Tyrosinase-Inhibitory Constituents from the Twigs of *Cinnamomum cassia*

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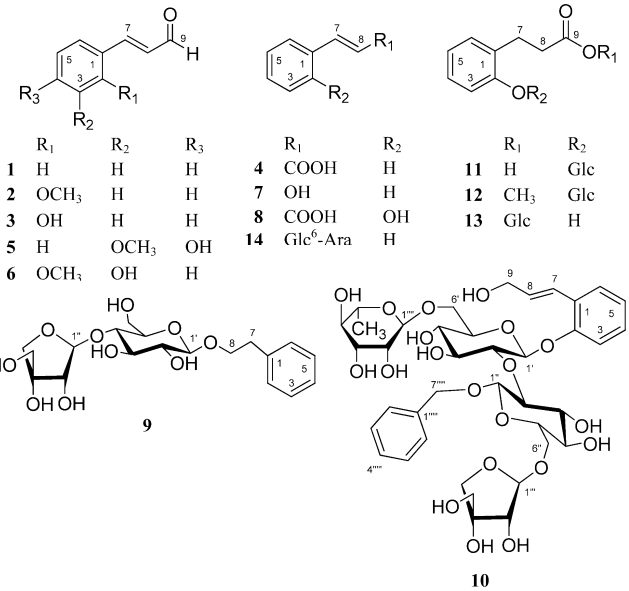
A methanol extract of the twigs of *Cinnamomum cassia* was found to possess inhibitory activity against tyrosinase. Purification of the MeOH extract afforded four new phenolics, cassiferaldehyde (**6**), icariside DC (**9**), cinnacassinol (**10**), and dihydrocinnacasside (**13**), together with 10 known compounds (**1**−**5**, **7**−**12**, and **14**). The structures of the new compounds were determined by spectroscopic data interpretation. Compounds **1**−**6** and **8**−**13** showed strong inhibitory activity against tyrosinase, with IC_{50} values ranging from 0.24 to 0.94 mM.

Cinnamomum cassia Blume (Lauraceae) is distributed in the southern part of mainland China, Myanmar, Laos, and Vietnam. The twigs are used in traditional Chinese medicine for treating dyspepsia, gastritis, blood circulation disturbances, diabetes, and inflammatory diseases.[1] Chemical and pharmacological investigations on *C. cassia* have resulted in the isolation of several bioactive compounds such as cinnamaldehyde and cinnamic acid, as well as coumarins, diterpenoids, and polyphenols,[2,3] which have been found to exhibit antifungal, cytotoxic, antipyretic, antioxidant, and antimicrobial activities.[4−9] In the course of a program to screen for tyrosinase inhibitors from plants, it was found that a MeOH extract of the twigs of *C. cassia* exhibited a strong inhibitory activity (>85% inhibition at 100 μg/mL). Since it has been reported that the tyrosinase inhibitors may have antimelanin synthesis activity and offer a potential treatment for Parkinson's disease,[10] a phytochemical investigation was carried out. The present report describes the isolation of 14 phenolics (**1**−**14**) from the MeOH extract of the twigs of *C. cassia*, including the characterization of four new compounds (**6**, **9**, **10**, and **13**). The inhibitory activity of compounds **1**−**14** against tyrosinase has been evaluated.



	R_1	R_2	R_3
1	H	H	H
2	OCH_3	H	H
3	OH	H	H
5	H	OCH_3	OH
6	OCH_3	OH	H

	R_1	R_2
4	COOH	H
7	OH	H
8	COOH	OH
14	Glc^6-Ara	H

	R_1	R_2
11	H	Glc
12	CH_3	Glc
13	Glc	H

9

10

The MeOH extract of the twigs of *C. cassia* was partitioned into hexane-, EtOAc-, and BuOH-soluble fractions. Repeated column chromatography of these fractions resulted in the purification of compounds **1**−**14**. The known compounds were identified as cinnamaldehyde (**1**),[11] 2-methoxycinnamaldehyde (**2**),[12] 2-hydroxycinnamaldehyde (**3**),[13] cinnamic acid (**4**),[14] coniferaldehyde (**5**),[15] cinnamic alcohol (**7**),[16] *o*-coumaric acid (**8**),[17] dihydomelilotoside (**11**),[18] methyl dihydromelilotoside (**12**),[19] and rosavin (**14**).[20]

