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Dalestones A and B, two anti-inflammatory cyclopentenones from *Daldinia eschscholzii* with an edited strong promoter for the global regulator *LaeA-like* gene

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[ABSTRACT] Replacement of the native promoter of the global regulator *LaeA-like* gene of *Daldinia eschscholzii* by a strong *gpdA* promoter led to the generation of two novel cyclopentenone metabolites, named dalestones A and B, whose structures were assigned by a combination of spectroscopic analysis, modified Mosher's reaction, and electronic circular dichroism (ECD). Dalestones A and B inhibit the gene expression of TNF-α and IL-6 in LPS-induced RAW264.7 macrophages.

[KEY WORDS] *Daldinia eschscholzii*; Global regulator; Cyclopentenone

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Introduction

Secondary metabolites (SMs) possess many pharmacological properties and play a basic role in drug discovery and development. Fungi are well-known producers of structurally diverse SMs with therapeutic significance, such as penicillin, caspofungin, and lovastatin [1-2]. Recent advances in fungal genome sequencing and bioinformatics analysis indicate that the SM biosynthetic potential of fungi is underappreciated as a greater number of SM biosynthetic gene clusters are present in the genome. This is because many of the SM gene clusters are silent or lowly expressed under the laboratory conditions [3-4]. Accordingly, great efforts have been made to activate the silent/cryptic biosynthetic gene clusters, leading to the discovery of novel SMs [5-7]. The activation strategy includes the simulation of environmental conditions, using chemical inhibitors to modify chromatin structures or genetic manipulation [8-9]. LaeA was first characterized as a global regulator in *Aspergillus nidulans* and was also found in phylogenetically diverse fungi such as *Trichoderma reese*, *Penicillium* sp., and *Fusarium* sp. [10-13]. *Daldinia eschscholzii* is a mantis-associated fungus which produces over 30 polyketides that are proposed to be generated from four biosynthetic pathways [14-15]. Bioinformatic analysis of the *D. eschscholzii* genome sequence indicates that it encodes 30 biosynthetic gene clusters including 27 polyketide synthases (PKSs), and 2 nonribosomal peptide synthases (NRPSs), indicating the biosynthetic potential of *D. eschscholzii* is underrepresented by the characterized SMs.

Cyclopentenones (CPs) possess important biological functions including but not limited to anti-inflammatory (e.g. prostaglandins) [16], anticancer (e.g. cyclopentenone and prostaglandins) [17], plant defense (e.g. jasmonic acid related precursors OPDA and dnOPDA) [18], phytotoxic activity (e.g. terrein) [19] (Fig. 1). Here, we present the isolation and structural elucidation of dalestones A and B (**1** and **2**), two new CPs induced through the promoter replacement of the global regu-

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lator *LaeA-like* gene in the *D. eschscholzii* genome. The biological evaluation showed that **1** and **2** are anti-inflammatory.

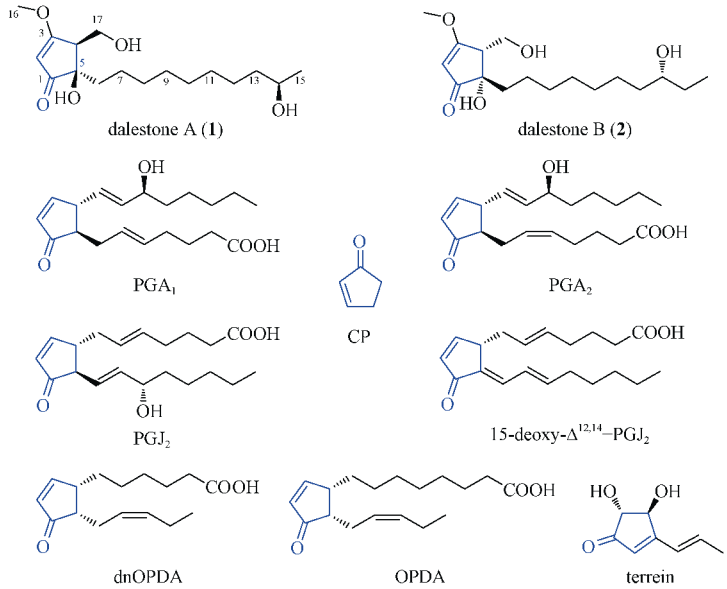

Fig. 1 Structures of dalestones and known cyclopentenones (CPs)

