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Eucalmaidials A and B, phloroglucinol-coupled sesquiterpenoids from the juvenile leaves of *Eucalyptus maideni*†

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Two new phloroglucinol-coupled sesquiterpenoids, eucalmaidials A and B (**1** and **2**), were isolated from the juvenile leaves of *Eucalyptus maideni*, along with eight known macrocarpals (**3–10**), eucalyptone (**11**), and three known triterpenoids (**12–14**). Eucalmaidials A and B represent a new skeleton of phloroglucinol-coupled iphionane. Their structures were elucidated by extensive NMR spectroscopic analysis and theoretical calculation of the ^{13}C NMR chemical shifts. The biosynthetic pathway of **1** and **2** was also postulated. Compounds **1**, **3**, **5**, **7**, **8**, and **10–14** were evaluated for their antifungal and antibacterial activities. Compound **1** exhibited antifungal activity against *Candida glabrata* with an IC_{50} value of 0.75 μg mL^{-1}.

Introduction

Phloroglucinol-coupled sesquiterpenoids, *e.g.* macrocarpals,[1–5] euglobals,[6,7] eucalyptone,[8] eucalyptals,[9] and eucalyptin,[10] are a group of typical secondary metabolites from the genus *Eucalyptus* (Myrtaceae).[6,11,12] They display a wide spectrum of significant bioactivities, *e.g.* antimicrobial,[1,3] antitumour,[9,10] and granulation[6,7] and HIV-Rase inhibitory effects.[2] These unusual types of natural products are considered to be formed through [4 + 2] cycloaddition of a *O*-quinone methide to a sesquiterpenoid double bond[11] or by nucleophilic addition of a sesquiterpenoid unit to an isoprenylated phloroglucinol.[9,11] Only a limited number of sesquiterpenoids, *e.g.* eudesmane, aromadendrene, guaiane and cadinane, are involved in the formation of these compounds, in comparison to the diversity of sesquiterpenoids found in the *Eucalyptus* volatile oil.[13]

E. maideni F. Muell is an introduced tall timber tree growing widely in the southern part of China. Preliminary TLC and HPLC analysis indicated that the juvenile leaves were rich in phloroglucinol derivatives. As part of a continuing study and search for new phloroglucinol-coupled sesquiterpenoids, the juvenile leaves of this plant were further chemically investigated.[14,15] Since macrocarpals are common components in genus *Eucalyptus*,[11,16] MS was employed for dereplication purposes. This led to the isolation of two new compounds (**1** and **2**) bearing an iphionane moiety, biogenetically related to eudesmane. Compounds **1** and **2** represent a new skeleton of 3,5-diformyl-isopentylphloroglucinol coupled sesquiterpenoids. In addition, nine known phloroglucinol-coupled sesquiterpenoids, macrocarpals A (**3**), B (**4**),[2] H (**5**),[3] K (**6**),[5] I (**7**), J (**8**),[3] E (**9**), G (**10**)[2] as well as eucalyptone (**11**),[8] and three known triterpenoids, 3β-hydroxy-11α-methoxyurs-12-en-28-oic acid (**12**),[17] 3β-hydroxyurs-9(11),12-dien-28-oic acid (**13**),[18] and 3β,13β-dihydroxyurs-11-en-28-oic acid (**14**)[19] were also identified during the process. We herein describe the isolation and structure elucidation of these compounds and their antifungal or antibacterial activities.

Results and discussion

The fresh juvenile leaves of *E. maideni* (7.8 kg) were extracted with 80% aq. acetone three times. The extracts were combined and concentrated to small volume (5 L), which was partitioned with $CHCl_3$. The $CHCl_3$ extract (50 g) was subjected to column chromatography on silica gel, MCI CHP20P, RP-8, and semi-preparative HPLC to yield 11 phloroglucinol-coupled sesquiterpenoids (**1–11**) and three triterpenoids (**12–14**).

