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Article

Two Novel Tyrosinase Inhibitory Sesquiterpenes Induced by CuCl_2 from a Marine-Derived Fungus *Pestalotiopsis* sp. Z233

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Abstract: Two new sesquiterpenes, $1\beta,5\alpha,6\alpha,14$ -tetraacetoxy- 9α -benzoyloxy- 7β -*H*-eudesman- $2\beta,11$ -diol (**1**) and $4\alpha,5\alpha$ -diacetoxy- 9α -benzoyloxy- 7β -*H*-eudesman- $1\beta,2\beta,11,14$ -tetraol (**2**), were produced as stress metabolites in the cultured mycelia of *Pestalotiopsis* sp. Z233 isolated from the algae *Sargassum horneri* in response to abiotic stress elicitation by CuCl_2 . Their structures were established by spectroscopic means. New compounds **1** and **2** showed tyrosinase inhibitory activities with IC_{50} value of $14.8 \mu\text{M}$ and $22.3 \mu\text{M}$.

Keywords: sesquiterpenes; *Pestalotiopsis* sp.; abiotic stress; tyrosinase inhibitory activities

1. Introduction

Marine-derived fungi, living in a stressful habitat, are of great interest as new promising sources of biologically active products. Since marine organisms live in a biologically competitive environment with unique conditions of pH, temperature, pressure, oxygen, light, nutrients and salinity, the chemical diversity of the secondary metabolites from marine fungi is considerably high [1–6]. New strategies of discovery of novel bioactive compounds including biotic [7–9] and abiotic [10] stress elicitations have been applied. Fungi of the genus *Pestalotiopsis* are characterized by their extensive distribution and

wide genetic, biological and chemical diversity [11]. Natural products from the *Pestalotiopsis* species exhibit considerable chemical diversity and various bioactivities [11–16]. In this study, two novel sesquiterpenes were produced as stress metabolites in the cultured mycelia of *Pestalotiopsis* sp. Z233 isolated from algae *Sargassum horneri* in response to abiotic stress elicitation by CuCl_2 .

Tyrosinase is a multifunctional copper-containing enzyme, which catalyzes the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and the subsequent oxidation of L-DOPA to dopaquinone, is widely distributed in microorganisms, animals and plants [17]. Tyrosinase inhibitors can be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation [18]. Several tyrosinase inhibitors have been studied in our previous studies [19]. In continuation of our search for bioactive natural products that can be used for the treatment of dermatological disorders associated with melanin hyperpigmentation, stress metabolites in the cultured mycelia of *Pestalotiopsis* sp. Z233 were investigated.

2. Results and Discussion

The *Pestalotiopsis* sp. Z233, isolated from algae *Sargassum horneri*, was grown in the absence and, in parallel experiment, in the presence of the abiotic stress agent, CuCl_2 . Upon comparison of TLC plates of mycelial extracts from both conditions, two additional spots in the extract of CuCl_2 treated culture were found. These two new compounds were presumably produced in response to abiotic stress. They were isolated by preparative TLC, and purified by Sephadex LH-20 column chromatography.