Compound **6** was obtained as a colorless, amorphous powder, and its positive HREIMS showed a molecular ion peak $[M]^+$ at *m/z* 178.0631, consistent with the molecular formula $C_{10}H_{10}O_3$. Its IR spectrum showed absorption bands for a hydroxy group and a carbonyl aldehyde, at ν_{max} 3280 and 1673 cm^{-1}, respectively. The ^1H NMR spectrum displayed a singlet methoxy resonance and signals for two *trans*-olefinic protons and three aromatic protons. The ^{13}C and DEPT NMR data (Table 1) of **6** were very similar to those of coniferaldehyde (**5**)[15] except for signals indicating the locations of a methoxy group at C-2 (δ_C 148.9) and a hydroxy group at C-3 (δ_C 152.1). This was supported by the long-range correlations in the HMBC spectrum from the methoxy protons and the H-7 olefinic proton to C-2 and from the aromatic protons H-4 and H-5 to C-3 (Figure 1). Therefore, **6** was determined as a new cinnamaldehyde derivative and has been named cassiferaldehyde.

Compound **9** was obtained as a colorless gum, with $[\alpha]^{25}_D$ +22.5. Its IR spectrum showed an absorption band at 1458 cm^{-1} due to an aromatic function and strong absorption bands at 3399 and 1075 cm^{-1} suggestive of a glycosidic structure. The molecular formula was established as $C_{19}H_{28}O_{10}$ from a molecular ion peak at *m/z* 416.1672 $[M]^+$ in the HREIMS. The ^1H and ^{13}C NMR data (Table 1) of **9** indicated the presence of a β-D-glucopyranosyl moiety, a β-D-apiofuranosyl moiety, and ethylphenyl resonances. The ^1H and ^{13}C NMR data were very similar to those of icariside D_1,[21] with a downfield shift of C-4′ (δ_C 78.8) indicating the linkage to C-1″ of an apiofuranosyl moiety. Analysis of the HBMC spectrum of **9** showed long-range correlations between the H-4′ proton and C-1″ carbon and between the H-1″ proton and the C-4′ carbon (Figure 1). Consequently, the structure of icariside DC (**9**) was determined as phenylethyl-1-*O*-β-D-apiofuranosyl-(1→4)-β-D-glucopyranoside.

Compound **10** was purified as an amorphous solid. The molecular formula, $C_{39}H_{54}O_{20}$, was observed from a molecular ion peak at *m/z* 842.8362 $[M]^+$ in the HREIMS. Combined analysis of the ^1H, ^{13}C, DEPT, and HMQC NMR spectra (Table 2) of **10** revealed the presence of an *o*-hydroxycinnamic alcohol skeleton possessing an oxygenated methylene signal, two *trans*-olefinic resonances, and *o*-hydroxyphenyl and phenyl methanol moieties. The ^1H and ^{13}C NMR spectra also suggested the presence of four sugar moieties of two β-D-glucopyranosyl units, one β-D-apiofuranosyl unit, and one α-L-rhamnopyranosyl unit. The HMBC spectrum displayed key correlations of the H-1′ aromeric proton to the C-2 carbon and the H-7‴″ oxymethine protons to the C-1″ carbon, showing the connectivities of the β-D-glucopyranosyl to the cinnamic alchohol

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Table 1. NMR Spectroscopic Data for **6**, **9**, and **13** (in MeOD)

	6		**9**		**13**	
position	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
1	129.5, qC		140.2, qC		131.5, qC	
2	148.9, qC		129.5, CH	7.26 d (1.6, 7.6)	157.1, qC	
3	152.1, qC		130.1, CH	7.26 d (1.6, 7.6)	123.5, CH	6.91 dt (2.8, 7.2)
4	120.9, CH	6.95 dd (1.5, 7.8)	127.3, CH	7.17 m	128.8, CH	7.14 d (7.2) overlap
5	125.9, CH	7.00t (7.8)	130.1, CH	7.26 d (1.6, 7.6)	116.4, CH	7.13 d (7.2) overlap
6	120.1, CH	7.17 dd(1.5, 7.8)	129.6, CH	7.26 d (1.6, 7.6)	131.1, CH	7.12 d (7.2) overlap
7	150.1, CH	7.90 d (16.2)	71.7, CH_2	3.74 dt (9.6, 7.2)	27.1, CH_2	2.97 m
				4.09 dt (9.6, 7.2)		
8	130.6, CH	6.79 dd (7.8, 16.2)	37.4, CH_2	2.92 t (7.2)	35.5, CH_2	2.62 t (7.6)
9	196.7, CHO	9.67 d (7.8)			177.7, qC	
Glc-1′			103.4, CH	4.35 d (7.6)	102.7, CH	4.92 d (7.6)
2′			78.7, CH	3.51 m	75.1, CH	3.51 m
3′			78.0, CH	3.42 m	78.3, CH	3.42 m
4′			78.8,CH	3.41 m	71.5, CH	3.41 m
5′			78.0, CH	3.49 m	78.2, CH	3.49 m
6′			62.8, CH_2	3.96 d (12.0)	62.6,CH_2	3.89 d (12.0) 3.71 dd (5.2, 12.0)
				3.85 dd (2.4, 12.0)		
Apio-1″			110.6, CH	5.38 d (1.6)		
2″			77.9, CH	3.93 d (1.6)		
3″			80.8, qC			
4″			75.4, CH_2	3.96 d (9.6)		
3.68 d (9.6)						
5″			66.3, CH_2	3.59 dd (11.2, 14.2)		
OCH_3	61.8, CH_3	3.85 s				