Compound **1** was obtained as a yellowish amorphous powder. The HRESIMS displayed a quasi-molecular ion peak at *m*/*z* 485.2531 $[M - H]^-$ (calcd 485.2539), corresponding to a molecular formula of $C_{28}H_{37}O_7$ with 10 degrees of unsaturation. The UV absorption bands at 272 and 387 nm suggested the presence of a 3,5-diformyl-phloroglucinol chromophore.[9] The ^{13}C NMR and DEPT spectra of **1** showed 28 carbon signals (Table 1), including six methyls, five methylenes, six methines, and 11 quaternary carbons. Of which, eight carbons [δ_C 192.0

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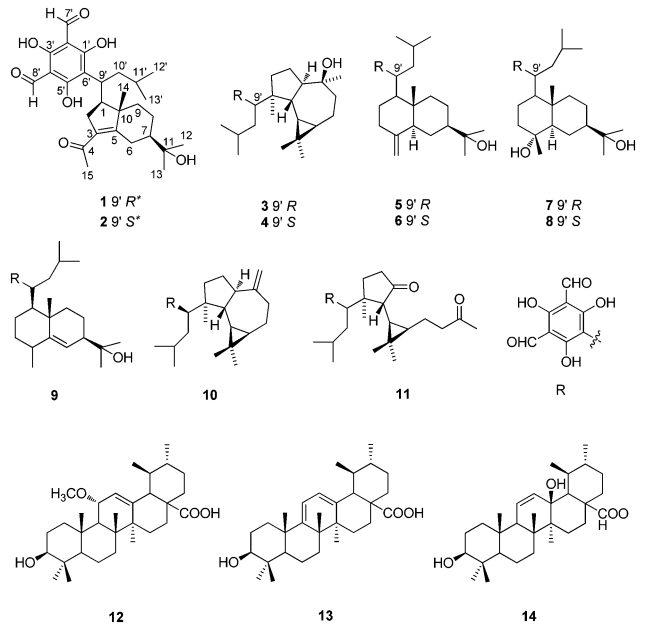
† Electronic supplementary information (ESI) available: UV, IR, MS, HRESIMS, and NMR spectra, as well as the calculated ^{13}C NMR data for new compounds **1** and **2**. See DOI: 10.1039/c4ra01078g

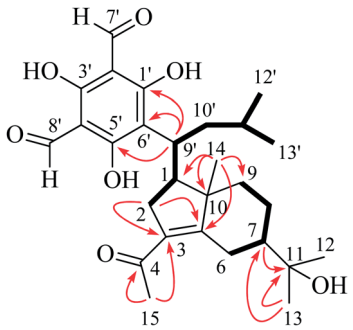


(CH), 191.9 (CH), 172.9 (C), 171.7 (C), 169.4 (C), 107.8 (C), 107.6 (C), and 105.8 (C)] arose from a typical 3,5-diformylphloroglucinol unit, and one ketone (δ_C 198.9) and one tetrasubstituted double bond (δ_C 163.2 and 132.6) were observed. These accounted for 8 out of the 10 degrees of unsaturation. The remaining two degrees of unsaturation therefore required that **1** possessed two additional rings.

First, the protonated C–C connectivity in the molecule were established from C-2 to C-13′ and C-6 to C-9 by the ^{1}H-^{1}H COSY spectrum (bold lines in Fig. 1). The structural fragments were then connected with each other and other functional groups by the HMBC experiment (Fig. 1). The correlations of H-1 with C-3 and H-2 with C-3 and C-5 linked a double bond $\Delta^{3,5}$ to C-2. The correlations of Me-14 with C-1, C-5, C-9, and C-10 allowed the connections of C-1, C-5, and C-9 to the quaternary carbon C-10. Thus, a five-membered A-ring was constructed. Me-15 correlated with C-3 and C-4, indicating an α,β-unsaturated ketone. The HMBC correlations of H-6 to C-3 and C-5, together with the correlation of H-9 with C-10 established the six-membered B-ring. A hydroxyisopropyl group was fixed at C-7 by the correlations of Me-12(13) with C-11 and C-7. An iphionane type

