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Anti-inflammatory Ingenane Diterpenoids from the Roots of *Euphorbia kansui*

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ABSTRACT

Bioassay-guided fractionation of the ethanolic extract of the roots of *Euphorbia kansui* led to the isolation of two new ingenane diterpenoids, euphorkans A (**1**) and B (**2**), together with 16 known analogues (**3**–**18**). Their structures were determined by combined spectral and chemical methods. All the isolates were evaluated for their inhibitory effects on lipopolysaccharide-induced nitric oxide production in RAW264.7 macrophage cells. Compounds **1**–**6** and **10**–**13** exhibited pronounced inhibitory activity with IC_{50} values in the range of 2.78–10.6 μM, and were more potent than the positive control, quercetin (IC_{50} = 15.8 μM). Compounds **1** and **5** were selected for further assays toward the key inflammation mediators TNF-α and IL-6, and showed a significant inhibition in a dose-dependent manner. The preliminary mechanistic study revealed that **1** and **5** inhibited NF-κB activity, which may exert a role in their anti-inflammatory activity.

Introduction

Inflammation is a defensive response of the organism to infection, physical injury, chemical insult, or radiation [1, 2]. Inflammation has been investigated intensively over the last two decades. However, owing to its involvement in devastating pathological conditions and the serious side effects of existing anti-inflammatory drugs, the discovery and development of novel anti-inflammatory agents remain a crucial challenge [3, 4]. The initiation, promotion, and progression of various inflammatory diseases are mediated by a number of proinflammatory mediators such as nitric oxide (NO), TNF-α, interleukin 1 (IL-1), and interleukin 6 (IL-6) [5]. During the inflammation process, nuclear factor-κB (NF-κB) is regarded as one of the most important transcriptional factors in regulating the expression of genes that encode proinflammatory mediators, inducible enzymes (iNOS and COX-2), adhesion molecules (e.g., ICAM, VCAM, and E-selectin), and growth factors [6, 7]. Therefore, compounds that downregulate the expression of these inflammatory mediators involved in the NF-κB signaling pathway might be potential anti-inflammatory candidates.

Euphorbia kansui L. (Euphorbiaceae) is a perennial herb widely distributed in the northwestern provinces of China. Its roots have been extensively used as a traditional medicine for the treatment of inflammation-related diseases, such as asthma, edema, and ascites [8]. In the past decades, a number of diterpenoids, triterpenes, and phenolic derivatives have been isolated from this plant, some of which exhibited anti-inflammatory, antileukemia, antitumor, antiallergic, antiviral, and antinematodal activities [9–12].

In our continuing efforts toward discovering structurally intriguing anti-inflammatory substances from Euphorbiaceae plants [13, 14], two new ingenane diterpenoids and sixteen analogues were isolated from the roots of *E. kansui*. These compounds were screened for anti-inflammatory activity by using the lipopolysaccharide (LPS)-induced NO production model, and the action mechanism of the active compounds was further explored. Herein, details of the isolation, structural elucidation, and anti-inflam-

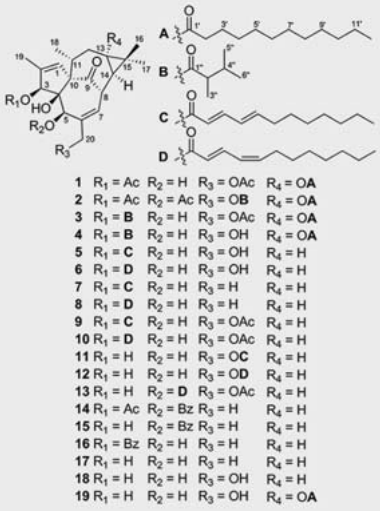
matory activities as well as preliminary structure-activity relationships (SARs) of these compounds are described.