at C-2, and another β-D-glucopyranosyl to phenylmethanyl at C-7‴″. The correlations from H-1‴″ to C-6′ and from H-1‴′ to C-6″ indicated the linkages of the α-L-rhamnopyranosyl and the β-D-apiofuranosyl to C-6′ and C-6″, respectively. The $^3J_{C,H}$ correlations from H-2′ to C-2″ and H-2″ to C-2′ were supportive of the (2−2) linkage of two the β-D-glucopyranosyl units (Figure 1). Accordingly, the structure of cinnacassiol (**10**) was assigned as 2-[1-*O*-(phenylmethanyl)-*O*-β-D-apiofuranosyl-(1−6)-β-D-glucopyranosyl]-(2−2)(*O*-α-L-rhamnopyranosyl)-(1−6)-β-D-glucopyranosyl-cinnanic alcohol.

Compound **13** was isolated as a white amorphous powder, with $[\alpha]^{25}_D$ +29.1, and showed a $[M]^+$ ion at *m/z* 328.1168 in the HREIMS, corresponding to the molecular formula $C_{15}H_{20}O_8$. The IR spectrum of **13** showed a glycosidic nature from absorptions at 3354 and 1087 cm^{-1}, and a band at 1735 cm^{-1} for a carbonyl ester group. The ^{1}H NMR spectrum displayed the following representative signals: a propanoyl proton, an *o*-hydroxyphenyl resonance, and an aromeric proton of a β-D-glucopyranosyl moiety. The ^{1}H and ^{13}C NMR data (Table 1) of **13** were closely comparable with those of dihydromelilotoside (**11**).[18] Analysis of the 2D NMR data allowed the assignment of the esterification location of the β-D-glucopyranosyl group in **13** at C-9, which was supported by the key HMBC correlation between the H-1′ and the carbonyl carbon C-9 (δ_C 177.7) (Figure 1). Accordingly, the structure of dihydrocinnacasside (**13**) was elucidated as *o*-hydroxyphenylpropanoyl-*O*-β-D-glucopyranoside.

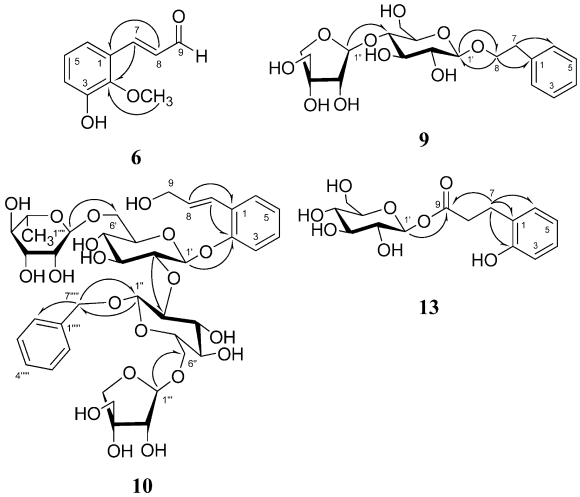

Figure 1. Selected HMBC correlations of compounds **6**, **9**, **10**, and **13**.

Compounds **1**−**14**, together with kojic acid and hydroquinone as positive controls, were examined for their inhibitory effects on tyrosinase activity in an in vitro assay.[22] The results are presented in Table 3, and it may be seen that compounds **1**−**6** and **8**−**13** exhibited strong inhibition of tyrosinase with IC_{50} values ranging from 0.24 to 0.94 mM. Of the positive controls used, hydroquinone is used as a commercial whitening agent in comestics.[23] The other compounds (**7** and **14**) were inactive (IC_{50} >10 mM).

