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NOTE

Aromatic constituents of *Cymbidium* Great Flower Marie Laurencin and their antioxidative activity

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Abstract Two novel aromatic glucosides, named marylaurecinosides B (**1**) and C (**2**), were isolated from *Cymbidium* Great Flower Marie Laurencin, together with six known aromatic compounds (**3**–**8**). These structures were determined on the basis of NMR experiments as well as chemical evidence. All of the isolated compounds (**1**–**8**) were tested for antioxidative activity using a superoxide dismutase-like assay.

Keywords *Cymbidium* Great Flower Marie Laurencin · Orchidaceae · Marylaurecinoside · 2-Benzyl-2-hydroxylsuccinic acid · Antioxidant activity · Superoxide dismutase

Introduction

The Orchidaceae family consists of more than 35,000 species of flowering plants in approximately 750 genera, which are widely distributed in temperate and tropical regions, except for the Antarctic. Since ancient times, the Orchidaceae have been used not only for ornamental purposes but also as medicinal plants, to treat paralysis, cholera, diarrhea, and sores [1]; therefore, the Orchidaceae can be said to be a prodigious source of potential new drugs. In the course of our study of bioactive substances from the Orchidaceae, we previously reported four new phenanthrene derivatives, ephemeranthoquinone B, marylaurencinols A and B, and marylaurencinoside A, together with six known phenanthrenes and their antibacterial and cytotoxic activities, isolated from the fresh roots of the well-known cultivated cymbidium, *Cymbidium* Great Flower Marie Laurencin [2]. In this paper we made a chemical study of the floral stem of *C*. Great Flower Marie Laurencin. Two new phenolic glucosides, marylaurecinosides B (**1**) and C (**2**) were isolated along with six known aromatic compounds (**3**–**8**). We describe here the isolation, purification, and structural elucidation of **1** and **2** determined primarily by extensive NMR experiments, and the antioxidant activity of all the isolated compounds using a superoxide dismutase (SOD)-like assay[1].

Results and discussion

Fresh floral stems were completely extracted with MeOH, and this extract was partitioned to give three soluble fractions in EtOAc, *n*-BuOH, and H_2O. The EtOAc-soluble portion was separated by ordinary-phase and reverse-phase silica gels to furnish two novel glucosides, marylaurecinosides B (**1**) and C (**2**), along with six known compounds, (2*R*)-2-benzyl-2-hydroxysuccinic acid (**3**) [3], 4-hydroxybenzoic alcohol (**4**) [4], protocatechualdehyde (**5**) [5], 4-hydroxybenzoic acid (**6**) [6], vanillic acid (**7**) [7], and sakakin (**8**) [8] (Fig. 1).

Marylaurecinoside B (**1**) was obtained as an amorphous powder. Negative-ion high-resolution (HR)-FAB-MS of **1** gave the molecular formula $C_{26}H_{29}O_{12}$ with a $[M - H]^-$

