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Two Novel Dicarboxylic Acid Derivatives and a New Dimeric Hydrolyzable Tannin from Walnuts

HIDEYUKI ITO,*,† TAKAHIRO OKUDA,† TOSHIYUKI FUKUDA,‡ TSUTOMU HATANO,† AND TAKASHI YOSHIDA§

Department of Pharmacognosy, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Tsushima, Okayama 700-8530, Japan; POLA Chemical Industries, Inc., Totsuka-ku, Yokohama 244-0812, Japan; and Faculty of Pharmaceutical Sciences, Matsuyama University, Bunkyo-cho, Matsuyama 790-8578, Japan

In addition to the 16 previously reported polyphenols including 3 new ellagitannins, 2 novel dicarboxylic acid derivatives, glansreginins A (**1**) and B (**2**), and a new dimeric hydrolyzable tannin, glansrin D (**3**), were isolated, together with 15 known compounds from walnuts, the seeds of *Juglans regia*. The structures of the new compounds were elucidated on the basis of 1D- and 2D-NMR analyses and chemical data. The antioxidant effect of these isolates was also evaluated by SOD-like and DPPH radical scavenging activities.

KEYWORDS: Walnuts; *Juglans regia*; Juglandaceae; glansreginin A; glansreginin B; glansrin D; 2-hydroxycinchoninic acid; antioxidant activity; SOD-like effect; radical scavenger

INTRODUCTION

Walnuts, the seeds of *Juglans* species, are a popular nutritious and delicious food. Among the numerous species of cultivated walnut trees, one of the main types is *Juglans regia* L. (Juglandaceae), which originated in India and its surrounding areas and is now widely cultivated in many European countries after its introduction by the ancient Romans. Walnuts have been used as folk remedies for kidney and stomach diseases and for cancer in Asia and Europe (*1*, *2*). Walnuts are an excellent source of unsaturated essential fat containing linoleic and α-linolenic acids (omega-6 and -3 fatty acids) and have attracted increasing attention for their human health benefits such as reducing cardiovascular risk (*3*). The cardioprotective benefit of walnuts was also suggested by the inhibitory effects of walnut polyphenols, ellagic acid, and gallic acid on the in vitro oxidation of human plasma and low-density lipoproteins (LDL) (*4*). Besides polyphenolics, the occurrence of other well-investigated quinones (*5*, *6*), phenylpropanoids (*6*), and volatile components (*7*) in walnuts has been reported. In a recent phytochemical study of walnuts, we reported 16 additional polyphenols, including 3 new hydrolyzable tannins, glansrins A−C (**4**−**6**) (*8*). The new tannins were characterized as ellagitannins having a tergalloyl group or a related acyl group. The isolated polyphenolic compounds were demonstrated to have an antioxidant effect by SOD-like and DPPH radical scavenging assays. These findings prompted us to further examine other antioxidants in walnuts, resulting in the isolation of two novel dicarboxylic acid derivatives, glansreginins A (**1**) and B (**2**), and a new ellagitannin, glansrin D (**3**), together with 15 known compounds. We also describe here the antioxidant effect of these isolates by the above two assays.

MATERIALS AND METHODS

General Experimental Procedures. Melting points were measured on a Yanaco micro-melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-1000 polarimeter. UV and CD spectra were recorded on Hitachi U-2001 and Jasco J-720W spectrometers, respectively. Electrospray ionization mass spectroscopy (ESIMS) and high-resolution (HR) ESIMS were performed with a Micromass AutoSpec OA-Tof using 50% aqueous MeOH containing 0.1% NH_4OAc as a solvent. ^1H and ^{13}C NMR spectra were recorded on a Varian INOVA AS600 (600 MHz for ^1H and 150 MHz for ^{13}C), and chemical shifts are given in δ (parts per million) values relative to that of the solvent [acetone-d_6 (δ_H 2.04; δ_C 29.8), $CDCl_3$ (δ_H 7.26; δ_C 77.0), pyridine-d_5 (δ_H 7.19; δ_C 123.5)] on a tetramethylsilane scale. The standard pulse sequences programmed for the instrument (INOVA AS600) were used for each 2D measurement (COSY, NOESY, HSQC, and HMBC). J_{CH} was set at 5 or 8 Hz in HMBC spectra. Normal-phase HPLC was conducted on a 250 × 4.6 mm i.d. YMC-Pack SIL A-003 (YMC Co., Ltd., Kyoto, Japan) column developed with *n*-hexane/MeOH/tetrahydrofuran/formic acid (55:33:11:1) containing oxalic acid (450 mg/L) (flow rate, 1.5 mL/min; detection, UV 280 nm) at ambient temperature. Reversed-phase HPLC was performed on a 150 × 4.6 mm i.d. YMC-Pack ODS-A A-302 (YMC Co., Ltd.) column developed with 10 mM H_3PO_4/10 mM KH_2PO_4/CH_3CN (44:44:12) (flow rate, 1.0 mL/min; detection, UV 280 nm) at 40 °C. Preparative HPLC was performed with 250 × 10 mm i.d. Inertsil SIL 100-5 (GL Sciences, Tokyo, Japan) for normal phase and/or 300 × 10 mm i.d. YMC-Pack ODS A-324 (YMC Co., Ltd.) for reversed phase. Column chromatography was conducted on Diaion HP-20 and MCI GEL CHP-20P (75−150 μm) (Mitsubishi Kasei Co., Tokyo, Japan), Toyopearl HW-40

* Corresponding author (fax +81-86-251-7926; e-mail hito@cc.okayama-u.ac.jp).
† Okayama University.
‡ POLA Chemical Industries.
§ Matsuyama University.

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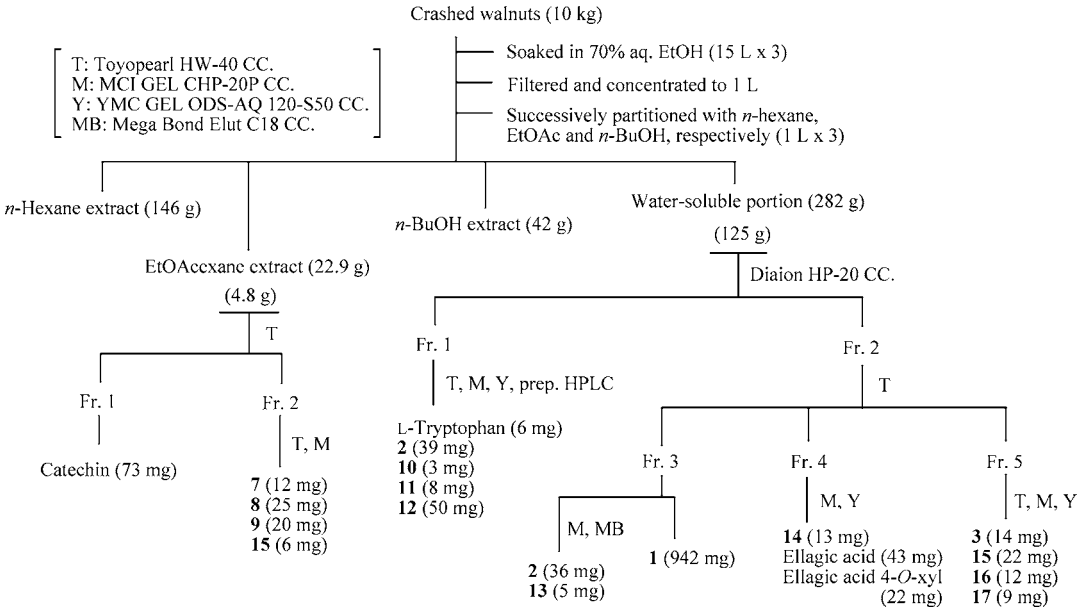
Chart 1



(coarse grade) (Tosoh Co., Tokyo, Japan), YMC-GEL ODS-AQ 120-50S (YMC Co., Ltd), and Mega Bond Elut C18 (1 g/6 mL) (Varian, CA).

