Constituents of Leaves of *Phellodendron japonicum* Maxim. and Their Antioxidant Activity

Chih-Yang Chiu,[a] Chia-Ying Li,[a] Chao-Chen Chiu,[a] Masatake Niwa,[b] Susumu Kitanaka,[c] Amooru Gangaiah Damu,[a] E-Jian Lee,[d] and Tian-Shung Wu*[,a,e]

[a] *Department of Chemistry, National Cheng Kung University; Tainan 701, Taiwan, R.O.C.:* [b] *Faculty of Pharmacy, Meijo University; Tempaku-ku, Nagoya 468–8502, Japan:* [c] *College of Pharmacy, Nihon University; Narashinodai, Funabashi, Chiba 274–8555, Japan:* [d] *Neurophysiology Laboratory, Neurosurgical Service, Department of Surgery and Anesthesiology, National Cheng Kung University Medical Center and Medical School; Tainan 701, Taiwan, R.O.C.: and* [e] *National Research Institute of Chinese Medicine; Taipei 112, Taiwan, R.O.C.*

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Three new flavonoid derivatives, 6‴-*O*-acetyl amurensin (1), 6‴-*O*-acetyl phellamurin (3) and (2*R*)-phellodensin-F (5), together with thirty known compounds have been isolated from the leaves of *Phellodendron japonicum* Maxim. Their structures were established by means of spectroscopic analysis, including extensive 2D NMR and Mass spectra. The known compounds were identified by comparison with published physical and spectral data. The isolated compounds were screened for their *in vitro* antioxidant activity through DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay. Compounds quercetin and phellodenin-A demonstrated significant radical scavenging activity.

Key words *Phellodendron japonicum*; flavonoid; antioxidant activity; DPPH assay

Phellodendron is a small genus of aromatic deciduous trees of East Asia often having thick corky bark. This bark has found application in Chinese traditional medicine for various diseases like meningitis, bacillary dysentery, pneumonia, tuberculosis, and liver cirrhosis.[1—3] Previous phytochemical work on the members of *Phyllodendron* has reported the isolation of berberine and aporphine type alkaloids, flavonoids, coumarins, lignans and limonoids.[3,4] During the course of our investigation on the bioactive chemical components of *Phyllodendron* species, we have focused on *Phellodendron japonicum* Maxim., which is a deciduous tree found widely in Honshu, Japan.[4] Few reports encountered on the isolation of flavonoids as major constituents from this plant.[5] The current study describes the isolation and structure determination of three new flavone derivatives as well as thirty known compounds from the methanol extract of the leaves of title plant and their DPPH radical scavenging activity.

Results and Discussion

6‴-*O*-Acetyl amurensin (**1**) was isolated as yellow powder with elemental composition $C_{28}H_{30}O_{12}$ from its HR-FAB-MS ($[M+1]^+$ *m/z* 559.1819). The IR absorption bands at 3361, 1719 and 1646 cm^{-1} indicated the presence of hydroxyl, ester and conjugated hydrogen-bonded carbonyl groups, respectively. The UV absorptions at 271, 327 and 373 nm were typical of a flavonol skeleton.[6] The ^{1}H-NMR of **1** revealed an A_2B_2 system of proton signals at δ 8.18 (2H, d, J=8.8 Hz, H-2′, 6′) and 7.03 (2H, d, J=8.8 Hz, H-3′, 5′), a D_2O exchangeable hydrogen-bonded hydroxyl signal at δ 12.14 (1H, s, 5-OH). A singlet at δ 6.67 was ascribed to H-6 of A-ring as it has HMQC with a carbon at δ_C 98.1 (C-6) and ^2J and ^3J HMBC correlations with carbons at δ_C 159.3 (C-5) and 109.0 (C-8), respectively. Also, characteristic prenyl proton signals were observed at δ 3.63 (2H, m, H-1″), 5.28 (1H, br t, J=6.8 Hz, H-2″), 1.65 (3H, s, CH_3-4″), and 1.82 (3H, s, CH_3-5″). The position of the prenyl group was determined at C-8 based on the HMBC correlations between H-1″ and C-7 (δ_C 160.8), C-8. The presence of an anomeric proton at δ 5.14 (d, J=7.6 Hz) and the carbon signals at δ 101.0, 77.3, 74.5, 74.0, 70.7, and 63.6 suggested the presence of one glucosyl moiety with β-configuration. From NOESY studies, glucose residue in **1** was found to be linked to C-7 as NOE of anomeric proton with H-6 were observed. There were very similar to those amurensin (**2**).[7] An acetyl methyl singlet at δ 2.04 (3H, s) in ^{1}H-NMR spectrum together with the ^{13}C-NMR signals at δ 170.3 and 20.0 inferred that **1** was an acetyl derivative of amurensin. The location of the acetyl group was determined to C-6‴ due to the ^3J correlation between H-6‴ (δ_H 4.21) and acetyl-carbonyl (δ_C 170.3) in the HMBC experiment. This was also supported by the down field shifts of C-6‴ to δ_C 63.6 and up field shift of C-5‴ to δ_C 74.5. Thus, the structure of **1** was elucidated as 6‴-*O*-acetyl amurensin.