Results and Discussion

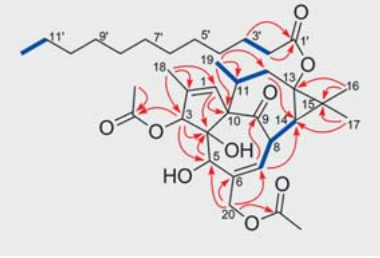
Roots of *E. kansui* (4 kg) were extracted with 95% EtOH at room temperature to give a residue, which was suspended in H_2O and successively partitioned with petroleum ether (PE), EtOAc, and *n*-BuOH. Each fraction was tested for inhibitory effects on NO generation in LPS-induced RAW264.7 cells. The PE-soluble fraction showed promising activity (> 80% inhibition at 50 µg/mL) and was selected for further chemical investigation, which afforded **1**–**18** (▶ **Fig. 1**).

Compound **1**, a colorless oil, had a molecular formula of $C_{36}H_{54}O_9$, as established by HRESIMS, corresponding to ten degrees of unsaturation. The IR absorption bands revealed the presence of OH (3471 cm^{-1}) and carbonyl (1729 cm^{-1}) groups. The ^1H and ^{13}C NMR data and HSQC spectra exhibited signals for one ketone (δ_C 205.2), three carbonyl groups (δ_C 174.0, 171.7, and 171.0), two trisubstituted double bonds [δ_H 6.09 (1H, d, J = 4.3 Hz) and 6.03 (1H, s); δ_C 136.3, 136.1, 131.6, and 128.5], and seven methyl groups [δ_H 2.16 (3H, s), 2.05 (3H, s), 1.79 (3H, s), 1.19 (3H, s), 1.06 (3H, s), 0.98 (3H, d, J = 7.2 Hz), and 0.88 (3H, t, J = 6.7 Hz); δ_C 22.5, 21.1, 21.0, 18.2, 16.7, 15.5, and 14.1]. As six of the ten degrees of unsaturation were accounted for by one ketone, three carbonyls, and two double bonds, the remaining degrees of unsaturation required that **1** be tetracyclic. The above-mentioned data were quite similar to that of the known ingenane diterpenoid 5,20-*O*-diacetyl-3-*O*-(2,3-dimethylbutanoyl)-13-*O*-dodecanoylingenol [15], except for the absence of an acetyl group and an upfield-shifted signal [δ_H 3.87 (1H, d, J = 6.5 Hz)], indicating that **1** was a deacetylated derivative of the previously described compound. HMBC correlations from the upfield-shifted signal (δ_H 3.87) to C-3, C-7, C-10, and C-20 indicated that the proton corresponding to this signal was at C-5. The methylene signals of the 13-*O*-dodecanoyl chain were overlapped (δ_H 1.23–1.30). The number of methylenes in this substituent was thus assigned from the MS data. The planar structure of **1** was further confirmed by detailed analysis of its 2D NMR data (▶ **Fig. 2**). The relative configuration of **1** was assigned to be the same as that of 5,20-*O*-diacetyl-3-*O*-(2,3-dimethylbutanoyl)-13-*O*-dodecanoylingenol by comparing their 1D NMR data and analyzing the NOESY data. In particular, NOE interactions of H-3/H-5 indicated that H-3 and H-5 were *cis*-oriented and were arbitrarily assigned in the α-orientation. The structure of **1** was further confirmed by the chemical correlation with the co-isolated known analogue 3-*O*-(2,3-dimethylbutyryl)-13-*O*-*n*-dodecanoyl-13-hydroxyingenol (**4**) [16]. The alkaline hydrolysis of **1** and **4** generated the same product, **19** (▶ **Fig. 3**), which was verified by comparison of their NMR and MS data. Thus, the structure of **1** was elucidated as depicted and was given the trivial name euphorkan A.

