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PHLOROGLUCINOLS FROM THE LEAVES OF *EUCALYPTUS GLOBULUS*

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Abstract – A new acylphloroglucinol sesquiterpene structure, euglobal-IX (**1**), together with five known compounds, euglobal-III (**2**), -IVa (**3**), -IVb (**4**), -Ia_2 (**5**) and robustadial B (**6**), were isolated from the leaves of *Eucalyptus globulus* extracted with methanol or methanolic ethylacetate. The chemical structure and relative configuration of **1** was determined by 1D, 2D NMR and MS spectroscopic analyses. New acylphloroglucinol **1** inhibited the catalytic activity of CYP3A4 (IC_{50} = 38.8 μM).

The genus *Eucalyptus* is native of Australia. It has been used in traditional medicine as an anti-septic, and against infections of the upper respiratory tract, including the common cold, influenza and sinus congestion.[1,2] Phytochemical analyses have established that the genus *Eucalyptus* contains monoterpenes,[3] cyanogenic glycosides[4] and triterpene cladocalol.[5]

We recently observed that monoterpene glycosides conjugated with gallic acid, globulusin A and B from the leaves of *Eucalyptus globulus* exhibited anti-oxidant, anti-inflammatory and anti-melanogenesis activities.[6] One major polyphenol, phloroglucinol, and its derivatives, have been found in the genus *Eucalyptus*, and some phloroglucinols have been reported to show biologic activities, such as HIV-RTase inhibition[7] as well as, anti-bacterial[8] and anti-viral[9] effects. We report here a new phloroglucinol, euglobal-IX (**1**), together with five known compounds isolated from the leaves of *E. globulus*.

