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A new phenanthrene with a spirolactone from *Dendrobium chrysanthum* and its anti-inflammatory activities

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Abstract—Investigation of phenolic patterns from the stems of *Dendrobium chrysanthum* by HPLC–PDA–MS has led to the isolation of a new phenanthrene derivative with a spirolactone ring, dendrochrysanene (**1**), that proved to suppress the mRNA level of TNF-α, IL8, IL10, and iNOS in murine peritoneal macrophages. The structure of **1** was characterized on the basis of various NMR (^{1}H, ^{13}C, ^{1}H–^{1}H COSY, HMQC, and HMBC), mass spectrometry, and X-ray crystal diffraction data.

1. Introduction

Dendrobium chrysanthum Wall. (Ochidaceae) is distributed widely in south China and has been recorded in Chinese Pharmacopoeia. The stems of this plant, locally known as 'ShiHu' or 'HuangCao', are used in traditional Chinese medicine, for antipyretic, eyes-benefiting, and immunomodulatory effects.[1] This species contributes one of the major sources of Caulis *Dendrobii* crude drug in herbal markets for its high demand due to its abundant source.

Dendrobium plants have been reported as containing alkaloids, phenols, and terpenes. Recently, we have isolated two new pyrrolidine-type alkaloids, *trans*- and *cis*-dendrochrysanines, from the chloroform extract of the stems of this plant,[2] whereas three alkaloids of the same type were reported from *D. chrysanthum* previously.[3] However, the phenolic compounds have proved to be the most characteristic as chemotaxonomic markers for the genus *Dendrobium*, over fifty phenols comprising bibenzyls, phenanthrenes, and fluorenones reported from this genus,[4,5] some of them displayed antitumoral,[6] anti-angiogenic,[7] and anti-inflammatory activities.[8] Thus, a comprehensive investigation of the potent chemical composition of phenols in *D. chrysanthum* is of significance for the understanding of therapeutic mechanisms and quality control of this herb.

As part of our ongoing studies on *Dendrobium*, nine phenols were identified or tentatively characterized from the ethyl acetate extracts of the stems of *D. chrysanthum* by the HPLC–PDA–MS analysis. From this fraction, a new phenanthrene phenol with a spirolactone ring, dendrochrysanene **1**, along with two known phenols moscatin **2** and chrysotoxin **3** was isolated by silica gel open column chromatography. Compounds **4**–**8** were previously obtained from the same part of this plant,[9] and compound **9** (2,5-dihydroxy-4,9-dimethoxylphenanthrene) was reported from *Bulbophyllum vaginatum* (Orchidaceae).[10]

As the stems of *Dendrobium* species are used to reduce fever or promote secretion of saliva and some phenols isolated from some *Dendrobium* plants were reported to exhibit anti-inflammatory effects,[8] furthermore,

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excess production of TNF-α, IL-8, IL-10, and NO may contribute to septic shock, tissue injury, local or systemic inflammatory disorders and autoimmune diseases,[11–13] compound **1** was investigated for the effects on the production of TNF-α, IL-8, IL-10 (interleukin-8 and 10), and iNOS in LPS-stimulated mouse peritoneal macrophages.

2. Results and discussion

Dendrobium chrysanthum Wall. (Ochidaceae) was collected in Yunnan province, People's Republic of China. Preliminary experiments by thin-layer chromatography indicated the presence of alkaloids using Dragendorff's reagent. Ethyl acetate and *n*-BuOH were successively used to partition against the acidic crude extract of the stems and yielded the non-alkaloids.

