Antioxidant Benzoylated Flavan-3-ol Glycoside from *Celastrus orbiculatus*

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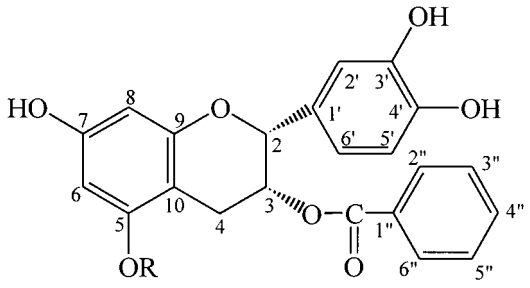
Received May 22, 2000

A new flavan-3-ol glycoside, (−)-epicatechin-5-*O*-*β*-D-glucosyl-3-benzoate (**1**), and two known compounds, (−)-epicatechin and (−)-epiafzelechin, were isolated from an EtOAc extract of *Celastrus orbiculatus* aerial parts that exhibited significant antioxidant effect in a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay. The structure of **1** was elucidated by spectroscopic analyses, and compound **1** and its aglycon, (−)-epicatechin-3-benzoate (**2**), were found to be moderately active as antioxidants in the DPPH assay.

Celastrus orbiculatus Thunb. (Celastraceae) is a perennial shrub that has been used in folk medicine as a treatment for rheumatoid arthritis and bacterial infection.[1] The family Celastraceae is well-known to produce various dihydro-*β*-agarofuran derivatives,[2] some of which exhibit insecticidal or insect antifeedant activity,[3] antitumor activity,[4] and antitumor-promoting activity.[5] Recently, we reported that dihydro-*β*-agarofuran compounds from this plant reversed multidrug resistance in cancer cells.[6,7] As part of our continuing search for plant-derived antiinflammatory agents, an ethyl acetate-soluble extract of the dried aerial parts of this plant was found to exhibit significant antioxidant effects, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical.[8,9] Bioassay-directed fractionation of this extract resulted in the isolation of a new benzoylated flavane glycoside, (−)-epicatechin-5-*O*-*β*-D-glucopyranosyl-3-benzoate (**1**), which was purified along with the known compounds (−)-epicatechin (**3**) and (−)-epiafzelechin (**4**). (−)-Epicatechin-3-benzoate (**2**) was also obtained from enzymatic hydrolysis of **1**. The present paper describes the isolation and structure elucidation of **1** and the antioxidant evaluation of the flavan-3-ol derivatives **1**−**4**.