# These authors contributed equally to this work.

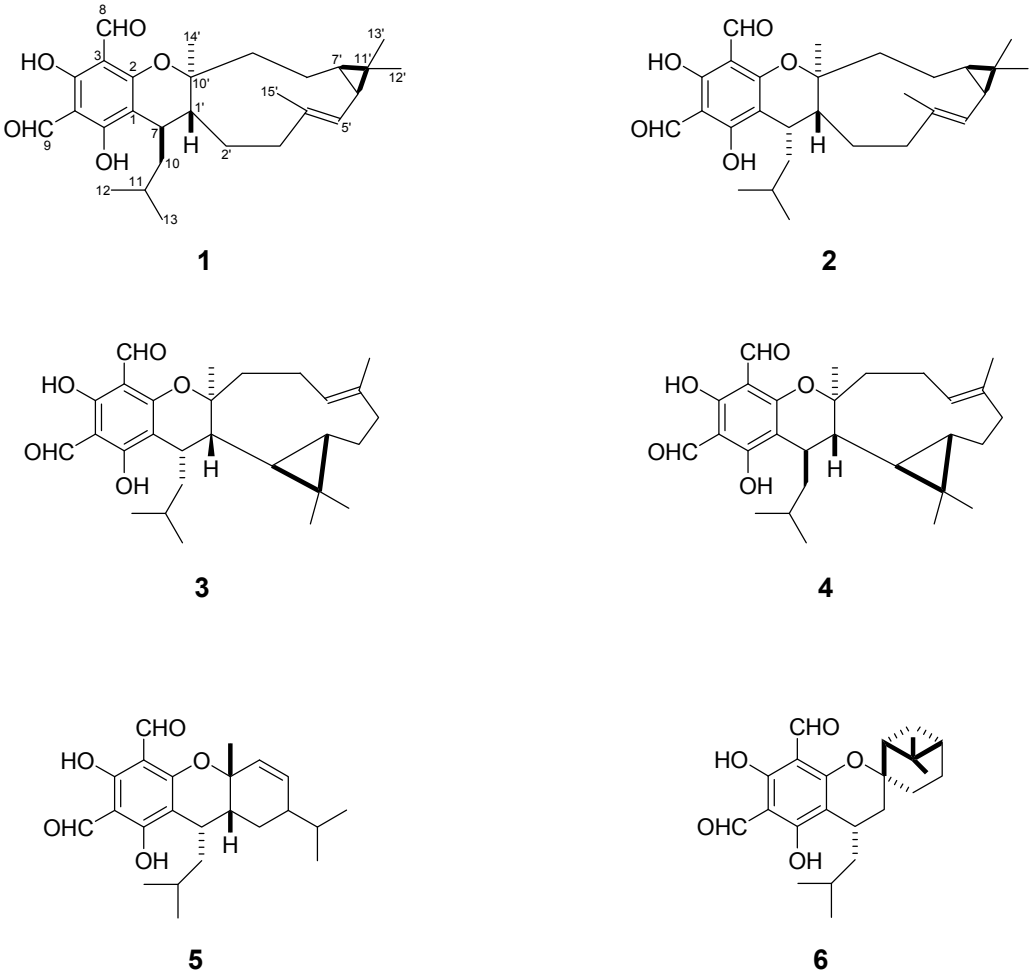

Figure 1. Chemical structures of phloroglucinols isolated from *E. globulus*

Compound **1** was obtained as a colorless solid, and its molecular formula of $C_{28}H_{38}O_5$ as determined by HRFABMS, which gave a pseudomolecular ion peak at *m/z* 455.2798 $[M+H]^+$. The 1D NMR spectral data for **1** revealed the presence of 2,4-diformylphloroglucinol moiety (two phenolic hydroxyl signals [δ_H 13.46 (1H, s), 13.32 (1H, s); δ_C 169.2, 168.0] and the two aldehyde signals [δ_H 10.13 (1H, s), 9.97 (1H, s); δ_C 191.9, 192.1]), and the spectral data for this moiety was similar to that of known compound **2**.[10] In addition, there were HMBC cross-peak from H-8 (δ 9.97) to C-3 (δ 104.7), H-9 (δ 10.13) to C-5 (δ 103.9), OH-4 (δ 13.32) to C-3 (δ 104.7) and C-4 (δ 168.0), OH-6 (δ 13.46) to C-1(δ 105.5) and C-5 (δ 103.9). Therefore, the partial structure of **1** was suggested for 2,4-diformylphloglucinol.

The 1D NMR and HMQC spectra of **1** showed the presence of an isobutyl [δ_H 2.25 (1H, m), 1.49 (1H, m), 1.49 (1H, m), 0.89 (3H, d, J = 6.59 Hz), 0.71 (3H, d, J = 6.59 Hz); δ_C 37.1, 25.3, 24.2, 24.0], four tertiary methyl [δ_H 1.71 (3H, s), 1.08 (3H, s), 1.05 (6H, s); δ_C 17.0, 14.9, 28.7, 21.0] and a double bond [δ_H 5.00 (1H, d, J = 8.79 Hz); δ_C 121.4, 136.3]. The sesquiterpene moiety of **1** was further determined by means of

2D NMR techniques, including ^{1}H-^{1}H COSY, HMQC and HMBC spectra. ^{1}H-^{1}H COSY correlations were observed between H-1′/H-2′, H-2′/H-3′, H-5′/H-6′, H-6′/H-7′, H-7′/H-8′ and H8′/H9′. Furthermore, there were HMBC cross-peaks from H-14′ (δ 1.05) to C-9′ (δ 35.4), from H-15′ (δ 1.71) to C-3′ (δ 41.1), C-4′ (δ 136.3) and C-5′ (δ 121.4), from H-12′ (δ 1.08) to C-11′ (δ 20.3) and C-7′ (δ 26.9). Therefore, the sesquiterpene moiety of **1** was suggested for bicyclogermacrene.

As illustrated in Figure 2, there were HMBC cross-peaks from H-7 (δ 2.72) to C-1 (δ 105.5), C-1′ (δ 39.0), C-10 (δ 37.1) and C-11 (δ 25.3), from H-1′ to C-2’ (δ 32.9), C-3′ (δ 41.1), C-10 (δ 37.1), C-10′ (δ 84.9) and C-14′ (δ 21.0), thereby establishing the planar structure of euglobal-IX (**1**). Although this planar structure of **1** was identical to that of euglobal III (**2**), ^{1}H and ^{13}C NMR spectra data of **1** differed from **3**. That is, **1** was considered to be diastereomer of euglobal III (**2**).

