

HETEROCYCLES, Vol. 83, No. 3, 2011, pp. 631 - 636. © The Japan Institute of Heterocyclic Chemistry
Received, 16th December, 2010, Accepted, 31st January, 2011, Published online, 8th February, 2011
DOI: 10.3987/COM-10-12126

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**), -Ia₂ (**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 (IC₅₀ = 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 glycosides⁴ 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 inhibition⁷ as well as, anti-bacterial⁸ and anti-viral⁹ 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.

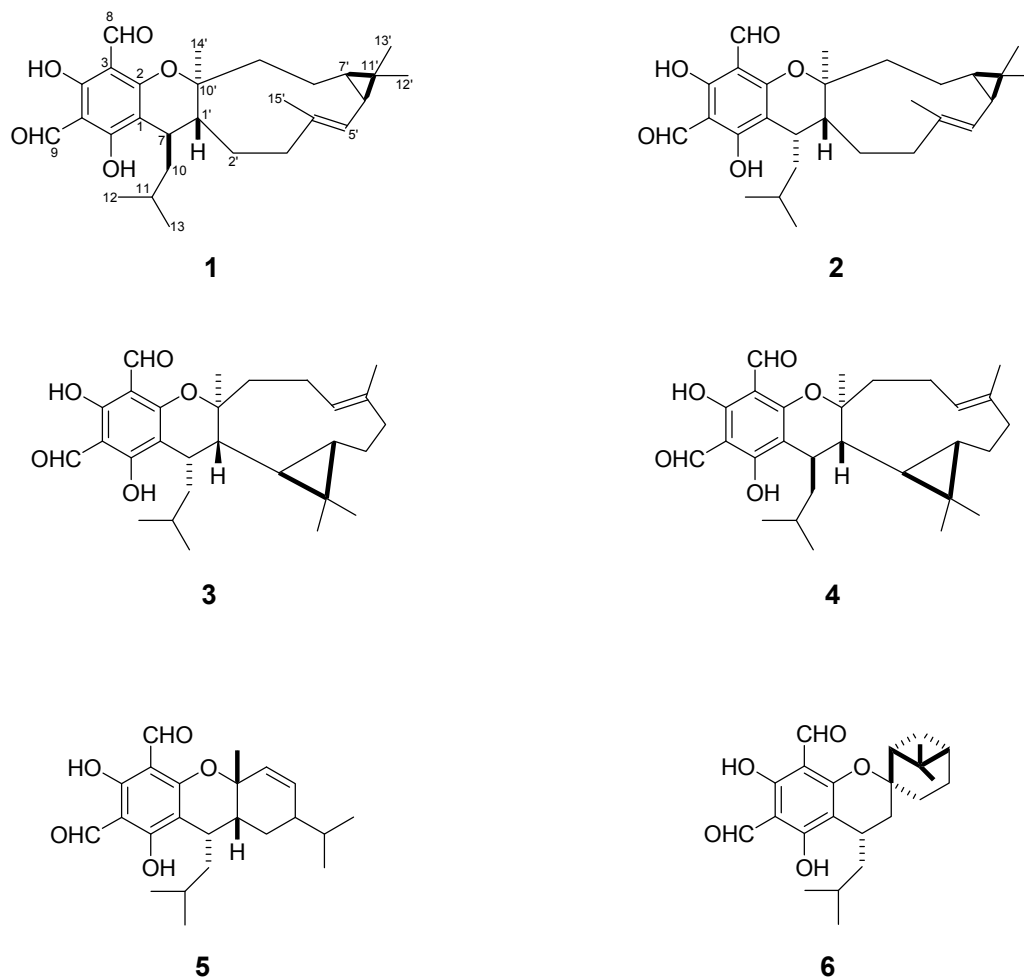


Figure 1. Chemical structures of phloroglucinols isolated from *E. globulus*

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 ^1H - ^1H COSY, HMQC and HMBC spectra. ^1H - ^1H 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**), ^1H and ^{13}C NMR spectra data of **1** differed from **3**. That is, **1** was considered to be diastereomer of euglobal III (**2**).

