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Eucalyptals D and E, new cytotoxic phloroglucinols from the fruits of *Eucalyptus globulus* and assignment of absolute configuration

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Abstract

Two new phloroglucinols, named eucalyptals D (**1**) and E (**2**), along with a related known compound (euglobal-In-3, **3**) were isolated from the fruits of *Eucalyptus globulus*. Their structures were established on the basis of extensive spectroscopic studies, revealing that they share a common 3,5-diformyl-isopentyl phloroglucinol unit, but each is instead coupled to a different sesquiterpenoid skeleton (aromadendrene in **1**, cadinene in **2**, and a spirosesquiterpene in **3**). Compound **1** possessed an unusual seven-membered D ring with an ether bridge between C-2 of the aromadendrene moiety and C-2' of the aromatic unit. The absolute configuration of the isolates was defined by the comparison of experimental and calculated electronic circular dichroism (ECD) spectra. Compounds **1–3** exhibited significant in vitro cytotoxicities against a few human cancer cell lines (Huh-7, Jurkat, BGC-823, and KE-97) using the CellTiter-GloTM luminescent cell viability assay method.

Keywords

Eucalyptus globulus; Myrtaceae; Phloroglucinols; Eucalyptals; Cytotoxicities

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Supplementary data

Supplementary data (NMR, HR-EIMS, CD/UV curves, and experimental/calculated electronic circular dichroism (ECD) spectra of compounds **1–3**) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tetlet.2012.03.073>.

Introduction

The phloroglucinol-terpene adducts are a class of secondary metabolites with interesting bioactive structures unique to plants in the family Myrtaceae, especially *Eucalyptus* species.^{1–3} The blue gum plant *Eucalyptus globulus* Labill was autochthonous to Australia.⁴ It was recorded that this plant was first introduced to China in late 1890s.⁵ Now it is widely cultivated in southern and south-western China, especially in Yunnan and Jiangxi provinces. The dried mature fruits, earned a common Chinese name ‘Yi-Kou-Zhong’, have been used as an herbal medicine for the treatment of influenza, inflammation, and eczema.⁶ The fruits have been documented to yield cytotoxic 3,5-diformyl-isopentyl phloroglucinol-coupled sesquiterpenes.^{7,8} In an ongoing project towards the discovery of new antitumor agents from natural products,^{9,10} two (**1,2**) new and one (**3**) related known phloroglucinol-coupled sesquiterpenes (Fig. 1) were isolated from ‘Yi-Kou-Zhong’ collected from the noted Jing-gang Mountain. We herein report the characterization of the new compounds and their cytotoxic effects against four human cancer cell lines (Huh-7 hepatocarcinoma, Jurkat T cell lymphoblast, BGC-823 and KE-97 gastric carcinoma).

Results and discussion

Air-dried and powdered fruits of *E. globulus* were extracted with 95% ethanol at room temperature (rt), and the extract was concentrated in vacuo. The crude extract was suspended in H₂O and then exhaustively extracted with EtOAc. Our phytochemical reinvestigation on the EtOAc fraction gave rise to three 3,5-diformyl-isopentyl phloroglucinol-coupled sesquiterpenes (**1**: 3.4 mg, **2**: 1.7 mg, **3**: 4.2 mg). The isolation was indeed guided by observing the typical signals of the formyl protons in the ¹H NMR spectrum. Comparison of the spectroscopic data and physical properties with those reported in the literature, compound **3** was identified as euglobal-In-3, a phloroglucinol possessing an unusual spirosesquiterpene skeleton previously isolated from the leaves of *Eucalyptus incrassate*.¹¹ The absolute configuration of **3** was deduced herein by means of the electronic circular dichroism (ECD) calculation for the first time (see below).