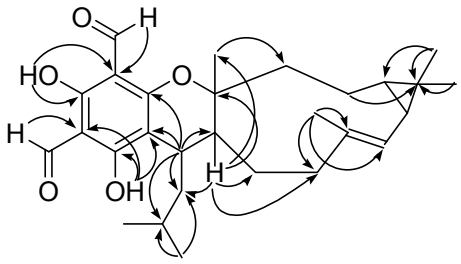

Figure 2. HMBC key correlations for **1**

The relative stereochemistry of **1** was elucidated on the basis of NOE data and ^{1}H-^{1}H couplings. The coupling constant of H-7 and H-1′ was 10.3 Hz. Thus, the orientation assigned was trans. As illustrated in Figure 3, the NOE key correlations were also observed between H-7/H-14′, H-14′/H-6′ and H-14′/H-7′.

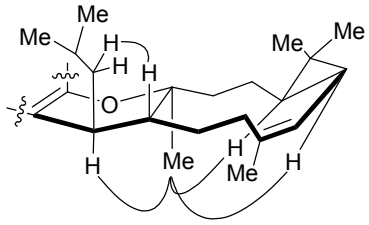

Figure 3. NOE correlations for **1**

The five known compounds euglobal-III (**2**), -IVa (**3**), -IVb (**4**), -Ia$_2$ (**5**) and robustadial B (**6**) were also isolated from the same fractions. They were identified by comparing their spectroscopic data with those reported in the literature.[10–12]

Table 1. ^{13}C and ^{1}H NMR spectroscopic data **1**

Euglobal- IX (**1**)							
Position	δ_C	δ_H		Position	δ_C	δ_H	
1	105.5			4'	136.3		
2	164.0			5'	121.4	5.00	(1H, *d*, 8.79)
3	104.7			6'	24.5	1.28	(1H, *m*)
4	168.0			7'	26.9	0.67	(1H, *m*)
5	103.9			8'	19.6	0.96	(1H, *m*)
6	169.2					1.67	(1H, *m*)
7	36.6	2.72	(1H, *ddd*, 2.01, 5.49, 10.25)	9'	35.4	1.24	(1H, *m*)
8	192.1	9.97	(1H, *s*)			1.73	(1H, *m*)
9	191.9	10.13	(1H, *s*)	10'	84.9		
10	37.1	1.49	(1H, *m*)	11'	20.3		
		2.25	(1H, *m*)	12'	14.9	1.08	(3H, *s*)
11	25.3	1.49	(1H, *m*)	13'	28.7	1.05	(3H, *s*)
12	24.0	0.71	(3H, *d*, 6.59)	14'	21.0	1.05	(3H, *s*)
13	24.2	0.89	(3H, *d*, 6.59)	15'	17.0	1.71	(3H, *s*)
1'	39.0	2.01	(1H, *td*, 2.93, 10.25)	OH		13.32	(1H, *s*)
2'	32.9	1.42	(2H, *m*)			13.46	(1H, *s*)
3'	41.1	2.08	(1H, *td*, 2.93, 12.08)				
		2.23	(1H, m)				

a) Values in parentheses indicate coupling constants in Hz

After establishing their structures, we examined the influence of the isolated acylphloroglucinols on the drug-metabolism activity of the P450 enzyme CYP3A4. Among six tested acylphloroglucinols, euglobal-IX (**1**) inhibited CYP3A4 enzymatic activity with an IC_{50} value of 38.8 μM. Interestingly, the inhibitory activity of the enzyme (IC_{50}: > 50 μM) of euglobal III (**2**), which is a diastereomer of **1**, was weaker than that of **1**. Further investigation is required for understanding the structural activity of acylphloroglucinols on the inhibitory activity of CYP3A4.

Sidana et al. recently reported that phloroglucinols, loxophlebal A and three sideroxylonals isolated from *E. loxophleba* showed moderate antimicrobial activity against *Escherichia coli* and its sub-strain at 3–100 μg/mL.[13] We therefore examined the anti-microbial activities of these compounds against *Staphylococcus* bacteria. No bactericidal activity was observed when bacteria were treated with compounds even at a higher concentration (50 μg/disk).

EXPERIMENTAL

General Optical rotations were measured using a Horiba SEPA-3000 high-sensitivity polarimeter. UV spectra were measured using a Shimadzu UV-1600 UV–visible spectrometer. IR spectra were recorded on a Shimadzu IR-460 IR spectrophotometer, whereas NMR spectra were obtained using a JEOL GSX-500 spectrometer in $CDCl_3$. Chemical shifts were referenced to the residual solvent peaks (δ_H 7.24

and δ_C 77.0 for $CDCl_3$). Mass spectra were measured on a JEOL SX-102 mass spectrometer. Reversed-phase HPLC was carried out on RP-23 (5 μm, Waters). Silica gel (63–210 μm, Kanto) and ODS (63–212 μm, Wako) were used for open-column chromatography. TLC was carried out on silica gel 60 F_{254} (Merck) and RP-18 F_{254S} (Merck).