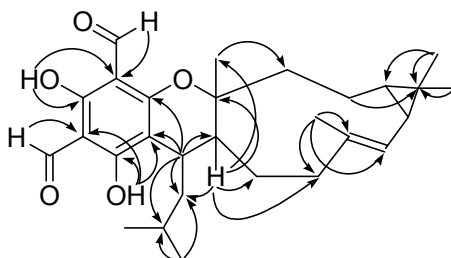


Figure 2. HMBC key correlations for **1**

The relative stereochemistry of **1** was elucidated on the basis of NOE data and ^1H - ^1H 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'.

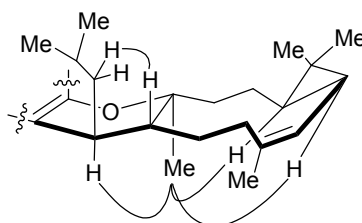


Figure 3. NOE correlations for **1**

The five known compounds euglobal-III (**2**), -IVa (**3**), -IVb (**4**), -Ia₂ (**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 ^1H 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, <i>d</i> , 8.79)
3	104.7		6'	24.5	1.28 (1H, <i>m</i>)
4	168.0		7'	26.9	0.67 (1H, <i>m</i>)
5	103.9		8'	19.6	0.96 (1H, <i>m</i>)
6	169.2				1.67 (1H, <i>m</i>)
7	36.6	2.72 (1H, <i>ddd</i> , 2.01, 5.49, 10.25)	9'	35.4	1.24 (1H, <i>m</i>)
8	192.1	9.97 (1H, <i>s</i>)			1.73 (1H, <i>m</i>)
9	191.9	10.13 (1H, <i>s</i>)	10'	84.9	
10	37.1	1.49 (1H, <i>m</i>)	11'	20.3	
		2.25 (1H, <i>m</i>)	12'	14.9	1.08 (3H, <i>s</i>)
11	25.3	1.49 (1H, <i>m</i>)	13'	28.7	1.05 (3H, <i>s</i>)
12	24.0	0.71 (3H, <i>d</i> , 6.59)	14'	21.0	1.05 (3H, <i>s</i>)
13	24.2	0.89 (3H, <i>d</i> , 6.59)	15'	17.0	1.71 (3H, <i>s</i>)
1'	39.0	2.01 (1H, <i>td</i> , 2.93, 10.25)	OH		13.32 (1H, <i>s</i>)
2'	32.9	1.42 (2H, <i>m</i>)			13.46 (1H, <i>s</i>)
3'	41.1	2.08 (1H, <i>td</i> , 2.93, 12.08)			
		2.23 (1H, <i>m</i>)			

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 sideroxytonals isolated from *E. loxophleba* showed moderate antimicrobial activity against *Escherichia coli* and its sub-strain at 3–100 $\mu\text{g/mL}$.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 $\mu\text{g/disk}$).

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 F₂₅₄ (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 temperature. The MeOH extract was partitioned between *n*-hexane:EtOAc = 1:1 and H₂O. The *n*-hexane:EtOAc = 1:1 layer (80 g/129 g) was separated by SiO₂ 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/H₂O and 95% MeCN/H₂O to give a robustadiol B (**6**) (4.7 mg). The *n*-hexane:EtOAc = 1:1 layer (43 g/129 g) was separated by SiO₂ 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/H₂O (80, 90, 95, 100%) to give seven fractions (C1–C7). Fraction C5 (50 mg) was further purified by ODS HPLC eluted with 95% MeOH/H₂O to give a euglobal-Ia₂ (**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 H₂O. The *n*-hexane layer was partitioned between *n*-hexane and 90% MeOH/H₂O. 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/H₂O 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/H₂O 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 ¹³C NMR spectroscopic data (125 MHz, $CDCl_3$). HRFABMS m/z 455.2798 $[M + H]^+$ (calcd for C₂₈H₃₉O₅).

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.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant number 20590004 for T.O.). The authors thank Ichimaru Pharcos Corporation for the generous gift of dried *E. globulus* leaves.

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