Results and Discussion

To begin this work, we first identified the *LaeA-like* gene (encoded by GME5509) by a BLAST search of the *D. eschscholzii* genome. *LaeA-like* encodes a 334-amino acid protein, which shares 38% and 50% sequence identities with those of *A. nidulans* (accession no: AAQ95166) [10] and *Botrytis cinerea* (accession no: A0A0B5L7R4) [20], respectively. To activate or overproduce SMs [10], the native promoter of *LaeA-like* in *D. eschscholzii* was substituted by a strong *gpdA* promoter (amplified from the *A. nidulans* genomic DNA) at the genome position (Fig. 2) [21]. The engineered strain was fermented in liquid ME media using wild-type (WT) strain as a control. After a 10-day cultivation, the culture broth was extracted with ethyl acetate. The HPLC analysis of extracts showed that the chemical profile of the P_{gpdA}: *LaeA-like* strain changed significantly in comparison to that of the WT strain (Fig. 3). Among them, the production of **3** increased dramatically [22]. Compound **3** is the reduced form of **4** that we isolated before, and was therefore proposed to be generated by

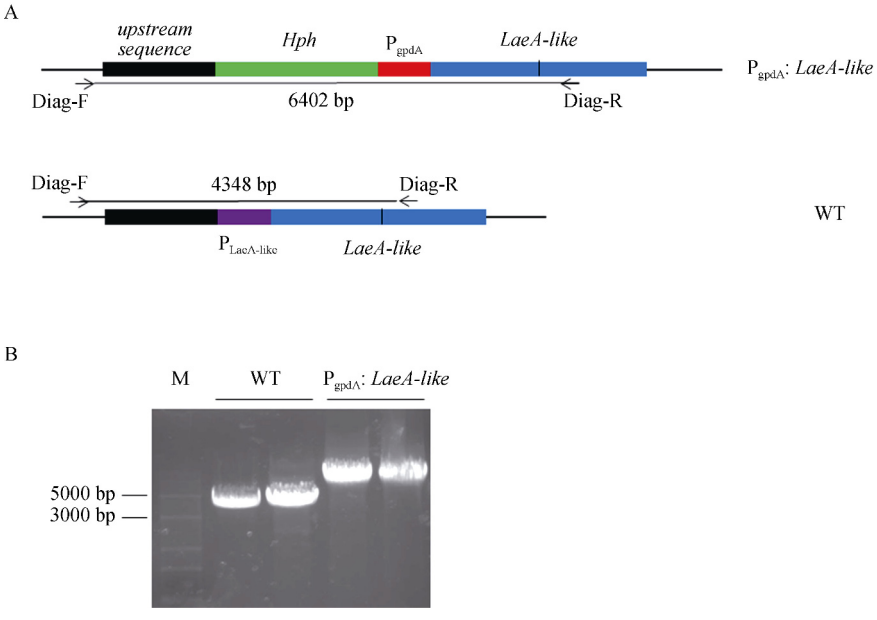

Fig. 2 Generation and verification of P_{gpdA}: *LaeA-like* strain of *D. eschscholzii*. A) Schematic illustration for constructing P_{gpdA}: *LaeA-like* strain. B) Verification of P_{gpdA}: *LaeA-like* strain using diagnostic PCR primers Diag-F and Diag-R