6′-*O*-Acetyl phellamurin (**3**) was obtained with HPLC as white powder. The ^{1}H- and ^{13}C-NMR spectra of **3** showed signals due to H-2 [δ 5.09 (d, J=12.0 Hz)], H-3 [δ 4.64 (dd, J=12.0, 4.4 Hz)] and 3-OH [δ 4.71 (d, J=4.4 Hz, D_2O exchangeable)] of a flavanonol suggested that **3** was a 2,3-*trans*-dihydroflavonol derivative.[6] A broad singlet at δ 11.64 exchangeable with D_2O was assigned to a chelated hydroxyl group at C-5. A set of A_2B_2 doublets at δ 7.43 (2H, J=8.8 Hz) and 6.91 (2H, J=8.8 Hz) was assigned to 2′-, 6′- and 3′-, 5′-protons of B-ring. A singlet at δ 6.36 was ascribed to H-6 of A-ring as it correlated to carbon at δ 96.4 in HMQC spectrum. Also, characteristic prenyl proton signals were observed at δ 3.13 (1H, dd, J=14.4, 7.6 Hz, H-1″), 3.31 (1H, m, H-1″), 5.16 (1H, m, H-2″), 1.58 (3H, s, CH_3-4″), and 1.61 (3H, s, CH_3-5″). The position of the prenyl group was determined at C-8, since H-1″ showed HMBC correlation with C-8. The presence of an anomeric proton at δ 5.08 (d, J=7.6 Hz) and the carbon signals at δ 101.2, 77.9, 75.1, 74.5, 71.2, and 64.2 suggested the presence of one glucosyl moiety with β-configuration. From ROESY studies, glucose residue in **3** was found to be linked to C-7 as of anomeric proton had NOE with H-6. An acetyl methyl singlet at δ 1.93

* To whom correspondence should be addressed. e-mail: tswu@mail.ncku.edu.tw

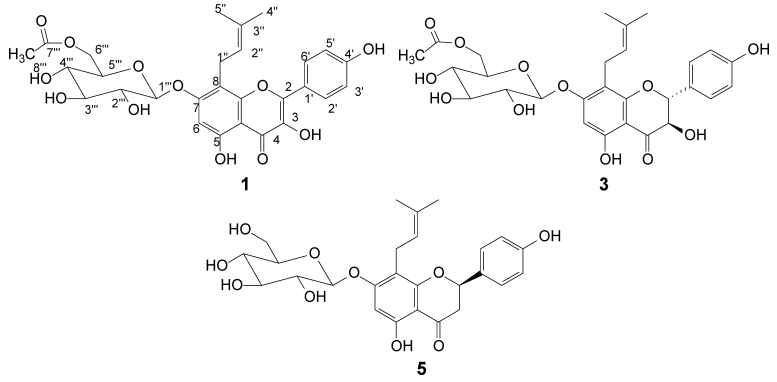

Fig. 1. Structures of New Compounds **1**, **3**, and **5**

(3H, s) in ^{1}H-NMR spectrum together with the ^{13}C-NMR signals at δ 170.9 and 20.6 were also observed. These data and coupling patterns were very close to those of phellamurin (**4**)[7] except for the acetyl signals. Thus, compound **2** was postulated as being an acetyl derivative of phellamurin. The location of the acetyl group was determined on C-6‴ due to the ^3J correlation between H-6‴ (δ_H 4.21) and an acetyl-carbonyl carbon (δ_C 170.9) in the HMBC experiment. The attachment of an acetyl group to C-6‴ was also evidenced by the down field shifts of C-6‴ to δ_C 64.2 and up field shift of neighbouring carbon C-5‴ to δ_C 75.1 in **3**, in comparison to those of **4**. Thus the structure of **3** was assigned to 6‴-*O*-acetyl phellamurin.