Plant material The dried leaves of *E. globulus* used in this study were donated by Ichimaru Pharcos Corporation and taxonomically identified by the authors, The plant sample was deposited in a database in our laboratory under registration number S-2006-07.

Extraction and isolation The dried leaves of *E. globulus* (2.5 kg) were extracted with MeOH at room tempature. The MeOH extract was partitioned between *n*-hexane:EtOAc = 1:1 and H_2O. The *n*-hexane:EtOAc = 1:1 layer (80 g/129 g) was separated by SiO_2 flash column chromatography with a stepwise gradient mixture of *n*-hexane/EtOAc/MeOH to give eight fractions (A1–A8). Fraction A1 (2.2 g) was rich in phloroglucinols and was further purified by ODS HPLC eluted with 95% MeOH/H_2O and 95% MeCN/H_2O to give a robustadial B (**6**) (4.7 mg). The *n*-hexane:EtOAc = 1:1 layer (43 g/129 g) was separated by SiO_2 flash column chromatography with a stepwise gradient mixture of *n*-hexane/EtOAc/MeOH to give seven fractions (B1–B8). Fraction B1 (595 mg) included phloroglucinol derivatives and was further purified by ODS column chromatography with MeOH/H_2O (80, 90, 95, 100%) to give seven fractions (C1–C7). Fraction C5 (50 mg) was further purified by ODS HPLC eluted with 95% MeOH/H_2O to give a euglobal-Ia_2 (**5**) (12.3 mg).

Next, the dried leaves (1.6 kg) of *E. globulus* were extracted with EtOAc:MeOH = 1:1 at room temperature. The EtOAc:MeOH = 1:1 extract was partitioned between *n*-hexane and H_2O. The *n*-hexane layer was partitioned between *n*-hexane and 90% MeOH/H_2O. The *n*-hexane layer was subjected to silica gel flash column chromatography with gradient mixtures of *n*-hexane and EtOAC, EtOAc and MeOH to give ten fractions (D1–D10). Fraction D3 was further separated by ODS flash column chromatography with a stepwise gradient of aqueous MeOH (60%, 70%, 80%, 90%, 95% and 100%), to give 17 fractions (E1–E17). Fraction E11 (870 mg/4.0 g) was purified by ODS HPLC with 95% MeCN/H_2O to give -III (**2**) (151.1 mg), -IVb (**4**) (47.5 mg) and fraction F (65.7 mg). Fraction F (65.7 mg) was purified by ODS HPLC with 95% MeCN/H_2O to give euglobal-IX (**1**) (6.4 mg) and -IVa, (**3**) (18.2 mg).

Euglobal IX Colorless solid; $[\alpha]_D^{20}$ –51.7 ($CHCl_3$, *c*0.10); UV ($CHCl_3$) λ_{max} (log ε) 348 (3.59), 282 (4.57) nm; IR ν_{max} (KBr) 3564, 2953, 1636, 1447, 1385, 1306, 1178, 1086, 843, 756, 608 cm^{-1}; for ^1H NMR spectroscopic data (500 MHz, $CDCl_3$) and ^{13}C NMR spectroscopic data (125 MHz, $CDCl_3$). HRFABMS *m/z* 455.2798 $[M + H]^+$ (calcd for $C_{28}H_{39}O_5$).

CYP3A4 inhibitory activity *In-vitro* CYP3A4 inhibition assays of compounds **1**–**6** were conducted using a Vivid® CYP3A4 Blue Screening Kit (Invitrogen Corp., Carlsbad, CA, USA) according to the

manufacturer's protocol.

Antimicrobial activity Antimicrobial activity was determined by the paper disk method.[14] A paper disk (ϕ 6 mm, Toyo Roshi Kaisha, Limited, Tokyo) with the sample was incubated on an agar plate containing *Staphylococcus aureus* subsp. (ATCC® Number: BAA1720™) at 37 °C.

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