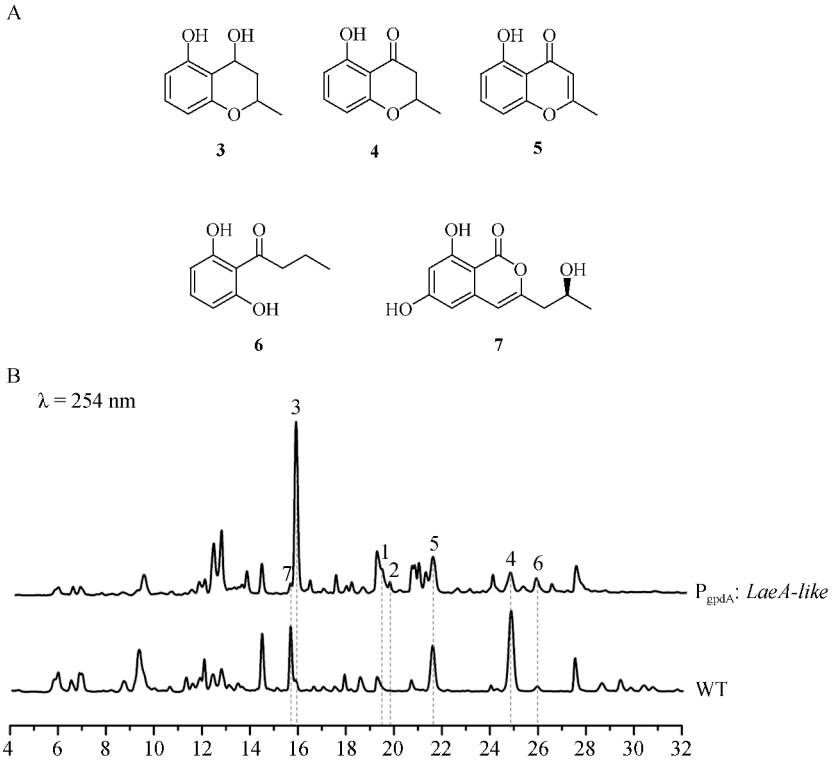

Fig. 3 HPLC profiles of the extracts from the wild-type (WT) and P$_{gpdA}$: *LaeA-like* strains of *D. eschscholzii*. (A) Known compounds regulated by LaeA-like. (B) The comparison between the crude extracts of the WT and P$_{gpdA}$: *LaeA-like* strains. Detection was carried out at 254 nm

the same biosynthetic pathway of **4**−**6** [14, 23]. Moreover, such a promotor replacement influenced simultaneously but differently the production of **4**−**5** (decreased), **6** (escalated) and the hexaketide **7** (declined). In particular, the HPLC profiling indicated two newly appeared peaks (arising from **1** and **2**) in the EtOAc extract derived from P$_{gpdA}$: *LaeA-like* strain (Fig. 3). To characterize the two compounds, a large-scale fermentation (20 L) was carried out. Compounds **1** and **2** were isolated from the extracts and their structures were determined by spectroscopic data, Mosher's reaction, and computational methods.

Dalestone A (**1**) was evidenced to have a molecular formula of $C_{17}H_{30}O_5$ from its HR-ESI-MS displaying its Na^+-liganded molecular ion at *m/z* 337.1986 ($C_{17}H_{30}O_5Na$ requires 337.1985). This molecular formula agrees with its ^{13}C NMR data and indicates its possession of three degrees of unsaturation. The ^1H NMR spectrum of **1** (Table 1) indicates the presence of two methyl protons at δ_H 1.02 (d, J = 6.0 Hz, H-15) and 3.82 (s, H-16). The ^{13}C NMR and DEPT spectra of **1** showed a total of 17 carbons ascribable to two methyl, nine methylene, three methine (one sp^2 and two sp^3) and three quaternary carbons (one ketone). The HMBC correlations of H-2 (δ_H 5.37, s)/C-1 (δ_C 205.9), C-3 (δ_C 188.5), C-4, C-5 and H-4/C-1, C-2 (δ_C 102.8) demonstrate the presence of a five-membered ring with a $\Delta^{2,3}$ double bond as shown (Fig. 4). The ^1H-^1H COSY correlations of H-4/H-17/17-OH, and HMBC correlations of H-17/C-3, C-4, C-5 indicate the 4-hydroxymethyl substituent (Fig. 4). The HMBC correlation of H-16/C-3 (δ_C 188.5) and the chemical shift of C-16 (δ_C 59.1) implied the 3-methoxy group. In conjunction with the molecular formula and residue signals, the HMBC correlations of 5-OH/C-1, C-4, C-5, H-6/C-1, C-4, C-5, C-7 revealed a long unbranched and saturated alkyl chain connected at C-5. The remaining hydroxyl group is connected at C-14 (δ_C 66.2) on the basis of the chemical shift and the ^1H-^1H COSY correlations of H-13/H-14/H-15, H-14/14-OH. Taken together, the planar structure of **1** was established as shown in Fig. 4. To address the stereochemistry, the NOESY spectrum of **1** was acquired. The relative configuration of C-4 and C-5 was evident from the NOESY correlations of H-4/H-6 and 5-OH/H-17 (Fig. 5). The absolute configuration of C-14 was determined by a modified Mosher's method [24-25]. Comparison of the ^1H NMR chemical shifts between (*S*)- and (*R*)-MTPA esters of **1** led to the assignment of 14*R*-configuration (Fig. 6). The absolute configuration of C-4 and C-5 was assigned by ECD calculations. The acquired CD spectrum of **1** was closely similar to the ECD spectrum computed for (4*S*, 5*R*, 14*R*)-**1** but contrary to that of (4*R*, 5*S*, 14*R*)-**1** (Fig. 7). This led to the assignment of the (4*S*, 5*R*, 14*R*)-configuration of **1**.