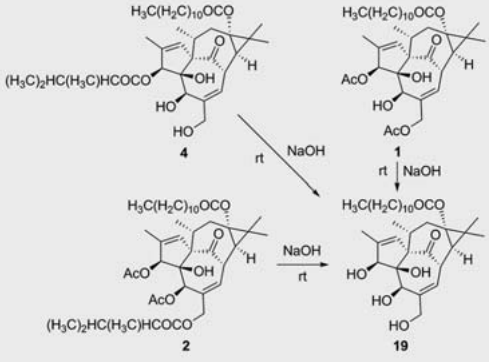
Compound **2** had a molecular formula of $C_{42}H_{64}O_{10}$, as determined by HRESIMS and ^{13}C NMR data. The 1D NMR spectra of **2** were very similar to those of 20-*O*-(2,3-dimethylbutanoyl)-13-*O*-dodecanoylingenol [17], except for the presence of two additional acetyl groups [δ_H 2.11 (s) and 2.26 (s); δ_C 20.8, 21.1, 170.7, and 172.5], indicating that **2** was a diacetylated derivative of the latter. The two acetyl groups were located at 3-OH and 5-OH on the basis of the HMBC correlations of H-3 (δ_H 4.97)/(δ_C 172.5) and H-5 (δ_H 5.39)/(δ_C 170.7), respectively, which was further supported by the strongly downfield-shifted H-3 and H-5 signals in **2** with respect to those in 20-*O*-(2,3-dimethylbutanoyl)-13-*O*-



▶ **Fig. 1** Structures of compounds **1**–**19**.



▶ **Fig. 2** Selected ^1H-^1H COSY (blue) and HMBC (→) correlations of **1**.



▶ **Fig. 3** Chemical correlations of compounds **1**, **2**, **4**, and **19**.

▶ **Table 1** IC_{50} values of the active compounds on LPS-induced NO production in RAW264.7 cells[a].

Compound	IC_{50} (μM)
1	4.90 (C.I. 4.67–5.13)
2	10.4 (C.I. 10.8–11.0)
3	5.69 (C.I. 5.44–5.94)
4	5.80 (C.I. 5.23–6.33)
5	2.78 (C.I. 2.72–2.84)
6	10.6 (C.I. 10.04–11.02)
10	2.86 (C.I. 2.40–3.30)
11	9.05 (C.I. 8.40–9.70)
12	9.45 (C.I. 8.54–10.33)
13	4.60 (C.I. 4.14–5.03)
19	8.86 (C.I. 7.40–9.10)
Quercetin[b]	15.8 (C.I. 14.4–16.2)

[a]Compounds with IC_{50} > 10 μM were not listed. [b]Positive control

dodecanoylingenol (H-3, δ_H 4.43 and H-5, δ_H 4.74). The structure of **2** was further confirmed by the chemical correlation of **2** to **19** via alkaline hydrolysis (▶ **Fig. 3**). Compound **2** was named euphorkan B.

The known compounds 3-*O*-(2,3-dimethylbutanoyl)-13-*O*-dodecanoyl-20-*O*-acetylingenol (**3**) [18], 3-*O*-(2.3-dimethylbutyryl)-13-*O*-*n*-dodecanoyl-13-hydroxyingenol (**4**) [16], 3-*O*-(2′*E*,4′*E*-decadienoyl) ingenol (**5**) [19], 3-*O*-(2′*E*,4′*Z*-decadienoyl) ingenol (**6**) [19], 3-*O*-(2*E*,4*E*-decadienoyl)-20-deoxyingenol (**7**) [18], 3-*O*-(2*E*,4*Z*-decadienoyl)-20-deoxyingenol (**8**) [18], 3-*O*-(2′*E*,4′*E*-decadienoyl)-20-*O*-acetylingenol (**9**) [19], 3-*O*-(2′*E*,4′*Z*-decadienoyl)-20-*O*-acetylingenol (**10**) [20], 20-*O*-(2′*E*,4′*E*-decadienoyl) ingenol (**11**) [19], 20-*O*-(2′*E*,4′*Z*-decadienoyl) ingenol (**12**) [19], 20-*O*-acetyl-[5-*O*-(2′*E*,4′*Z*)-decadienoyl]-ingenol (**13**) [20], kansuiphorin C (**14**) [21], 5-*O*-benzoyl-20-deoxyingenol (**15**) [22], 3-*O*-benzoyl-20-deoxyingenol (**16**) [22], 20-deoxyingenol (**17**) [23], ingenol (**18**) [24], and 13-*O*-docecanoylingenol (**19**) [15] were identified by comparison of their spectroscopic data with those in the literature.