Table 1 ^{13}C and ^{1}H NMR data of **1** and **2** in pyridine-d_5

	1		**2**	
No.	δ_C	δ_H (mult., J_{Hz})	δ_C	δ_H (mult., J_{Hz})
1	52.4	2.93 (dd, 3.1, 7.8)	51.9	3.12 (ddd, 3.4, 7.6, 11.6)
2α	38.4	2.73 (dd, 7.8, 15.0)	38.9	3.01 (dd, 7.6, 14.5)
2β		2.65 (dd, 3.1, 15.0)		2.52 (dd, 3.4, 14.5)
3	132.6		132.1	
4	198.9		198.7	
5	163.2		164.5	
6α	27.1	3.96 (brd, 11.7)	26.9	4.01 (brd, 12.5)
6β		2.12 (dd, 6.4, 11.7)		2.09 (dd, 8.0, 12.5)
7	49.8	1.67 (m)	49.6	1.55 (m)
8α	23.9	1.97 (m)	23.8	1.46 (m)
8β		1.67 (m)		1.36 (m)
9α	43.0	1.67 (m)	40.1	1.46 (m)
9β		2.34 (m)		1.82 (m)
10	49.9		50.3	
11	71.4		71.4	
12	27.8	1.41 (s)	28.1	1.33 (s)
13	27.6	1.41 (s)	27.3	1.32 (s)
14	16.9	1.26 (s)	17.1	1.20 (s)
15	30.7	2.09 (s)	30.9	2.31 (s)
1′	172.9		173.7	
2′	107.6		107.8	
3′	171.7		173.0	
4′	107.8		107.8	
5′	169.4		171.6	
6′	105.8		105.0	
7′	192.0	10.58 (s)	191.9	10.59 (s)
8′	191.9	10.57 (s)	191.6	10.59 (s)
9′	32.9	3.90 (dd, 3.3, 11.3)	33.0	3.66 (ddd, 3.0, 11.6, 11.6)
10′a	41.7	2.49 (dd, 11.3, 11.3)	42.3	2.39 (dd, 11.6, 11.6)
10′b		1.53 (ddd, 3.3, 11.3, 11.3)		1.38 (m)
11′	27.2	1.67 (m)	26.5	1.67 (m)
12′	24.9	1.10 (d, 6.1)	25.0	1.11 (d, 6.3)
13′	22.2	0.94 (d, 6.1)	22.5	0.93 (d, 6.3)



1

Fig. 1 Key ^{1}H-^{1}H COSY (—) and HMBC (→) correlations of **1**.

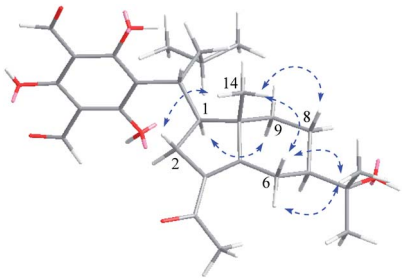


Fig. 2 Key ROESY (H ↔ H) correlations of **1**.

sesquiterpenoid moiety thus emerged in **1**.[20,21] Finally, the HMBC correlations of H-9′ with C-1′, C-5′, and C-6′ confirmed the presence of 3,5-diformyl-isopentylphloroglucinol unit, which was attached to the iphionane sesquiterpenoid moiety *via* the C-1–C-9′ bond. The planar structure of **1** was therefore constructed.

The relative configuration of **1** was established on the basis of the ROESY experiment (Fig. 2). The strong NOE correlations of Me-14 with H-6β, H-8β and H-9β indicated that these protons were co-facially oriented on the B-ring with a chair conformation, assuming Me-14 adopted a β-configuration as shown in the previously reported iphionanes.[21] In addition, Me-14 correlated with H-2β, while H-1 showed strong correlation with H-9α, indicating that H-1 was α-oriented. The obvious NOE correlations of H-7 with H-6α, and Me-12(13) with both H-6α and H-6β indicated that the hydroxyisopropyl group at C-7 adopted the β-equatorial orientation, which was supported by the chemical shift of C-7 at δ 49.8.[22] Thus, the relative configurations of C-1, C-7 and C-10 in **1** were assigned as (1*S**,7*R**,10*S**).