(2*R*)-Phellodensin-F (**5**) was isolated as white powder with elemental composition $C_{26}H_{30}O_{10}$ from its HR-FAB-MS ($[M+1]^+$ *m/z* 503.1919). The IR absorption bands at 3380 and 1635 cm^{-1} consistent with the presence of hydroxyl group and hydrogen-bonded carbonyl group, respectively. The UV absorptions at 286, and 342 nm were characteristic of a flavanone skeleton.[6] The ^{1}H-NMR spectrum displayed hydrogen-bonded hydroxyl singlet at δ 12.09 (1H, s, 5-OH). A singlet at δ 6.29 was assumed to be H-6 as it showed correlation with carbon at δ 95.6 in HMQC spectrum. One set of A_2B_2 signals at δ 7.41 (2H, d, J=8.4 Hz) and 6.90 (2H, d, J=8.4 Hz) were attributed to H-2′, -6′ and H-3′, -5′ of *para* substituted B-ring, which was further supported by a D_2O exchangeable downfield proton signal at δ 8.52 for 4′-OH in the ^{1}H-NMR spectrum. Besides, the characteristic H-2 and H-3 proton signals of flavnone were observed at δ 5.48 (1H, dd, J=12.8, 3.6 Hz, H-2), 3.18 (1H, m, H-3α) and 2.79 (1H, m, H-3β). The presence of an anomeric proton at δ 5.06 (1H, d, J=7.6 Hz) and the carbon signals at δ 100.8, 77.3, 77.2, 74.0, 70.5 and 61.8 suggested the presence of one glucosyl moiety with β-configuration. Also, the signals at δ 3.18 (1H, m, H-1″), 3.36 (1H, dd, J=13.6, 7.6 Hz, H-1″), 5.20 (1H, br t, J=7.6 Hz, H-2″), 1.61 (6H, s, CH_3-4″ and CH_3-5″) suggested that **5** contained a prenyl group located. The above data were superimposable on those of phellodensin-F.[8] However, the CD spectrum of **5** showed negative Cotton effect at the region of 340 nm and a positive effect at 284 nm established *R*-configuration at C-2 position. For this reason, the structure of **5** can be represented as (2*R*)-phellodensin-F.

In addition, thirty known compounds, amurensin (**2**),[7] phellamurin (**4**),[7] quercetin (**6**),[9] phellodensin-A (**7**),[10] phellodensin-B (**8**),[11] rutaecarpine (**9**),[12] 3-formylindole (**10**),[13] umbelliferone (**11**),[14] esculetin (**12**),[15] scopoletin (**13**),[14] scoparone (**14**),[14] columbianetin (**15**),[16] *p*-hydroxybenzaldehyde (**16**),[17] methylparaben (**17**),[18] *p*-hydroxybenzoic acid (**18**),[17] methyl *p*-anisate (**19**),[19] vanillic acid (**20**),[17] methyl *p*-hydroxycinnamate (**21**),[17] methyl caffeate (**22**),[20] benzoic acid (**23**),[21] *p*-coumaric acid (**24**),[22] caffeic acid (**25**),[23] phellodenin-A (**26**),[24] lupenone (**27**),[25] β-sitosterol (**28**),[26] friedelin (**29**),[27] 3-*epi*lupeol (**30**),[28] *N*-*p*-coumaroyltyramine (**31**),[29] *N*-*trans*-cinnamoyltyramine (**32**),[29] and grasshopper-ketone (**33**)[30] were also isolated from the leaves of *P. japonicum*. Among these compounds, all except **2** and **4** were isolated for the first time form *P. japonicum*. All the known compounds were identified by comparison of their spectroscopic data (UV, IR, NMR, MS spectrometry) with the authentic samples or literature data.