Materials. The kernels of walnut (cv. Chandler), which were air-dried, crushed, and passed through a 5 mm mesh sieve, were obtained from TABATA (Tokyo, Japan). DPPH, xanthine, L-ascorbic acid, and gallic acid of reagent grade were purchased from Wako Pure Chemical Industries (Osaka, Japan). XOD from bovine milk was obtained from Sigma (St. Louis, MO).

Extraction and Isolation. The crushed kernels of walnut (10 kg) were soaked in 70% aqueous EtOH (15 L × 3) for 24 h at ambient temperature. The filtrate was concentrated to 1 L and extracted with *n*-hexane (1 L × 3), EtOAc (1 L × 3), and *n*-BuOH (1 L × 3), successively, to give the respective extracts and the water-soluble portion. Fractionations were collected by monitoring normal- and/or reversed-phase HPLC. Part (4.8 g) of the EtOAc extract (22.9 g) was chromatographed over Toyopearl HW-40 (45 × 2.2 cm i.d.) with aqueous MeOH (30% → 40% → 50% → 60% → 70% MeOH) → MeOH/H_2O/acetone (7:2:1) → 70% aqueous acetone, to afford (+)-catechin (73 mg) from the 50% MeOH eluate (**Figure 1**). The eluate of MeOH/H_2O/acetone (7:2:1) (**Figure 2**) was further chromatographed over MCI GEL CHP-20P with aqueous MeOH and/or Toyopearl HW-40 (30 × 1.1 cm i.d.) with aqueous MeOH, to give 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose (**7**) (12 mg), 1,2,4,6-tetra-*O*-galloyl-*β*-D-glucose (**8**) (25 mg), 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**9**) (20 mg), and rugosin F (**15**) (6 mg). Part (125 g) of the aqueous extract (282 g) was fractionated by column chromatography over Diaion HP-20 (43 × 6.0 cm i.d.) with H_2O and increasing amounts of EtOH (H_2O → 10% → 20% → 40% → 100% EtOH) in a stepwise gradient mode. The 10% and 20% EtOH eluates (**Figure 1**) were separately subjected to column chromatography over Toyopearl HW-40 (40 × 2.2 cm i.d.) with aqueous MeOH, MCI GEL CHP-20P (40 × 1.1 cm i.d.) with aqueous MeOH, and YMC-GEL ODS-AQ 120-S50 (20 × 1.1 cm i.d.) with aqueous MeOH and/or purified by preparative reversed-phase HPLC to furnish L-tryptophan (6 mg), glansreginin B (**2**) (39 mg), gemin D (**10**) (3 mg), casuariin (**11**) (8 mg), and pterocarinin A (**12**) (56 mg). The 40% EtOH eluate (**Figure 2**) from Diaion HP-20 separation was subjected to column chromatography over Toyopearl HW-40 (50 × 2.2 cm i.d.) with aqueous MeOH (30% → 40% → 50% → 60% → 70% MeOH) → MeOH/H_2O/acetone (7:2:1) → MeOH/H_2O/acetone (7:1:2) → 70% aqueous acetone. The 30% MeOH eluate (**Figure 3**) afforded glansreginin A (**1**) (942 mg) and was further purified by MCI GEL CHP-20P (50 × 1.1 cm i.d.) and Mega Bond Elut C18 with aqueous MeOH, to give glansreginin B (**2**) (36 mg) and platycaryanin A (**13**) (5 mg). The 60% MeOH eluate (**Figure 4**) was further chromatographed over MCI GEL CHP-20P (50 × 1.1 cm i.d.) and YMC-GEL ODS-AQ 120-S50 (20 × 1.1 cm i.d.) with aqueous MeOH, to yield euprostin A (**14**) (13 mg), ellagic acid (43 mg), and ellagic acid 4-*O*-xylopyranoside (22 mg). The eluate of 70% acetone (**Figure 5**) from the separation by column chromatography over Toyopearl HW-40 was similarly fractionated and purified by a combination of MCI GEL CHP-20P and/or YMC GEL ODS-AQ 120-S50 with aqueous MeOH, to afford glansrin D (**3**) (14 mg), rugosin F (**15**) (22 mg), 1-desgalloylrugosin F (**16**) (12 mg), and heterophylliin D (**17**) (9 mg) (**Chart 1**).

*Glansreginin A (**1**)* was obtained as a pale yellow amorphous powder: $[\alpha]_D^{23}$ −37.9° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 265.5 (4.45) nm; CD (MeOH) [θ] (nm) -6.7×10^4 (212), $+3.1 \times 10^4$ (238), -1.7×10^4 (263), $+2.8 \times 10^3$ (306); ^{1}H and ^{13}C NMR data, see **Table 1**; ESIMS, *m*/*z* 594 $[M + H]^+$, 611 $[M + NH_4]^+$, 616 $[M + Na]^+$; HRESIMS, *m*/*z* 594.2171 $[M + H]^+$, $C_{28}H_{35}NO_{13}$ + H, requires 594.2187. Anal.: C, 54.3%; H, 6.16%; N, 2.09%; Calcd for $C_{28}H_{35}NO_{13}\cdot 1.5H_2O$: C, 54.2%; H, 6.13%; N, 2.26%.

*Acid Hydrolysis of **1**.* A solution of **1** (20 mg) in 1 M HCl (2 mL) was heated in a boiling water bath for 7 h. The deposit was collected by centrifugation and recrystallized to yield 2-hydroxycinchoninic acid (**1a**) (2.9 mg). The filtrate was evaporated and subjected to chromatography over Mega Bond Elut C18 with aqueous MeOH, to give glansreginic acid (**1b**) (1.6 mg) and monomethyl glansreginate (**1c**) (3.9 mg). Further amounts of compounds **1a** (13 mg) and **1b** (24 mg) to secure sufficient quantities for structure elucidation were obtained by repeated hydrolysis of **1** (69 mg). The aqueous portion gave D-glucose, which was identified on the basis of HPLC analysis and D-glucose oxidation reaction (*9*).

*Glansreginic acid (**1b**)* was obtained as a pale brown amorphous powder: $[\alpha]_D^{23}$ +21.2° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 212.5 (3.65), 262.5 (3.52) nm; ^{1}H NMR (acetone-d_6 + D_2O) δ 7.18 (1H, br d, J = 10.8 Hz, H-3), 6.48 (1H, br dd, J = 10.8, 15.0 Hz, H-4), 6.16 (1H, dt, J = 15.0, 7.8 Hz, H-5), 4.01 (1H, dt, J = 8.4, 4.2 Hz, H-8), 2.46 (1H, dd, J = 4.2, 15.0 Hz, H-9), 2.44 (1H, m, H-6), 2.42 (1H, dd, J = 8.4, 15.0 Hz, H-9), 2.12 (1H, dt, J = 14.4, 7.2 Hz, H-6), 1.89 (3H, d, J = 1.2 Hz, H-11), 1.72 (1H, m, H-7), 0.93 (3H, d, J = 7.2 Hz, H-12); ESIMS, *m*/*z* 260 $[M + NH_4]^+$.