Compound **1** was obtained as a yellowish oil. The HR-TOF-MS exhibited an ion peak at m/z 593.2595 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{30}\text{H}_{41}\text{O}_{12}$, 593.2593), corresponding to the molecular formula, $\text{C}_{30}\text{H}_{40}\text{O}_{12}$. The ^{13}C NMR spectrum showed the presence of eight signals for four acetoxyl moieties, seven signals for a benzoyloxyl moiety, with the remaining 15 resonances corresponding to a sesquiterpene skeleton. The ^1H and ^{13}C NMR spectra indicated compound **1** to be a highly oxygenated eudesmane derivative (Table 1). The 15 signals for the eudesmane backbone comprised three methyls (δ_{C} 25.5, 30.1 and 17.3), one oxymethylene (δ_{C} 64.4), two methylenes (δ_{C} 30.3 and 34.2), two methines (δ_{C} 32.5 and 47.9), four oxymethines (δ_{C} 70.9, 68.5, 76.9 and 68.8), a quaternary carbon (δ_{C} 52.7), two oxygenated quaternary carbons (δ_{C} 88.8 and 82.4). Two of the three methyls (δ_{C} 25.5 and 30.1) were assigned to an oxygenated isopropyl group (carbinol signal at δ_{C} 82.4), with the third (δ_{C} 17.3) being Me-15. In the COSY spectrum of **1** (Figure 1), the oxymethine proton at δ_{H} 5.38 (m, H-2) was coupled with the oxymethine proton at δ_{H} 5.50 (d, $J = 3.5$ Hz, H-1) and methylene protons at δ_{H} 1.75 (dd, 14.5, 2.0 Hz, H-3a) and 2.13 (dd, 14.5, 3.2 Hz, H-3b). The methine proton at δ_{H} 2.22 (m, H-4) exhibited cross peaks with methylene protons of H₂-3 and methyl protons at δ_{H} 1.12 (d, $J = 7.5$ Hz, Me-15) in the COSY spectrum of **1**. A sequence of H-1/H-2/H-3/H-4/Me-15 was deduced from above ^1H ^1H COSY analyses. Another sequence of H-6/H-7/H-8/H-9 was inferred from the observation of COSY cross peaks from the methine proton at δ_{H} 2.19 (m, H-7) to the oxymethine proton at δ_{H} 5.88 (d, $J = 1.0$ Hz, H-6) and methylene protons at δ_{H} 2.40 (m, H-8a) and 2.16 (m, H-8b), and cross peaks from H₂-8 to the oxymethine proton at δ_{H} 5.34 (m, H-9). The benzoyloxyl moiety was assigned at C-9 from the observation of HMBC correlations from the oxymethine proton at δ_{H} 5.34 (m, H-9) and aromatic protons at δ_{H} 7.90 (dd, $J = 8.0, 2.0$ Hz) to the benzylic ester carbon resonance at

δ_C 164.4 (s, C-16) and the oxygenated methylene carbon resonance at δ_C 64.4 (t, C-14). The HMBC peaks from two methyl groups at δ_H 1.41 (s, Me-12) and 1.42 (s, Me-13) to two oxygenated quaternary carbons at δ_C 88.8 (s, C-7) and 82.4 (s, C-11) positioned the oxygenated isopropyl group at C-7 of ring B. Three of four acetoxy groups were assigned to C-1, C-6 and C-14 from analysis of the HMBC cross peaks of H-1/C-25, H-6/C-29 and H-14/C-23. The NOESY correlations from acetoxy Me-28 to H-1 and H-2 at ring A positioned the remaining acetoxy group at oxygenated C-5. The relative configuration of **1** was determined from the analyses of NOESY data. The oxygenated H-9 showed NOESY correlations with H₂-14, indicating that benzoyloxy moiety was α -oriented. The H-6 and H-7 protons at δ_H 5.88 (m) and 2.19 (m) showed NOESY correlations with the methylene protons at δ_H 5.00 and 4.25 (d, $J = 12.7$, H₂-14), contributing an α -oriented acetoxy unit and an α -oriented oxygenated isopropyl group in **1** as drawn. The NOESY cross peak of H₂-14 β /Me-15 implied a β -oriented CH₃ at C-4. H-1 showed strong NOESY cross peak with aromatic H-18/22, revealing a β -oriented OH at C-1. The 3.5 Hz coupling constant between H-1 and H-2 indicated an equatorial H-2 proton, assigning the hydroxyl group at C-2 as a β configuration. This inference was also confirmed by the observation of the NOESY correlation from the axial proton of H-3 α to H-2 α . Therefore, the structure of this isolate was elucidated as 1 β ,5 α ,6 α ,14-tetraacetoxy-9 α -benzoyloxy-7 β H-eudesman-2 β , 11-diol (Figure 2). The assignment of NMR signals of **1** is listed in Table 1.

Table 1. NMR Data (500 MHz) for Compound **1** and **2** in DMSO-*d*₆.