HPLC–PDA–MS analysis of the ethyl acetate fraction showed the presence of nine major phenols. The structures of peak 4–8 were identified as moscatilin (**4**), gigantol (**5**), crepidatin (**6**), chrysotobibenzyl (**7**), and dengibsin (**8**) by a direct comparison with the authentic samples based on the retention behavior, UV and MS data obtained on-line.[9] Compounds **2**, **3**, and **9** were putatively characterized as moscatin (**2**), chrysotoxin (**3**), and 2,5-dihydroxy-4,9-dimethoxylphenanthrene (**9**), respectively, by referring to the UV and MS data in the literature (Fig. 1). Moreover, in the ESI-MS spectrum of *D. chrysanthum*, compound **1** was first detected which exhibited a $[M+H]^+$ ion at *m*/*z* 509 and the unambiguous identification of the structure initiated our effort for further phytochemical investigation.

Sequential applications of Sephadex LH-20 and silica gel column chromatography of this fraction gave the new compound **1** (26 mg), the known phenanthrene **2** (6.5 mg), and bibenzyl **3** (2 mg). Compound **1** was characterized by high-resolution mass spectrometry, NMR (^1H, ^{13}C, ^1H–^1H COSY, HMQC, and HMBC) spectroscopy, and X-ray crystal diffraction analysis. Compounds **2** and **3** were identified by comparing to the reported data.[6] Moreover, the retention behavior, UV and MS data of compounds **1**–**3**, together with those of compounds **4**–**8** isolated previously, were investigated, and consistent with the LC/MS results obtained, which confirmed the presence of these compounds identified in *D. chrysanthum*.

Compound **1** was obtained as yellow needles from an acetone/petroleum ether solution. As described above, the ESI-MS showed an $[M+H]^+$ ion at *m*/*z* 509 and a $[M-H]^-$ ion at *m*/*z* 507, while HRESI-MS of **1** in the