*Monomethyl glansreginate (**1c**)* was obtained as a pale yellow amorphous powder: ^{1}H NMR (acetone-d_6 + D_2O) δ 7.18 (1H, d, J = 10.8 Hz, H-3), 6.48 (1H, br dd, J = 10.8, 15.0 Hz, H-4), 6.15 (1H, dt, J = 15.0, 7.8 Hz, H-5), 4.00 (1H, m, H-8), 3.61 (3H, s, -OC$\underline{H}_3$), 2.46 (1H, dd, J = 4.2, 15.0 Hz, H-9), 2.44 (1H, m, H-6), 2.42 (1H, dd, J = 9.0, 15.0 Hz, H-9), 2.09 (1H, dt, J = 13.8, 7.2 Hz, H-6), 1.89 (3H, s, H-11), 1.70 (1H, m, H-7), 0.92 (3H, d, J = 6.6 Hz, H-12); ^{13}C NMR (acetone-d_6 + D_2O) δ 173.1 (C-10), 169.5 (C-1), 142.4 (C-5), 139.3

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for **1** and **2** in Acetone-d_6 + D_2O

	1		**2**	
position	δ_H	δ_C	δ_H	δ_C
1			3.59 (1H, d, 12.6) 3.64 (1H, d, 12.6)	64.4
2		177.0		105.0
3	3.14 (1H, d, 15.0)[a] 3.19 (1H, d, 15.0)	41.2	4.10 (1H, m)	80.1
4		78.4	4.07 (1H, t, 9.8)	75.3
4a		126.8		
5	7.41 (1H, d, 7.5)	126.2	3.76 (1H, m)	83.7
6	6.98 (1H, t, 7.5)	122.3	3.66 (2H, m)	62.4
7	7.24 (1H, t, 7.5)	131.0		
8	6.78 (1H, d, 7.5)	110.8		
8a		144.4		
9		170.4		
1′	4.44 (1H, d, 7.5)	99.1	5.40 (1H, d, 4.2)	93.0
2′	3.22 (1H, br t, 8.0)	74.3	3.47 (1H, dd, 4.2, 9.6)	71.7
3′	3.31 (1H, br t, 9.0)	77.5	3.77 (1H, m)	74.0
4′	3.29 (1H, br t, 9.0)	70.7	3.42 (1H, t, 9.6)	70.7
5′	3.16 (1H, m)	75.0	4.10 (1H, m)	72.7
6′	4.11 (1H, dd, 5.5, 12.0) 4.24 (1H, dd, 1.5, 12.0)	64.3	4.29 (1H, dd, 4.8, 11.4) 4.39 (1H, dd, 2.4, 11.4)	64.2
1″		168.8		168.8
2″		125.7		125.5
3″	7.17 (1H, d, 11.5)	139.6	7.22 (1H, d, 11.4)	139.6
4″	6.48 (1H, dd, 11.5, 15.0)	128.1	6.47 (1H, dd, 11.4, 15.0)	128.0
5″	6.20 (1H, dt, 15.0, 8.0)	142.9	6.21 (1H, m)	143.1
6″	2.10 (1H, dt, 14.0, 8.0) 2.43 (1H, m)	37.7	2.10 (1H, m) 2.40 (1H, m)	37.8
7″	1.71 (1H, m)	39.0	1.71 (1H, m)	39.0
8″	3.99 (1H, dt, 8.0, 4.5)	70.8	3.98 (1H, m)	70.9
9″	2.43 (2H, m)	39.8	2.42 (2H, m)	39.9
10″		174.3		174.2
11″	1.91 (3H, s)	12.8	1.90 (3H, s)	12.7
12″	0.93 (3H, d, 7.0)	14.0	0.90 (3H, d, 6.6)	14.0

[a] *J* values (hertz) are in parentheses.

(C-3), 128.3 (C-4), 125.9 (C-2), 70.8 (C-8), 51.6 (−OCH$_3$), 40.2 (C-9), 39.2 (C-7), 37.8 (C-6), 14.0 (C-12), 12.7 (C-11); ESIMS, *m*/*z* 274 [M + NH$_4$]$^+$.

*Methylation of **1c***. A solution of **1c** (3.6 mg) in EtOH was treated with ethereal diazomethane (1 mL) at ambient temperature for 6 h. After removal of the solvent, the residue gave dimethyl glansreginate (**1d**) (3.5 mg) as a pale yellow amorphous powder: ^{1}H NMR ($CDCl_3$) δ 7.16 (1H, br d, J = 11.4 Hz, H-3), 6.38 (1H, dd, J = 11.4, 15.0 Hz, H-4), 6.05 (1H, dt, J = 15.0, 7.2 Hz, H-5), 3.98 (1H, ddd, J = 3.0, 3.6, 9.6 Hz, H-8), 3.75 (3H, s, H-13), 3.72 (3H, s, H-14), 2.50 (1H, dd, J = 9.6, 16.2 Hz, H-9), 2.44 (1H, dd. J = 3.0, 16.8 Hz, H-9), 2.41 (1H, dt, J = 14.4, 7.2 Hz, H-6), 2.08 (1H, dt, J = 14.4, 7.2 Hz, H-6), 1.93 (3H, d, J = 1.2 Hz, H-11), 1.68 (1H, m, H-7), 0.93 (3H, d, J = 6.6 Hz, H-12); ^{13}C NMR ($CDCl_3$) δ 173.1 (C-10), 169.0 (C-1), 142.7 (C-5), 139.2 (C-3), 128.1 (C-4), 125.7 (C-2), 70.9 (C-8), 51.8 (C-14), 51.6 (C-13), 40.2 (C-9), 39.2 (C-7), 37.8 (C-6), 14.0 (C-12), 12.7 (C-11); ESIMS, *m*/*z* 288 [M + NH$_4$]$^+$.

*MTPA Esters of Dimethyl Glansreginate (**1d**)*. A mixture of **1d** (1.7 mg) and (+)- or (−)-MTPA chlorides (Tokyo Chemical Industry, Tokyo, Japan) (each 10 μL) in dry pyridine (200 μL) was kept overnight at ambient temperature. The respective reaction mixture was purified by preparative TLC (Si gel, $CHCl_3$/MeOH, 19:1) to yield (*R*)- and (*S*)-MTPA esters (each 1.1 mg).

*(R)-MTPA ester of **1d*** was obtained as a pale yellow amorphous powder: ^{1}H NMR ($CDCl_3$) δ 7.35−7.55 (5H, MTPA phenyl-H), 7.14 (1H, d, J = 12.0 Hz, H-3), 6.32 (1H, dd, J = 11.4, 15.0 Hz, H-4), 5.97 (1H, dt, J = 15.0, 6.6 Hz, H-5), 5.50 (1H, dt, J = 8.4, 4.2 Hz, H-8), 3.76 (3H, s, H-13), 3.63 (3H, s, H-14), 3.50 (3H, s, MTPA-OCH$_3$), 2.67 (1H, dd, J = 8.4, 16.2 Hz, H-9), 2.56 (1H, dd. J = 4.8, 16.2 Hz, H-9), 2.31 (1H, m, H-6), 1.98 (1H, m, H-6), 1.95 (1H, m, H-7), 1.92 (1H, m, H-11), 0.94 (3H, d, J = 6.6 Hz, H-12).