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peak at *m/z* 533.2006, indicating twelve unsaturations. The IR spectrum of **1** suggested the presence of hydroxyl (3375, 1070 cm^{-1}), carbonyl (1730 cm^{-1}), and aromatic ring groups (1613 cm^{-1}). The ^{1}H NMR spectrum of **1** showed AB-type aromatic protons at δ 7.05 (2H, d, $J = 8.8$ Hz) and δ 7.27 (2H, d, $J = 8.8$ Hz), five overlapped aromatic protons at δ 7.06 (2H, m) and δ 7.17 (3H, m), three sets of isolated methylene protons at δ 2.59, 2.96 (each 1H d, $J = 16.2$ Hz), δ 2.92, 2.98 (each 1H d, $J = 13.5$ Hz), and δ 5.04, 5.07 (each 1H d, $J = 11.9$ Hz), and one anomeric proton at δ 4.91 (d, $J = 7.7$ Hz), together with one acetyl methyl signal at δ 2.02 (3H, s). The ^{13}C NMR spectrum, in combination with HMQC data, showed 26 carbon resonances, of which 20 were assignable to two aromatic rings, with a hexose and acetyl group. The other six carbon resonances could be assigned to two carboxyl carbons at δ 174.0 and 175.5, a quaternary carbon at δ 77.1, an oxymethylene carbon at δ 67.9, and two methylene carbons at δ 44.1 and 46.3. The COSY correlation of **1** revealed the presence of phenyl, benzyloxy, and β-glucopyranosyl moieties (Fig. 2). Acid hydrolysis of **1** liberated D-glucose, confirmed by optical rotation using chiral detection in HPLC analysis (see "Experimental" section) [9]. The HMBC correlations from H_2-7 (δ 5.07, 5.04) to C-3 (δ 131.5), C-4 (δ 131.0), C-5 (δ 131.5), C-8 (δ 175.5), from H_2-10 (δ 2.59, 2.96) to C-9 (δ 77.1), and C-11 (δ 174.0), from H_2-12 (δ 2.92, 2.98) to C-8 (δ 175.5), C-9 (δ 77.1), C-13 (δ 136.6), C-14, and C-18 (δ 131.5) revealed that the 4-hydroxybenzyloxy and 2-benzyl-2-hydroxysuccinyl moieties were connected between the C-7 and C-8 positions in each unit. Furthermore, the HMBC correlation between C-1 (δ_C 159.0) and H-1′ (δ_H 4.91) indicated that the β-glucopyranosyl moiety combined with C-1 (Fig. 2).

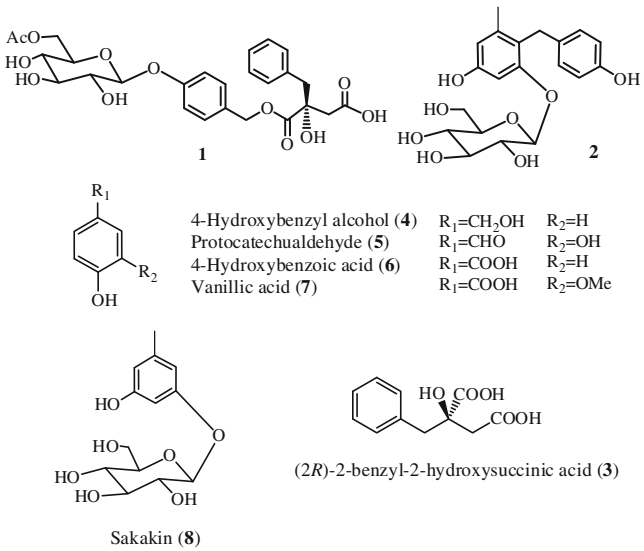

Fig. 1 Chemical structures of compounds **1–8**

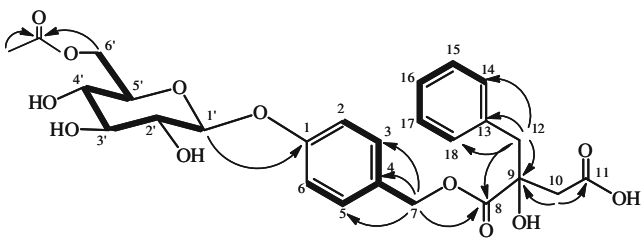

Fig. 2 COSY (*thick lines*) and HMBC (*curved arrows*) correlations for **1**

The remaining location of the acetyl moiety was determined at H_2-6 of glucose by their acylation shifts at δ 4.23 (dd, $J = 12.0$, 6.6 Hz) and δ 4.40 (dd, $J = 12.0$, 2.2 Hz), and HMBC correlations, as shown in Fig. 2. On alkaline hydrolysis, **1** afforded 2-benzyl-2-hydroxysuccinic acid (**9**), and optical roration showed $[\alpha]_D^{25}$ −20.5° ($c = 0.3$, MeOH), suggesting an *R* configuration [3]. Thus, from the above findings, the structure of **1** was formulated as shown.