Experimental Section

General Experimental Procedures. Specific rotations were measured on a JASCO DIP-370 polarimeter. UV spectra were obtained using a Beckman DU-650 recording spectrophotometer. FT-IR spectra were carried out using a JASCO Report-100 infrared spectrometer. FT-NMR spectra were recorded on a Bruker DRX-300 spectrometer (^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz) using CD_3OD as the solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts (δ) are expressed in ppm with reference to the TMS signals. Two-dimensional (2D) NMR (HMQC, HMBC) experiments were performed on a Bruker Avance 500 spectrometer. HRMS was measured on a JMS-700 Mstation mass spectrometer. Semipreparative HPLC was conducted on Trilution LC with a UV/vis-151 detector, a 321 pump, a 402 syringe pump, and a GX-271 liquid handler (Gilson, Inc.), using a YMC-pack Pro C_{18} (250 × 20 mm, i.d.) column. Column chromatography was performed using silica gel (Kieselgel 60, 70−230 mesh and 230−400 mesh, Merck). Thin-layer chromatography (TLC) was performed on Merck precoated silica gel 60 F_{254} and/or RP-18 F_{254s} plates (0.25 mm), and compounds were observed under UV light at 254 and 365 nm or visualized by spraying the dried plates with 10% H_2SO_4, followed by heating at 180 °C. Optical density (OD) values in the tyrosinase inhibitory activity assays were read on an Emax Precision microplate reader.

Plant Material. The dried twigs of *C. cassia* were purchased from a pharmacy in Daejeon, Korea, in November 2006, and were identified by one of us (K.B.). A voucher specimen (CN 95125) was deposited at the herbarium in the College of Pharmacy, Chungnam National University.

Table 2. NMR Spectroscopic Data for **10** (in MeOD)

position	δ_C, mult.	δ_H (J in Hz)	position	δ_C, mult.	δ_H (J in Hz)
1	128.5, qC		1‴	111.7, CH	4.97 d (2.5)
2	156.3, qC		2‴	78.2, CH	3.90 d (2.5)
3	117.4, CH	7.18 dd (1.5, 7.5)	3‴	80.6, qC	
4	129.9, CH	7.21 dd (1.5, 7.5)	4‴	75.3, CH	3.95 d (10.0)
					3.74 d (10.0)
5	123.7,CH	6.98 br t (7.5)	5‴	65.8, CH_2	3.58 dd (1.5, 11.0)
6	127.4, CH	7.48 dd (1.5, 7.5)	1⁗	102.5, CH	4.78 d (1.0)
7	126.6, CH	7.07 d (16.0)	2⁗	72.4, CH	3.87 m
8	130.6, CH	6.34 dd (5.5, 16.0)	3⁗	72.6, CH	3.70 m
9	64.2, CH_2	4.25 dd (1.5, 5.5)	4⁗	74.2, CH	3.39 m
1′	103.0, CH	4.48 d (8.0)	5⁗	70.0, CH	3.70 m
2′	75.1, CH	3.51 m	6⁗	18.2, CH_3	1.27 d (6.3)
3′	78.2, CH	3.45 m	1⁗′	139.0, qC	
4′	71.9, CH	3.29 m	2⁗′	129.5, CH	7.42 brd (7.5)
5′	77.2, CH	3.58 m	3⁗′	129.4, CH	7.33 d (7.5)
6′	68.3, CH_2	3.67 m	4⁗′	128.9, CH	7.27 br t (7.5)
		4.01 dd (2.0, 11.5)			
1″	103.3, CH	4.33 d (7.5)	5⁗′	129.4, CH	7.33 d (7.5)
2″	75.1, CH	3.23 m	6⁗′	129.5, CH	7.42 brd (7.5)
3″	78.3, CH	3.30 m	7⁗′	71.9, CH_2	4.87 d (12.5)
					4.46 d (12.5)
4″	71.8, CH	3.36 m			
5″	77.1, CH	3.38 m			
6″	68.9, CH	3.61 m			
		4.02 dd (1.5, 11.0)			