Eucalyptal D (**1**)¹² was obtained as a pale yellow gum. The molecular formula of **1** was determined to be C₂₈H₃₆O₅ based on a prominent molecular ion peak at *m/z* 452.2562 [M]⁺ in its HR-EIMS. The IR and UV data of compound **1** were in good agreement with those of euglobal-In-3 (**3**),¹¹ indicating that **1** also has a similarly substituted phloroglucinol moiety. The ¹H NMR spectral data (in CDCl₃, Table 1) of **1** showed two D₂O-exchangeable hydrogen-bonded phenolic hydroxyls [δ 13.35 (1H, s, 4'-OH), 13.22 (1H, s, 6'-OH)], two aldehyde protons [δ 10.09 (1H, s, H-7'), 10.20 (1H, s, H-8')], one oxymethine [δ 5.36 (1H, br d, *J* = 4.5 Hz, H-2)], a methine proton [δ 3.19 (1H, br d, *J* = 11.0 Hz, H-9')] adjacent to the aromatic ring and isobutyl side chain, four singlet methyls [δ 1.60 (vinylic Me-14), 1.18 (Me-15), 1.06 (Me-12), 0.99 (Me-13)], two doublet methyls [δ 0.95 (3H, d, *J* = 6.3 Hz, Me-13'), 0.77 (3H, d, *J* = 6.6 Hz, Me-12')], and two typical methine protons resonating at δ 0.68 (1H, dd, *J* = 11.3, 9.9 Hz, H-6) and 0.49 (1H, ddd, *J* = 9.9, 9.5, 6.3 Hz, H-7) attributed to a *gem*-dimethyl cyclopropane ring. ¹³C and DEPT NMR spectra of **1** exhibited twenty-eight signals classified as six sp³ methyl, four sp³ methylene, six sp³ (one oxygenated at δ 86.5) and two sp² (δ 193.3, 192.1) methine, two sp³, and eight sp² (δ 169.5, 168.3, 167.2,

138.0, 135.5, 115.9, 108.2, 104.6) quaternary carbons (Table 1). These results demonstrated that **1** was a phloroglucinol-coupled sesquiterpene, and the 3,5-diformyl-isopentyl phloroglucinol unit of which was secured by 2D NMR experiments. An obvious isobutyl spin system was found in the COSY spectrum. In the HMBC spectrum, methine proton H-9' was correlated with C-1', C-2', C-6', and C-11', while formyl proton H-7' was correlated with C-2', C-3', and H-8' was correlated with C-5' and C-6' (Fig. 2).

Two separated spin systems of $\text{-OCHCH}_2\text{-}$ (H-2 and H₂-3) and $\text{-CHCHCHCH}_2\text{CH}_2\text{-}$ (H-5, H-6, H-7, H₂-8, and H₂-9) were found for the sesquiterpene unit in the COSY spectrum of **1**. One bond proton-carbon connectivities and the long range proton-carbon couplings in this unit were then ascertained by HSQC and HMBC NMR experiments (Fig. 2), which led to the determination of an aromadendrene-type sesquiterpene moiety involving a 5-membered ring (A), 7-membered ring (B), and 3-membered ring (C). The molecular formula of **1** accounted for eleven degrees of unsaturation. Apart from six double-bond equivalents in the 3,5-diformyl-isopentyl phloroglucinol unit, and the tricyclic sesquiterpene unit bearing an unambiguous double-bond (olefinic carbons resonating at δ 138.0 and 135.5), the remaining one degree of unsaturation must be an additional ring connecting between the two units. The linkage positions could be thereafter easily elucidated by a key HMBC correlation between the oxymethine proton H-2 at δ 5.36 and C-2' at δ 168.3. Meanwhile, H-9' at δ 3.19 was further correlated with C-3 (δ 39.9), and C-15 (δ 22.4), H-5 at δ 2.08 (1H, d, J = 11.5 Hz) was correlated with C-9' at δ 43.0 in the HMBC NMR spectrum.

In fact, similar phloroglucinol-aromadendrene coupled compounds (e.g. macrocarpals A and B, euglobal V) were previously obtained from the fruits and leaves of *E. globulus*.^{8,13-16} However, such a seven-membered D ring constructed with an ether bridge between C-2 of the tricyclic aromadendrene moiety and C-2' of the aromatic E ring in compound **1** has never been encountered.