Compounds **1**–**19** were evaluated for their inhibitory effects on NO production in LPS-activated RAW264.7 macrophages. Quercetin, a well-known natural NO inhibitor, was used as the positive control (IC_{50} = 15.8 μM) [25]. As shown in ▶ **Table 1**, compounds **1**–**6**, **10**–**13**, and **19** exhibited pronounced inhibitory activity with IC_{50} values ranging from 2.78 to 10.6 μM, while the others with IC_{50} values greater than 25 μM were considered to be inactive. To exclude that the inhibitory activities of the active compounds on RAW264.7 cells were due to cytotoxicity, cell viability was measured using the MTT method. No obvious cytotoxicity (> 90% cell survival) was observed at the concentration of 50 μM (Data not shown). Preliminary SARs revealed that the oxidation of C-20 and C-13 may be important to the activity, as all active compounds, except compound **9**, contain a hydroxyl group or an ester residue at these positions.

An excess of proinflammatory mediators such as TNF-*α*, IL-1, IL-6, NO, and PGE_2 produced by macrophages can trigger pathological conditions and lead to multiple chronic inflammatory diseases. Particularly, TNF-*α* and IL-6 are regarded as potent biomarkers to assess the inflammatory process [26]. To further explore the anti-inflammatory potential of **1** and **5**, their inhibitory effects on the LPS-induced expression of TNF-α and IL-6 in RAW264.7 cells were investigated. As shown in ▶ **Fig. 4A, B**, the enhanced levels of TNF-*α* and IL-6 via the stimulation of LPS were dramatically decreased ($p < 0.01$) by pretreatment with **1** and **5**. In particular, **1** and **5** inhibited TNF-*α* in a dose-dependent manner. These results suggest that **1** and **5** were able to reduce the inflammatory response in RAW264.7 cells. As NF-*κ*B plays a key role in inflammation via transcription of proinflammatory genes [27], whether **1** and **5** could suppress the activation of NF-*κ*B was also investigated. A luciferase assay was performed on a RAW264.7 cell line stably transfected with NF-*κ*B-luciferase reporter plasmid. As shown in ▶ **Fig. 5**, compound **5** exhibited about a 2-fold more potent inhibition on NF-*κ*B activation (IC_{50} = 11.0 μM) than the positive control pyrrolidine dithiocarbamate (PDTC), a well-known NF-*κ*B inhibitor (IC_{50} = 20.9 μM) [25], while compound **1** (IC_{50} = 17.9 μM) was comparable to PDTC. This study demonstrated that the effects of **1** and **5** on the production of NO, TNF-*α*, and IL-6 in LPS-induced RAW264.7 macrophages were associated with the inhibition of NF-*κ*B transcriptional activity.

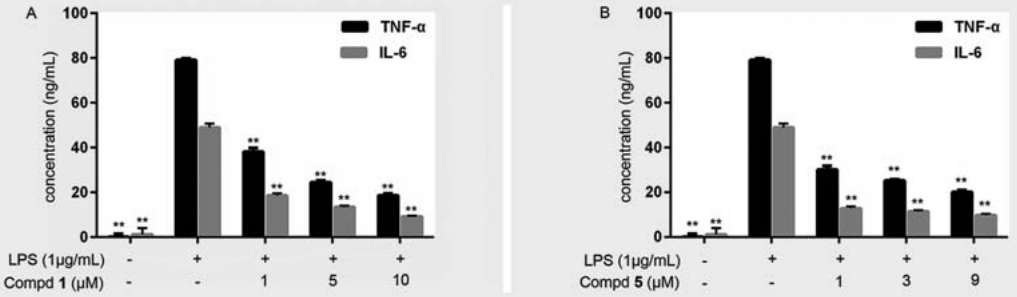
The current study supports the use of *E. kansui* in folk medicine for the treatment of inflammation-related diseases. While the anti-inflammatory activity of ingenanes has been previously reported [28, 29], this is the first study on the anti-inflammatory mechanism of this group of compounds. Based on the present results, ingenane diterpenes deserve further investigation as potential therapeutic candidates for inflammation-related conditions.