Position	1		2	
	$\delta_C^{a,b}$, mult.	δ_H^c , mult. (J in Hz)	$\delta_C^{a,b}$, mult.	δ_H^c , mult. (J in Hz)
1	70.9, CH	5.50 d (3.5)	70.9, CH	5.50 d (3.5)
2	68.5, CH	5.38, m	68.4, CH	5.36, m
3a	30.3, CH ₂	1.75, dd (14.5, 2.0)	39.0, CH ₂	1.83 dd (14.0, 2.0)
3b		2.13, dd (14.5, 3.2)		2.09, dd (14.0, 3.0)
4	32.5, CH	2.22, m	68.8, C	
5	88.8, C		88.8, C	
6a	76.9, CH	5.88, d (1.0)	30.7, CH ₂	2.18, m
6b				1.80, m
7	47.9, CH	2.19, m	42.4, CH	2.06, m
8a	34.2, CH ₂	2.40, m	32.2, CH ₂	2.16, m
8b		2.16, m		2.02, m
9	68.8, CH	5.34, m	68.8, CH	5.69, m
10	52.7, C		50.9, CH	
11	82.4, C		83.1, C	
12	25.5, CH ₃	1.41, s	24.1, CH ₃	1.44, s
13	30.1, CH ₃	1.42, s	29.1, CH ₃	1.29, s
14a	64.4, CH ₂	5.00, d (12.7)	65.5, CH ₂	5.11, d (12.5)
14b		4.25, d (12.7)		4.79, d (12.5)
15	17.3, CH ₃	1.12, d (7.5)	26.0, CH ₃	1.37, s
16	164.4, C		164.6, C	
17	128.8, C		128.7, C	
18/22	129.5, CH	7.90, dd (8.0, 2.0)	129.5, CH	7.93, dd (8.0, 2.0)
19/21	128.5, CH	7.52, t (8.0)	128.5, CH	7.53, t (8.0)

Table 1. Cont.

20	133.6, CH	7.65, t (8.0)	133.5, CH	7.64, t (8.0)
23	169.9, C		168.3, C	
24	20.8, CH ₃	2.19, s	20.7, CH ₃	1.98, s
25	168.6, C		169.3, C	
26	20.0, CH ₃	1.46, s	20.0, CH ₃	1.38, s
27	169.4, C			
28	20.8 CH ₃	2.20, s		
29	169.6, C			
30	21.0, CH ₃	2.09, s		

^a Recorded at 125 MHz; ^b Multiplicities inferred from DEPT and HMQC experiments; ^c Recorded at 500 MHz.