The relative stereochemistry of **1** was determined by the analysis of the proton coupling constants (Table 1) and the NOE correlations in the NOESY NMR spectrum (Fig. 3). A large coupling constant (11.5 Hz) between H-5 and H-6 indicated that both H-5 and H-6 took opposite axial positions. Clear NOE correlations were observed between H-5 and Me-12 at δ 1.06, between H-5 and H-9', between H-6 at δ 0.68 and H-7 at δ 0.49, between H-6 and Me-15 at δ 1.18, between Me-15 and H-3a at δ 1.53, between H-3b at δ 2.32 and H₂-10' at δ 1.42/1.30, as well as between H₂-3 and H-2. These data revealed that H-2, H-6, H-7, Me-15, and the isobutyl group adopted the same orientation, whereas H-5, H-9' and the cyclopropane ring assumed the same orientation as depicted in Fig. 1.

Eucalyptal E (**2**)¹² was also obtained as a pale yellow gum. The molecular weight of **2** and its chemical formula of C₂₈H₃₈O₇ were deduced from its HR-EIMS, which resulted in an $[\text{M-H}_2\text{O}]^+$ ion peak at m/z 468.2516. The IR and UV data of **2** closely resembled those of compounds **1** and **3**,¹¹ indicating that **2** also has a substituted phloroglucinol moiety. The ¹H and ¹³C NMR data (Table 1) of **2** showed general features similar to those of compound **4** (eucalyptal B, Fig. 1), a known 3,5-diformyl-isopentyl phloroglucinol-coupled cadinene previously also isolated from the fruits of *E. globulus* in 2007.⁷ The ¹H NMR spectral data of **2** exhibited two D₂O-exchangable phenolic hydroxyls [δ 13.35 (1H, s, 4'-OH), 13.38

(1H, s, 6'-OH)], two aldehyde protons [δ 10.06 (1H, s, H-7'), 10.17 (1H, s, H-8')], an olefinic proton resonating at δ 5.62 (1H, brs, H-2), one oxymethine [δ 4.55 (1H, d, J = 10.0 Hz, H-5 α)], a methine proton [δ 2.66 (1H, t, J = 5.6 Hz, H-9')] adjacent to the aromatic ring and isobutyl side chain, four tertiary methyl groups with singlets at δ 1.26 (3H, Me-12), 1.30 (3H, Me-13), 1.28 (3H, Me-14), and 0.88 (3H, Me-15), and two secondary methyl doublets at δ 0.95 (3H, d, J = 6.5 Hz, Me-12') and 0.97 (3H, d, J = 6.5 Hz). Similar to **1**, the ^{13}C NMR and DEPT NMR spectra of **2** also gave 28 signals [six sp^3 methyl, four sp^3 methylene, five sp^3 (one oxygenated at δ 77.3) and three sp^2 (δ 191.7 $\times$ 2, 115.4) methine, three sp^3 (two oxygenated at δ 74.9 and 70.4) and seven sp^2 (δ 169.3, 167.9, 162.2, 138.5, 107.0, 104.0, 103.4) quaternary carbons] (Table 1).

The obvious difference between **2** and **4** was in the cadinene moiety. A tertiary methyl group (geminal to a carbon bearing an oxygen) at δ 1.28 (3H, Me-14) was present in **2**, instead of the $^{10(14)}$ exomethylene group in **4**.⁷ Moreover, a trisubstituted double bond at C-1 was present in **2**. These structural features were confirmed by 2D NMR experiments, and the planar structure of **2** was determined. The relative stereochemistry of **2** was determined by a combination of analysis of the proton coupling constants (Table 1) and the NOE correlations in the NOESY NMR spectrum (Fig. 3). In the proposed structure of **2**, a large coupling constant (10.0 Hz) between H-5 at δ 4.55 and H-6 at δ 2.62 indicated that both H-5 and H-6 took opposite axial positions. Clear NOE correlations were observed between H-5 and Me-12 at δ 1.26, between H-5 and H-3a at δ 2.51, between H-6 and H-7 at δ 2.01, between H-6 and Me-15 at δ 0.88, between Me-15 and H-3b at δ 2.05, between Me-15 and H-9' at δ 2.66, as well as between H_{ax}-9 at δ 1.83 (br d, J = 16.1, 11.3 Hz) and Me-14 at δ 1.28, but not between Me-14 and H-6 (otherwise they should have a strong NOE correlation). The above data mentioned that H-5, the 2-hydroxyisopropyl group at C-7, Me-14, and the isobutyl group at C-9' took the same orientation, whereas H-6, H-7, 10-OH, Me-15, and H-9' adopted the same orientation as illustrated in Figure 1.