Materials and Methods

General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a Shimadzu UV-2450 spectrophotometer. IR spectra were determined on a Bruker Tensor 37 infrared spectrophotometer with KBr disks. NMR spectra were measured on a Bruker AM-400 spectrometer at 25 °C. ESIMS and HRESIMS were carried out on a Finnigan LCQ Deca instrument. Absorbance was recorded at 490 nm and 540 nm using a microplate reader (Molecular Devices) with Soft Max Pro 5 software (Molecular Devices). Semipreparative HPLC was performed on an A Shimadzu LC-20AT equipped with an SPD-M20A PDA detector and a YMC-pack ODS-A column (250 × 10 mm, S-5 μm, 120 Å). Silica gel (300–400 mesh, Qingdao Haiyang Chemical Co. Ltd.), reversed-phase C_{18} (RP-C_{18}) silica gel (S-50 μm, 120 Å, YMC Co. Ltd.), and MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd.) were used for column chromatography (CC). All solvents were of analytical grade.

LPSs (from *Escherichia coli* serotype 055:B5), PDTC (98%), and quercetin (98%) were purchased from Sigma-Aldrich. MTT, DMSO, and geneticin (G418) were obtained from Millipore. DMEM and FBS were purchased from Gibco. A Griess reagent kit was pro-



▶ **Fig. 4** **A** Effects of **1** on the production of TNF-α in LPS-induced RAW264.7 macrophages. **B** Effects of **5** on the production of TNF-α in LPS-induced RAW264.7 macrophages. RAW264.7 macrophages were treated with increasing concentrations of **1** (1, 5, and 10 μM) and **5** (1, 3, and 9 μM) for 30 min followed by LPS stimulation for another 24 h. The expression of TNF-α and IL-6 was measured by an ELISA assay. (**p < 0.01 versus the group treated with LPS only)

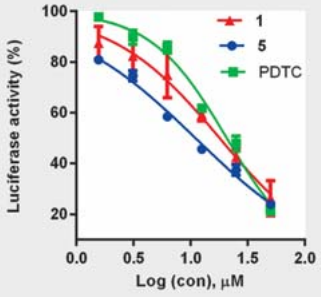
vided by Beyotime. Luciferase assay system reagent was from Promega. Mouse TNF-α and IL-6 kits were purchased from Neobioscience.

Plant material

The roots of *E. kansui* were purchased in January 2016 from Bozhou Medicine Market, Anhui Province, P.R. China, and were identified by Dr. Gui-Hua Tang of Sun Yat-sen University. A voucher specimen (accession number: GS201607) has been deposited at the School of Pharmaceutical Sciences, Sun Yat-sen University.