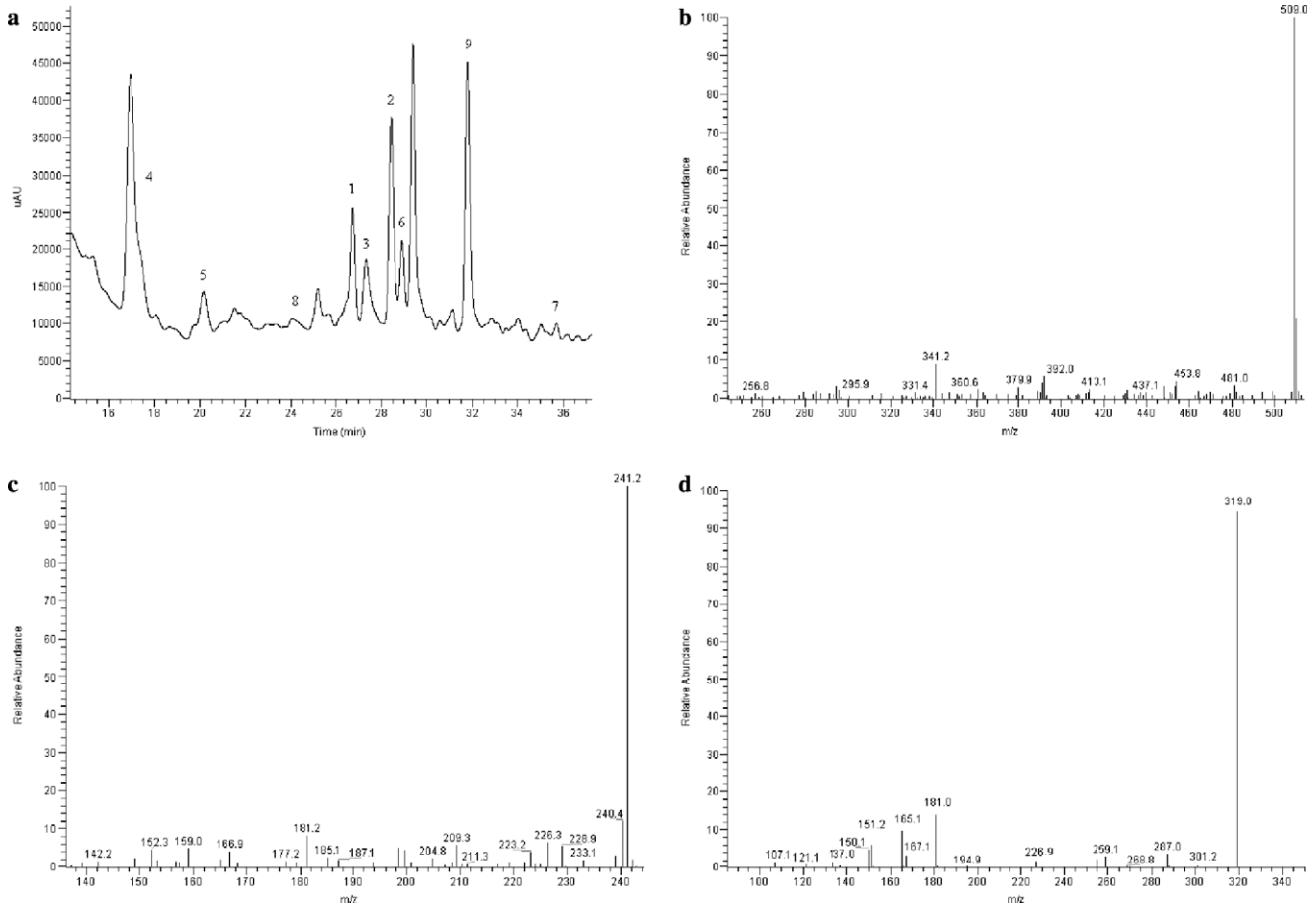


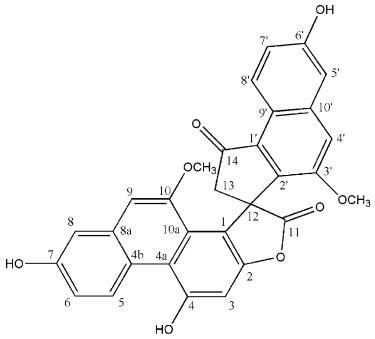
Figure 1. (a) HPLC/UV chromatograms (280 nm) for the extracts of *D. chrysanthum* and ESI-MS spectra of (b) dendrochrysanene, (c) moscatin, and (d) chrysotoxin.

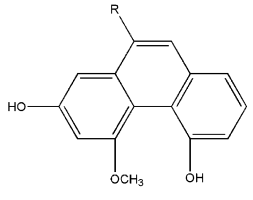
negative mode showed a $[M-H]^-$ ion at *m/z* 507.1102 (calculated for 507.1080), which indicated a molecular formula of $C_{30}H_{20}O_8$. The IR spectrum exhibited absorption at 3429 cm^{-1} (OH), 1785 cm^{-1} (carbonyl), and 1627, 1589, 1460, 977, 829, and 675 cm^{-1} (aromatic rings), while the UV spectrum of **1** showed absorption maxima at 223, 281, and 318 nm, similar to those of phenanthrene derivatives.[10] The ^1H and ^{13}C NMR spectra of **1** (acetone-d_6, Table 1) were analyzed with the help of the 2D COSY and HMQC experiments, suggesting the presence of a phenanthrene moiety and a naphthalene moiety. In the phenanthrene unit, there were three aromatic protons of an ABX spin system assigned to H-5 [δ 9.61 (1H, d, J = 9.4 Hz)], H-6 [δ 7.03 (1H, dd, J = 2.8, 9.4 Hz)], and H-8 [δ 7.11 (1H, d, J = 2.8 Hz)], and two aromatic proton singlets at δ 7.23 and δ 6.90 for H-3 and H-9, respectively.[10] For the naphthalene unit, there was an aromatic proton singlet at δ 7.34 (H-4′) together with three aromatic protons at δ 7.25 (1H, d, J = 2.4 Hz), δ 7.27 (1H, m), and δ 9.08 (1H, d, J = 9.1 Hz) of another ABX spin system, assigned to H-5′, H-7′, and H-8′, indicating a 1,2,3,6-tetra-substituted naphthalene moiety.[14] In addition, two methoxyl groups at δ 3.24 (3H, s) and δ 3.59 (3H, s), and three hydroxyl groups at δ 8.49 (1H, br s), δ 8.81 (1H, br s), and δ 10.13 (1H, br s), all exchangeable with D_2O, were observed. The ^{13}C NMR resonance spectrum of **1** gave 30 carbon signals, among them 24 aromatic carbons in the downfield were consistent with the assumption of the phenanthrene and naphthalene moieties, and the signal at δ 53.7 and 55.1 attributed to the two methoxyl carbons. The remaining four carbons were observed as two carbonyl carbons at δ 178.7 (C-11) and δ 204.0 (C-14), a quaternary carbon at a δ 53.1 (C-12), and a methylene carbon at δ 49.7 (C-13) bearing two protons (H-13a, δ 3.21 and H-13b, 3.76), which was similar to the spirolactone moiety of blespirol, a compound isolated from *Bletilla striata*.[15] The significant difference between them was found that the methylene carbon signal (C-14) at δ 39.1 in blespirol was replaced by the carbonyl carbon (δ 204.0) in **1**, corresponding to the downfield shift of the methylene protons (H-13a and H-13b). Thus, compound **1** was proposed to be a phenanthrene derivative connected to a naphthalene moiety linked with a spirolactone.

