NMR (500 MHz, DMSO-$d_6$) $\delta$: given in Table 1. $^{13}$C-NMR (125 MHz, DMSO-$d_6$) $\delta_C$: given in Table 1. Positive-ion FAB-MS $m/z$: 619 $(M+Na)^+$.

Arenariumoside III (**3**): A pale yellow powder, $[\alpha]_D^{25}$ −62.1° ($c$=0.32, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{27}H_{32}O_{15}Na$ $(M+Na)^+$ 619.1639; Found 619.1646. UV [$\lambda_{max}$ (log $\varepsilon$), MeOH]: 349 (4.15) nm. IR (KBr, cm$^{-1}$): 3569, 1687, 1655, 1509, 1458, 1075. $^1$H-NMR (500 MHz, DMSO-$d_6$) $\delta$: given in Table 2. $^{13}$C-NMR (125 MHz, DMSO-$d_6$) $\delta_C$: given in Table 2. Positive-ion FAB-MS $m/z$: 619 $(M+Na)^+$.

Arenariumoside IV (**4**): A pale yellow powder, $[\alpha]_D^{27}$ −58.7° ($c$=0.15, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{27}H_{32}O_{15}Na$ $(M+Na)^+$ 619.1639; Found 619.1646. CD (nm, $\Delta\varepsilon$, MeOH): 298 (−7.39), 331 (+3.82). UV [$\lambda_{max}$ (log $\varepsilon$), MeOH]: 282 (4.45) nm. IR (KBr, cm$^{-1}$): 3568, 1647, 1611, 1509, 1458, 1071, 1032. $^1$H-NMR (500 MHz, DMSO-$d_6$) $\delta$: given in Table 1. $^{13}$C-NMR (125 MHz, DMSO-$d_6$) $\delta_C$: given in Table 1. Positive-ion FAB-MS $m/z$: 619 $(M+Na)^+$.

**Acid Hydrolysis of 1—4** A solution of **1**—**4** (6.0, 6.3, 4.0, 4.0 mg) in 1 M HCl (1.0 ml) was heated at 80 °C for 1 h. After cooling, the reaction mix-

ture 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 ($H_2O \rightarrow MeOH$). The $H_2O$-eluted fraction was subjected to HPLC analysis under the following conditions: HPLC column, Kaseisorb LC $NH_2$-60-5, 4.6 mm i.d.×250 mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan); mobile phase, $CH_3CN$–$H_2O$ (85 : 15, v/v); flow rate 0.8 ml/min]. Identification of D-glucose from **1**—**4** present in the $H_2O$-eluted fraction was carried out by comparison of its retention time and optical rotation with that of an authentic sample. $t_R$: 13.9 min (positive optical rotation). On the other hand, the MeOH-eluted fraction was purified by HPLC [$CH_3CN$–1% aqueous AcOH (40 : 60, v/v)] to furnish naringenin[44] (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-$\alpha$-Induced Cytotoxicity in L929 Cells** Inhibitory effect on TNF-$\alpha$-induced cell death in L929 cells was assayed by the method described in our previous report.[19] Briefly, a suspension of $2\times10^4$ cells [obtained from Dainippon Pharmaceutical (Osaka, Japan)] in 100 $\mu$l of minimum essential medium Eagle supplemented with 1% non-essential amino acid solution (Invitrogen), fetal calf serum (FCS, 10%), penicillin G (100 units/ml), and streptomycin (100 $\mu$g/ml) was incubated in a 96-well microplate. After 44 h of incubation in the medium containing TNF-$\alpha$ (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. Probability ($p$) values less than 0.05 were considered significant.

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