Compound **2**, a yellowish amorphous powder, has the same molecular formula $C_{28}H_{37}O_7$ as that of **1**, which was determined by HRESIMS at *m*/*z* 485.2544 $[M - H]^-$ (calcd 485.2539) and its ^{13}C NMR data. The ^{1}H and ^{13}C NMR spectra of **2** closely resemble those of **1**. Detailed 2D NMR analysis (HSQC, ^{1}H-^{1}H COSY, and HMBC) suggested compound **2** was a C-9′ epimer of **1**, resulting from the coupling of an iphionane type sesquiterpenoid moiety with an isopentylphloroglucinol moiety in an opposite stereochemical fashion. It is evident that the chemical shift of C-9 and the carbons of the phloroglucinol moiety for **2**

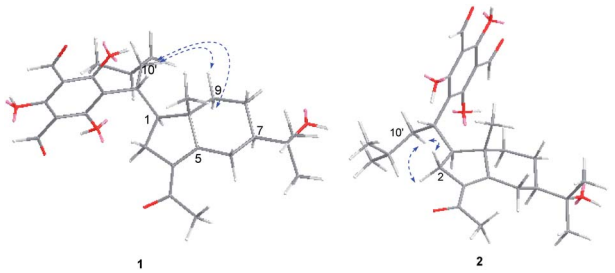
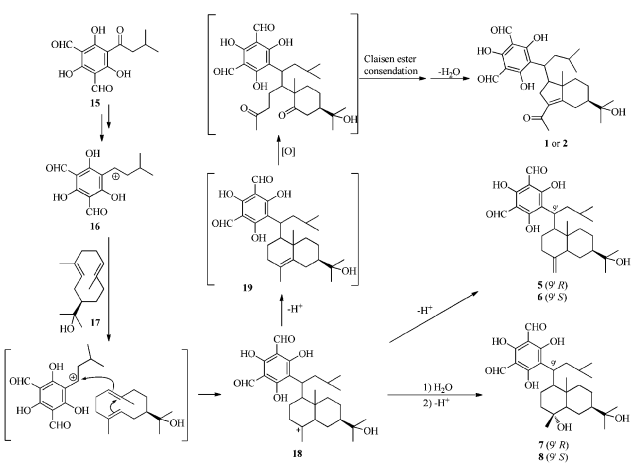


Fig. 3 Energy-minimized (MM2) structures of **1** and **2** showing key ROESY (H ↔ H) correlations.

differ from those for **1**, which may be attributed to the different orientations of the aromatic ring system. Different shielding effects of the phloroglucinol ring to H-1, H-2 and H-9 in **1** and **2** were also noticeable by their chemical shifts (Table 1).

The relative configuration of C-9′ in **1** and **2** was assigned by comparison of their ROESY spectra. First, two isomers with the same configurations at C-1, C-7 and C-10, but a opposite configuration at C-9′, that is, 1*S**,7*R**,10*S**,9′*R** and 1*S**,7*R**,10*S**,9′*S** isomers, were optimized by the MM2 energy minimization. In the optimized models, only H-10′ from the 1*S**,7*R**,10*S**,9′*R** isomer showed NOE correlations with H-9α and H-9β. While H-10′ from the 1*S**,7*R**,10*S**,9′*S** isomer showed NOE correlations with H-2α and H-2β (Fig. 3). Careful analysis of the ROESY spectral data of **1** and **2** revealed that H-10′ showed correlations with H-9α and H-9β, but no correlation with H-2α or H-2β in **1**; while correlations of H-10′ with H-2α or H-2β were observed in **2**. Thus, the relative configurations of **1** and **2** were determined as (1*S**,7*R**,10*S**,9′*R**)-**1** and (1*S**,7*R**,10*S**,9′*S**)-**2**, respectively. These should cause H-1 and H-2 in **1** being upfield-shifted in comparison with those of in **2**, due to the shielding effects of the phloroglucinol ring; while H-9 in **2** should be upfield-shifted for the same reason. This was consistent with the ^{1}H NMR data (Table 1) of H-1, H-2 and H-9 for **1** and **2**.