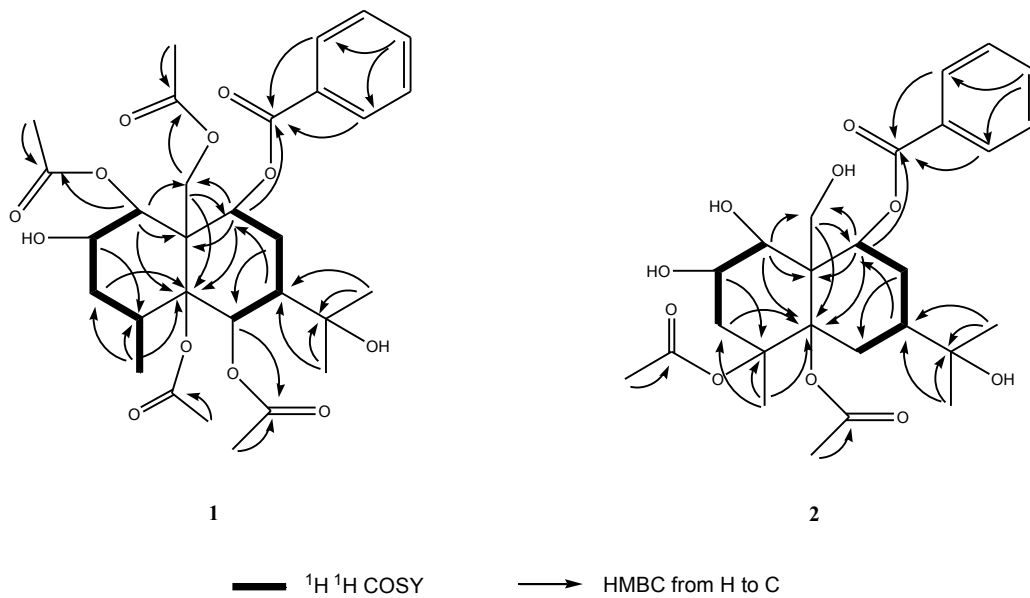
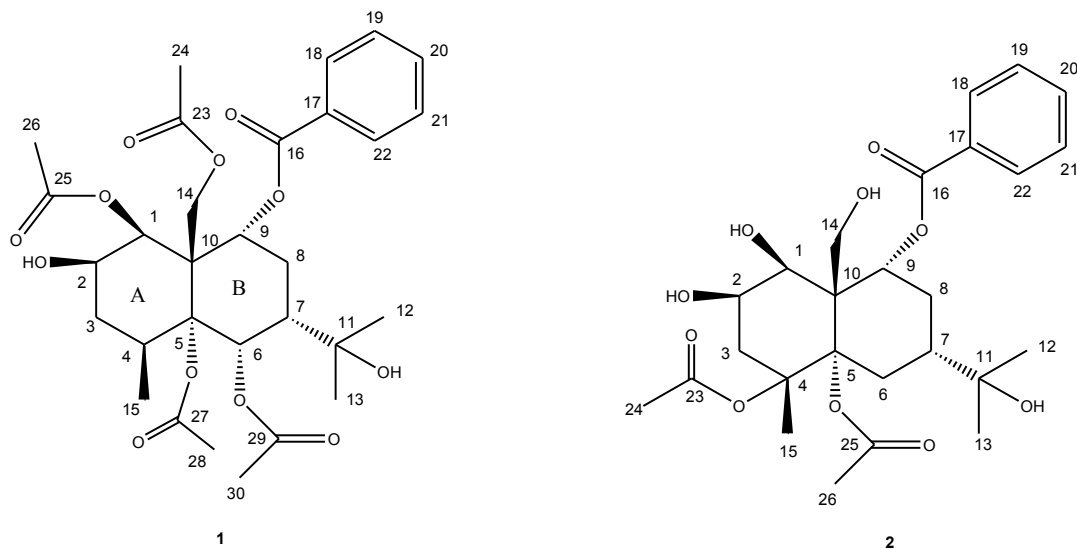
Figure 1. Key ¹H ¹H COSY and HMBC correlations of compounds 1 and 2.

Figure 2. Structures of compounds 1 and 2.



Compound **2** was obtained as a yellow gum. The HR-TOF-MS exhibited an ion peak at m/z 509.2385 $[M + H]^+$ (calcd. for $C_{26}H_{37}O_{10}$, 509.2381), indicating that the molecular formula was $C_{26}H_{36}O_{10}$ with nine degrees of unsaturation. The NMR data (Table 1) in the downfield region of **2** were similar to those of the compound **1**, 1 β ,5 α ,6 α ,14-tetraacetoxy-9 α -benzoyloxy-7 β H-eudesman-2 β ,11-diol, which was isolated from the same fungus. However, the deshielded oxymethine proton of H-6 was absent in **2**. In the upfield region of the NMR spectra of **2**, two methylene protons at δ_H 2.18 and 1.80 (2 m, H₂-6) were added, whereas two methyl were absent (Table 1), when compared with those of compound **1**. The doublet Me proton signal was downshifted to δ_H 1.37, and showed itself as a singlet in the upfield region of the NMR spectra of **2** as compared with that of compound **1**. These differences suggested that C-4 was substituted in **2**, and two acetoxy groups were transformed to hydroxyl groups. No long range correlations were observed from oxymethine protons of H-1 and H-2, oxymethylene protons of H₂-14 to any of the two acetoxy ester carbonyl carbons, indicating that C-1, C-2 and C-14 were hydroxylated. The oxygenated quaternary carbon at δ_C 68.8 (s) was attributed to C-4 from the observation of long range correlations from the oxymethine proton signal at δ_H 5.36 (m, H-2) and the singlet Me proton signal at δ_H 1.37 (s, Me-15) to the carbon signal at δ_C 68.8 (s) (Figure 1). NOESY correlations from two acetoxy Me proton signals to H-1 and H-2 at ring A positioned two acetoxy groups at C-4 and C-5. The oxygenated isopropyl group was assigned at C-7 from the analyses of HMBC cross peaks of Me-12/C-7, M-13/C-7 and H-9/C-7. C-7 was proved to be linked to oxygenated quaternary carbon C-5 via a methylene bridge from analysis of the HMBC cross peaks from H₂-6 and H-7 to C-5. The relative configuration of **2** was deduced to be the same as in **1**. The NOESY cross peak of Me-26/H-1 α , Me-15 β /H₂-14 β implied a *trans* eudesmane. The hydroxyl groups at C-1 and C-2 were β -oriented as shown by the observation of NOESY cross peaks of H-1 α /H-3 α and H-2 α /H-3 α in the NOESY experiment. H-7 and H-9 were proved to be β -oriented from the analyses of NOESY peaks of H₂-14 β /H-7 β and H₂-14 β /H-9 β . The new eudesmane derivative was elucidated as 4 α ,5 α -diacetoxy-9 α -benzoyloxy-7 β H-eudesman-1 β ,2 β ,11,14-tetraol. The assignment of NMR signals of **2** is listed in Table 1.

