Reinvestigation of Structures of Robustasides B and C, and Isolation of (*E*)-2,5-Dihydroxycinnamic Acid Esters of Arbutin and Glucose from the Leaves of *Grevillea robusta*

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From the leaves of *Grevillea robusta*, compounds whose NMR data were superimposable on those of robustasides B and C were isolated along with two new compounds, (*E*)-2,5-dihydroxycinnamic acid esters of arbutin and D-glucose, and two known compounds, robustaside A and (*E*)-2,5-dihydroxycinnamic acid. The structures of robustasides B and C were not arbutin caffeates, being revised to arbutin (*E*)-2,5-dihydroxycinnamic acid esters.

Key words *Grevillea robusta*; Proteaceae; robustaside B; robustaside C; (*E*)-2,5-dihydroxycinnamic acid; grevilloside

Grevillea robusta A. CUNN., which belongs to Proteaceae, originates from subtropical areas of eastern Australia and has been planted in Japan. It is an evergreen tree between 20–35 m in height with dark green delicately dented bipinnatifid leaves reminiscent of fronds. The leaves are 15–30 cm long with grey-white or rusty undersides. Phytochemical investigation of the same plant, collected in Egypt, has been reported and several phenolic glucosides were isolated.[1] Cytotoxic 5-alkylresorcinol metabolites were also isolated from the title plant[2] and a MeOH extract of its timber exhibited potent leishmanicidal activity.[3] In previous papers, the isolation of glucosides of 5-alkylresorcinol derivatives was reported.[4,5] Further phytochemical work resulted in the isolation of two known compounds, robustasides B (**1**) and C (**2**), and two new (*E*)-2,5-dihydroxycinnamic acid esters of arbutin and D-glucose (**3**, **4**) along with arbutin 6′-*O*-*p*-coumarate, robustaside A (**5**)[1] and (*E*)-2,5-dihydroxycinnamic acid (**6**).[6] The structures of robustasides B′ (**7**) and C′ (**8**) reported by Ahmed et *al.*[1] must be revised according to this investigation (Fig. 1).

Robustaside B (**1**), $[\alpha]_D^{26}$ −52.7, was isolated as an amorphous powder and its elemental composition was determined to be $C_{21}H_{22}O_{10}$ by high-resolution electrospray-ionization mass-spectrometry (HR-ESI-MS). The IR spectrum exhibited absorption bands ascribable to hydroxy (3363 cm^{-1}) and ester carbonyl (1697 cm^{-1}) groups, and aromatic rings (1629, 1509 cm^{-1}). In the ^{1}H-NMR spectrum, four protons coupled with an AA′BB′ system, three aromatic protons [δ_H 6.72 (2H) and 6.93], two olefinic protons with a *trans* geometry and an anomeric proton (δ_H 4.73) were observed, and D-glucose was identified by sugar analysis using a chiral detector. The ^{13}C-NMR spectral data as well as the ^{1}H-NMR data were the same as those reported for robustaside B′ (**7**), which was isolated from the same plant. In the heteronuclear multiple bond correlation (HMBC) spectrum, however, the H-7‴ proton [δ_H7.97 (d, J=16 Hz)] showed correlation peaks with δ_C 151.7 (C-2‴, s) and 114.9 (C-6‴, d) (Fig. 2a). Thus, the acyl moiety may not be a caffeate. In comparison of its ^{13}C-NMR resonances with those of methyl (*E*)-2,5-dihydroxycinnamate (**9**) and caffeate (**10**)[7] (Table 1), it is obvious that the acyl moiety is (*E*)-2,5-dihydroxycinnamate. Therefore, the structure of **1** was elucidated to be arbutin 6′-*O*-2,5-dihydroxycinnamic acid ester, as shown in Fig. 1, and the structure of robustaside B′ (**7**) was revised to **1**.