The nine compounds of the isolates **2**, **4**, **6**, **16**, **17**, **18**, **20**, **22**, and **26** were examined for their antioxidant properties using the α,α-diphenyl-β-picrylhydrazyl free radical (DPPH) scavenging assay. The results were compared with α-tocopherol, which was commonly used in the food industry as antioxidant (IC_{50}, 27.0 μM). Among them, compounds **6** and **26** showed strong DPPH radical-scavenging activity with an IC_{50} values of 17.5 and 21.5 μM, respectively. Compounds **2**, **18** and **20** showed moderate scavenging activity with an IC_{50} values of 94.0, 87.6, 59.4 μM, respectively. These results implied that *P. japonicum* might be able to afford protection against oxidative damage.

Experimental

General Procedures Melting points were recorded on Yanaco MP-S3 melting point apparatus without correction. The UV spectra were recorded on a Hitachi UV-3210 spectrophotometer. The IR spectra were measured on a Jasco IR Report-100 spectrophotometer as KBr discs. ^{1}H, ^{13}C, HMQC, HMBC, and NOESY NMR spectra were recorded on Bruker AC-200, AMX-400 and Varian-400 Unity Plus spectrometers, using tetramethylsilane (TMS) as internal standard; all chemical shifts are reported in parts per million (ppm, δ). Mass spectra (EI or FAB) were performed on a VG 70-250 S spectrometer. Optical rotations were recorded on a Jasco DIP-370 polarimeter. CD spectra were recorded with a Jasco J-720 spectropolarimeter.

Plant Material The leaves of *P. japonicum* Maxim. were collected in August 2000 from Japan, and authenticated by Prof. C. S. Kuoh (Department of Life Science, National Cheng Kung University, Tainan, Taiwan). A voucher specimen of the plant (NCKU Wu 20000809) has been deposited at the herbarium of National Cheng Kung University, Tainan, Taiwan.

Extraction and Separation The air-dried and powdered leaves of *P. japonicum* (2.3 kg) were extracted with hot methanol (5 l×6) and concentrated to give dark brown syrup and colorless crystals **2** (23.6 g). The syrup was allowed to recrystallization in acetone to get **4** (15.7 g). Then the remaining syrup was partitioned between water and chloroform and the resulted chloroform solubles (90 g) were chromatographed over silica gel using a gradient of chloroform and methanol to afford 12 fractions. Fraction

