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NOTE

Two novel aromatic glucosides, marylaurencinosides D and E, from the fresh flowers of *Cymbidium* Great Flower 'Marylaurencin'

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Abstract Two novel aromatic glucosides, named marylaurencinosides D (**1**) and E (**2**), were isolated from the fresh flowers of *Cymbidium* Great Flower 'Marylaurencin'. In addition, eight known aromatic compounds (**3**–**10**) were isolated. These structures were determined on the basis of NMR experiments as well as chemical evidence.

Keywords *Cymbidium* Great Flower 'Marylaurencin' · Orchidaceae · Marylaurencinoside · (*R*)-2-benzylmalic acid · (*R*)-eucomic acid

Introduction

The Orchidaceae family consists of more than 35,000 species, found in 820 genera, of flowering plants, which are widely distributed in temperate and tropical regions. However, only a few have been investigated for their chemical composition and biological activities [1]. 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 from the fresh root, and marylaurencinosides B and C, along with six known aromatic compounds and their antioxidant activity, from the fresh floral stems of the well-known cultivated cymbidium, *Cymbidium* Great Flower 'Marylaurencin' [2, 3]. In our continuing search for bioactive substances from the Orchidaceae, we became interested in the chemical study of the fresh flowers of this plant. Fresh flowers were completely extracted with MeOH. This extract was partitioned to give three soluble fractions in EtOAc, *n*-BuOH, and H_2O. The *n*-BuOH-soluble portion was separated by ordinary-phase and reversed-phase silica gels to furnish two novel aromatic glucosides, marylaurencinosides D (**1**) and E (**2**), along with eight known compounds, grammatophylloside B (**3**) [4], cronupapine (**4**) [5], 2-*O*-(*trans*-coumaroyl) malic acid (**5**) [6], coniferoside (**6**) [7], sinapic acid 4-*O*-glucoside (**7**) [8], sakakin (**8**) [9], arbutin (**9**) [10], and gastrodin (**10**) [11] (Fig. 1). This paper describes the isolation, purification, and structural elucidation of **1**–**10** determined primarily by extensive NMR experiments and chemical degradation.

Results and discussion

Marylaurencinoside D (**1**) was obtained as an amorphous powder. HR-FAB-MS of **1** gave molecular formula $C_{25}H_{30}O_{12}$ with a $[M + Na]^+$ peak at *m/z* 545.1639, indicating 11 unsaturations. The IR spectrum of **1** suggested the presence of hydroxyl (3370 cm^{-1}), carbonyl (1720 cm^{-1}), and aromatic ring (1600 cm^{-1}) groups. The ^{1}H-NMR spectrum of **1** showed 1,3,4-trisubstituted benzene ring protons at δ 7.02 (dd, $J = 8.3$, 2.0 Hz), 7.15 (d, $J = 2.0$ Hz), and 7.52 (d, $J = 8.3$ Hz), one phenyl group proton at δ 7.25 (1H, dd, $J = 8.0$, 8.0 Hz), 7.30 (2H, dd, $J = 8.0$, 8.0 Hz), and 7.51 (2H, d, $J = 8.0$ Hz), three sets of isolated methylene protons at δ 3.16, 3.48 (each 1H d, $J = 15.8$ Hz), 3.35, 3.41 (each 1H d, $J = 13.4$ Hz), and 5.33, 5.37 (each 1H d, $J = 12.1$ Hz), and one methoxyl

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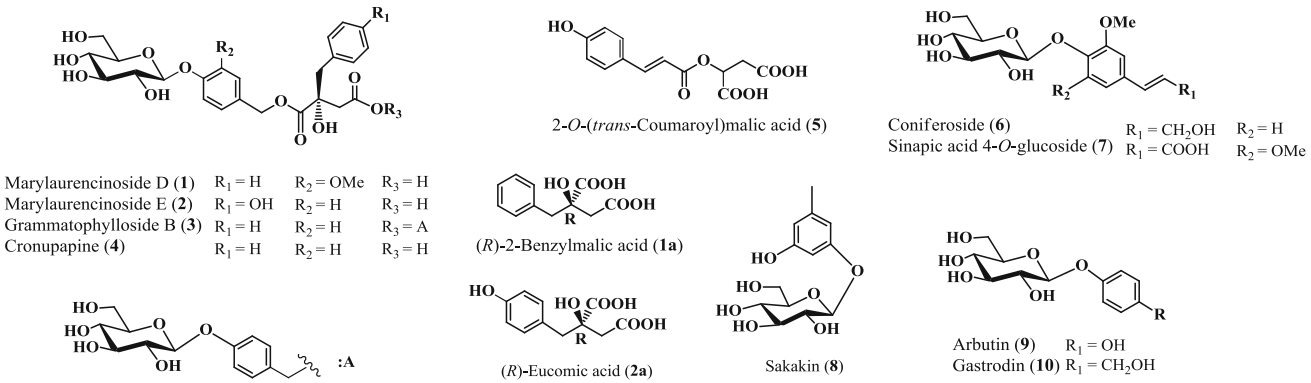