Robustaside C (**2**), $[\alpha]_D^{26}$ −75.7, was also isolated as an amorphous powder and its elemental composition was determined to be $C_{27}H_{32}O_{15}$ by HR-ESI-MS. The IR and UV spectra were similar to those of **1**, and both the ^{1}H- and ^{13}C-NMR spectra also showed good similarity, except for the

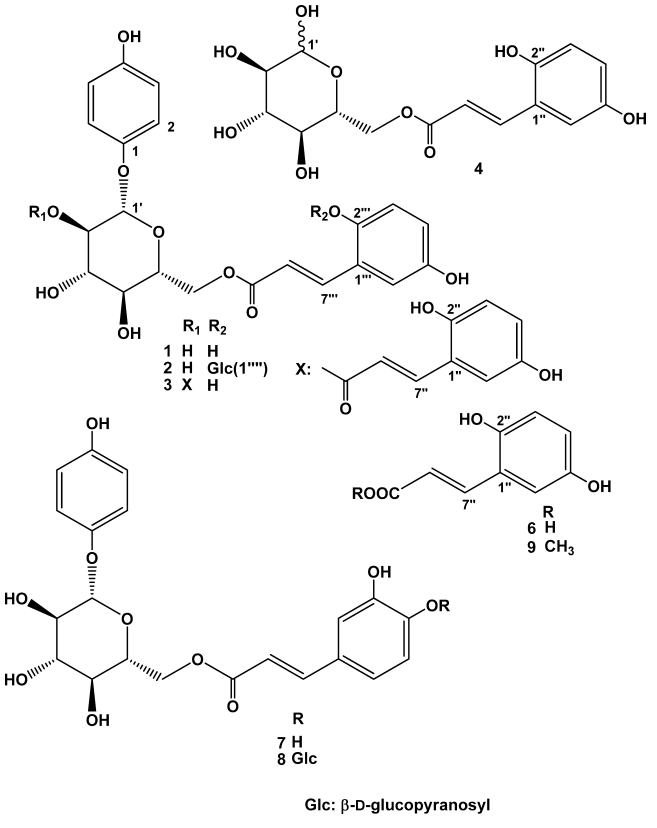

Fig. 1. Structures of Isolated and Reference Compounds

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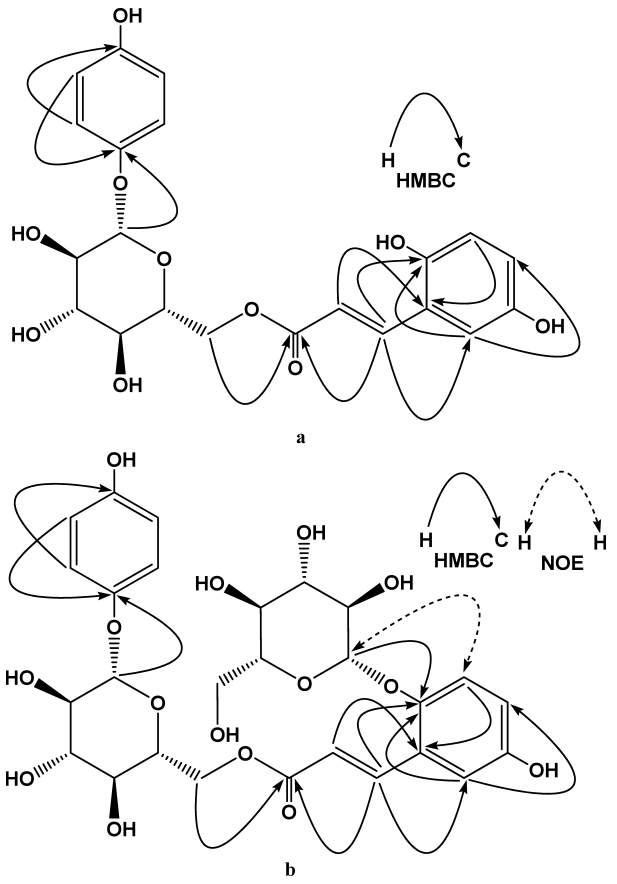

Fig. 2. Diagnostic HMBC Correlations for **1** (a) and **2** (b)
The dashed line curve in (b) shows the results of difference NOE experiments.