*(S)-MTPA ester of **1d*** was obtained as a pale yellow amorphous powder: ^{1}H NMR ($CDCl_3$) δ 7.35−7.55 (5H, MTPA phenyl-H), 7.11 (1H, d, J = 10.8 Hz, H-3), 6.25 (1H, dd, J = 10.8, 15.6 Hz, H-4), 5.87 (1H, dt, J = 15.6, 7.2 Hz, H-5), 5.49 (1H, dt, J = 8.4, 4.2 Hz, H-8), 3.76 (3H, s, H-13), 3.67 (3H, s, H-14), 3.54 (3H, s, MTPA-OCH$_3$), 2.70 (1H, dd, J = 8.4, 15.6 Hz, H-9), 2.59 (1H, dd. J = 4.2, 15.6 Hz, H-9), 2.15 (1H, m, H-6), 1.95 (1H, m, H-7), 1.92 (1H, m, H-11), 1.87 (1H, m, H-6), 0.89 (3H, d, J = 7.2 Hz, H-12).

*Enzymatic Hydrolysis of **1***. A solution of **1** (157 mg) in H_2O (10 mL) was treated with naringinase (20 mg) (Sigma-Aldrich, St. Louis, MO) at 40 °C for 2 h. After the addition of MeOH and then centrifugation (10000*g*), the supernatant was evaporated and purified by Mega Bond Elut C18 cartridge column chromatography and preparative reversed-phase HPLC to yield **1b** (75 mg), **1e** (38 mg), and **1f** (5.3 mg).

1e was obtained as a pale yellow amorphous powder: $[\alpha]_D^{23}$ −31.3° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 255.0 (3.69), 294.0 (3.13) nm; CD (MeOH) [θ] (nm) -7.3×10^4 (212), $+4.3 \times 10^4$ (238), -2.1×10^4 (263), $+3.1 \times 10^3$ (300); ^{1}H NMR (acetone-d_6 + D_2O) δ 7.42 (1H, br d, J = 7.8 Hz, H-5), 7.27 (1H, dt, J = 1.2, 7.8 Hz, H-7), 7.00 (1H, dt, J = 1.2, 7.8 Hz, H-6), 6.92 (1H, br d, J = 7.8 Hz, H-8), 4.15 (1H, d, J = 7.8 Hz, H-1′), 3.61 (1H, dd, J = 2.4, 12.0 Hz, H-6′), 3.49 (1H, dd, J = 5.4, 12.0 Hz, H-6′), 3.27 (1H, t, J = 9.0 Hz, H-4′), 3.21 (1H, dd, J = 7.8, 9.0 Hz, H-2′), 3.20 (1H, d, J = 15.6 Hz, H-3), 3.17 (1H, t, J = 9.0 Hz, H-3′), 3.11 (1H, d, J = 15.6 Hz, H-3), 2.80 (1H, ddd, J = 2.4, 5.4, 9.0 Hz, H-5′); ESIMS, *m*/*z* 370 [M + H]$^+$, 387 [M + NH$_4$]$^+$; HRESIMS, *m*/*z* 370.1143 [M + H]$^+$, $C_{16}H_{19}NO_9$ + H, requires 370.1138.

1f was obtained as a pale yellow amorphous powder: $[\alpha]_D^{23}$ −2.2° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 253.5 (3.54), 288.0 (2.94) nm; CD (MeOH) [θ] (nm) -5.0×10^4 (*211*), $+3.1 \times 10^4$ (239), -1.1×10^4 (264); ^{1}H NMR (acetone-d_6 + D_2O) δ 7.37 (1H, dd, J = 1.2, 7.8 Hz, H-5), 7.17 (1H, dt, J = 1.2, 7.8 Hz, H-7), 6.94 (1H, dt, J = 1.2, 7.8 Hz, H-6), 6.87 (1H, dt, J = 1.2, 7.8 Hz, H-8), 2.88 (1H, d, J = 16.2 Hz, H-3), 2.83 (1H, d, J = 16.2 Hz, H-3); ESIMS, *m*/*z* 208 [M + H]$^+$, 225 [M + NH$_4$]$^+$, 230 [M + Na]$^+$; HRESIMS, *m*/*z* 208.0607 [M + H]$^+$, $C_{10}H_9NO_4$ + H, requires 208.0610.

*Glansreginin B **(2)*** was obtained as a pale yellow amorphous powder: $[\alpha]_D^{23}$ +15.9° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 265.5 (3.95) nm; ^{1}H and ^{13}C NMR data, see **Table 1**; ESIMS, *m*/*z* 584 [M + NH$_4$]$^+$; HRESIMS, *m*/*z* 584.2556 [M + NH$_4$]$^+$, $C_{24}H_{38}O_{15}$ + NH$_4$, requires 584.2554.

*Methanolysis of **2***. A solution of **2** (2 mg) in 1% NaOMe/MeOH (0.2 mL) was kept at ambient temperature for 2 h. After the addition of a few drops of AcOH, the concentrated solution was subjected to a Bond Elut C18 cartridge column with H_2O and MeOH elution to give sucrose from the aqueous eluate, which was identified by cochromatography with an authentic specimen on HPLC equipped with a refractive index detector.

*Acid Hydrolysis of **2***. A solution of **2** (11 mg) in 1 M HCl (1 mL) was heated in a boiling water bath for 6 h. The reaction mixture was extracted with EtOAc, and then the EtOAc layer was evaporated. The residue was separated by a Mega Bond Elut C18 cartridge column followed by preparative TLC (Si gel, $CHCl_3$/MeOH, 9:1) to afford **1b** (2.7 mg).

*Glansrin D **(3)*** was obtained as a pale brown amorphous powder: $[\alpha]_D^{23}$ +58.9° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 216.0 (5.14), 265.5 (4.73) nm; CD (MeOH) [θ] (nm) $+2.0 \times 10^5$ (237), -5.4×10^4 (262), $+2.9 \times 10^4$ (282), -6.3×10^4 (311); ^{1}H NMR (acetone-d_6 + D_2O) δ 7.07, 7.13 [each 2H, s, galloyl (gal)-H], 7.09 [1H, s, valoneoyl (val)-H$_C$], 6.69 (1H, s, HHDP-H$_A$), 6.59 (1H, s, HHDP-H$_B$), 6.51 (1H, s, val-H$_A$), 6.43 (1H, s, HHDP-H$_D$), 6.38 (1H, s, HHDP-H$_C$), 6.18 (1H, s, val-H$_B$), and sugar protons, see **Table 2**; ^{13}C NMR (acetone-d_6 + D_2O) δ 169.4 (HHDP C-7‴), 168.8 (HHDP C-7″), 168.6 (HHDP C-7′), 168.2 (val C-7), 168.1 (HHDP C-7), 167.9 (val C-7′), 166.6 (gal C-7), 165.2 (gal C-7′), 162.6 (val C-7″), 146.7 (val C-4′), 146.1 (2C, gal C-3′, 5′), 145.9 (2C, gal C-3, 5), 145.3 (val C-4), 145.1 (2C, HHDP C-4″, 4‴), 145.0 (2C, HHDP C-4, 4′), 137.7, 140.3, 144.1, 144.2, 144.3, 144.4, 144.7, 144.9 (HHDP C-6, 6′, 6″, 6‴, val C-6, 6′, 2″, 3″), 143.1 (val C-5″), 141.2 (val C-4″), 140.0 (HHDP C-4′), 139.3 (gal C-4), 137.0 (val C-5′), 136.6 (val C-5), 136.5 (HHDP C-5), 136.4 (HHDP C-5″), 136.2 (HHDP C-5‴), 136.1 (HHDP C-5′), 125.1, 125.6, 125.7, 125.9, 126.0, 126.4 (HHDP C-2, 2′, 2″, 2‴, val C-2, 2′), 120.4 (gal C-1), 119.3 (gal C-1′), 117.7 (val C-1′), 116.2 (HHDP C-1), 115.7 (val C-1),

Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data for the Sugar Moieties of **3** in Acetone-d_6 + D_2O

	3	
position	δ_H	δ_C
1	6.13 (1H, d, 9.0)[a]	92.0
2	5.14 (1H, t, 9.0)	75.8
3	5.38 (1H, dd, 9.0, 10.2)	77.2
4	5.07 (1H, t, 10.2)	69.0
5	4.41 (1H, dd, 6.6, 13.2)	73.1
6	5.22 (1H, dd, 6.6, 13.2)	63.0
	3.75 (1H, d, 13.2)	
1′	5.84 (1H, d, 7.8)	93.3
2′	5.27 (1H, dd, 7.8, 9.6)	74.1
3′	4.12 (1H, t, 9.6)	73.0
4′	4.96 (1H, t, 9.6)	72.4
5′	4.16 (1H, dd, 6.6, 9.6)	73.0
6′	5.15 (1H, dd, 6.6, 13.2)	63.3
	3.73 (1H, d, 13.2)	

[a] *J* values (hertz) are in parentheses.

115.6 (HHDP C-1′), 114.9 (HHDP C-1″), 114.4 (HHDP C-1‴), 112.6 (val C-1″), 110.2 (2C, gal C-2′, 6′), 110.0 (2C, gal C-2, 6), 109.6 (val C-6″), 108.1 (HHDP C-3), 107.8 (HHDP C-3′), 107.3 (val C-3), 107.15 (HHDP C-3‴), 107.14 (HHDP C-3″), 104.8 (val C-3′), and sugar carbons, see **Table 2**; ESIMS, *m*/*z* 1738 $[M + NH_4]^+$. Anal.: C, 47.0%; H, 4.02%. Calcd for $C_{75}H_{52}O_{48}\cdot 11H_2O$: C, 46.9%; H, 3.86%.

*Methylation of **3** followed by Methanolysis.* A solution of **3** (1 mg) in EtOH was treated with ethereal diazomethane (1 mL) overnight at ambient temperature. After removal of the solvent, the residue was directly methanolyzed with 1% NaOMe/MeOH (1 mL) overnight at ambient temperature. After the addition of a few drops of AcOH, the evaporated solution was analyzed by reversed-phase HPLC developed with 60% aqueous MeOH to detect methyl tri-*O*-methylgallate (**18**) (t_R 4.3 min), dimethyl hexamethoxydiphenate (**19**) (t_R 5.6 min), and trimethyl octa-*O*-methylvaloneate (**20**) (t_R 13.2 min), which were shown to be identical to authentic specimens.

*Partial Hydrolysis of **3**.* An aqueous solution of **3** (4 mg/mL) was heated in a boiling water bath for 2 h. Reversed-phase HPLC analysis of the reaction mixture exhibited the production of rugosin C (**21**) (t_R 14.8 min). Furthermore, the reaction mixture was submitted to preparative normal-phase HPLC to afford hippomanin A (**22**) (0.8 mg) as an off-white amorphous powder: ^{1}H NMR (acetone-d_6 + D_2O) δ 7.12, 7.14 (each 2H, s, gal-H), 6.54, 6.55, 6.61, 6.64 (each 1H, s, HHDP-H), 5.42 (1H, d, J = 3.6 Hz, H-1α), 5.22 (1H, dd, J = 6.6, 12.6 Hz, H-6β), 5.19 (1H, dd, J = 6.6, 12.6 Hz, H-6α), 5.03 (1H, dd, J = 8.4, 9.6 Hz, H-2β), 4.97 (1H, t, J = 9.6 Hz, H-4β), 4.94 (1H, t, J = 10.2 Hz, H-4α), 4.86 (1H, dd, J = 3.6, 10.2 Hz, H-2α), 4.85 (1H, d, J = 8.4 Hz, H-1β), 4.48 (1H, m, H-5α), 4.23 (1H, t, J = 10.2 Hz, H-3α), 4.00 (1H, m, H-5β), 3.96 (1H, t, J = 9.6 Hz, H-5α), 3.77 (1H, dd, J = 1.8, 12.6 Hz, H-6β), 3.70 (1H, dd, J = 1.8, 12.6 Hz, H-6α); ESIMS, *m*/*z* 652 $[M + NH_4]^+$, 657 $[M + Na]^+$.

Radical Scavenging Effect on DPPH Radical. EtOH solutions of each walnut polyphenol at various concentrations (0.5−10 μg/mL) were added to a solution of DPPH (30 μM) in EtOH (0.6 mL), and the reaction mixture (total volume, 1.2 mL) was shaken vigorously. After being held at ambient temperature for 30 min, the remaining DPPH was determined by colorimetry at 520 nm, and the radical scavenging activity of each compound was expressed using the ratio of the absorption of DPPH (percent) relative to the control DPPH solution (100%) in the absence of a sample. The mean values were obtained from triplicate experiments.

SOD-like Activity by Nitrite Method. A mixture consisting of the sample solution (10−500 μg/mL), 0.5 mM xanthine solution (pH 8.2) (0.2 mL), buffer solution (65 mM KH_2PO_4, 35 mM $Na_2B_4O_7$, and 0.5 mM EDTA·2Na, pH 8.2) (0.2 mL), 10 mM hydroxylamine (0.1 mL), water (0.2 mL), and XOD (12.4 mU/mL in buffer) (0.2 mL) was incubated for 30 min at 37 °C, and the reagent was added (30 μM *N*-naphthylethylenediamine, 3 mM sulfanilic acid, and 25% acetic acid) (2.0 mL). The final mixture was kept for 30 min at ambient temperature, and the absorption was measured at 550 nm. A blank was measured in the same way with no enzyme. Water was used as a control. SOD-like assay (percent) (EC_{50}) was calculated using the following equation and was given as the mean value of three experiments.

$$\text{SOD-like activity (\%)} = \left\{1 - \frac{(\text{sample } A_{550}) - (\text{blank}_{\text{sample}}\ A_{550})}{(\text{control } A_{550}) - (\text{blank}_{\text{control}}\ A_{550})}\right\} \times 100$$

RESULTS AND DISCUSSION

Structure Elucidation of New Compounds. A concentrated aqueous EtOH extract of crushed walnuts was successively extracted with *n*-hexane, EtOAc, and *n*-BuOH, to afford the respective extracts. The EtOAc extract was fractionated by an extensive series of chromatographic procedures to furnish five known compounds: (+)-catechin (*10*); 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**7**) (*11*); 1,2,4,6-tetra-*O*-galloyl-β-D-glucose (**8**) (*11*); 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**9**) (*11*); and rugosin F (**15**) (*12*). Similarly, repeated column chromatography of the water-soluble portion yielded 2 novel dicarboxylic acid derivatives, glansreginins A (**1**) and B (**2**), and a new dimeric tannin, glansrin D (**3**), together with 11 known compounds: L-tryptophan; ellagic acid (*13*); ellagic acid 4-*O*-xylopyranoside (*14*); gemin D (**10**) (*15*); casuariin (**11**) (*16*); pterocarinin A (**12**) (*17*); platycaryanin A (**13**) (*18*); euprostin A (**14**) (*19*); **15**; 1-desgalloylrugosin F (**16**) (*20*); and heterophylliin D (**17**) (*21*) (**Figure 1**; **Chart 1**). These known compounds were identified by direct comparison with authentic specimens or by comparison of spectroscopic data with those reported in the literature.