Table 1. ^{13}C and ^1H NMR data of **1** in acetone-d_6[a,b]

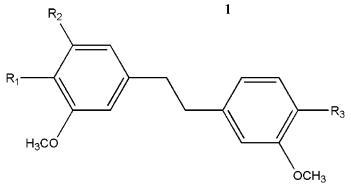
Pos.	^{13}C	^1H
1	112.9	
2	152.2	
3	98.0	7.23 s
4	156.9	
5	129.6	9.61 d (9.4)
6	114.2	7.03 dd (2.8, 9.4)
7	155.1	
8	109.9	7.11 d (2.8)
9	104.9	6.90 s
10	152.5	
4a	118.7	
4b	120.4	
8a	134.0	
10a	124.4	
11	178.7	
12	53.1	
13	49.7	3.21 d (18.4)
		3.76 d (18.4)
14	204.0	
1′	146.6	
2′	132.4	
3′	154.0	
4′	111.3	7.34 s
5′	109.0	7.25 d (2.4)
6′	156.4	
7′	118.0	7.27 m
8′	124.8	9.08 d (9.1)
9′	118.8	
10′	137.3	
10-OCH_3	53.7	3.24 s
3′-OCH_3	55.1	3.59 s
4-OH		8.81 br s
7-OH		8.49 br s
6′-OH		10.13 br s

[a] Chemical shifts in parts per million, J values in hertz in parentheses.
[b] Assignments were made using COSY, HMQC, and HMBC techniques.

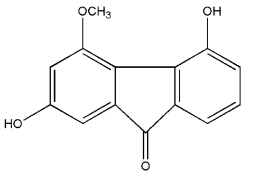




2 R=H
9 R=OMe



3 R_1=OH, R_2=R_3= OMe
4 R_1=R_3=OH, R_2= OMe
5 R_1=H, R_2=R_3=OH
6 R_1= R_2= OMe, R_3=OH
7 R_1= R_2=R_3= OMe



8

In order to establish the ring substitution patterns and the linkage sites, a careful examination of HMBC was performed (Fig. 2), which provided detailed information to link the partial structure, obtained by the analysis of the COSY and HMQC spectra. The correlation between H-9 and C-8 (109.9), H-8 and C-9 (104.9), indicated the assignment of H-9, while the presence of the cross-peaks between C-4a (118.7) and H-5, H-3, and the correlation between H-3 and C-2 (152.2) confirmed the assignment of H-3. The methoxyl group proton signal at δ_H 3.24 had ^3J correlations to the oxygenated aromatic carbon at δ 152.5 (C-10) and the correlation between H-9 and C-10 indicated that the methoxyl located at C-10. Furthermore, the hydroxyl locations at C-4 (156.9) and C-7 (155.1) were decided on the basis of the HMBC cross-peaks between OH-4 (δ_H 8.81) and C-4, C-3 (98.0), OH-7 (δ_H 8.49) and C-7, C-8, respectively. The assignment and substitution patterns of the naphthalene part were also deduced by the analysis of the HMBC spectra.