Fig. 1 Chemical structures of compounds **1**–**10**, **1a**, and **2a**

Table 1 ^{1}H- and ^{13}C-NMR spectral data for **1** and **2** (600 MHz and 150 MHz, in C_5D_5N)

No.	**1**		**2**	
	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)
1	175.2		175.4	
2	76.8		77.1	
3	44.5	3.16 (d, 15.8)	44.5	3.18 (d, 15.8)
		3.48 (d, 15.8)		3.48 (d, 15.8)
4	173.7		173.9	
5	45.9	3.35 (d, 13.4)	45.3	3.31 (d, 13.6)
		3.41 (d, 13.4)		3.37 (d, 13.6)
6	136.9		127.0	
7/11	131.2	7.51 (d, 8.0)	132.4	7.42 (d, 8.8)
8/10	128.4	7.30 (dd, 8.0, 8.0)	115.9	7.13 (d, 8.8)
9	127.1	7.25 (dd, 8.0, 8.0)	157.9	
1′	130.5		130.0	
2′	113.5	7.15 (d, 2.0)	130.5	7.40 (d, 8.8)
3′	149.8		116.8	7.31 (d, 8.8)
4′	147.9		158.5	
5′	116.0	7.52 (d, 8.3)	116.8	7.31 (d, 8.8)
6′	121.9	7.02 (dd, 8.3, 2.0)	130.5	7.40 (d, 8.8)
7′	67.2	5.33 (d, 12.1)	66.9	5.33 (d, 12.1)
		5.37 (d, 12.1)		5.35 (d, 12.1)
Glc-1″	102.1	5.67 (d, 7.4)	102.0	5.61 (d, 7.7)
2″	74.8	4.34 (dd, 8.8, 7.4)	74.9	4.31 (dd, 8.8, 7.7)
3″	78.5	4.36 (dd, 8.8, 8.8)	78.5	4.37 (dd, 8.8, 8.8)
4″	71.2	4.36 (dd, 8.8, 8.8)	71.2	4.35 (dd, 8.8, 8.5)
5″	78.9	4.11 (ddd, 8.8, 5.1, 2.5)	78.9	4.11 (ddd, 8.5, 5.2, 2.3)
6″	62.3	4.40 (dd, 12.0, 5.1)	62.3	4.40 (dd, 12.1, 5.2)
		4.52 (dd, 12.0, 2.5)		4.54 (dd, 12.1, 2.3)
OMe	55.8	3.66 (s)		

signal at δ 3.66 (3H, s), together with one anomeric proton at δ 5.67 (d, J = 7.4 Hz) (Table 1). The ^{13}C-NMR spectrum, in combination with HMQC data, showed 25 carbon resonances, of which 19 were assignable to two benzene