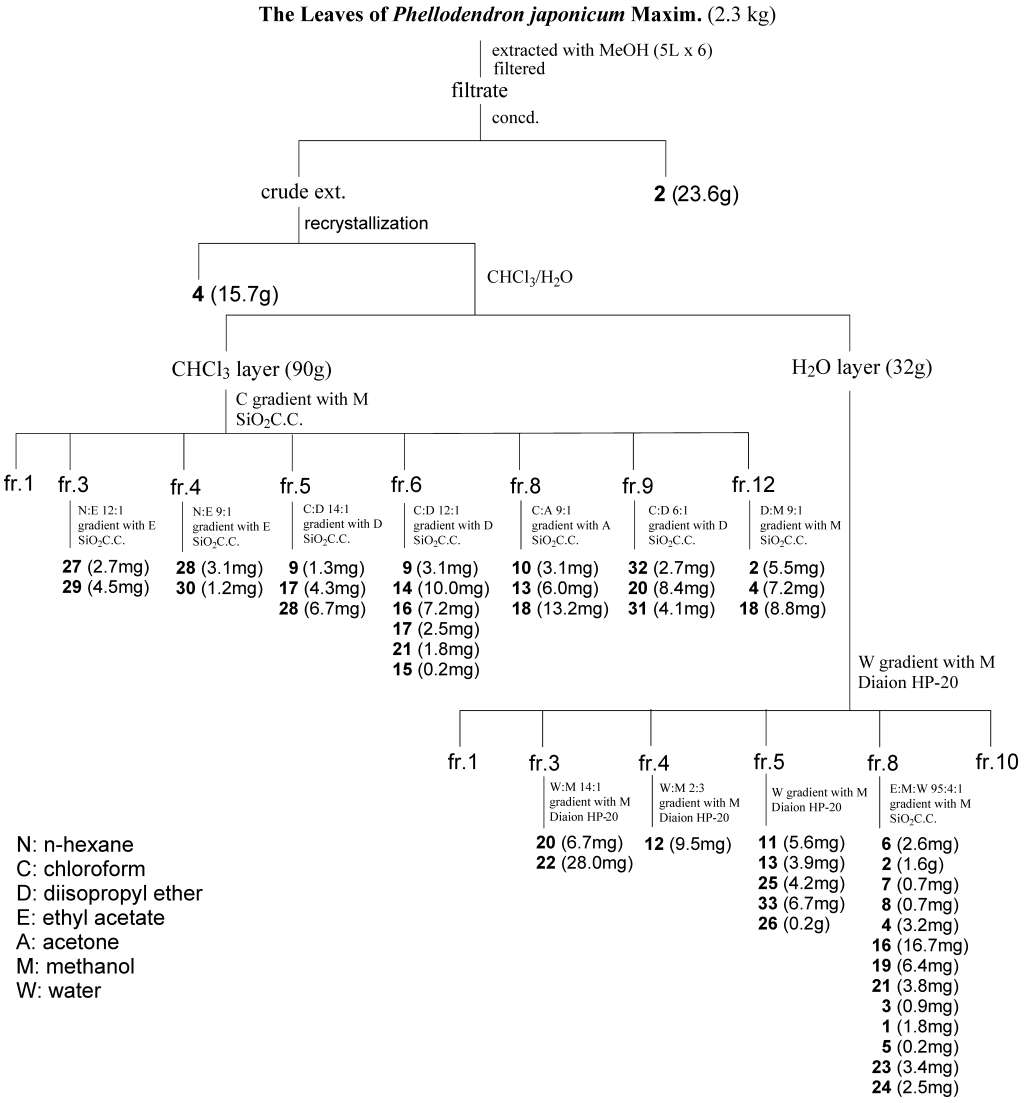

Chart 1

3 was rechromatographed over silica gel using mixture of *n*-hexane and ethyl acetate as eluents, and purified by preparative TLC to yield **27** (2.7 mg) and **29** (4.5 mg). Fraction 4 on column chromatography with *n*-hexane and ethyl acetate (9 : 1) yielded **28** (3.1 mg) and **30** (1.2 mg). Fraction 5 was chromatographed over silica gel using a gradient of chloroform and diisopropyl ether to afford **9** (1.3 mg), **17** (4.3 mg), and **28** (6.7 mg). Fraction 6 was subjected to column chromatography over silica gel with chloroform–diisopropyl ether (12 : 1) to give **9** (3.1 mg), **14** (10.0 mg), **16** (7.2 mg), **17** (2.5 mg), **21** (1.8 mg), and **15** (0.2 mg). Similarly, fraction 8 was also chromatographed over silica gel with chloroform–acetone (9 : 1) to give **10** (3.1 mg), **13** (6.0 mg), and **18** (13.2 mg). Silica gel column chromatography of fraction 9 by chloroform–diisopropyl ether (6 : 1) resulted in **32** (2.7 mg), **20** (8.4 mg), **31** (4.1 mg). The last fraction of chloroform layer was separated with silica gel column chromatography using diisopropyl ether and methanol in 9 : 1 ratio to afford **2** (5.5 mg), **4** (7.2 mg), and **18** (8.8 mg). The water-soluble fraction (32.0 g) was chromatographed over Diaion HP-20 using water–methanol gradients, which yielded 10 fractions. Fraction 3 was column chromatographed with Diaion HP-20 gel using gradients of water and methanol to afford **20** (6.7 mg) and **22** (28.0 mg). Fraction 4 on column chromatography over Diaion HP-20 with water–methanol (2 : 3) yielded **12** (9.5 mg). Column chromatography of fraction 5 over Diaion HP-20 with water–methanol gradients afforded **11** (5.6 mg), **13** (3.9 mg), **25** (4.2 mg), **33** (6.7 mg), and **26** (205.2 mg). Fraction 8 was chromatographed over silica gel using mixture of ethyl acetate, methanol and water as eluents (95 : 4 : 1) and successively purified by HPLC [Cosmosil 5C-18-AR-II waters (5 μm)] with methanol–water (35 : 65) to afford **6** (2.6 mg), **2** (1.6 g), **7** (0.7 mg), **8** (0.7 mg), **4** (3.2 mg), **16** (16.7 mg), **19** (6.4 mg), **21** (3.8 mg), **3** (0.9 mg), **1** (1.8 mg), **5** (0.2 mg), **23** (3.4 mg), and **24** (2.5 mg).