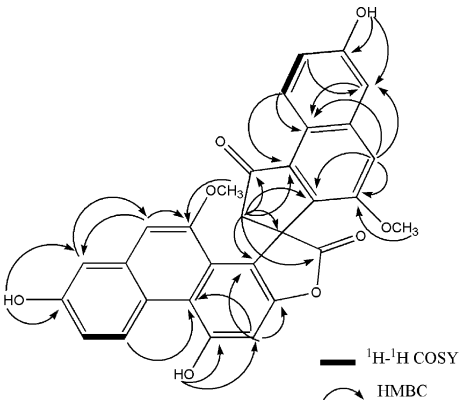


Figure 2. Selected HMBC and COSY correlations of **1**.

The correlations between H-4′ and C-5′ (109.0), C-9′ (124.8) allowed the assignment of H-4′, while the second methoxyl signal at δ 3.59 substituted on C-3′ (154.0) was according to the cross-peaks between these methoxyl protons and C-3′, H-4′ and C-3′, and the correlations from the OH-6′ (δ_H 10.13) to C-6′, C-5′ (109.0) suggested that C-6′ was substituted by the third hydroxyl group.

Significant correlations between the two methylene protons (H-13a and H-13b) and both the carbonyl carbons (C-11 and C-14) and the quaternary carbon (C-12) were observed in the HMBC spectrum, which confirmed the structure of the spirolactone rings. Moreover, their locations were determined to be at C-1 and C-2 on the phenathrene rings, while at C-1′, C-2′ on the naphthalene part, respectively, based on the HMBC correlations between the two methylene protons (H-13a and H-13b) and C-1, C-1′, and C-2′, between H-3 and C-1, C-2, between H-8′ and C-1′, and between H-4′ and C-2′. Consequently, the above spectral data established the structure of compound **1**, dendrochrysanene, and systematically named as 2-hydro-7,7′,10′-trihydroxy-4-4′-dimethoxylspiro[(1*H*)-cyclopenta[*a*]naphthalene-3,3′-(2′*H*)-phenanthro[2,1-*b*]furan]-1,2′-dione, which was also confirmed by X-ray diffraction analysis (Fig. 3).

Extract of Caulis *Dendrobii* and its chemical constituents have been reported to display wide spectral pharmacological activities such as immunostimulation, anti-inflammation, antitumor, and antiangiogenesic.[3] To disclose the biological active components and the related mechanisms, the inhibition effects of compound **1** on the production of pro-inflammatory cytokines and iNOS were investigated. The effects of compound **1** on the mRNA level of TNF-α, IL-8, IL-10, and iNOS from mouse peritoneal macrophages when stimulated by LPS were examined, respectively. As the RT-PCR analysis results shown, all of TNF-α, IL-8, IL-10, and iNOS mRNAs were induced readily from mouse peritoneal macrophages by LPS. However, these inductions were inhibited by compound **1** in a concentration-dependent manner: 1.1 μg/ml of compound **1** partially suppressed the mRNA level of TNF-α and IL-8 from LPS activated macrophages, respectively, and 11.2 μg/ml of compound **1** significantly inhibited the amount of TNF-α and IL-8 mRNAs (Figs. 4a and b). Addition of compound **1**