Marylaurenoside C (**2**) was obtained as an amorphous powder and showed a $[M + Na]^+$ peak at *m/z* 415.1389 in HR-FAB-MS, which corresponded to the molecular formula $C_{20}H_{24}O_8$. The IR spectrum of **2** showed absorptions at 3375, 1613 and 1070 cm^{-1}. The COSY and HMQC spectra showed the presence of olefinic methyl, 4-monosubstituted benzyl, 2,4,6-tri-substituted phenyl, and β-glucopyranosyl groups (Fig. 3). On acid hydrolysis, **2** liberated D-glucose, identified by optical rotation using chiral detection in HPLC analysis [9]. The gross structure of **2** was determined by the same strategy as **1**. In the HMBC data, the connectivity from H_2-7 (δ 3.82, 4.01) to C-3 (δ 157.9), C-4 (δ 112.2), C-5 (δ 140.1), C-8 (δ 133.7), C-9 (δ 130.2), from H-9 (δ 6.93) and H-10 (δ 6.61) to C-11 (δ 156.0), from H_3-14 (δ 2.10) to C-4, C-5, and C-6 (δ 121.6), and from H-1′ (δ 4.82) to C-3 revealed that 4-(4-hydroxybenzyl)-5-methylbenzene-1,3-diol, in which β-glucopyranosyl was attached at C-3 (Fig. 3). The following NOEs between H_3-14/H-6 (δ 6.31), /H_2-7, and H-2 (δ 6.55) /H-1′ confirmed the substituent positions in the tetra-substituted aromatic ring. Thus, from the above findings, the structure of **2** was formulated as shown.

To our knowledge, compound **3**, which was identified as (2*R*)-2-benzyl-2-hydroxysuccinic acid by optical rotation $[\alpha]_D^{25}$ −14.4° ($c = 0.6$, MeOH), is here isolated for the first time as a natural product.

The antioxidant activities of **1–8** were studied with a SOD assay kit. Vitamin C was used as a positive control (IC_{50} 66.2 μM). Compounds **4** and **5** exhibited marked SOD-like activity (IC_{50} 24.2 and 11.9 μM, respectively).

In conclusion, we identified eight compounds, including two new phenolic glucosides, from *C.* Great Flower Marie

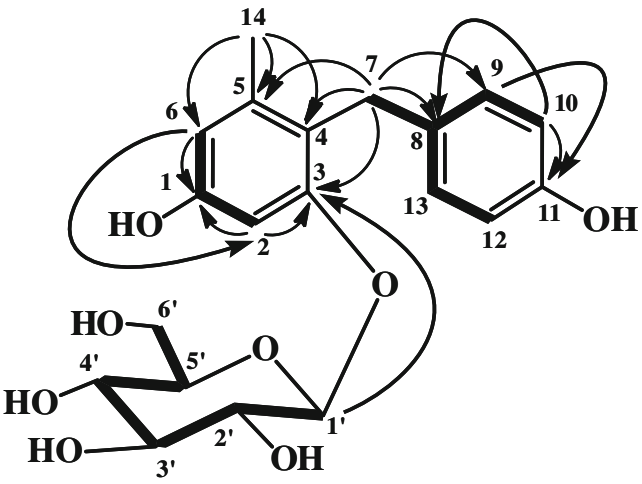

Fig. 3 COSY (*thick lines*) and HMBC (*curved arrows*) correlations for **2**

Laurencin. The major antioxidative compounds were identified as 4-hydroxybenzoic alcohol (**4**), and protocatechualdehyde (**5**). These results suggested that the antioxidative activities of **1**–**8** were partly attributed to the catechols, or the ratio of the phenols in the molecular compounds. The goal of this study is to identify functional ingredients for the development and utilization of health-care drugs.The study of the active ingredient is therefore in progress.