Table 1 ^{1}H and ^{13}C NMR data of 1 and 2 (δ in ppm, *J* in Hz, DMSO-d_6)

No.	1		2	
	δ_H [a]	δ_C [b]	δ_H [c]	δ_C [d]
1		205.9		205.3
2	5.37, s	102.8	5.37, s	102.3
3		188.5		187.9
4	2.69, t, 4.3	51.7	2.69, t, 4.0	51.3
5		78.9		78.4
6	1.52, m	38.7	1.52, m	38.2
	1.41, m		1.41, m	
7	1.27, m	23.4	1.29, m	22.9
	1.13, m		1.17, m	
8	1.16–1.26, overlap	29.7 [e1]	1.21–1.29, overlap	29.4 [e]
9	1.16–1.26, overlap	29.4 [e2]	1.21–1.29, overlap	29.0
10	1.16–1.26, overlap	29.5 [f2]	1.21–1.29, overlap	29.2 [f]
11	1.16–1.26, overlap	29.9 [f1]	1.32, m	25.2
			1.21, m	
12	1.29, m	25.8	1.30, m	36.6
	1.21, m		1.25, m	
13	1.24, m	39.5	3.28, m	70.9
14	3.54, m	66.2	1.37, m	29.8
			1.26, m	
15	1.02, d, 6.0	24.1	0.83, t, 7.4	10.0
16	3.82, s	59.1	3.82, s	58.6
17	3.66, m	59.4	3.66, m	58.9
5-OH	5.08, s		5.07, s	
13-OH			4.21, d, 5.3	
14-OH	4.29, d, 4.4			
17-OH	4.24, t, 5.8		4.23, t, 5.9	

[a] 600 MHz, [b] 150 MHz, [c] 400 MHz, [d] 100 MHz; [e] and [f] are interchangeable.

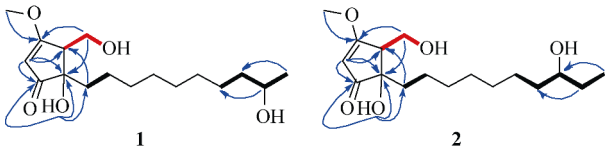

Fig. 4 ^{1}H–^{1}H COSY (black bond) and key HMBC (blue arrow) correlations of 1 and 2

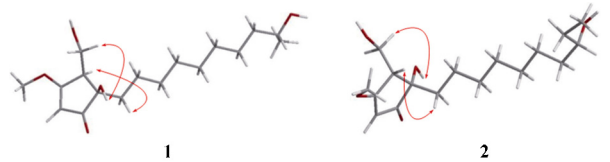

Fig. 5 Key NOESY correlations of 1 and 2

Dalestone B (**2**) has a molecular formula of $C_{17}H_{30}O_5$, too, as derived from the $[M+Na]^+$ ion at *m/z* 337.1981, in its HR-ESI-MS (calcd. for $C_{17}H_{30}O_5Na$: 337.1985). The ^{1}H and ^{13}C NMR data of **2** are closely similar to those of **1**. However, the presence of the 13-hydroxyl group in **2** was required by the ^{1}H-^{1}H COSY correlations of H-12/H-13/H-14/H-15 (δ_H 0.83, t, 7.4) and the HMBC correlations of H-15/C-13 (δ_C 70.9) (Fig. 4). Again by the NOESY correlations, modified Mosher's reaction, and ECD calculations, **2** was addressed to have the (4*R*, 5*S*, 13*R*)-configuration (Figs. 5 and 7).