Since no suitable crystals could be obtained for X-ray analysis in the study, a computational calculation using the gauge-



Scheme 1 Hypothetical biogenetic route of **1** and **2**.

Table 2 Antifungal and antibacterial activities of 1, 3, 5, 7, 8, and 10–14

Compounds	*C. glabrata*[a]	*S. aureus*	MRS[b]
1	0.75	—[c]	—
3	1.38	1.87	1.16
5	1.71	3.29	2.74
7	<0.8	8.23	4.53
8	1.93	—	—
10	1.59	0.53	<0.8
11	0.62	8.05	6.29
12	—	—	14.88
13	—	5.94	8.44
14	—	—	—
Amphotericin B	0.14		
Ciprofloxacin		0.08	0.08

[a] IC_{50} value of antifungal or antibacterial activities, μg mL^{-1}. [b] Methicillin-resistant *S. aureus*. [c] No activity at the conc. of 20 μg mL^{-1}.

independent atomic orbital (GIAO) method was carried out to confirm the relative configuration of **1** and **2**. Conformational searches were performed for (1*S*,7*R*,10*S*,9′*R*)-**1** and (1*S*,7*R*,10*S*,9′*S*)-**2** using MMFF94S force field and the stable geometries were optimized at the B3LYP/6-31G (d) level using the reported methods.[23–27] The lower energy conformations were used for ^{13}C NMR calculations at the B3LYP/6-311++G (2d, p) level. After Boltzmann statistics, the obtained ^{13}C NMR data were compared with corresponding experimental data (ESI†). The experimental ^{13}C NMR of C-9 in **1** and **2** showed major difference (δ_C 43.0 in **1**; δ_C 40.1 in **2**), which matched with the calculated ^{13}C NMR (δ_C 44.2 in **1**; δ_C 41.1 in **2**) of C-9. In addition, The calculated ^{13}C NMR of C-10, C-14, and C-9′ in (1*S*,7*R*,10*S*,9′*R*)-**1** were slighter upfield-shifted than those in calculated (1*S*,7*R*,10*S*,9′*S*)-**2**, more than 1.0 ppm difference. These were further supported by the experimental ^{13}C NMR data (Table 1).

Eucalmaidials A (**1**) and B (**2**) represent a novel chemo-type of phloroglucinol-coupled sesquiterpenoids. Iphionane-type sesquiterpenoid have only reported from family Asteraceae and Cyperaceae.[21] Their biogenetic precursor was considered to be eudesmane-type sesquiterpenoids.[21] The pathway to account for the plausible formation of **1** and **2** was postulated (Scheme 1). 4,6-Diformyl-2-isopentanoyl-phloroglucinol (**15**), the most abundant compound in the titled genus, was reported to biosynthetically produce the carbocationic intermediate **16**.[28] Then, the intermediate **16** attacks the germacrol (**17**) to generate the intermediate **18**. Macrocarpals I and J (**7** and **8**) are generated by ensuing hydration. Alternatively, deprotonation of intermediate **18** led to the macrocarpal H (**5**), K (**6**), and intermediate **19**, from which compounds **1** and **2** are generated by subsequent oxidation, Claisen condensation and dehydration.[21]

The known compounds, macrocarpals A (**3**), B (**4**), H (**5**), K (**6**), I (**7**), J (**8**), E (**9**), G (**10**), eucalyptone (**11**), 3β-hydroxy-11α-methoxyurs-12-en-28-oic acid (**12**), 3β-hydroxyurs-9(11),12-dien-28-oic acid (**13**), and 3β,13β-dihydroxyurs-11-en-28-oic acid (**14**), were determined on the basis of ESI-MS and ^1H, ^{13}C and 2D NMR data, as well as by comparison with data reported in the literature. Compounds **10–12** were reported from the genus *Eucalyptus* for the first time.