The overall absolute configuration of phloroglucinol-coupled sesquiterpenes has never been determined. To our knowledge, the absolute configuration at C-9' of macrocarpals H, I, and J was once determined by a computational chemical method involving Monte Carlo (MC) and semiempirical molecular orbital calculations.¹⁷ The absolute stereochemistry at C-10 of a derivative of macrocarpals A and C was established by chemical transformation and modified Mosher's method; however, the absolute configuration of entire structure of the macrocarpals A and C was still unknown.¹⁴ To date, the calculated experimental electronic circular dichroism (ECD) spectral data have been successfully utilized to determine the absolute configuration of complex naturally occurring compounds (e.g. sorbiterrin A,¹⁸ psiguadials,¹⁹ and discorhabdins²⁰). To further determine the absolute configuration of compounds **1–3**, a computational approach was applied, and the simulated ECD curves were then overlaid with the experimental CD spectra for comparison. Briefly, conformational analyses were carried out by the Amber force field method followed by the PM6 semi-empirical method in tandem to acquire pre-optimized structures. As a result, a group of low energy conformations of compounds **1–3** were generated. Each low energy conformation was further optimized by the density functional theory (DFT) method at the B3LYP/6-31g(d,p) level in the gas phase and applying the Polarizable Continuum Model (PCM)

solvation model, respectively, and the optimized geometries were then followed by excited state calculations using the time-dependent density functional theory (TDDFT) method at the same theoretical level. Resulting from the simulated ECD curves overlaid with the experimental CD spectra of each compound (Fig. 4) (see Supplementary data), the absolute configuration of compounds **1–3** was thus finally assigned as (2*R*, 4*R*, 5*R*, 6*R*, 7*R*, 9' *S*)-eucalyptal D (**1**), (4*R*, 5*S*, 6*R*, 7*R*, 10*R*, 9' *S*)-eucalyptal E (**2**), and (1*S*, 4*R*, 5*S*, 10*R*, 9' *S*)-euglobal-In-3 (**3**), respectively.

From a structural point of view, the above three isolates (**1–3**) have a common 3,5-diformyl-isopentyl phloroglucinol unit, but each is instead coupled to a different sesquiterpenoid framework. With regard to their biosynthetic pathway, these phloroglucinol-sesquiterpene adducts (**1–3**) are thought to be derived biogenetically from a phloroglucinol precursor (**a**) and a bicyclogermacrene (**b**) (Fig. 5). In fact, biogenetic pathways have been previously proposed for phloroglucinol-coupled aromadendrenes (e.g. macrocarpals A–C,¹⁴) cadinenes (e.g. eucalyptals A–C,⁷) and spirosesquiterpenes (e.g. euglobal-In-2¹¹) obtained from the plant *E. globulus*. Since compound **1** contained an unusual seven-membered D ring with an ether bridge between C-2 of the aromadendrene moiety and C-2' of the aromatic unit, a possible biosynthetic pathway is therefore highlighted in Fig. 5. The key step is producing a stable intermediate (**c**) possessing an allyl cation with a *p*- π conjugated effect, followed by a carbocation-induced cyclization under acidic condition to form such a seven-membered D ring in compound **1**.