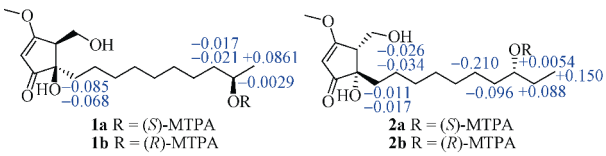

Fig. 6 δ values (δ_S–δ_R) for the MTPA esters of the methyl derivatives of 1 and 2

Compounds **1** and **2** were tested for the anti-inflammatory activity using the Gtiess method [26]. Macrophage cells were cultured (NC group), followed by being stimulated with 100 ng·mL^{-1} LPS (LPS group) or with 100 ng·mL^{-1} LPS

plus 20 μmol·L^{-1} **1** or **2** (**1** or **2** treated groups). The mRNA levels were measured by qPCR after the 6-hour stimulation. Compound **2** was demonstrated to be more inhibitory than **1** on the gene expression of TNF-*α* and IL-6 in LPS-induced RAW264.7 macrophages (Fig. 8). The 4, 5-configurations of **1** and **2** might contribute to the activity.

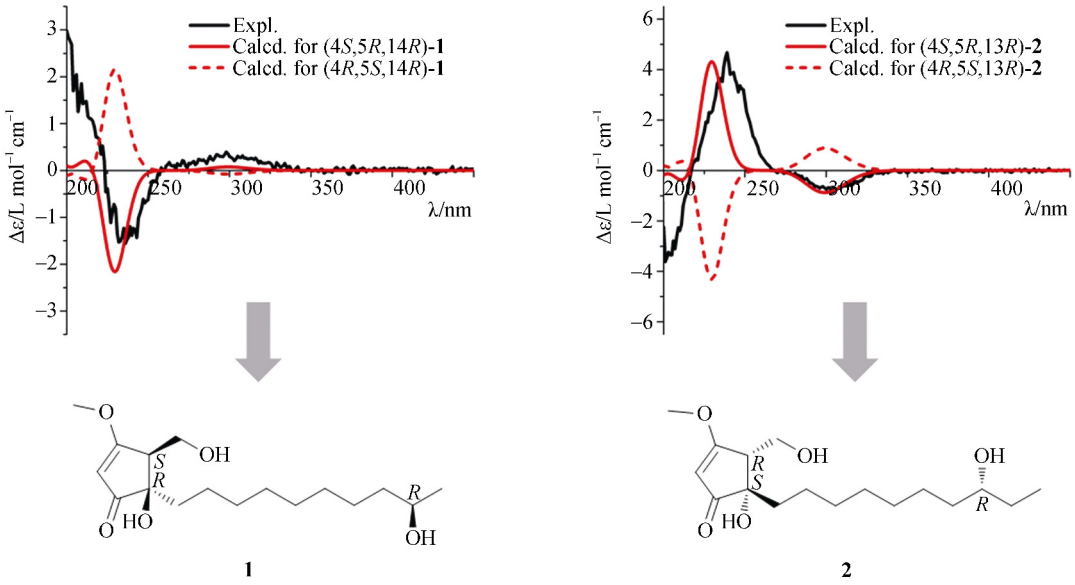

Fig. 7 Experimental and calculated ECD spectra of 1 and 2

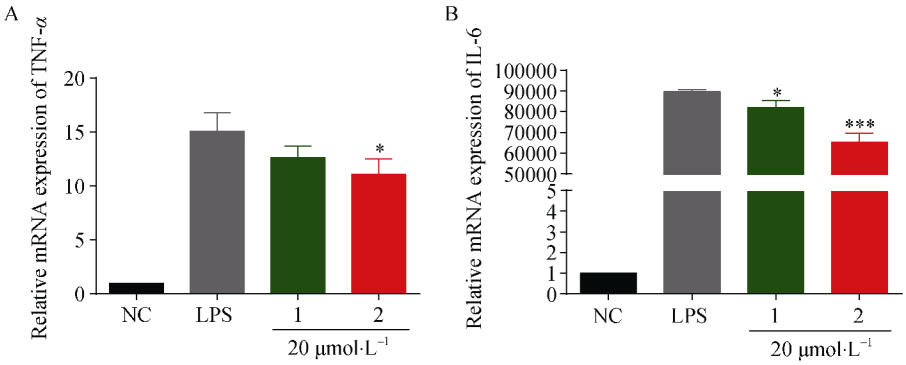

Fig. 8 The effect of 1 and 2 on the mRNA expression of TNF-*α* (A) and IL-6 (B) in LPS stimulated RAW 264.7 cells. Data were collected from three independent experiments and presented as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs* the LPS group

Conclusion

D. eschscholzii is made to produce the new anti-inflammatory cyclopentenones, dalestones A (**1**) and B (**2**), by replacing its native promoter of the global regulator *LaeA-like* gene by a strong *gpdA* promoter. The study showcases the discovery of novel small molecules by manipulating global regulator genes.

Experimental

General procedures

Optical rotations were measured in MeOH on a Rudolph Research Analytical Autopol IV automatic polarimeter. CD spectra were recorded on a JASCO-810 spectropolarimeter, and UV curves on a NANODROP 2000c (Thermo Scientific, USA). IR spectra were acquired on a Nexus 870 FT-IR Spectrometer, and mass spectra on an Agilent Accurate-Mass Q-TOF LC/MS 6530 instrument with a Poroshell 120 EC-C_{18} column (2.7 μm, 4.5 mm × 50 mm). All NMR experiments were performed in DMSO-d_6 on a Bruker DRX-400 (or DRX-600) spectrometer using TMS as an internal standard. Column (CC) and thin-layer chromatographies (TLC) were accomplished on silica gel (200–300 mesh) and GF254 (10–20 μm), respectively, both being purchased from Qingdao Marine Chemical Company, China. Gel filtration were performed on Sephadex LH-20 (Pharmacia Biotech, Sweden). Semi-preparative reversed-phase HPLC (RP-HPLC) purifications were accomplished on an ODS-2 Hypersil column (5 μm, 250 mm × 10 mm).