Two new compounds were tested for tyrosinase inhibitory activities [19]. 1 β ,5 α ,6 α ,14-tetraacetoxy-9 α -benzoyloxy-7 β H-eudesman-2 β ,11-diol and 4 α ,5 α -diacetoxy-9 α -benzoyloxy-7 β H-eudesman-1 β ,2 β ,11,14-tetraol showed IC₅₀ values of 14.8 μ M and 22.3 μ M, when the active compounds were compared to the standard tyrosinase inhibitor kojic acid (IC₅₀ = 21.2 μ M).

3. Experimental Section

3.1. General Experimental Procedures

Optical rotations were recorded on a Perkin-Elmer-341 polarimeter. The IR spectra (CHCl₃) were run on a NicoletAvatar-360FT-IR spectrometer. ¹H NMR (500 MHz) and ¹³C NMR (125 MHz) spectra were measured at 25 °C on a Bruker AVANCE DMX 500 NMR spectrometer with TMS as internal standard. TOF-MS were recorded on a GCT-Premier GC-TOF-MS spectrometer. ESIMS were recorded on an Agilent 6460 Triple Quad LC/MS. TLC was performed using Merck precoated plates (Silica gel 60 F254) of 0.25 mm thickness. Sephadex LH-20 (Amersham) was used for column chromatography.

3.2. Fungal Cultivation and Stress Applications

The fungus *Pestalotiopsis* sp. Z233 was separated from the marine algae *Sargassum horneri*, which was collected from seashore in Wenzhou, China, and identified by its ITS-5.8s rDNA sequences. Subcultures of the organism are deposited at the Department of Ocean Science and Engineering, Zhejiang University. Cultures were separated into control (5 L) and stressed groups (5 L). The control fungus was grown in the culture medium consisting of ($\text{g}\cdot\text{L}^{-1}$) sucrose (66.0 g), yeast extract (10.0 g), silkworm chrysalis (30.0 g), $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ (0.4 g), and KH_2PO_4 (0.4 g) in sterilized and filtrated natural seawater collected from Wenzhou, China. The stressed culture medium consisted of additional $50\ \mu\text{mol/L}$ CuCl_2 . The fermentations were carried out at $24\ ^\circ\text{C}$ for 10 days.