6‴-*O*-Acetyl Amurensin (**1**): Yellow powder, mp: 235—237 °C. HR-FAB-MS *m/z*: 559.1819 $[M+H]^+$ (Calcd for $C_{28}H_{31}O_{12}$: 559.1816). $[\alpha]_D^{25}$ −91.3° (*c*=0.05, MeOH). UV λ_{max} (MeOH) (log ε) nm: 271 (4.46), 327 (4.20), 373 (4.36). IR (KBr) cm^{-1}: 3361 (OH), 2923, 1719 (C=O), 1646 (C=O), 1599, 1259, 1081. FAB-MS *m/z* (%): 559 $[M+H]^+$ (17), 355 (24), 299 (16), 185 (100), 149 (20). ^{1}H-NMR (400 MHz, acetone-d_6) δ: 1.65 (3H, s, 4″-CH_3), 1.82 (3H, s, 5*0*-CH_3), 2.04 (3H, s, 8‴-CH_3), 3.45 (1H, m, H-4‴), 3.52—3.74 (4H, m, H-1″, 2‴, 3‴), 3.86 (1H, td, *J*=8.6, 2.0 Hz, H-5‴), 4.21 (1H, m, H-6‴), 4.46 (1H, d, *J*=9.6 Hz, H-6‴), 4.51 (1H, d, *J*=4.4 Hz, OH), 4.55 (1H, br s, OH), 4.65 (1H, br s, OH), 5.14 (1H, d, *J*=7.6 Hz, H-1‴), 5.28 (1H, br t, *J*=6.8 Hz, H-2″), 6.67 (1H, s, H-6), 7.03 (2H, d, *J*=8.8 Hz, H-3′, 5′), 8.07 (1H, br, 3-OH), 8.18 (2H, d, *J*=8.8 Hz, H-2′, 6′), 9.05 (1H, br s, 4′-OH), 12.14 (1H, s, 5-OH). ^{13}C-NMR (100 MHz, acetone-d_6) δ: 17.5 (C-5″), 20.0 (C-8‴), 21.7 (C-1″), 25.1 (C-4″), 63.6 (C-6‴), 70.7 (C-4‴), 74.0 (C-2‴), 74.5 (C-5‴), 77.3 (C-3‴), 98.1 (C-6), 101.0 (C-1‴), 105.1 (C-10), 109.0 (C-8), 115.7 (C-3′, 5′), 122.9 (C-2″), 129.9 (C-2′, 6′), 131.4 (C-3″), 136.1 (C-3), 147.8 (C-2), 153.5 (C-9), 159.3 (C-5), 159.6 (C-4′), 160.8 (C-7), 170.3 (C-7‴), 176.4 (C-4).

6‴-*O*-Acetyl Phellamurin (**3**): White powder. HR-FAB-MS *m/z*: 561.1969 $[M+H]^+$ (Calcd for $C_{28}H_{33}O_{12}$: 561.1972). ^{1}H-NMR (400 MHz, acetone-d_6) δ: 1.58 (3H, s, 4″-CH_3), 1.61 (3H, s, 5″-CH_3), 1.93 (3H, s, 8‴-CH_3), 3.13 (1H, dd, *J*=14.4, 7.6 Hz, H-1″), 3.31 (1H, m, H-1″), 3.43 (1H, m, H-4‴), 3.55—3.58 (2H, m, H-2‴, 3‴), 3.84 (1H, m, H-5‴), 4.21 (1H, dd, *J*=11.6, 7.2 Hz, H-6‴), 4.44 (1H, m, H-6‴), 4.51 (1H, m, OH), 4.60 (1H, m, OH), 4.64 (1H, dd, *J*=12.0, 4.4 Hz, H-3), 4.71 (1H, d, *J*=4.4 Hz, 3-OH), 5.08 (1H, d, *J*=7.6 Hz, H-1‴), 5.09 (1H, d, *J*=12.0 Hz, H-2), 5.16 (1H, m, H-2″),

6.36 (1H, s, H-6), 6.91 (2H, d, J=8.8 Hz, H-3′, 5′), 7.43 (2H, d, J=8.8 Hz, H-2′, 6′), 8.51 (1H, s, 4′-OH), 11.64 (1H, br s, 5-OH); ^{13}C-NMR (100 MHz, acetone-d_6) δ: 17.8 (C-5″), 20.6 (C-8‴), 22.2 (C-1″), 25.8 (C-4″), 64.2 (C-6‴), 71.2 (C-4‴), 73.4 (C-3), 74.5 (C-2‴), 75.1 (C-5‴), 77.9 (C-3‴), 84.2 (C-2), 96.4 (C-6), 101.2 (C-1‴), 102.6 (C-10), 110.5 (C-8), 115.8 (C-3′, 5′), 123.4 (C-2″), 129.1 (C-1′), 130.1 (C-2′, 6′), 131.3 (C-3″), 158.6 (C-4′), 160.1 (C-9), 162.5 (C-5), 164.4 (C-7), 170.9 (C-7‴), 199.3 (C-4).