Strains, media and growth conditions

D. eschscholzii was cultured on PDA plates at 28 °C for 3 days. The fresh mycelium of the strain was inoculated into 1 L

flask containing 400 mL malt extract (ME) media (20 g·L^{-1} malt extract, 1 g·L^{-1} peptone and 20 g·L^{-1} sucrose). After fermentation at 28 °C with agitation (140 r·min^{-1}) for 10 days, the supernatant was harvested and extracted with EtOAc (*V*/*V*, 1 : 1) for three times.

Aspergillus nidulans LO4389 was cultured on YG medium (5 g yeast extract, 20 g glucose and 400 μL trace element solution) as described by Szewczyk *et al* [27].

Construction of gene overexpression mutants

About 1.5 kb fragments upstream (up) and downstream (down) homologous arms of the *LaeA-like* promoter (approximately 1.0 kb upstream of ATG) were amplified from genomic DNA of *D. eschscholzii*. The split-marker of hygromycin B gene (*hph-1* and *hph-2*) was amplified from plasmid pSH75. The strong *gpdA* promoter (P_{gpdA}) was amplified from genomic DNA of *A. nidulans* LO4389 to substitute the native promoter of *LaeA-like* in *D. eschscholzii*. Two P_{gpdA}: *LaeA-like* cassettes (up + *hph-1* and *hph-2* + P_{gpdA} + down) containing split *hph* marker gene was constructed by fusion PCR.

Promoter replacement was obtained by PEG-mediated protoplast transformation of two fusion PCR cassettes. Fungal protoplast preparation and transformation were performed as described [27]. Positive overexpression strains were selected on PDA plates containing 200 μg·mL^{-1} hygromycin B and verified by diagnostic PCR.

HPLC analysis

The crude extracts derived from the cultures of *D. eschscholzii* were dissolved in methanol and analyzed by HPLC at the following conditions: a linear gradient from 10% to 70% CH_3CN in water for 40 min, 100% CH_3CN for 3 min, and 10% CH_3CN in water for 2 min (flow rate: 0.5 mL·min^{-1}).