1 R = β- D-glucose

2 R=H

Compound **1** was obtained as bright pink needles. It showed dark-blue with $FeCl_3$ and orange-red with anisaldehyde−sulfuric acid. The molecular formula of **1** was determined to be $C_{28}H_{28}O_{12}$ from the positive HRFABMS. The ^{1}H NMR spectrum of **1** shows 10 aromatic signals arising from three different aromatic moieties. The two characteristic *meta*-coupled doublets (AB type) at *δ* 6.17 and 6.06 (J = 2.1 Hz) were attributed to the H-6 and H-8 protons of the flavonoid A-ring structure. Another three signals at *δ* 6.92 (J = 1.9 Hz), *δ* 6.63 (J = 8.2 Hz), and *δ* 6.73 (J = 8.2, 1.9 Hz) originated from 2′, 5′, and 6′ protons of the flavonoid B ring. The remaining five signals, corresponding to aromatic protons at *δ* 7.79 (2H, br d, J = 7.5 Hz), *δ* 7.47 (2H, t, J = 7.5 Hz), and *δ* 7.61 (1H, br t, J = 7.5 Hz), indicated that **1** contains a monosubstituted aromatic ring. The mass fragmentation ion at *m*/*z* 435 [M − $C_6H_5CO_2$]$^+$ supports the presence of a benzoyl group. The lower field shifted signals at *δ* 5.51 (1H, m, H-3) and *δ* 5.09 (1H, br s, H-2) suggested the presence of a flavan-3-ol moiety with 2,3-*cis* type stereochemistry. The small coupling constant between H-2 (*δ* 5.09) and H-3 (*δ* 5.51) and the ^{13}C NMR chemical shift for C-2 (*δ* 77.0) and C-3 (*δ* 68.7) were in agreement with those of epigallocatechin structure.[10] The CD spectrum of **1** showed a negative Cotton effect in the 280 nm region (^1L_b transition), indicating that **1** has (*R*)-configuration at the C-2 position.[11] Together with the $^3J_{2,3}$ coupling constant and the optical rotation, $[\alpha]^{25}_D$ −95°, the absolute configuration was determined to be 2*R*,3*R*. The placement of the benzoyl group at C-3 was confirmed by the HMBC spectrum, in which the carbonyl carbon at *δ* 164.8 showed long-range correlations with H-3 (*δ* 5.51, m) and the *ortho*-, *meta*-coupled benzoyl protons, H-2″ and H-6″ at *δ* 7.79. The presence of a sugar moiety was revealed by an anomeric proton at *δ* 4.71 (d, J = 6.9 Hz) and overlapping signals at *δ* 3.10−3.30, attributable to four hydroxylated methine protons. Therefore, the structure of **1** was deduced as a benzoylated flavan-3-ol glycoside, in agreement with the molecular formula, $C_{28}H_{28}O_{12}$. On enzymatic hydrolysis with *β*-glucosidase, compound **1** afforded glucose and an aglycon identified as compound **2**. The configuration at the glucose C-1 position was concluded to be *β* on the basis of the J value (6.9 Hz) of the anomeric proton signal. The position of the glucose linkage in compound **1** was determined by the HMBC spectrum, in which the anomeric proton (*δ* 4.71) showed a long-range correlation with the oxygenated quaternary carbon at *δ* 156.5, assignable to C-5 on the basis of the long-range correlations with H-4 (*δ* 2.99) and H-6 (*δ* 6.17). The ^{1}H NMR signal at *δ* 9.32 due to a hydroxyl proton showed a long-range correlation with the quaternary carbon at *δ* 155.1, which was assigned to C-7 based on the long-range correlation with H-6 and H-8 (*δ* 6.17 and 6.06, respectively). Therefore, we concluded that the glucopyranosyl group is

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10.1021/np000251l CCC: $20.00
Published on Web 12/02/2000

attached at C-5, and the structure of **1** was established as (−)-epicatechin-5-*O*-*β*-D-glucopyranosyl-3-benzoate. The stereochemistry of **1** was confirmed by spectral analyses of the aglycon **2**. The vicinal coupling patterns of the heterocyclic C ring of **2** were in agreement with a *cis* relative substitution at C-2 and C-3, and a negative Cotton effect in the 280 nm region (^1L_b transition) in the CD spectrum indicated that **2** has the (*R*)-configuration at the C-2 position.[11] Together with the $^3J_{2,3}$ coupling constant and the optical rotation, $[\alpha]^{25}_D$ −100°, the absolute configuration of **2** was determined to be 2*R*,3*R*. This is the first report of the occurrence of **1** as a natural product, although **2** has been reported previously as a semisynthetic compound.[12]

Compounds **3** and **4** were also obtained from the EtOAc fraction and identified as (−)-epicatechin and (−)-epiafzelechin, respectively, by comparison of their physical and spectral data with those previously reported.[13−15]

Compounds **1** and **2**, with IC_{50} values of 25 and 17 *μ*g/mL, respectively, exhibited moderate activities as free-radical scavengers in the DPPH assay, in comparison with reference antioxidants such as 2(3)-*tert*-butyl-4-hydroxyanisole (IC_{50} 4.5 *μ*g/mL), vitamin E (IC_{50} 6.2 *μ*g/mL), and (−)-epigallocatechin-3-*O*-gallate (IC_{50} 5.6 *μ*g/mL). Compounds **3** and **4**, with IC_{50} values of 8.5 *μ*g/mL and 7.5 *μ*g/mL, respectively, exhibited more potent antioxidant activity than compounds **1** and **2**.

Experimental Section

General Experimental Procedures. Melting points were measured without correction on an Electrothermal model 9100. Optical rotations were determined on a JASCO DIP-370 polarimeter at 25 °C. CD spectra were recorded on a JASCO J-720 spectropolarimeter. IR and UV spectra were obtained on a JASCO IR Report-100 and Milton Roy 3000 spectrometer, respectively. ^{1}H NMR (300 MHz), ^{13}C NMR (75 MHz), and HMBC spectra were obtained on a Varian Unity NMR spectrometer using DMSO-d_6 as a solvent. EIMS were measured on a Hewlett-Packard 5989A mass spectrometer, HR-FABMS on a JEOL HX 110 mass spectrometer, and ESIMS on a Finnigan Navigator mass spectrometer. Kieselgel 60 (Merck no. 9385 and 7729) was used for column chromatography.