Experimental

General

Optical rotation was performed on a JASCO P-1030 digital polarimeter. The UV spectra were recorded using a Shimadzu UV-6000 spectrophotometer. IR spectra were measured on a Shimadzu FT/IR-8400S instrument. NMR spectra were recorded on a Varian UNITY 600 spectrometer. The chemical shifts are given as δ (ppm) in CD_3OD solution, using tetramethylsilane (TMS) as the internal standard. NMR experiments included COSY, HMQC, HMBC, and ROESY. Coupling constants (*J* values) are given in Hz. HR-FAB-MS was measured on a JEOL JMS-700 MStation. For HPLC column chromatography, COSMOSIL 5C18-AR-II (Nacalai Tesque, Inc., Kyoto, Japan, 20 mm i.d. × 250 mm) was used. TLC was performed on pre-coated silica gel $60F_{254}$ (Merck). Spots were detected by examining plates sprayed with *p*-anisaldehyde/H_2SO_4/MeOH reagent followed by heating on a hot plate.

Plant material

Fresh floral stems of *Cymbidium* Great Flower Marie Laurencin (Ministry of Agriculture, Forestry and Fisheries of Japan, seed registration No. 2841) were cultivated and harvested in February 2008 at Kawano Mericlone Co., Ltd. (Tokushima Prefecture, Japan), and identified by one of the authors (S.K.). A voucher specimen (TB 5430) has been deposited in the Herbarium of the Department of Pharmacognosy, Tokushima Bunri University, Tokushima, Japan.

Extraction and isolation

Fresh floral stems of *C*. Great Flower Marie Laurencin (1.4 kg) were completely extracted with MeOH at room temperature for 3 weeks. The methanolic extract was partitioned between EtOAc and H_2O. The EtOAc-soluble portion (21 g) was subjected to silica gel column chromatography with hexane–EtOAc–MeOH (10:1:0 → 0:1:10) to afford seven fractions (frs. 1–7). Fraction 2 (4.2 g) was passed through silica gel with hexane–EtOAc (9:1) and purified by preparative HPLC (ODS, 95 % MeOH) to afford 4-hydroxybenzyl alcohol (**4**, 32.6 mg). Fraction 3 (0.62 g) was passed through silica gel with hexane–EtOAc (1:1) and purified by preparative HPLC (ODS, 50 % MeOH) to yield protocatechualdehyde (**5**, 5.2 mg), 4-hydroxybenzoic acid (**6**, 8.1 mg), and vanillic acid (**7**, 2.7 mg). Fraction 6 (1.9 g) was purified by preparative HPLC (ODS, 40–50 % MeOH) to afford marylaurecinosides B (**1**, 15.8 mg), C (**2**, 7.7 mg), (2*R*)-2-benzyl-2-hydroxysuccinic acid (**3**, 33.6 mg), and sakakin (**8**, 6.4 mg).

Marylaurecinoside B (**1**)

An amorphous powder; $[\alpha]_D^{25}$ −40.3° (c = 1.6, MeOH); FT-IR (dry film) cm^{-1}: 3375 (OH), 1730 (C=O), 1613 (C=C), 1070 (OH). UV λmax (MeOH) nm (log ε): 215 (4.13), 269 (2.83); ^1H NMR and ^{13}C NMR data see Table 1; HR-FAB-MS *m/z* 533.2006 (calcd for $C_{26}H_{29}O_{12}$: 533.2001)

Marylaurecinoside C (**2**)

An amorphous powder; $[\alpha]_D^{25}$ −34.7° (c = 0.8, MeOH); FT-IR (dry film) cm^{-1}: 3375 (OH), 1613 (C=C), 1070 (OH).UV λmax (MeOH) nm (log ε): 272 (3.37), 290 (3.12); ^1H NMR and ^{13}C NMR data see Table 1; HR-FAB-MS *m/z* 415.1389 (calcd for $C_{20}H_{24}O_8Na$: 415.1369).