(2*R*)-Phellodensin-F (**5**): White powder, mp: 220—221 °C. HR-FAB-MS *m/z*: 503.1919 $[M+H]^+$ (Calcd for $C_{26}H_{31}O_{10}$: 503.1917). $[\alpha]_D^{25}$ −67.5° (c=0.15, MeOH). UV λ_{max} (MeOH) nm: 286, 342. IR (KBr) cm^{-1}: 3380 (OH), 2921, 1635 (C=O), 1371, 1074. FAB-MS *m/z* (%): 503 $[M+H]^+$ (32), 341 (100), 285 (38), 219 (22), 185 (71), 165 (58), 149 (29). ^{1}H-NMR (400 MHz, acetone-d_6) δ: 1.61 (6H, s, H-4″, 5″), 2.79 (1H, m, H-3$_\beta$), 3.15—3.22 (2H, m, H-3$_\alpha$, 1″), 3.36 (1H, dd, J=13.6, 7.6 Hz, H-1″), 3.46 (1H, m, H-4‴), 3.52—3.54 (2H, m, H-2‴, 3‴), 3.60 (1H, m, H-5‴), 3.71 (1H, m, H-6‴), 3.89 (1H, dd, J=8.4, 2.8 Hz, H-6‴), 4.29 (1H, d, J=4.4 Hz, OH), 4.38 (1H, d, J=1.6 Hz, OH), 4.47 (1H, d, J=2.8 Hz, OH), 5.06 (1H, d, J=7.6 Hz, H-1‴), 5.20 (1H, br t, J=7.6 Hz, H-2″), 5.48 (1H, dd, J=12.8, 3.6 Hz, H-2), 6.29 (1H, s, H-6), 6.90 (2H, d, J=8.4 Hz, H-3′, 5′), 7.41 (2H, d, J=8.4 Hz, H-2′, 6′), 8.52 (1H, s, 4′-OH), 12.09 (1H, s, 5-OH). ^{13}C-NMR (100 MHz, acetone-d_6) δ: 17.2 (C-5″), 21.8 (C-1″), 25.2 (C-4″), 42.9 (C-3), 61.8 (C-6‴), 70.5 (C-4‴), 74.0 (C-2‴), 77.2 (C-3‴), 77.3 (C-5‴), 79.1 (C-2), 95.6 (C-6), 100.8 (C-1‴), 103.7 (C-10), 109.6 (C-8), 115.5 (C-3′, 5′), 123.1 (C-2″), 128.2 (C-2′, 6′), 130.2 (C-1′), 130.5 (C-3″), 157.9 (C-4′), 159.6 (C-9), 162.3 (C-5), 163.7 (C-7), 197.6 (C-4). CD (MeOH: c=8.36×10^{-4} M) $[\theta]_{340}$ −0.23922, $[\theta]_{322}$ 0, $[\theta]_{306}$ +0.175312, $[\theta]_{284}$ +0.150993, $[\theta]_{262}$ +0.0223585, $[\theta]_{250}$ +0.15031, $[\theta]_{240}$ +0.0781038, $[\theta]_{220}$ +0.150084.

Free Radical-Scavenging Activity Assay The effect of isolated compounds on the scavenging of DPPH radical was estimated according to the method of Yamaguchi *et al.*[31] with minor modifications. A sample was dissolved in 0.1 ml DMSO and then added to 0.1 ml of 0.1 mM DPPH in ethanol. The mixture was shaken vigorously and allowed to stand for 30 min at room temperature in the dark. The absorbance at 517 nm by DPPH was measured by a μ Quant universal microplate spectrophotometer. α-Toc (Sigma Chemical Co.) was used as a standard agent. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{scavenging effect (\%)} = [1 - (\text{absorbance of sample at 517 nm} / \text{absorbance of control at 517 nm})] \times 100$$

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