Extraction, bioassay-guided fractionation, and isolation

The air-dried powder of the roots of *E. kansui* (4 kg) was extracted with 95% EtOH (3 × 3 L) at room temperature to give 120 g of crude extract. The extract was suspended in H_2O (3 L) and partitioned successively with PE (3 × 3 L), EtOAc (3 × 3 L), and *n*-BuOH (3 × 3 L). Each of these fractions was tested for its inhibitory activity against NO production induced by LPS in RAW264.7 macrophage cells. The PE extract (90 g, 80.2% inhibition at 50 μg/mL) was subjected to MCI gel CC eluted with a MeOH/H_2O gradient (3 : 7 → 10 : 0) to afford four fractions (I–IV). Fr. I (8.8 g) was subjected to silica gel CC (PE/EtOAc, 5 : 1 → 0 : 1) to give five fractions (Ia-Ie). Fr. Ic (1.1 g) was separated by silica gel CC (PE/acetone, 10 : 1) followed by semipreparative HPLC (MeCN/H_2O, 70/30, 3 mL/min) to give **7** (15 mg, t_R 8.0 min) and **8** (13 mg, t_R 9.8 min). Fr. Id (128 mg) was purified on silica gel CC (CH_2Cl_2/MeOH, 100 : 1) to give **13** (6.7 mg). Fr. Ie (550 mg) was separated by RP-C_{18} silica gel CC (MeOH/H_2O, 6 : 4 → 10 : 0) to yield **6** (14.2 mg) and **12** (48 mg). Fr. II (16.5 g) was subjected to silica gel CC (CH_2Cl_2/MeOH, 0 : 1 → 100 : 1) to give five fractions (IIa-IIe). Fr. IIb (6.1 g) was separated by RP-C_{18} silica gel CC (MeOH/H_2O, 5 : 5 → 10 : 0) followed by silica gel CC (PE/acetone, 10 : 1 → 4 : 1) to give **11** (4.8 mg) and **15** (9.6 mg). Fr. III (2.5 g) was subjected to silica gel CC (CH_2Cl_2/MeOH, 1 : 0 → 100 : 1) to give two fractions (IIIa and IIIb). Fr. IIIb (0.8 g) was subjected to a Sephadex LH-20 column using MeOH as the eluent followed by semipreparative HPLC (MeOH/H_2O, 80/20, 3 mL/min) to give **3** (3.1 mg, t_R 12.6 min), **14** (4.3 mg, t_R 15.3 min), and **16** (6.3 mg, t_R 15.6 min).



▶ **Fig. 5** Inhibitory effect of **1** and **5** on LPS-induced NF-κB-dependent promoter activity in RAW264.7 macrophage stably transfected with NF-κB-luciferase reporter plasmid. Pyrrolidine dithiocarbamate (PDTC) was used as a positive control. Cells were pretreated with the indicated concentration (1.56–50 μM) for 1 h prior to treatment with LPS (1 μg/mL) for 6 h. Then, the activity was measured using the Luciferase system assay.

Fr. IV (7 g) was applied to silica gel CC (PE/acetone, 400 : 1 → 3 : 1) to give four fractions (IVa-IVd). Fr. IVb (862 mg) was separated by RP-C_{18} silica gel CC (MeOH/H_2O, 5 : 5 → 10 : 0) followed by semipreparative HPLC (MeOH/H_2O, 85/15, 3 mL/min) to give **9** (9.5 mg, t_R 11.1 min) and **10** (8.3 mg, t_R 12.2 min). Fr. IVc (121 mg) was further purified by a Sephadex LH-20 column using MeOH as the eluent to give **5** (8 mg) and **1** (4.8 mg). Fr. IVd (2.3 g) was separated by RP-C_{18} silica gel CC (MeOH/H_2O, 7 : 3 → 10 : 0) to give three subfractions (Fr. IVd1-IVd3). Fr. IVd2 was further separated by semipreparative HPLC (MeOH/H_2O, 85/15, 3 mL/min) to give **18** (5 mg, t_R 8.6 min) and **17** (8.1 mg, t_R 9.3 min). Fr. IVd3 was applied to silica gel CC (PE/acetone, 8 : 1) to give **4** (5.2 mg) and **2** (11 mg).