presence of terminal glucopyranose. Glucose was determined to be D-series by the same method as used for **1**. Since the ^{13}C-NMR resonances of the arbutin moiety were superimposable on those of **1**, the terminal β-D-glucopyranose must be attached to one of the phenolic hydroxy groups on the acyl group. In the HMBC spectrum, the anomeric proton (δ_H 4.79) showed a cross peak with δ_C 151.3 (C-2‴), and significantly different nuclear Overhauser effect (NOE) correlations were observed between the anomeric proton and the H-3‴ (δ_H 7.16) aromatic proton (Fig. 2b). Further HMBC correlations between H-7‴ (δ_H 141.9) and C-2‴ and C-6‴, and H-6‴ [δ_H 7.04 (d, J=3 Hz)] and C-2‴, and three aromatic protons of the acyl group were well resolved and clearly coupled in an ABX system. Therefore, the structure of **2** was elucidated to be 2″-*O*-β-D-glucopyranoside of robustaside B (**1**), as shown in Fig. 1. On the basis of the aforementioned spectroscopic data, the structure of robustaside C′ (**8**) must be revised to **2**.

Compound **3**, $[\alpha]_D^{22}$ −23.5, was isolated as an amorphous powder and its elemental composition was determined to be $C_{30}H_{28}O_{13}$ by HR-ESI-MS. The IR and UV spectral data were similar to those of **1** and **2**, and the NMR spectra also showed close resemblance to those of **1**. In the ^{13}C-NMR spectrum, signals (δ_C 168.6, 169.2) for two carbonyl carbons were observed and some of the sp^2 carbon signals assignable to the acyl moieties appeared at two close frequencies. In the ^{1}H-NMR spectrum, the integrals of the protons assignable to the acyl moieties also implied that two units of 2,5-dihydroxycinnate were present in **3**. One of the acyl moieties was linked to the hydroxy group at the 6′-position judging from the HMBC correlation between δ_H 4.60 and 4.41 on C-6′ and δ_C 169.2 (C-9‴), whereas the other carbonyl carbon (δ_C 168.6) showed a HMBC correlation with the proton shifted downfield at δ_H 5.08, which was assigned to H-2′ from the correlation with the anomeric proton (δ_H 4.97) in the ^{1}H–^{1}H correlation spectrum. Therefore, the structure of compound **3** was elucidated to be arbutin 2′,6′-di-*O*-(*E*)-2,5-dihydroxycinnamate, as shown in Fig. 1, and it was given the trivial name, grevilloside I.

Compound **4**, $[\alpha]_D^{28}$ +28.8, was isolated as an amorphous powder and its elemental composition was determined to be $C_{15}H_{18}O_9$. Although compound **4** gave distinct two peaks on HPLC separation, the NMR spectra for these two peaks were identical. Thus, the compounds isolated from these two peaks were interconvertible with each other and **4** must exist as an equilibrium mixture. On acid hydrolysis, **4** gave D-glucose as a sugar component and in the NMR spectra of **4**, two anomeric carbons (δ_C 94.8, 98.3) were observed with the respective anomeric protons [δ_H 5.12 (½H, d, J=4 Hz, H-1′α), and 4.53 (½H, d, J=8 Hz, H-1′β)]. The abundance ratio of α- and β-isomers was estimated to be nearly 1 : 1 from the integrals of isolated signals in the ^{1}H-NMR spectrum.

Experimental

General Experimental Procedures The general experimental procedures used in this study were the same as those used in a previous paper.[4] Methyl (*E*)-2,5-dihydroxycinnamate (**9**) was purchased from Sigma-Aldrich Co., LLC (St. Louis, MO, U.S.A.).

Plant Material The plant material was the same as that used in our previous experiment (accession No. 05-GR-Okinawa-0629).[4]

Extraction and Isolation Dried leaves of *G. robusta* (6.35 kg) were extracted three times with MeOH (30 L) at 25°C for one week and then concentrated to 3 L *in vacuo*. The extract was washed with *n*-hexane (3 L, 32.6 g) and then the MeOH layer was concentrated to a gummy mass. The latter was suspended in water (3 L) and then extracted with EtOAc (3 L) to give 160 g of an EtOAc-soluble fraction. The aqueous layer was extracted with 1-BuOH (3 L) to give a 1-BuOH-soluble fraction (405 g), and the remaining water-layer was concentrated to yield 475 g of a water-soluble fraction. A portion (237 g) of the 1-BuOH-soluble fraction was applied to a Diaion HP-20 column (Φ=75 mm, L=50 cm) using H_2O–MeOH (4 : 1, 8 L), (2 : 3, 8 L), (3 : 2, 8 L), and (1 : 4, 8 L), and MeOH (6 L), 1 L fractions being collected. The residue (19.9 g in fractions 4–6) of the 20% MeOH eluent was subjected to silica gel (450 g) CC, with elution with $CHCl_3$ (3 L) and $CHCl_3$–MeOH [(49 : 1, 3 L), (24 : 1, 3 L), (23 : 2, 3 L), (9 : 1, 3 L), (7 : 1, 3 L), (17 : 3, 3 L), (4 : 1, 3 L), (3 : 1, 3 L), and (3 : 2, 3 L)]. The fractions collected were 500 mL.