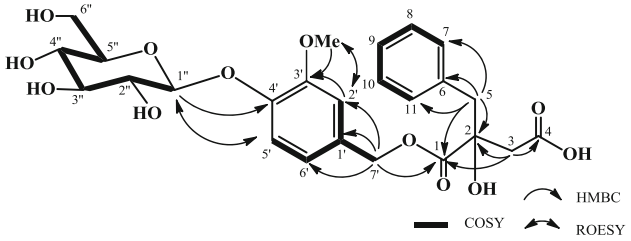

Fig. 2 COSY, selected HMBC, and ROESY correlations of **1**

rings, two carboxyl carbons at δ 173.7 and 175.2, a quaternary carbon at δ 76.8, an oxymethylene carbon at δ 67.2, two methylene carbons at δ 44.5 and 45.9, and a methoxyl group at δ 55.8. The remaining six carbon resonances at δ 102.1, 78.9, 78.5, 74.8, 71.2, and 62.3 could be assigned to a hexose sugar moiety. The COSY correlations of **1** revealed the presence of phenyl, 3,4-disubstituted benzyl, and β-glucopyranosyl moieties (Fig. 2). Acid hydrolysis of **1** liberated D-glucose, identified by optical rotation using chiral detection in HPLC analysis (see Experimental section) [12]. The HMBC correlations from three sets of isolated methylene to carbons, H_2-3 (δ 3.16, 3.48)/C-1 (δ 175.2),/C-2 (δ 76.8), and/C-4 (δ 173.7), H_2-5 (δ 3.35, 3.41)/C-1,/C-2,/C-6 (δ 136.9),/C-7 (δ 131.2), and/C-11 (δ 131.2), H_2-7′ (δ 5.33, 5.37)/C-1,/C-1′ (δ 130.5),/C-2′ (δ 113.5), and/C-6′ (δ 121.9), revealed that the C-1 position of 2-benzylmaloyl moiety was connected through an ester bond at the C-7′ position of 3-methoxy-4-hydroxybenzyl alcohol unit (Fig. 2). Furthermore, the HMBC correlation from H-1″ (δ 5.67) to C-4′ (δ 147.9) obviously indicated that the glucopyranosyl moiety combined with the C-4′ position. The following NOEs between H-2′ (δ 7.15)/OMe (δ 3.66) and H-5′ (δ 7.52)/H-1″ confirmed the substituent positions of the methoxy group and the glucopyranosyl linkage. On alkaline hydrolysis, **1** afforded 2-benzylmalic acid (**1a**), whose optical rotation showed $[\alpha]_D^{25}$ –17.4 (c 0.2, MeOH), suggesting an R configuration [13]. Thus, from the above findings, the structure of **1** was formulated as shown in Fig. 1.

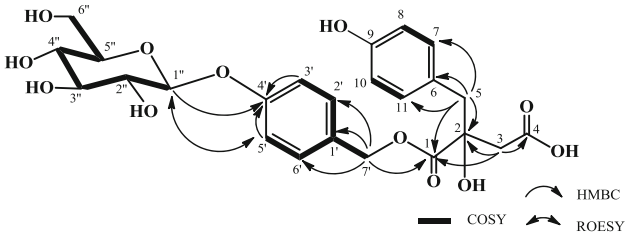

Fig. 3 COSY and selected HMBC correlations of **2**

Marylaurencinoside E (**2**) was obtained as an amorphous powder and showed an $[M + Na]^+$ peak at *m/z* 531.1455 in HR-FAB-MS, which corresponded to the molecular formula $C_{24}H_{28}O_{12}$. The IR spectrum of **2** showed absorptions at 3327, 1720, and 1590 cm^{-1}. The COSY and HMQC spectra showed the presence of a *para*-substituted phenyl, *para*-substituted benzyl, two sets of isolated methylene, and β-glucopyranosyl moieties (Fig. 3). On acid hydrolysis, **2** liberated D-glucose identified by optical rotation using chiral detection in HPLC analysis. The gross structure of **2** was determined by the same strategy as **1**. In the HMBC data, the correlations of H_2-3/C-1,/C-2, and/C-4, of H_2-5/C-1,/C-2,/C-6,/C-7, and/C-11, of H_2-7′/C-1,/C-1′,/C-2′, and/C-6′ revealed that eucomic acid [14] and 4-hydroxybenzyl alcohol were connected through an ester bond at C-1 and C-7′ in each unit, in which β-glucopyranosyl moiety was also attached at C-4′ (Fig. 3). On alkaline hydrolysis, **2** afforded eucomic acid (**2a**), $[\alpha]_D^{25}$ –10.9 (*c* 0.3, MeOH), suggesting an *R* configuration [14]. Thus, from the above findings, the structure of **2** was formulated as shown in Fig. 1.

The isolated compound (**8**) and its aglycone, orcinol (**8a**), were tested for antimicrobial activity against *Bacillus subtilis* and *Klebsiella pneumonia*. However, neither was active against the two strains.

The present study reported four glucosyloxybenzyl derivatives of (*R*)-2-benzylmalic acid (**1**, **3**, and **4**) and of (*R*)-eucomic acid (**2**), which are very specific types of compounds obtained from the orchid family [3–5]. The main skeletons of **1**–**4** seem to be formed from one molecule each of phenylpyruvic acid and acetate unit. These specific compounds provided further confirmation of the typical profile of secondary metabolites found in this family, and might be useful for further biological studies.