Table 3. Inhibitory Activity of Isolated Compounds **1**−**14** against Tyrosinase

compound	inhibitory activity IC_{50} (mM)[a]
cinnamaldehyde (**1**)	0.52 ± 0.03
2-methoxy cinnamaldehyde (**2**)	0.42 ± 0.02
2-hydroxy cinnamaldehyde (**3**)	0.47 ± 0.03
cinnamic acid (**4**)	0.41 ± 0.01
coniferaldehyde (**5**)	0.24 ± 0.03
cassiferaldehyde (**6**)	0.26 ± 0.04
cinnamic alcohol (**7**)	>10
O-coumaric acid (**8**)	0.67 ± 0.03
icariside DC (**9**)	0.71 ± 0.03
cinnacassinol (**10**)	0.94 ± 0.04
dihydromelilotoside (**11**)	0.57 ± 0.01
methyl dihydromelilotoside (**12**)	0.63 ± 0.02
dihydrocinnacaside (**13**)	0.67 ± 0.03
rosavin (**14**)	>10
kojic acid[b]	0.14 ± 0.02
hydroquinone[b]	4.21 ± 0.05

[a] Values present mean ± SD of triplicate experiments. [b] Positive control.

Extraction and Isolation. The sliced twigs of *C. cassia* (30 kg) were extracted with hot MeOH (60 L × 3 times) for two days. The MeOH extracts were filtered, combined, and concentrated in vacuo, resulting in the production of a residue (1650 g). This residue was suspended in H_2O and then fractionated successively with hexane, EtOAc, and *n*-BuOH, producing a hexane-soluble (410 g), EtOAc-soluble (230 g), and *n*-BuOH-soluble (135 g) fraction, respectively. The hexane-soluble fraction (410 g) was subjected to silica gel column chromatography, eluted with hexane and EtOAc gradient mixtures (100:1−0:100), to give seven fractions, H1−H7. Fraction H1 was chromatographed on silica gel, eluting with hexane−EtOAc (25:1), as well as ODS silica gel with MeOH−H_2O (55:45), to afford **1** (6.0 g) and **2** (1.4 g). Fraction H6 was subjected to silica gel column chromatography, eluting with hexane−EtOAc (10:1), and then purification by medium-pressure liquid chromatography (MPLC) (YMC-ODS 15 × 30 mm, 5 μm), eluted with MeOH−H_2O (60:40), to give **3** (1.2 g). Compound **4** (2.2 g) was isolated and purified from fraction H7 using YMC-ODS column chromatography, with MeOH−H_2O (60:40) as eluant, and MPLC (YMC-ODS 15 × 300 mm, 5 μm), eluted with MeOH−H_2O (50:50).

The EtOAc-soluble fraction (230 g) was chromatographed on a silica gel column with a stepwise gradient of $CHCl_3$ and MeOH (60:1 to 0:1), to separate eight fractions, E1−E8. Subfraction E2 was chromatographed on a silica gel column, with mixtures of hexane−EtOAc (10:1 to 2:1) as the eluting solvent systems, and semipreparative HPLC (YMC-ODS, 10 × 250 mm, 5 μm), eluted with MeOH−H_2O (40:60), to afford **5** (516 mg) and **6** (4.2 mg). Compound **7** (120 mg) was purified from fraction E4 using silica gel column chromatography, eluted with hexane−EtOAc (3:1), and then purified by MPLC (YMC-ODS 15 × 300 mm, 5 μm), with MeOH−H_2O (60:40) as mobile phase. Subfraction E5 was subjected to silica gel column chromatography, eluted with $CHCl_3$−MeOH−H_2O (3:1:0.1), to afford **8** (52 mg).

The *n*-BuOH-soluble fraction (135 g) was suspended in H_2O and then applied to a Diaion HP-20 column with a H_2O and MeOH gradient eluent to give fractions B1−B14. Fraction B5 was subjected to passage over silica gel, using CH_3Cl−MeOH−H_2O (15:1:0.1 to 1:1:0.1) for elution, to yield nine subfractions, B5.1−B5.9. Subfraction B5.5 was applied to a Sephadex LH-20 column, eluted with MeOH−H_2O (1:5), and then YMC-ODS column chromatography with the mobile phase system MeOH−H_2O (1:4) to give **9** (87 mg) and **10** (2.7 mg). Fraction B6 was chromatographed on a Sephadex LH-20 column with MeOH−H_2O (1:5) as solvent and then purified by reversed-phase HPLC (YMC-ODS, 10 × 250 mm, 5 μm), with MeOH−H_2O (1:5) as mobile phase, yielding **11** (15 mg), **12** (10 mg), **13** (12 mg), and **14** (43 mg).