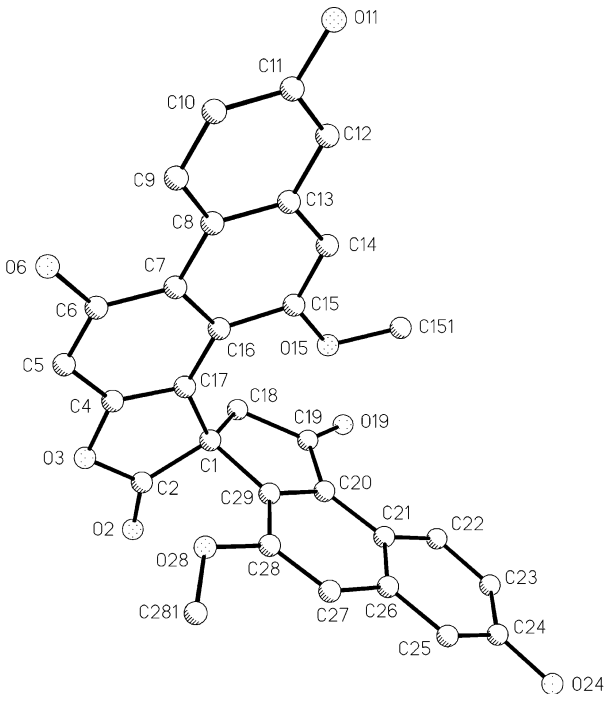


Figure 3. A perspective view of **1** by X-ray diffraction.

partially suppressed IL-10 and iNOS mRNAs at 0.11 and 1.1 μg/ml, and 11.2 μg/ml of compound **1** significantly inhibited IL-10 and iNOS at the mRNA level (Figs. 4c and d). The inhibition on production of these inflammatory mediators may explain, in part, the beneficial effects of compound **1** suggested as an anti-inflammatory agent.

3. Experimental

3.1. General experimental procedures

Optical rotations were determined with Horiba SEPA-300 polarimeter. UV spectra were measured with a Shimadzu UV-2200 spectrophotometer. IR spectra were measured with a Jasco FT/IR-230 infrared spectrometer. NMR spectra were recorded on a Bruker ACF-500 instrument with acetone-d_6 as solvent. Low-resolution ESI-MS (negative ions) were detected on an Agilent 1100 MSD and high-resolution ESI-MS were performed on a mass spectrometer with a negative mode. Finnigan DECA XP Plus LC/MS with a Zorbax SB-C_{18} column (5 μm, 250 × 4.6 mm) was used for the analysis of compounds **1**–**9** using a gradient elution of solvent A: acetonitrile and solvent B: 5‰ formic acid in water at a flow rate of 0.8 ml/min as follows: 32% A (15 min), 15–65% A (30 min), and 65–32% (10 min).

3.2. Plant material

Dendrobium chrysanthum was collected in Yunnan province, PR China, in August 2002 and authenticated by Prof. Luoshan Xu, Department of Pharmacognosy, China Pharmaceutical University. A voucher specimen (No. DC-YN0208-1) is deposited at the Herbarium of China Pharmaceutical University (CPU).