Experimental

General experimental procedures

NMR spectra were recorded on a Varian UNITY 600 spectrometer. The chemical shifts are given as δ (ppm) in C_5D_5N 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, Develosil RPAQUEOUS-AR-5 (Nomura Chemical Co., Ltd., 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 flowers of *Cymbidium* Great Flower 'Marylaurencin' (Ministry of Agriculture, Forestry and Fisheries of Japan, seed registration No. 2841) were cultivated and harvested in February 2012 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 flowers of *Cymbidium* Great Flower 'Marylaurencin' (10 kg) were completely extracted with MeOH at room temperature for 2 weeks. The methanolic extract was partitioned between EtOAc, *n*-BuOH, and H_2O. The *n*-BuOH-soluble portion (88 g) was subjected to silica gel column chromatography with $CHCl_3$–MeOH–H_2O (25:6:0.1 → 6:4:1) to afford eight fractions (frs. 1–8). Fraction 2 (1.2 g) was passed through Sephadex LH-20 with MeOH and purified by preparative HPLC (ODS, 30–60 % MeOH) to afford marylaurencinoside D (**1**, 15.8 mg), and 2-*O*-(*trans*-coumaroyl) malic acid (**5**, 10 mg). Fraction 3 (9.23 g) was passed through silica gel with $CHCl_3$–MeOH–H_2O (65:30:5) and purified by preparative HPLC (ODS, 20–50 % MeOH) to afford cronupapine (**4**, 350 mg), coniferoside (**6**, 7.6 mg), sinapic acid 4-*O*-glucoside (**7**, 6.6 mg), and sakakin (**8**, 25.4 mg). Fraction 4 (17.25 g) was passed through Sephadex LH-20 to afford six subfractions (frs. 4-1–4-6). Fraction 4-3 was purified by preparative HPLC (ODS, 20–50 % MeOH) to yield grammatophylloside B (**3**, 14.7 mg), arbutin (**9**, 35.6 mg), and gastrodin (**10**, 145.4 mg). Fraction 7 (2.43 g) was purified by preparative HPLC (ODS, 40 % MeOH) to afford marylaurencinoside E (**2**, 70.8 mg).

Marylaurencinoside D (**1**)

An amorphous powder; $[\alpha]_D^{25}$ –45.8 (*c* 0.7, MeOH); FT-IR (dry film) λ_{max} 3370 (OH), 1720 (C=O), 1600 (C=C) cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 218 (3.90), 255 (3.21), 261 (3.21). ^{1}H-NMR (600 MHz, C_5D_5N) and ^{13}C-NMR

(150 MHz, C_5D_5N) data see Table 1; HRMS-FAB *m/z* 545.1639 $[M + Na]^+$ (calcd for $C_{25}H_{30}O_{12}Na$: 545.1635).

Marylaurencinoside E (**2**)

An amorphous powder; $[\alpha]_D^{25}$ –29.0 (*c* 3.7, MeOH); FT-IR (dry film) λ_{max} 3327 (OH), 1720 (C=O), 1590 (C=C) cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 225 (3.91), 256 (3.38), 278 (3.33). ^{1}H-NMR (600 MHz, C_5D_5N) and ^{13}C-NMR (150 MHz, C_5D_5N) data see Table 1; HRMS-FAB *m/z* 531.1455 $[M + Na]^+$ (calcd for $C_{24}H_{28}O_{12}$ Na: 531.1478).

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 (Shimadzu RID-10A) and chiral detection (ShodeOR-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 mM of D-glc). The sugar portion gave the following peak of D-(+)-glucose at 20.6 min.

Alkaline hydrolysis of compounds **1** and **2**

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 (*R*)-2-benzylmalic acid (**1a**, 2 mg). Compound **1a**: amorphous solid; $[\alpha]_D^{25}$ –17.4 (*c* 0.2, MeOH); FAB-MS *m/z* 223 $[M–H]^-$. Compound **2** (10 mg) was treated in the same way as described for **1** to give (*R*)-eucomic acid (**2a**, 3 mg). Compound **2a**: amorphous solid; $[\alpha]_D^{25}$ –10.9 (*c* 0.3, MeOH); FAB-MS *m/z* 239 $[M–H]^-$.

Antimicrobial assay

Bacillus subtilis NBRC 3134 and *Klebsiella pneumoniae* NBRC 3512 were used for testing antimicrobial activity. These strains were tested by using microdilution assays. Bacterial strains were inoculated on YP agar plates [0.5 % peptone (Becton, Dickinson and Co., USA), 0.3 % yeast extract (Becton, Dickinson and Co.), 0.1 % $MgSO_4 \cdot 7H_2O$ and 2 % agar (Nacalai Tesque, Japan)] and incubated at 37 °C (*B. subtilis*) and 27 °C (*K. pneumoniae*) for 12 h. A stock solution of samples was prepared at 10 mg/mL in DMSO and further diluted to varying concentrations in 96-well plates which contained microbial strains incubated in YP medium. Each plate was further incubated at 37 °C (*B. subtilis*) and 27 °C (*K. pneumoniae*) overnight and ampicillin (Nacalai Tesque) was used as a reference reagent for the bacterial strains.

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