The residue (45.1 g) of fractions 7–12 obtained on Diaion HP-20 CC was subjected to silica gel (450 g) CC, with elution with $CHCl_3$ (4.5 L) and $CHCl_3$–MeOH [(49 : 1, 4.5 L), (24 : 1, 4.5 L), (23 : 2, 4.5 L), (9 : 1, 4.5 L), (7 : 1, 4.5 L), (17 : 3, 4.5 L), (4 : 1, 4.5 L), (3 : 1, 4.5 L), and (3 : 2, 4.5 L)]. The fractions collected were 500 mL. An aliquot (1.76 g) of combined fractions 61–66 (2.61 g) of the 12.5–15% MeOH eluate was separated by octadecyl silica (ODS) open CC to give two residues in fractions 88–99 (204 mg) and fractions 133–159 (435 mg).

Tab1e 1. ^{13}C-NMR Spectroscopic Data for Robustasides B (**1**) and C (**2**), Grevilloside I (**3**), Compound **4**, (*E*)-2,5-Dihydroxycinnamic Acid (**6**), Methyl (*E*)-2,5-Dihydroxycinnamate (**9**), and Caffeate (**10**)

C	**1**	**2**	**3**	**4**		**6**	**9**	**10**[f]
1	152.5	152.4	152.3					
2,6	119.7	119.8	119.9					
3,5	116.8	116.8	116.9					
4	153.9	154.0	154.1	*α*	*β*			
1′	103.9	103.9	102.4	94.0	98.3			
2′	75.0	75.0	75.2	73.8	76.3			
3′	78.0	78.2	76.1	75.5	78.0			
4′	71.9	71.4	72.0	72.1	71.8			
5′	75.6	75.5	75.7	70.9	74.8			
6′	64.8	64.8	64.7	64.9	65.0			
1″			122.97[a]	122.94	122.96	123.1	123.1	127.3
2″			151.6	151.6		151.4	151.6	115.3
3″			118.09[b]	118.1		118.5	118.0	149.8
4″			120.4	120.32	120.34	120.1	120.3	146.8
5″			151.35[c]	151.3		151.4	151.4	116.4
6″			114.86[d]	114.9		114.8	114.8	123.3
7″			142.6	142.46	142.5	142.3	142.3	148.2
8″			117.91[e]	117.93	117.99	118.0	117.9	114.7
9″			168.6	169.44	169.53	171.3	170.0	168.0
-OMe							52.0	
1‴	123.0	126.8	123.00[a]					
2‴	151.7	151.3	151.7					
3‴	118.1	120.09	118.11[b]					
4‴	120.4	120.06	120.4					
5‴	151.4	154.1	151.36[c]					
6‴	114.9	113.7	114.95[d]					
7‴	142.5	141.9	142.7					
8‴	117.9	118.7	117.99[e]					
9‴	169.3	169.1	169.2					
1⁗		104.0						
2⁗		75.5						
3⁗		78.2						
4⁗		71.7						
5⁗		78.0						
6⁗		62.7						

a–e) Maybe exchangeable. *f*) Data from ref. 5.

The former was separated by DCCC to give 35.6 mg of **6** in fractions 39–46. The latter was purified by DCCC to afford 227 mg of **1** in fractions 24–29. An aliquot (1.85 g) of combined fractions 67–73 (2.01 g) of the 12.5–15% MeOH eluate was separated by ODS open CC to give a residue in fractions 70–88 (273 mg), which was then separated by DCCC. The residue (130 mg) in fractions 12–18 was purified by HPLC (H_2O–MeOH, 4 : 1) to give two interconvertible peaks at 10 min and 13 min (a total of 9.2 mg of **4**). An aliquot (1.74 g) of combined fractions 67–73 (3.85 g) of the 12.5–15% MeOH eluate was separated by ODS open CC to give 25.3 mg of **2** in fractions 100–115.