The cytotoxicities of compounds **1–3** against human BGC-823 and KE-97 gastric, Huh-7 hepatocarcinoma, and Jurkat T lymphoma cancer cell lines were tested by the CellTiter-GloTM luminescent cell viability assay method.¹⁰ The results were summarized in Table 2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The structures of phloroglucinol-coupled sesquiterpenes **1–4**.

Figure 2.
Key COSY (—) and HMBC (H→C) correlations of **1**.

Figure 3.
Observed key NOE (H...H) correlations of **1** and **2**.

Figure 4. Simulated ECD spectra of **1–3** (after Boltzmann averaging) compared with the experimental CD spectra.

Figure 5.
Hypothetical biosynthetic pathway of **1**.

Table 1

¹H and ¹³C NMR data of **1** and **2** (in CDCl₃, δ in ppm, *J* in Hz)

No.	1^a		2^b	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1		138.0		138.5
2	5.36 (br d, 4.5)	86.5	5.62 (br s)	115.4
3	1.53 (dd, 14.2, 4.5, H-3a) 2.32 (br d, 14.2, H-3b)	39.9	2.51 (br d, 18.0, H-3a) 2.05 (dd, 18.0, 4.3, H-3b)	34.6
4		43.7		43.1
5	2.08 (d, 11.5)	47.1	4.55 (d, 10.0)	77.3
6	0.68 (dd, 11.5, 9.9)	32.4	2.62 (br d, 10.0)	43.5
7	0.49 (ddd, 10.0, 9.9, 5.3)	24.9	2.01 (m)	29.6
8	1.41 (overlapped, H-8a) 1.58 (overlapped, H-8b)	21.3	2.23 (m) 1.65 (overlapped)	25.1
9	2.14 (overlapped, H-9a) 2.04 (overlapped, H-9b)	36.4	1.83 (br dd, 16.0, 10.5, H _{ax}) 1.66 (overlapped, H _{eq})	31.1
10		135.5		70.4
11		18.6		74.9
12	1.06 (s, 3H)	15.6	1.26 (s, 3H)	30.0
13	0.99 (s, 3H)	28.1	1.30 (s, 3H)	29.6
14	1.60 (s, 3H)	22.0	1.28 (s, 3H)	22.3
15	1.18 (s, 3H)	22.4	0.88 (s, 3H)	20.9
1'		115.9		107.0
2'		168.3		162.2
3'		108.2		104.0
4'		167.2		167.9
5'		104.6		103.4
6'		169.5		169.3
7'	10.09 (s)	193.3	10.06 (s)	191.7
8'	10.20 (s)	192.1	10.17 (s)	191.7
9'	3.19 (br d, 11.0)	43.0	2.66 (t, 5.6)	36.3
10'	1.30 (overlapped, H-a) 1.42 (overlapped, H-b)	41.7	1.13 (m) 1.38 (m)	43.5
11'	1.11 (m)	25.8	1.70 (m, overlapped)	27.6
12'	0.95 (d, 6.3)	21.4	0.95 (d, 6.5)	23.0
13'	0.77 (d, 6.6)	24.5	0.97 (d, 6.5)	23.1
4'-OH	13.35 (s)		13.35 (s)	
6'-OH	13.22 (s)		13.38 (s)	

^aRecorded at 500 and 125 MHz for ¹H and ¹³C, respectively.^bRecorded at 400 and 100 MHz for ¹H and ¹³C, respectively.

Table 2Cytotoxicities of compounds **1–3**

Compound	IC ₅₀ (μM, mean ± SEM, <i>n</i> = 4)			
	KE-97	Jurkat	BGC-823	Huh-7
1	5.20 ± 1.16	7.16 ± 2.84	6.65 ± 1.36	11.73 ± 1.81
2	7.22 ± 2.06	10.71 ± 4.21	10.16 ± 0.89	24.57 ± 4.45
3	4.63 ± 0.86	10.50 ± 3.25	14.27 ± 0.78	10.77 ± 3.55
ST ^a	0.13 ± 0.01	0.14 ± 0.03	0.17 ± 0.02	0.23 ± 0.08

^aStaurosporine (ST): Positive control.