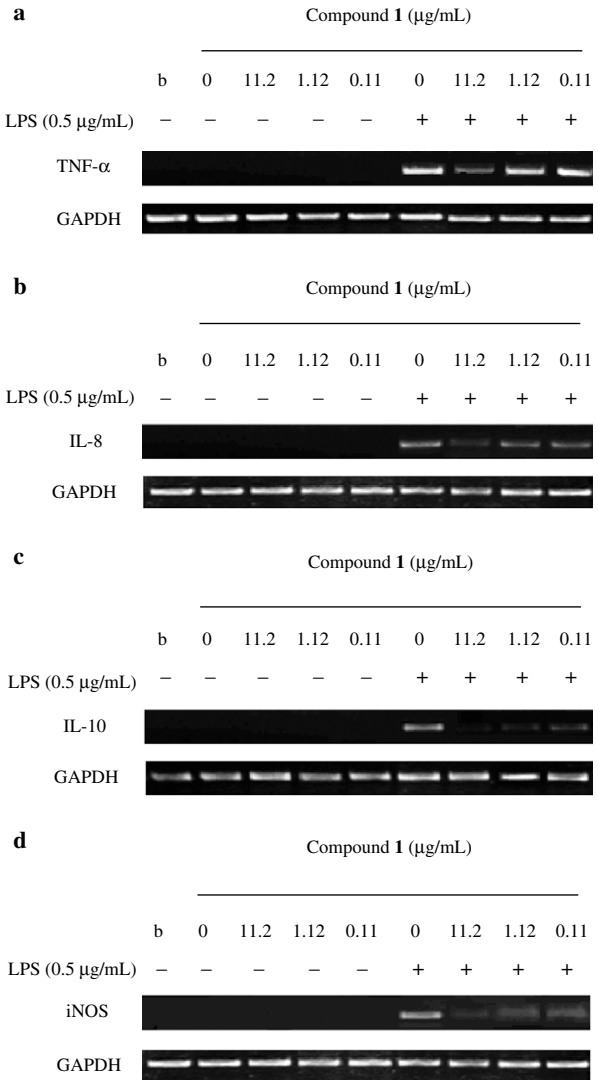


Figure 4. Inhibitory effects of compound **1** on TNF-α, IL-8, IL-10, and iNOS production. Compound **1** inhibited TNF-α, IL-8, IL-10, and iNOS production in LPS induced mouse peritoneal macrophages. Mouse peritoneal macrophages were first treated with compound **1** for 1 h, then were further cultured in medium with 0.2 μg/ml LPS and/or with various concentrations of compound **1** for 1 h to detect TNF-α (a), IL-8 (b), IL-10 (c), and iNOS (d) mRNA in the cells by RT-PCR, respectively. Similar results were obtained in four independent experiments. Data reported are means ± SEM of four independent experiments. b, blank.

3.3. Extraction and isolation

The air-dried stems of *D. chrysanthum* (10 kg) were cut into small pieces and extracted with 95% EtOH under reflux (3 × 50 L) for 3 h each. After removal of solvent in vacuo, the extract (500 g) was suspended in 0.1 mol/l HCl and extracted with EtOAc and *n*-BuOH successively to yield the non-alkaloids. The ethyl acetate extract (280 g) was washed with H_2O to pH 7 and subjected to silica gel column chromatography eluting with petroleum ether–EtOAc, affording seven fractions. Fraction IV from the eluate of petroleum ether–EtOAc (9:1) was subjected to silica gel column chromatography with CH_2Cl_2–acetone gradient system to provide compounds **2** (6.5 mg) and **3** (2 mg), and further Sephadex LH-20 column chromatography with CH_2Cl_2–MeOH (1:1) of fraction *V* from the eluate of petroleum ether–EtOAc (8:2) gave compound **1** (26 mg).

3.4. Compound 1

Yellow needles. $[\alpha]_D^{24}$ ±0 (*c* 0.25, MeOH). UV (MeOH) λ_{max} (log ε) 223, 281, 318 nm. IR (KBr) υ_{max} 3429, 1785, 1627, 1589, 1460, 977, 829, 675 cm^{-1}. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra (acetone-d_6): Table 1. ESI-MS (negative ions): *m/z* 507 $[M-H]^-$; HRESI-MS: *m/z* 507.1102 (calculated for $C_{30}H_{20}O_8$, *m/z* 507.1080). Finnigan LTQ LC/MS *m/z* 509 $[M+H]^+$.