The residue (39.0 g) of fractions 13–18 obtained on Diaion HP-20 CC was subjected to silica gel (400 g) CC, and eluted with $CHCl_3$ (4.5 L) and $CHCl_3$–MeOH [(49 : 1, 4.5 L), (24 : 1, 4.5 L), (23 : 2, 4.5 L), (9 : 1, 4.5 L), (7 : 1, 4.5 L), (17 : 3, 4.5 L), (4 : 1, 4.5 L), (3 : 1, 4.5 L), and (3 : 2, 4.5 L)]. Each fraction collected was 500 mL. An aliquot (1.90 g) of combined fractions 48–54 (4.97 g) of the 10% MeOH eluate was separated by ODS open CC and the residue (297 mg) in fractions 171–180 was purified by DCCC to give 19.5 mg of **5** in fractions 37–40. An aliquot (1.87 g) of combined fractions 55–62 (2.80 g) of the 12.5% MeOH eluate was separated by ODS open CC and the residue (167 mg) in fractions 129–138 by DCCC to give 23.9 mg of **1** in fractions 21–27. The residue (170 mg) in fractions 175–182 was separated by DCCC and the residue (31.6 mg) in fractions 28–35 was purified by HPLC (H_2O–MeOH, 3 : 2) to give 13.9 mg of **3** from the peak at 24 min.

Robustaside B (**1**): Amorphous powder. $[\alpha]_D^{26}$ −52.7 (c=0.96, MeOH). IR ν_{max} (film) cm^{-1}: 3363, 2953, 2837, 1697, 1629, 1509, 1455, 1341, 1267, 1211, 1074, 1028. UV λ_{max} (MeOH) nm (logε): 280 (4.15), 220 (4.18). ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.97 (1H, d, J=16 Hz, H-7‴), 6.96 (2H, d, J=9 Hz, H-2, 6), 6.93 (1H, br s, H-6‴), 6.72 (2H, m, H-3‴, 4‴), 6.67 (2H, d, J=9 Hz, H-3, 5), 6.54 (1H, d, J=16 Hz, H-8‴), 4.73 (1H, d, J=7 Hz, H-1′), 4.54 (1H, dd, J=12, 2 Hz, H-6′a), 4.36 (1H, dd, J=12, 6 Hz, H-6′b), 3.68–3.33 (4H, m, H-2′, 3′, 4′, 5′). ^{13}C-NMR (100 MHz, CD_3OD): Table 1. HR-ESI-MS (positive-ion mode) m/z: 457.1112 $[M+Na]^+$ (Calcd for $C_{21}H_{22}O_{10}Na$: 457.1105).

Robustaside C (**2**): Amorphous powder. $[\alpha]_D^{26}$ −75.5 (c=0.75, MeOH). IR ν_{max} (film) cm^{-1}: 3363, 2921, 1697, 1629, 1558, 1510, 1454, 1392, 1289, 1212, 1072. UV λ_{max} (MeOH) nm

(logε): 280 (4.20), 216 (4.27). ^{1}H-NMR (400 MHz, CD_3OD) δ: 8.20 (1H, d, J=16 Hz, H-7‴), 7.16 (1H, d, J=9 Hz, H-3‴), 7.04 (1H, d, J=3 Hz, H-6‴), 6.95 (2H, d, J=9 Hz, H-2, 6), 6.83 (1H, dd, J=9, 3 Hz, H-4″), 6.66 (2H, d, J=9 Hz, H-3, 5), 6.47 (1H, d, J=16 Hz, H-8‴), 4.79 (1H, m, H-1⁗), 4.75 (1H, m, H-1′), 4.53 (1H, dd, J=12, 2 Hz, H-6′a), 4.40 (1H, dd, J=12, 6 Hz, H-6′b), 3.87 (1H, dd, J=12, 2 Hz, H-6⁗a), 3.70 (1H, dd, J=12, 6 Hz, H-6⁗b), 3.73–3.35 (8H, m, H-2′, 3′, 4′, 5′, 2⁗, 3⁗, 4⁗, 5⁗). ^{13}C-NMR (100 MHz, CD_3OD): Table 1. HR-ESI-MS (positive-ion mode) m/z: 619.1636 $[M+Na]^+$ (Calcd for $C_{27}H_{32}O_{15}Na$: 619.1633).