Acid hydrolysis of compounds **1** and **2**

Each sample (1 mg) in 5 % H_2SO_4-dioxane (1:1) was heated at 100 °C for 2 h. The reaction mixture was diluted with H_2O and then neutralized with Amberlite IRA-35 and evaporated in vacuo to dryness. The identification and the D or L configuration of glucose was determined by using RI detection

Table 1 ^{1}H and ^{13}C NMR spectral data for **1** and **2** in CD_3OD

Position	Marylaurenoside B (**1**)		Position	Marylaurenoside C (**2**)	
	δ_C	δ_H (*J* Hz)		δ_C	δ_H (*J* Hz)
1	159.0		1	157.1	
2/6	117.7	7.05 (d, 8.8)	2	101.8	6.55 (d, 2.3)
3/5	131.3	7.27 (d, 8.8)	3	157.9	
4	131.0		4	112.2	
7	67.9	5.04 (d, 11.9)	5	140.1	
		5.07 (d, 11.9)	6	121.6	6.31 (d, 2.2)
8	175.5		7	31.2	3.82 (d, 15.4)
9	77.1				4.01 (d, 15.4)
10	44.1	2.59 (d, 16.2)	8	133.7	
		2.96 (d, 16.2)	9	130.2	6.93 (d, 8.8)
11	174.0		10	115.8	6.61 (d, 8.8)
12	46.3	2.92 (d, 13.5)	11	156.0	
		2.98 (d, 13.5)	12	115.8	6.61 (d, 8.8)
13	136.6		13	130.2	6.93 (d, 8.8)
14/18	131.5	7.06 (m)	14	20.1	2.10 (s)
15/17	129.1	7.17 (m)			
16	127.9	7.17 (m)			
1′	102.1	4.91 (d, 7.7)	1′	103.0	4.82 (d, 7.7)
2′	74.8	3.46 (m)	2′	75.0	3.41 (m)
3′	77.8	3.47 (m)	3′	78.1	3.41 (m)
4′	71.6	3.39 (m)	4′	71.3	3.39 (m)
5′	75.3	3.65 (m)	5′	78.2	3.40 (m)
6′	64.7	4.23 (dd, 12.0, 6.6)	6′	62.6	3.71 (dd, 11.8, 4.7)
		4.40 (dd, 12.0, 2.2)			3.87 (dd, 11.8, 1.5)
Ac	172.8	2.02 (s)			
	20.8				

(Shimadzu RID-10A) and chiral detection (Shodex OR-1) by HPLC (Shodex RSpak NH_2P-50 4D, CH_3CN–H_2O–H_3PO_4, 95:5:1, 1 mL/min, 47 °C), by comparison with an authentic sugar (10 mmol D-glc). The sugar portion gave the following peak of D-(+)-glucose at 20.6 min.

Alkaline hydrolysis of compound **1**

Compound **1** (10 mg) in MeOH (1 mL) was treated with 5 % KOH (1 mL) and heated at 90 °C for 2 h. The reaction mixture was adjusted to pH 4.0 with 5 % HCl and extracted with EtOAc. The EtOAc layer was subjected to silica gel column chromatography, eluting with hexane–EtOAc (4:6) to afford (2*R*)-2-benzyl-2-hydroxysuccinic acid (**9**, 3 mg). Compound **9**: amorphous solid; $[\alpha]_D^{25}$ −20.5° ($c = 0.3$, MeOH); FAB-MS *m/z* 223 $[M - H]^-$.

Superoxide dismutase-like activity assay

SOD-like activity was determined according to the method of Ukeda [10] using a SOD Assay Kit-WST (Dojindo Lab., Kumamoto, Japan). A test sample was dissolved in DMSO to give a final DMSO concentration of 0.8 % (v/v).

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