Glansreginin A (**1**) was isolated as a pale yellow amorphous powder. The molecular formula, $C_{28}H_{35}NO_{13}$, was established from an $[M + H]^+$ peak at *m*/*z* 594.2171 in HRESIMS and by elemental analysis. The ^{1}H NMR spectrum of **1** (**Table 1**) in the aromatic proton region displayed signals attributed to 1,2-disubstituted benzene ring protons (δ 6.78, 6.98, 7.24, and 7.41) and three mutually coupled olefin protons (δ 6.20, 6.48, and 7.17). The proton signals due to mutually coupled methine (δ 1.71, 3.99) and two methylene [δ 2.10, 2.43 (3H)] and one isolated methylene (δ 3.14, 3.19), besides those due to a β-glucopyranoside, were observed in the aliphatic proton region of the ^{1}H NMR spectrum. The ^{13}C NMR spectrum of **1** (**Table 1**) showed resonances of 14 sp^2 carbons, including 4 carbonyl carbons, and 14 sp^3 carbons, among which 6 resonances were attributable to β-glucopyranoside. Acid hydrolysis of **1** gave glucose, 2-hydroxycinchoninic acid (**1a**) (*22*, *23*), and a dicarboxylic acid derivative with 12 carbons (**1b**), together with a byproduct (**1c**) corresponding to the monomethyl ester of **1b** (**Figure 2**). The liberated glucose was of the D-series on the basis of a positive response to the reaction with D-glucose oxidase (*9*).

Compound **1b**, $[\alpha]_D^{23}$ +21.2°, was isolated as a pale brown amorphous powder. ^{1}H and ^{13}C NMR spectra of **1b** exhibited signals comparable to those of **1**, except for a lack of the signals of aromatic, glucose, and isolated methylene protons, suggesting the presence of two carbonyl carbons, three olefinic protons, two methines, two methylenes, and two methyl units in **1b**. In addition, the ^{1}H−^{1}H COSY spectrum of **1b** revealed sequential couplings of olefinic, methylene, methine, and methylene protons. On the other hand, NMR spectroscopic data of **1c** were very similar to that of **1b**, except for the presence of a carboxymethyl signal. The ESIMS of **1c** showed an $[M + NH_4]^+$ ion peak at *m*/*z* 274, which was 14 mass units larger than that of **1b**, implying that compound **1c** corresponds to the monomethyl ester of **1b**. The HMBC spectrum of **1c** exhibited

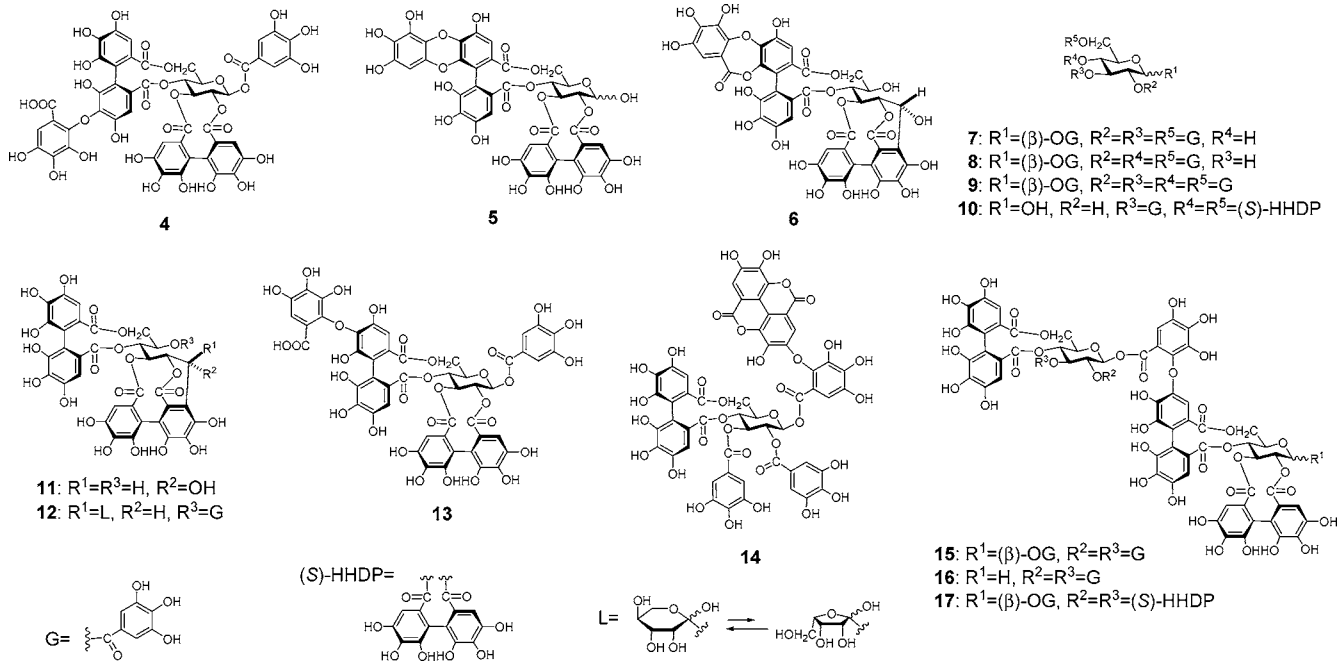


Figure 1. Structures of glansrins A–C (**4**–**6**) and isolated known tannins. G, galloyl; L, lyxose.