Cassiferaldehyde (6): colorless, amorphous powder; mp 78−80 °C; UV (MeOH) λ_{max} (log ϵ) 214 (3.30) and 350 (3.24) nm; IR (KBr) ν_{max} 3280, 1673, 1586, 1331, 1206, 1106, and 727 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HREIMS *m*/*z* 178.0631 $[M]^+$ (calcd for $C_{10}H_{10}O_3$, 178.0630).

Icariside DC (9): colorless gum; $[\alpha]^{25}_D$ +22.5 (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ϵ) 214 (3.00) nm; IR (KBr) ν_{max} 3399, 2936, 1458, and 1066 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HREIMS *m*/*z* 416.1672 $[M]^+$ (calcd for $C_{19}H_{28}O_{10}$, 416.1682).

Cinnacassiol (10): white, amorphous powder; $[\alpha]^{25}_D$ −57.2 (*c* 0.35, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (2.31), 248 (1.82), and 284 (1.33) nm; IR (KBr) ν_{max} 3506, 3354, 2900, 1735, 1240, and 1087 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HREIMS *m*/*z* 842.8362 $[M]^+$ (calcd for $C_{39}H_{54}O_{20}$, 842.8368).

Dihydrocinnacasside (13): colorless, amorphous powder; mp 174−175 °C; $[\alpha]^{25}_D$ −29.1 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 214 (2.55) nm; IR (KBr) ν_{max} 3354, 2900, 1735, 1240, and 1087 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HREIMS *m*/*z* 328.1168 $[M]^+$ (calcd for $C_{19}H_{28}O_{10}$, 328.1158).

Acid Hydrolysis of Compounds 9, 10, and 13. Solutions of **9** (5.2 mg), **10** (1.2 mg), and **13** (2.5 mg) in 0.5 N H_2SO_4 (dioxane−H_2O, 1:1, 2 mL) were each heated at 100 °C for 2 h. Each reaction mixture was diluted with H_2O (3 mL) and extracted with $CHCl_3$ (5 mL × 3 times). The H_2O layer was dried in vacuo after neutralization with 1 N NaOH and passed through a Sep-Pak C_{18} cartridge (Waters, Milford,

MA). Sugars were identified as glucose (R_f 0.16), rhamnose (R_f 0.32), and apiose (R_f 0.38) by TLC in $CHCl_3$−MeOH−H_2O (12:6:1), using the authentic samples D-glucose, L-rhamnose, and D-apiose. The remaining eluate was concentrated to dryness, and the residue was stirred with D-cysteine methyl ester hydrochloride, hexamethyldisilazane, and trimethylsilylchloride in pyridine using the same procedures as in a previous report.[24] After the reaction, the supernatant was analyzed by GC [column: capillary column BD-5, 0.25 mm × 30 m, detector: FID, detector temperature: 300 °C, injector temperature: 270 °C, carrier gas, N_2; column temperature, 210 °C]. The peaks corresponding to the D-glucosyl, L-rhamnosyl, and D-apiosyl derivatives appeared with t_R 8.55, 5.31, and 4.03 min, respectively.

Tyrosinase Inhibitory Activity Assay. Mushroom tyrosinase and L-dopa used for the bioassay were purchased from Sigma Chemical Co. Antityrosinase activity was measured by spectrophotometry, according to the method of Mason and Peterson[25] with minor modifications. Each test substance was dissolved in 0.1 mL of 10% DMSO in aqueous solution and incubated with 0.1 mL of 135 U/mL mushroom tyrosinase in phosphate buffer solution (PBS, pH 6.8) at 25 °C for 10 min, and then 0.1 mL of L-dopa (0.5 mM, PBS pH 6.8) was added. The reaction mixture was incubated for 5 min. The amount of dopachrome in the mixture was determined by the optical density (OD) at 475 nm using an Emax Precision microplate reader. Kojic acid and hydroquinone (Sigma) were used as positive control agents. The inhibitory percentage of tyrosinase was calculated as follows: % inhibition = $\{[(A - B) - (C - D)]/(A - B)\} \times 100$ (A: OD at 475 nm without test substance; B: OD at 475 nm without test substance and tyrosinase; C: OD at 475 nm with test substance; D: OD at 475 nm with test substance, but without tyrosinase).

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Supporting Information Available: ^1H, ^{13}C, HMQC, and HMBC spectra of the four new compounds (**6**, **9**, **10**, and **13**). This material is available free charge via the Internet at http://pubs.acs.org.

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