Grevilloside I (**3**): Amorphous powder. $[\alpha]_D^{22}$ −23.5 (c=0.93, MeOH). IR ν_{max} (film) cm^{-1}: 3354, 2930, 2860, 1699, 1627, 1507, 1206, 1076. UV λ_{max} (MeOH) nm (logε): 359 (4.01), 279 (4.34), 213 (4.14). ^{1}H-NMR (400 MHz, CD_3OD) δ: 8.01 (2H, J=16 Hz, H-7″, 7‴), 6.94 (2H, brs, H-6″, 6‴), 6.87 (2H, d, J=9 Hz, H-2, 6), 6.73–6.71 (4H, m, H-3,″ 4″, 3‴, 4‴), 6.65 (2H, d, J=9 Hz, H-3, 5), 6.57 (2H, d, J=16 Hz, H-8″, 8‴), 5.08 (1H, dd, J=8, 8 Hz, H-2′), 4.97 (1H, d, J=8 Hz, H-1′), 4.60 (1H, dd, J=12, 2 Hz, H-6′a), 4.41 (1H, d, J=12, 6 Hz, H-6′b), 3.78–3.70 (2H, m, H-3′, 5′), 3.56 (1H, dd, J=8 Hz, H-4′). ^{13}C-NMR (100 MHz, CD_3OD): Table 1. HR-ESI-MS (positive-ion mode) m/z: 619.1425 $[M+Na]^+$ (Calcd for $C_{30}H_{28}O_{13}Na$: 619.1422).

Compound **4**: Amorphous powder, $[\alpha]_D^{28}$ +28.8 (c=0.91, MeOH). IR ν_{max} (film) cm^{-1}: 3388, 2924, 2860, 1698, 1630, 1504, 1457, 1188, 1028. UV λ_{max} (MeOH) nm (logε): 358 (3.68), 281 (3.86). ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.94 (1H, d, J=16 Hz, H-7″), 6.91 (1H, brs, H-6″), 6.72 (2H, m, H-3″, 4″), 6.52 (1H, d, J=16 Hz, H-8″), 5.12 (½H, d, J=4 Hz, H-1′α), 4.53 (½H, d, J=8 Hz, H-1′β), 4.52 (½H, dd, J=12, 2 Hz, H-6′aβ), 4.47 (½H, dd, J=12, 2 Hz, H-6′aα), 4.34 (½H, dd, J=12, 6 Hz, H-6′bβ), 4.31 (½H, dd, J=12, 6 Hz, H-6′bα), 4.05 (½H, ddd, J=9, 6, 2 Hz, H-5′α), 3.71 (½H, dd, J=9, 9 Hz, H-3′α), 3.56 (½H, dd, J=9, 3 Hz, H-2′α), 3.43–3.36 (2H, m, H-3′β, 4′α, 4′β, 5′β), 3.18 (½H, brdd, J=8, 8 Hz, H-2′β). ^{13}C-NMR (100 MHz, CD_3OD): Table 1. HR-ESI-MS (positive-ion mode) m/z: 369.0859 $[M+Na]^+$ (Calcd for $C_{15}H_{18}O_9Na$: 365.0843).

Sugar Analysis About 500 μg of each compound (**3**, **4**) was hydrolyzed with 1 M HCl (0.1 mL) at 90°C for 2 h. The reaction mixtures were partitioned with an equal amount of EtOAc (0.1 mL), and the water layers were analyzed with a chiral detector (JASCO OR-2090*plus*) on an amino column [Asahipak NH_2P-50 4E, Φ=4.6 mm, L=25 cm, CH_3CN–H_2O (3 : 1), 1 mL/min]. The hydrolyzates of **3** and **4** each gave a peak for D-glucose at 14.0 min with a positive optical rotation sign. The peaks were identified by co-chromatography with authentic D-glucose.

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