3.3. Extraction and Isolation

The whole culture of the control and stressed broth of *Pestalotiopsis* sp. Z233 (5 L) were filtered. The air-dried mycelia of control (48.4 g) and the stressed group (45.4 g) were extracted at room temperature with MeOH ($3 \times 1\ \text{L}$), respectively. The extracts were evaporated in *vacuo* to afford a gummy residue for both Cu treated (2.2 g) and corresponding control (2.7 g). The residues were partitioned in H_2O (500 mL) and extracted successively with EtOAc ($3 \times 500\ \text{mL}$) and *n*-butanol ($3 \times 500\ \text{mL}$). The EtOAc and *n*-butanol extracts of the treated and control cultures were subjected to TLC examination on aluminium sheets pre-coated with Silica gel 60 F 254 (Merck). The spots were applied in as equal amounts as possible. The plates were developed in the following developing solvent systems: benzene–acetone (6:1), benzene–EtOAc (5:1) petroleum ether–EtOAc (5:1) for the EtOAc extract; CHCl_3 –MeOH (3:1), CH_2Cl_2 –MeOH (4:1) and benzene– CHCl_3 –MeOH (1:3:1) for the *n*-butanol extract. After development, the plates were examined under UV light (250 nm) to locate any additional spots in the different extracts of the treatments in comparison with those of the corresponding control extracts. The spots on the plates were also visualized by spraying with an EtOH– H_2SO_4 solution. Two additional compounds were detected on the plates developed in benzene–acetone (6:1) solvent system in the EtOAc extract of stress elicited mycelium. Several prep-TLC plates were prepared and the target compounds were isolated by preparative TLC in the developing solvent systems of benzene–acetone (6:1). The crude compounds were applied to a Sephadex LH-20 column ($1 \times 80\ \text{cm}$, 38 g, Amersham), and eluted with acetone to yield pure compounds **1** (8.4 mg) and **2** (7.0 mg).

1 β ,5 α ,6 α ,14-Tetraacetoxy-9 α -benzoyloxy-7 β *H*-eudesman-2 β ,11-diol (**1**): yellowish oil; $[\alpha]_{\text{D}}^{24} -22$ (*c* 0.001, CHCl_3); UV (MeOH) λ_{max} (log ϵ) 230 (4.11), 254 (4.23), 280 (4.56) nm; IR ν_{max} 3422, 1766, 1614, 1515, 1333, 1111, 721 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Table 1; ESIMS m/z 593 $[\text{M} + \text{H}]^+$; HR-TOF-MS m/z 593.2595 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{30}\text{H}_{41}\text{O}_{12}$, 593.2593).

4 α ,5 α -Diacetoxy-9 α -benzoyloxy-7 β *H*-eudesman-1 β ,2 β ,11,14-tetraol (**3**): yellow gum; $[\alpha]_{\text{D}}^{24} -26$ (*c* 0.001, CHCl_3); UV (MeOH) λ_{max} (log ϵ) 230 (4.30), 254 (4.17), 280 (4.43) nm; IR ν_{max} 3416, 1766, 1615, 1513, 1324, 1113, 824 cm^{-1} ; ^1H NMR and ^{13}C NMR, see Table 1; ESIMS m/z 509 $[\text{M} + \text{H}]^+$; HR-TOF-MS m/z 509.2385 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{26}\text{H}_{37}\text{O}_{10}$, 509.2381).

3.4. Tyrosinase Inhibition Assay

Tyrosinase inhibition assay were performed in 96-well microplate format using SpectraMax 340 microplate reader according to the previously developed method [19]. The compounds were initially screened for the *O*-diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All active inhibitors from the preliminary screening were subjected to IC₅₀ studies. Compounds were dissolved in methanol to a concentration of 2.5%. Mushroom tyrosinase (28 nM) were preincubated with the compounds in 50 nM Na-phosphate buffer (pH 6.8) for 10 min at 25 °C. Then the L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 37 °C) due to the formation of the DOPACHROME for 10 min. The percent inhibition of the enzyme was calculated as follows:

$$\text{Percent inhibition (\%)} = [(B - S)/B] \times 100$$

The *B* and *S* are the absorbance for the blank and samples, respectively. After screening of the compounds, median inhibitory concentration (IC₅₀) was calculated. Kojic acid was used as standard inhibitors for the tyrosinase.

4. Conclusions

In this study, two novel sesquiterpenes were produced as stress metabolites by the mycelia of cultured *Pestalotiopsis* sp. Z233 in response to abiotic stress elicitation by CuCl₂. These new metabolites possess interesting eudesmane skeleton, and exhibited potent tyrosinase inhibitory activities, indicating that metal stress elicitation on marine fungi was a promising strategy to discover new bioactive natural compounds.

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Conflict of Interest

The authors declare no conflict of interest.

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