3.5. Crystallographic data of compound 1

$C_{30}H_{20}O_8$, monoclinic, spacegroup C2/c, a = 28.696(5), b = 12.005(2), c = 19.001(4) Å, β = 123.010(11)°, Z = 8, M = 508.46, T = 183(2) K, λ = 0.71073 Å, U = 5489.4(17) Å^3, D_c = 1.230 Mg/m^3, μ(Mo K_α) = 0.090 mm^{-1}, $F(000)$ = 2112, reflections collected = 45,840, independent reflections = 4965, R_{int} = 0.0398, refinement method = full-matrix least squares on F^2, data/restraints/parameters = 4965/0/348, goodness-of-fit on F^2 = 0.895, final R indices $[I > 2\sigma(I)]$, R_1 = 0.0605, wR_2 = 0.1230; R indices (all data) R_1 = 0.1304, wR_2 = 0.1440, largest difference peak and hole being 0.265 and −0.214 e Å^{-3}.

Suitable yellow crystals were grown from an acetone/petroleum ether solution. X-ray intensity data were collected using a crystal of size 0.42 × 0.18 × 0.10 mm^3 on a Siemens P4 diffractometer using Mo K_α monochromated radiation in the 2θ range 3.8°–50.5°. The remaining hydrogen atoms were placed in idealized positions with isotropic displacement parameters set equal to 1.2 U_{eq} (or 1.5 U_{eq} for methyl groups) of the parent carbon atoms. In the final cycles of refinement, the non-hydrogen atoms were assigned anisotropic displacement parameters. A disordered acetone solvent molecule was eliminated from the refinement using the Squeeze option in the program Platon as it was not possible to model it successfully.[16] A total of 260 electrons were recovered in agreement with there being a acetone molecule in the unit cell. The bond lengths and angles are normal within experimental error. Crystallographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data Centre as Supplementary Publication No. CCDC 290179. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44 (0) 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

4. Biological assays

4.1. Reagents

LPS (*Escherichia coli* 055:B5) and thioglycollate were purchased from Sigma. RPMI 1640 medium was from Hyclone. Trizol Reagent and reverse transcriptase were from Invitrogen. Prestained SDS–PAGE markers were

from Bio-Rad. Nitrocellulose membranes were from Schleicher & Schuell.

4.2. Preparation of mouse peritoneal macrophages

Mouse peritoneal macrophages were harvested from 2-month-old male C57BL/6J mice that had been injected i.p. four days earlier with 1 ml of 10% thioglycollate. Cells were seeded in 24-well plates and cultured at 37 °C in a humidified atmosphere containing 5% CO_2 with RPMI 1640 medium. After incubation for 2 h, non-adherent cells were removed and adherent cells were cultured in medium with compound **1** for 1 h and were further cultured in medium with LPS (200 ng/ml) and various concentrations of compound **1** for different times as described.

4.3. Reverse transcriptase-mediated PCR (RT-PCR) analysis

Mouse peritoneal macrophages treated with compound **1** were further cultured in medium with LPS and/or with various concentrations of compound **1** for 1 h to detect TNF-α, iNOS mRNAs, and for 2 h to detect IL-8, IL-10 mRNAs. Cells were washed in PBS and then used to isolate RNA. Total RNA was isolated according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using reverse transcriptase and primer oligo(dT)15. Primers for TNF-α, IL-8, IL-10, iNOS, and GAPDH were as follows: TNF-α (5′-ATGGATCCACCATGAGCACAG AAAGC-3′ and 5′-ATGAATTCTCACAGAGCAATG ACTCC-3′), IL-8 (5′-ATGGATCCATGATCCCAGC CACCCG-3′ and 5′-ATGGATCCTTACTTGGGGA CACCTTTTAG-3′), IL-10 (5′-ATGGATCCATGCC TGGCTCAGCACTG-3′, 5′-ATCTCGAGTTAGCTT TTCATTTTGAT-3′), iNOS (5′-CGCTCTGGAAGT TCTCAG-3′ and 5′-ACCACATCTGTCTCCCAG-3′), GAPDH (5′-TTTGTGATGGGTGTGAACCACGA G-3′ and 5′-GGAGACAACCTGGTCCTCAGTGTA-3′). Amplification for TNF-α, IL-8, IL-10, iNOS, and GAPDH was 30, 30, 30, 35, and 30 cycles, respectively.

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