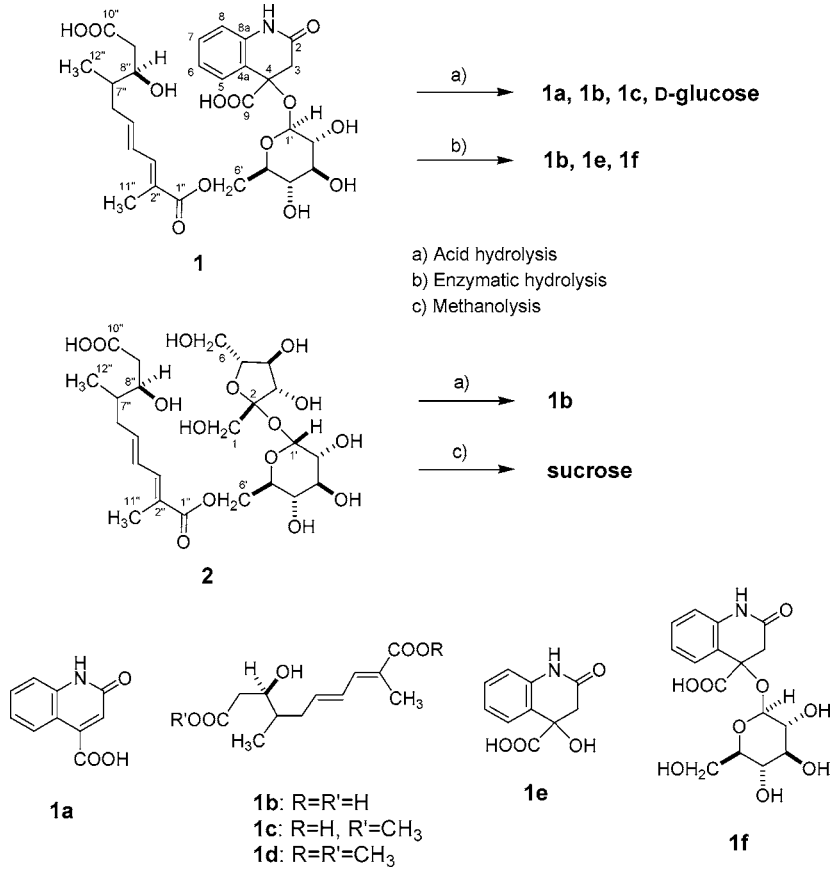


Figure 2. Structures and reactions of glansreginins A (**1**) and B (**2**).

correlations between carboxymethyl protons (δ 3.61) and methylene protons (H-9, δ 2.42, 2.46) through one carbonyl carbon (C-10, δ 173.1). Both methyl (H-11, δ 1.89) and vinyl (H-3, δ 7.18) protons showed three-bond correlations with another carbonyl carbon (C-1, δ 169.5). The structures of **1b** and **1c** were thus assigned to the formulas shown and named glansreginic acid and its monomethyl ester, respectively. Compound **1c** is probably an artifact formed during the separation procedure of **1b**. Compound **1b** was previously reported as a component of *Phaseolus multiflorus* (*24*), whereas its stereostructure has remained unassigned. The *E*-orientation at H-4 and -5 in **1b** and **1c** was consistent with their coupling constant (J = 15 Hz). The absolute configuration at C-8 in **1b** was determined according to the modified Mosher's method (*25*). Dimethyl glansreginate (**1d**), which was prepared from **1c** by methylation, was treated with (+)- or (−)-MTPA chlorides

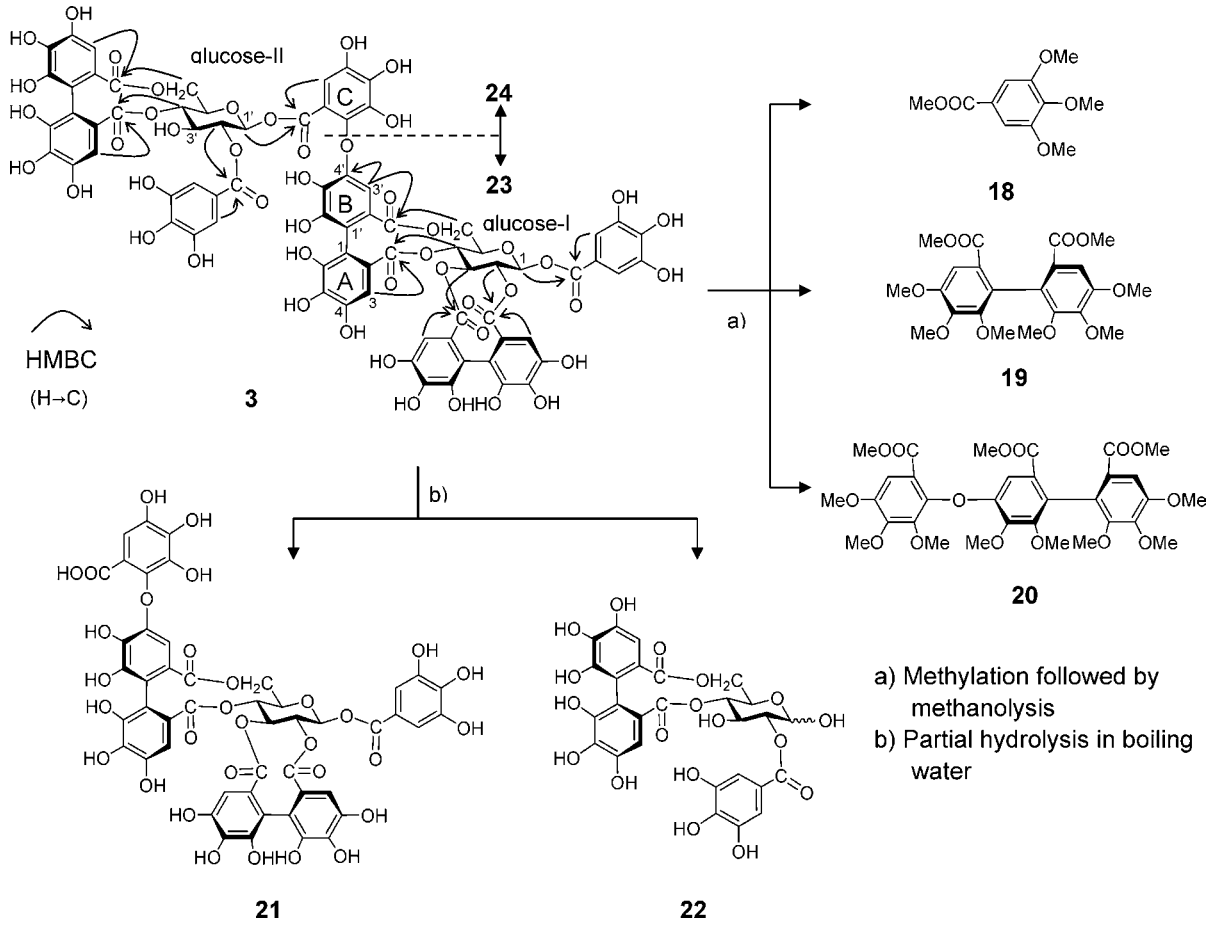


Figure 3. Structure and reactions of glansrin D (**3**).

in anhydrous pyridine to furnish (*R*)- and (*S*)-MTPA ester derivatives, respectively. Negative values ($\Delta\delta_{S-R}$) were obtained for H-3, -4, -5, -6, -7, -11, and -12, and positive differences for H-9 and -14 confirmed that the absolute configuration of the chiral center at C-8 is of the *R*-series. The linkage mode of quinolone, dicarboxylic acid, and glucose moieties in **1** was established by the HMBC experiment. The anomeric proton (δ 4.44) was correlated with a quaternary carbon bearing an oxygen function (C-4, δ 78.4). H-6′ signals (δ 4.11, 4.24) on glucose showed cross-peaks with ester carbonyl resonance (δ 168.8), which in turn correlated with H-3″ (δ 7.17) and CH_3-11″ (δ 1.91). Furthermore, distinct cross-peaks between the anomeric proton and both H-3 and H-5 were observed in the NOESY experiment. These key 2D NMR correlations clarified the glucosidic linkage to the 4-position on the quinolone moiety and the ester bond between the 6′-position on glucose and the 1″-position on glansreginic acid moieties. These structural features were substantiated by the production of compounds **1b**, **1e**, and **1f** upon the enzymatic hydrolysis of **1** with naringinase (**Figure 2**). Compound **1a** obtained by acid hydrolysis of **1** was considered to be a product formed by β-elimination from compound **1f**. On the basis of these findings, the gross structure of glansreginin A was established as formula **1**, although the absolute configuration at C-4 and -7″ remains undetermined.