*Isolation and characterization of **1** and **2***

The P_{gpdA}: *LaeA-like* strain was cultivated in 20 L ME media at 28 °C with agitation (140 r·min^{-1}) for 10 days. Then, the fermentation was extracted with EtOAc (*V*/*V*, 1 : 1) for three times. The crude extract was fractionated by CC eluted with petroleum ether/EtOA (100 : 1→1 : 1), followed by CH_2Cl_2/MeOH (100 : 1→1 : 1) to yield 23 fractions (Frs. 1–23). Fr. 15 was gel filtrated over Sephadex LH-20 (MeOH) to afford 6 parts (Frs. 15.1–15.6). Fr. 15.6 was finally purified by semi-preparative HPLC using MeCN/H_2O (34 : 66) to yield **1** (16.8 mg, t_R 14 min) and **2** (19.4 mg, t_R 16 min).

Dalestone A (**1**). Colourless oil; [α]25 D –7.5 (*c* 0.08, MeOH); CD (MeOH) $\Delta\varepsilon_{200}$ +2.84, $\Delta\varepsilon_{236}$ –1.48; UV (MeOH) λ_{max} (log ε) 248 (3.34), 229 (3.29) nm; IR (KBr) ν_{max}: 3389, 2928, 2854, 1692, 1590, 1460, 1439, 1384, 1347, 1248, 1204, 1171, 1101, 1025, 832 cm^{-1}; HR-ESI-MS *m/z* 337.1986 [M + Na]$^+$ (calcd. for $C_{17}H_{30}O_5Na$, 337.1985). ^1H and ^{13}C NMR data, see Table 1.

Dalestone B (**2**). Colourless oil; [α]25 D –10.0 (*c* 0.16, MeOH); CD (MeOH) $\Delta\varepsilon_{201}$ –6.87, $\Delta\varepsilon_{239}$ +8.89, $\Delta\varepsilon_{303}$ –1.43; UV (MeOH) λ_{max} (log ε) 248 (3.33), 229 (3.07) nm; IR (KBr) ν_{max}: 3386, 2931, 2855, 1693, 1591, 1460, 1440, 1384, 1346, 1247, 1205, 1111, 1025, 831 cm^{-1}; HR-ESI-MS *m/z* 337.1981 [M + Na]$^+$ (calcd. for $C_{17}H_{30}O_5Na$, 337.1985). ^1H and ^{13}C NMR data, see Table 1.

Computational details.

For **1** and **2**, the geometries of the lowest-energy conformations were optimized with density functional theory (DFT) at CAM-B3LYP/6–31G (d, p) level. The corresponding excited-state calculations in CH_3OH solvent were performed at the ground-state optimized geometries. Time-dependent DFT (TDDFT) with the same functional and basis set was carried out to calculate the spin-allowed excitation energy and rotatory strength of the lowest 100 excited states. The electronic circular dishroism (ECD) spectra were generated using the program SpecDis [28] by applying a Gaussian band shape with the width of 0.25 eV, from oscillator strengths and dipole-velocity rotational strengths, respectively. The solvent effects on the electronic structures of the studied systems were evaluated by quantum chemistry method through the polarizable continuum model (PCM, dielectric constants ε = 32.64 for CH_3OH). All the ECD calculations were performed with the Gaussian 09 program [29].

Real-time quantitative PCR analysis for the TNF-α and IL-6 gene expression

The mouse macrophage cells were cultured in 24 well plates containing LPS (100 ng·mL^{-1}) and 20 μmol·L^{-1} **1** or **2**. Macrophages incubated with culture medium alone and with LPS (100 ng·mL^{-1}) were used as negative and positive controls, respectively. After 6 h stimulation, macrophages cells were collected and total RNA was extracted. Reverse transcribes were performed by using First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instruction. Real-time quantitative PCRs were carried out on iCycler Thermal Cycler system (Bio-Rad, USA) using SYBR Green master mix (Roche, Germany). Relative mRNA expression of *TNF-α* and *IL-6* were normalized to control group using GAPDH as an internal control and calculated by $2^{-\Delta\Delta CT}$ method.

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