Plant Material. The dried aerial parts of *C. orbiculatus* were collected at the herbal garden of Chungbuk National University, Cheongju, Korea, in October 1998, and identified by Dr. Kyong Soon Lee, Chungbuk National University. A voucher specimen (no. 98101) is deposited at our institute.

Extraction and Isolation. Dried aerial parts of *C. orbiculatus* (3 kg) were ground and extracted with MeOH (5 L) three times at room temperature. MeOH extract (250 g) was partitioned between CH_2Cl_2 and H_2O. The resulting aqueous layer was extracted 3 times with EtOAc and concentrated under vacuum. When tested for antioxidant activity, the EtOAc extract exhibited activity, with an IC_{50} value of 45 *μ*g/mL. The EtOAC extract (20 g) was subjected to Si gel column (4 × 55 cm) chromatography eluting with a step gradient of CH_2Cl_2−MeOH (10:1, 5:1, 2:1, 100% MeOH each 1.5 L) to afford 4 fractions. Fraction 1 (5.2 g) was further fractionated on a Si gel column (4 × 48 cm) eluting with a step gradient of CH_2Cl_2−MeOH (10:1, 8:1, 5:1, 2:1, 100% MeOH each 1.2 L) to obtain 10 fractions. Fraction 1−8 was chromatographed on a Si gel column (3 × 45 cm) eluted with EtOAc−MeOH (9:1) to afford compound **1** (78 mg). Compounds **3** (1.5 g) and **4** (75 mg) were isolated by column chromathography as described for compound **1**, but from fractions 1−5 and 1−7, respectively.

Enzymatic Hydrolysis of Compound 1. A solution of compound **1** (10 mg) in H_2O (5 mL) was incubated at 37 °C overnight with *β*-glucosidase (20 mg) from almonds (Sigma Chemical Co., St Louis, MO). The reaction mixture was filtered and extracted with EtOAc. The EtOAc extract was evaporated in vacuo and purified on preparative TLC by elution with CH_2Cl_2−MeOH (5:1) to give compound **2** (6.3 mg). The presence of glucose in the aqueous phase was determined by TLC (*n*-BuOH−pyridine−H_2O; 6:4:3, R_f = 0.70).

(−)-Epicatechin-5-*O*-*β*-D-glucopyranosyl-3-benzoate (1): bright pink needles from 1:1 mixture of CH_2Cl_2 and MeOH; $FeCl_3$, dark-blue; mp 191−192 °C; $[\alpha]^{25}_D$ −95° (*c* 0.3, MeOH); CD MeOH $\Delta\epsilon$ (λ, nm), −1.83 (275), −5.50 (236); UV (MeOH) λ_{max} (log ϵ) 213 (4.67), 229 (4.47), 283 (3.78) nm; IR (KBr) ν_{max} 3400, 1700, 1620, 1600, 1520, 1450, 1360, 1280 cm^{-1}, FABMS (positive-ion mode) *m*/*z* 557 $[M + H]^+$; HRFABMS (positive-ion mode) calcd for $C_{28}H_{29}O_{12}$ 557.1659, found 557.1653, ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.32 (1H, br s, OH-7), 7.79 (2H, br d, *J* = 7.5 Hz, H-2″, H-6″), 7.61 (1H, br t, *J* = 7.5 Hz, H-4″), 7.47 (2H, t, *J* = 7.5 Hz, H-3″, H-5″), 6.92 (1H, d, *J* = 1.9 Hz, H-2′), 6.73 (1H, dd, *J* = 8.2, 1.9 Hz, H-6′), 6.63 (1H, d, *J* = 8.2 Hz, H-5′), 6.17 (1H, d, *J* = 2.1 Hz, H-6), 6.06 (1H, d, *J* = 2.1 Hz, H-8), 5.51 (1H, m, H-3), 5.09 (1H, br s, H-2), 4.71 (1H, d, *J* = 6.9 Hz, anomeric H), 3.69 (1H, dd, *J* = 10.7, 5.3 Hz, glc H-6), 3.50 (1H, m, glc H-6), 3.10−3.30 (4H, glc H-2, -3, -4, -5) 2.99 (2H, m, H-4); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 25.5 (t, C-4), 60.6 (t, glc C-6), 68.7 (d, C-3), 69.5 (d, glc C-4), 73.2 (d, glc C-2), 76.3 (d, glc C-3), 76.5 (d, glc C-5), 77.0 (d, C-2), 95.5 (d, C-6), 96.6 (d, C-8), 99.5 (s, C-10), 100.5 (d, glc C-1), 114.0 (d, C-2′), 115.1 (d, C-5′), 117.2 (d, C-6′), 128.8 (2d, C-3″, C-5″), 129.0 (s, C-1′), 129.1 (2d, C-2″, C-6″), 129.5 (s, C-1″), 133.3 (d, C-4″), 144.7 (s, C-3′), 144.8 (s, C-4′), 155.1 (s, C-7), 156.5 (s, C-5), 156.8 (s, C-9), 164.8 (s, COO)