The molecular formula of glansreginin B (**2**) was determined as $C_{24}H_{38}O_{15}$ by an $[M + NH_4]^+$ peak at *m*/*z* 584.2556 in HRESIMS. ^1H and ^{13}C NMR data displayed signals characteristic of sucrose in addition to those of glansreginic acid (**1b**) (**Table 1**). The presence of a glansreginic acid moiety in **2** was established by acid hydrolysis of **2** yielding **1b**, and sucrose was detected in the methanolysates of **2**, respectively (**Figure 2**). The linkage of each moiety was manifested by the HMBC spectrum of **2**, in which the signal of H-6′ (δ 4.29, 4.39) on sucrose was associated with both H-3″ (δ 7.22) and H-11″ (δ 1.90) through a common ester carbonyl carbon resonance (δ 168.8). On the basis of the above data, the structure of glansreginin B (**2**) was assigned to the sucrose ester of glansreginic acid. A novel C_{12} dicarboxylic acid derivative, glansreginic acid, is plausibly formed from tetraterpenoids such as violaxanthin in higher plants in a similar way to abscisic acid (*26*).

Glansrin D (**3**) is a dimeric hydrolyzable tannin with the molecular formula $C_{75}H_{52}O_{48}$, which was indicated by ESIMS, elemental analysis, and NMR analyses. The ^1H NMR spectrum of **3** revealed two 2H singlets (δ 7.07, 7.13) and seven 1H singlets (δ 6.18, 6.38, 6.43, 6.51, 6.59, 6.69, 7.09), ascribable to one valoneoyl, two galloyl, and two HHDP groups. These acyl components were chemically identified by methylation of **3** followed by methanolysis yielding methyl tri-*O*-methylgallate (**18**), dimethyl hexamethoxydiphenate (**19**), and trimethyl octa-*O*-methylvaloneate (**20**) (**Figure 3**). The presence of two sets of *C1* glucopyranose moieties was indicated by two anomeric proton signals (δ 6.13, d, $J = 9.0$ Hz and δ 5.84, d, $J = 7.8$ Hz) and the coupling pattern and chemical shifts of other aliphatic proton signals, which were assigned by the $^1H-^1H$ COSY spectrum (**Table 2**). A remarkable upfield shift of the H-3′ signal (δ 4.12) showed that a hydroxyl group at the 3′-position of the glucose II core was not acylated. These data thus implied that the monomeric components of **3** were casuarictin (**23**) (*16*) and 1,2-di-*O*-galloyl-4,6-HHDP-β-D-

Table 3. SOD-like Activity and DPPH Radical Scavenging Activity of Walnut Constituents

compound	SOD-like activity EC_{50} (μM)	DPPH radical scavenging activity EC_{50} (μM)	MW
glansreginin A (**1**)	>1000	>25	593.6
glansreginin B (**2**)	>1000	>25	566.6
glansrin D (**3**)	69.7	1.63	1721.2
1,2,3,6-tetra-*O*-galloylglucose (**7**)	76.1	0.53	788.6
1,2,4,6-tetra-*O*-galloylglucose (**8**)	133.1	0.87	788.6
1,2,3,4,6-penta-*O*-galloylglucose (**9**)	50.0	0.47	940.7
pterocarinin A (**12**)	70.2	0.44	1068.8
platycaryanin A (**13**)	108.6	0.63	1104.8
euprostin A (**14**)	64.6	0.81	1238.1
rugosin F (**15**)	331.0	0.53	1873.3
1-desgalloylrugosin F (**16**)	63.9	0.38	1721.2
heterophylliin D (**17**)	160.3	0.59	1871.3
ellagic acid 4′-*O*-xylopyranoside	82.9	3.45	434.3
L-ascorbic acid	34.6	6.25	176.1
gallic acid	31.7	5.88	170.1

glucose (**24**) (*27*). The position and orientation of the valoneoyl group were established by the HMBC spectrum of **3** (**Figure 3**). The H-6 signal at δ 5.22 of the glucose I core showed a correlation with an aromatic proton signal at δ 6.18 through a common ester carbonyl carbon resonance at δ 167.9. This aromatic proton signal was assigned to H-3′ on the B-ring of the valoneoyl group on the basis of the two-bond correlation with an ethereal phenyl carbon (C-4′ on the B-ring of the valoneoyl group) at δ 146.7. Key HMBC correlations disclosed the binding mode of other acyl groups to sugar moieties as shown in **Figure 3**. Atropisomerisms of both HHDP and valoneoyl groups were confirmed as (*S*)-configuration by the intensive positive Cotton effect at 237 nm ($[\theta]$ $+2.0 \times 10^5$) in the CD spectrum of **3** (*28*). Furthermore, partial hydrolysis of **3** in boiling water gave rugosin C (**21**) and hippomanin A (**22**) (*27*) to provide definite evidence of the binding mode and stereochemistry of the valoneoyl group as shown in the formula. The ^{1}H NMR data of hippomanin A (**22**) were fully assigned in this study. On the basis of these data, the structure of glansrin D was represented by formula **3**.

It is noteworthy that dimeric hydrolyzable tannins, glansrin D (**3**), rugosin F (**15**) (*12*), 1-desgalloylrugosin F (**16**) (*20*), and heterophylliin D (**17**) (*21*), were isolated for the first time from walnuts.

Antioxidant Activity of the Compounds from Walnuts. The antioxidant activity of the isolates in the present study was evaluated by SOD-like activity and DPPH radical scavenging effects. The results are summarized in **Table 3**. In the SOD-like assay, polyphenolic compounds **7**, **9**, **12**, **14**, **16**, ellagic acid 4-*O*-xylopyranoside, and glansrin D (**3**) exhibited EC_{50} values in the range of 50.0−82.9 μM, which were comparable to those of L-ascorbic acid and gallic acid as positive controls. On the other hand, all tested polyphenols had more potent DPPH radical scavenging effect than positive controls. Among these active polyphenols, dimeric hydrolyzable tannins (**15**−**17**) and gallotannins (**7**−**9**) showed more potent activity with EC_{50} below 0.9 μM. Non-polyphenolic new compounds, glansreginins A (**1**) and B (**2**), had no effect in either assay.

As walnuts contain only a low amount of α-tocopherol (*29*), walnut hydrolyzable tannins might contribute as effective antioxidants not only to protection against the autoxidation of unsaturated essential fatty acids in the nut but also to health benefits such as improvement of cardiovascular functions. The biological activity of walnut polyphenols is under further investigation.

ABBREVIATIONS USED

SOD, superoxide dismutase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; COSY, ^{1}H−^{1}H correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; HSQC, heteronuclear single-quantum coherence; HMBC, heteronuclear multiple-bond correlation; HHDP, hexahydroxydiphenoyl; MTPA, 2-methoxy-2-trifluoromethyl-2-phenylacetyl.

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