Compounds **1, 3, 5, 7, 8** and **10–14** were tested for their antifungal and antibacterial activity against *Candida glabrata*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (Table 2). Eucalmaidial A (**1**), macrocarpal I (**7**), and eucalyptone (**11**) showed antifungal activity against *C. glabrata* with an IC_{50} of 0.75, less than 0.8, 0.62 μg mL^{-1}, respectively; while macrocarpals A (**3**), H (**5**), J (**8**), and G (**10**) showed moderate antifungal activity, in comparison with corresponding positive controls, amphotericin B (IC_{50} = 0.14 μg mL^{-1}). Triterpenoids (**11–12**) showed no antifungal activity against *C. glabrata*. Of all the tested compounds, only macrocarpal G (**10**) showed moderate antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus*, in comparison with corresponding positive controls, Ciprofloxacin (IC_{50} = 0.08 μg mL^{-1}).

Conclusions

In summary, two new phloroglucinol-coupled iphionanes, eucalmaidial A and B (**1**, **2**) were isolated from the juvenile leaves of *E. maideni*, along with nine known phloroglucinol coupled sesquiterpenoids (**3–11**) and three known triterpenoids (**12–14**). Iphionane moiety is rare in the family Myrtaceae. They were proposed to be biogenetically derived from phloroglucinol coupled eudesmane. Triterpenoids **10–12** were reported from the genus *Eucalyptus* for the first time. Eucalmaidial A (**1**) macrocarpal I (**7**), and eucalyptone (**11**) showed antifungal activity against *C. glabrata* with an IC_{50} of 0.75, less than 0.8, 0.62 μg mL^{-1}, respectively. And only macrocarpal G (**10**) showed moderate antibacterial activities against *S. aureus* and methicillin-resistant *S. aureus*.

Experimental section

General procedures

Column chromatography was performed on Silica gel, 200–300 mesh (Qingdao Haiyang Chemical Co.), MCI-gel CHP20P, 75–100 μm (Mitsubishi Chemical Co., Ltd.), and RP-8 (40–63 μm, Merck). Preparative HPLC (Waters 600, USA) was performed using a C-18 column (ZORBAX, 9.4 mm i.d. × 250 mm, USA) and developed isocratically at room temperature with MeOH–H_2O (85/15); detection wavelength (276 nm). Thin-layer chromatography (TLC) was performed on precoated silica gel H plates, 0.2–0.25 mm thick (Qingdao Haiyang Chemical Co.), with $CHCl_3$–MeOH–H_2O [9 : 1 : 0.1 (a) or 8 : 2 : 0.2 (b), v/v/v], and spots were detected by spraying with anisaldehyde–H_2SO_4 reagent followed by heating. Optical rotations were obtained on a JASCO P-1020 polarimeter. UV spectroscopic data were measured on a Shimadzu-210A double-beam spectrophotometer. IR spectra of samples in KBr discs were recorded on a Bruker-Tensor-27 spectrometer with KBr pellets. NMR spectra were carried out on either a Bruker DRX-500 or an Avance 600 MHz spectrometer. ESI-MS or HR-ESI-MS was recorded on an API QSTAR Pular-1 mass spectrometer.

Plant materials

The juvenile leaves of *E. maideni* were collected in the Botanical Garden of Kunming Institute of Botany, Chinese Academy of Sciences, Yunnan, China, in June 2009, and identified by Prof. Xiao Cheng (Botanical Garden, Kunming Institute of Botany, Chinese Academy of Sciences). A voucher specimen (KIB-ZL-200901) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation

The fresh juvenile leaves of *E. maideni* (7.8 kg) were extracted with 80% aqueous acetone at room temperature (30 L × 3, each 1 week). After removal of organic solvents, the extracts were concentrated to small volume (5 L) and partitioned with $CHCl_3$ (2.5 L × 5) after filtration of the precipitate. The $CHCl_3$ fraction (50 g) was subjected to silica gel column chromatography (CC), eluting with $CHCl_3$–MeOH (0 : 1–7 : 3) to afford six fractions. TLC analysis in neutral ($CHCl_3$–MeOH–H_2O 8 : 2 : 1) and acidic (benzene/HCOOEt/HCOOH 4/5/0.5) system revealed that compounds in fractions 3–6 contained phenolic hydroxyl group or carboxyl groups. Further HPLC analysis [separation and detection modules: Waters 2695, Waters 2996; column: agilent C18 (4.6 i.d. × 250 mm); gradient: 0–5 min: 10% MeOH, 5–35 min 10–100%, 35–40 min, 100%; flow rate: 1.0 min mL^{-1} these fractions revealed that in late eluted parts, around 30 min, peaks contained characteristic UV profile of acylphloroglucinol group, 270 nm and 380 nm. Fractions 3–6 were further monitored by ESIMS. Fraction 3 ($ESIMS^-$ *m*/*z* 453, 485), fraction 4 ($ESIMS^-$ *m*/*z* 471), fraction 5 ($ESIMS^-$ *m*/*z* 471, 485), and fraction 6 ($ESIMS^-$ *m*/*z* 471, 489) contained the molecular ion of possible phloroglucinol sesquiterpenoids. Further purification on fraction 3 (2.5 g) over silica gel ($CHCl_3$–MeOH–H_2O, 9 : 1 : 0.1–7 : 3 : 0.5), MCI gel CHP20P (70–100% MeOH), and RP-8 (70–90% MeOH) to yield **10** (60 mg, MW 454), **11** (60 mg, MW 486), and **12** (40 mg), **13** (9 mg), and **14** (8 mg). Fraction 5 (5.1 g) was subjected to chromatography on MCI gel CHP20P (60–100% MeOH), silica gel ($CHCl_3$–MeOH–H_2O, 9 : 1 : 0.1–7 : 3 : 0.5), and semipreparative HPLC (80% MeOH–H_2O, water was acidified with 0.1% CF_3COOH) to yield **1** (9.0 mg), and **2** (2.0 mg), together with **3** (20 mg, MW 472), **4** (18 mg, MW 472), **5** (20 mg, MW 472), and **6** (8 mg, MW 472). Fraction 6 (2.5 g) was applied to MCI gel CHP20P (70–100% MeOH), silica gel ($CHCl_3$–MeOH–H_2O, 9 : 1 : 0.1–7 : 3 : 0.5), and RP-8 (70–90% MeOH) to yield **7** (16 mg), and **8** (25 mg).

Eucalmaidial A (**1**): yellow amorphous powder; $[\alpha]_D^{20}$ + 8.3 (*c* 0.2, MeOH); UV (MeOH), λ_{max} (log ε) 272 (3.86), 387 (3.32) nm; IR (KBr) ν_{max} 3431, 2923, 2581, 1630, 1511, 1384, 1273, 1128, 1033 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz), see Table 1; ^{13}C NMR (pyridine-d_5, 125 MHz), see Table 1; ESIMS (negative ion mode) *m*/*z* 485 $[M - H]^-$; HRESIMS *m*/*z* 485.2531 $[M - H]^-$ (calcd for $C_{28}H_{37}O_7$, 485.2539).

Eucalmaidial B (**2**): yellow amorphous powder; $[\alpha]_D^{20}$ − 21.1 (*c* 0.3, MeOH); UV (MeOH), λ_{max} (log ε) 274 (4.20), 388 (3.81) nm; IR (KBr) ν_{max} 3432, 2926, 2865, 1632, 1454, 1311, 1148, 1097 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz), see Table 1; ^{13}C NMR (pyridine-d_5, 125 MHz), see Table 1; ESIMS (negative ion mode) *m*/*z* 485 $[M - H]^-$; HRESIMS *m*/*z* 485.2544 $[M - H]^-$ (calcd for $C_{28}H_{37}O_7$, 485.2539).

Antifungal and antibacterial assay

Susceptibility testing was performed using a modified version of the NCCLS method using organisms obtained from the American Type Culture Collection (Manassas, VA) including *Candida glabrata* ATCC 90030, *Staphylococcus aureus* ATCC 29213, and methicillin-resistant *Staphylococcus aureus* ATCC 33591. Detailed procedures have been described in a previous paper.[29]

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