(−)-Epicatechin-3-benzoate (2): light brown needles from 1:1 mixture of CH_2Cl_2 and MeOH; $FeCl_3$, dark-blue; mp 168−170 °C; $[\alpha]^{25}_D$ −100° (*c* 0.6, MeOH); CD MeOH $\Delta\epsilon$ (λ, nm), −0.88 (281), −5.69 (237); UV (MeOH) λ_{max} (log ϵ) 212 (4.53), 230 (4.35), 284 (3.57) nm; IR (KBr) ν_{max} 3400, 1710, 1610, 1600, 1520, 1450, 1360, 1280 cm^{-1}, ESIMS (negative-ion mode) *m*/*z* 393 $[M - H]^+$; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.79 (2H, br d, *J* = 7.5 Hz, H-2″, H-6″), 7.61 (1H, br t, *J* = 7.5 Hz, H-4″), 7.47 (2H, t, *J* = 7.5 Hz, H-3″, H-5″), 6.92 (1H, d, *J* = 1.8 Hz, H-2′), 6.73 (1H, dd, *J* = 8.1, 1.8 Hz, H-6′), 6.64 (1H, d, *J* = 8.1 Hz, H-5′), 5.93 (1H, d, *J* = 2.4 Hz, H-6), 5.85 (1H, d, *J* = 2.4 Hz, H-8), 5.44 (1H, m, H-3), 5.10 (1H, br s, H-2), 2.99 (2H, dd, *J* = 16.8, 4.5 Hz, H-4*α*), 2.76 (1H, dd, *J* = 16.8, 2.0 Hz, H-4*β*); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 25.5 (t, C-4), 69.2 (d, C-3), 76.2 (d, C-2), 94.2 (d, C-6), 95.6 (d, C-8), 97.0 (s, C-10), 114.0 (d, C-2′), 115.1 (d, C-5′), 117.2 (d, C-6′), 128.8 (2d, C-3″, C-5″), 129.0 (s, C-1′), 129.2 (2d, C-2″, C-6″), 129.6 (s, C-1″), 133.3 (d, C-4″), 144.8 (s, C-3′), 144.9 (s, C-4′), 155.5 (s, C-7), 156.5 (s, C-5), 156.9 (s, C-9), 165.0 (s, COO)

(−)-Epicatechin (3): colorless needles from 1:1 mixture of CH_2Cl_2 and MeOH; $FeCl_3$, dark-blue; mp 240−243 °C; $[\alpha]^{20}_D$ −59.0° (*c*, 1.0, MeOH). The compound exhibits spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) data comparable to published values.[13]

(−)-Epiafzelechin (4): bright yellow needles from 1:1 mixture of CH_2Cl_2 and MeOH; $FeCl_3$, dark-blue; mp 239−240 °C; $[\alpha]^{20}_D$ −41.0° (*c*, 1.0, MeOH);. The compound exhibits spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS) data comparable to published values.[15]

Evaluation of DPPH Radical Scavenging Activity. The antioxidant activities of the plant extract and pure compounds were assessed on the basis of the radical scavenging effect of the stable DPPH free radical.[8,9] The tested samples (2 *μ*L of DMSO solution and 200 *μ*L of DPPH in EtOH solution, 100 *μ*M) were added in a 96-well plate. After incubation at 37 °C for 30 min, the absorbance of each solution was determined at 490 nm using a microplate reader in comparison with a DMSO control group, and the remaining DPPH was calculated. IC_{50} value is the concentration of sample required to scavenge 50% DPPH free radicals.

Acknowledgment. This work was supported by a grant from the Korea Ministry of Science and Technology.

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NP000251L