Euphorkan A (**1**): colorless oil; $[\alpha]_D^{25}$ + 33.7 (*c* 0.2, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 204 (4.17) nm; IR (KBr) ν_{max} 3471, 1729, 1377, 1231, 1022 cm^{-1}; HRESIMS *m/z* 653.3658 $[M + Na]^+$ (calcd. for $C_{36}H_{54}O_9Na^+$, 653.3660); ^1H NMR ($CDCl_3$, 400 MHz): δ_H 6.09 (1H, d, *J* = 4.3 Hz, H-7), 6.03 (1H, s, H-1), 5.47 (1H, s, H-3), 4.75

(1H, d, J = 12.6 Hz, H-20a), 4.48 (1H, d, J = 12.6 Hz, H-20b), 4.05 (1H, m, H-8), 3.87 (1H, d, J = 6.5 Hz, H-5), 2.72 (2H, m, H_2-12), 2.60 (1H, m, H-11), 1.79 (3H, s, H_3-19), 1.19 (3H, s, H_3-16), 1.06 (3H, s, H_3-17), 1.05 (1H, m, H-14), 0.98 (3H, d, J = 7.2 Hz, H_3-18), 3-OAc: 2.05 (3H, s), 13-OCO$(CH_2)_{10}CH_3$: 0.88 (3H, t, J = 6.7 Hz), 1.23–1.30 (H-3′–H-10′, overlapped); 20-OAc: 2.16 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 205.2 (C, C-9), 136.3 (C, C-2), 136.1 (C, C-6), 131.6 (CH, C-1), 128.5 (CH, C-7), 84.5 (C, C-4), 82.9 (CH, C-1), 74.7 (C, C-5), 71.9 (C, C-10), 68.9 (C, C-13), 66.6 (CH_2, C-20), 42.8 (CH, C-8), 37.6 (CH, C-11), 35.2 (CH_2, C-12), 30.4 (C, C-15), 28.3 (CH, C-14), 22.5 (CH_3, C-16), 18.2 (CH_3, C-18), 16.7 (CH_3, C-17), 15.5 (CH_3, C-19), 3-OAc: 171.7, 21.1, 20-OAc: 171.0, 21.0; 13-OCO$(CH_2)_{10}CH_3$: 174.0 (C, C-1′), 34.4 (CH_2, C-2′), 31.9–29.2 (CH_2 × 8, C-3′–C-10′ overlapped), 22.7 (CH_2, C-11′), 14.1 (CH_3, C-12′).

Euphorkan B (**2**): colorless oil; $[\alpha]_D^{25}$ + 41.4 (c 0.3, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 208 (3.47) nm; IR (KBr) ν_{max} 2925, 1732, 1369, 1232, 1023 cm^{-1}; HRESIMS m/z 727.4425 $[M - H]^-$ (calcd for $C_{42}H_{63}O_{10}^-$, 727.4427); ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 6.22 (1H, brs, H-7), 6.07 (1H, brs, H-1), 5.39 (1H, s, H-5), 4.97 (1H, s, H-3), 4.53 (1H, d, J = 15.2 Hz, H-20a), 4.25 (1H, d, J = 15.2 Hz, H-20b), 4.20 (1H, m, H-8), 2.70 (1H, m, H-12a), 2.60 (1H, m, H-11), 2.27 (1H, m, H-12b), 1.76 (3H, s, H_3-19), 1.26 (1H, m, H-14), 1.18 (3H, s, H_3-16), 1.06 (3H, s, H_3-17), 0.99 (3H, d, J = 7.0 Hz, H_3-18), 3-OAc: 2.11 (3H, s), 5-OAc: 2.26 (3H, s), 13-OCO$(CH_2)_{10}CH_3$: 0.88 (3H, t, J = 6.8 Hz), 1.21–1.32 (H-3′–H-10′, overlapped); 20-OCOCH(CH_3)CH$(CH_3)_2$: 2.19 (1H, m, H-2″), 1.86 (1H, m, H-4″), 1.06 (3H, d, J = 7.0 Hz), 0.88 (3H, d, J = 6.9 Hz), 0.86 (3H, d, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 204.4 (C, C-9), 135.8 (C, C-2), 133.9 (C, C-6), 131.6 (CH, C-1), 130.4 (CH, C-7), 85.6 (C, C-4), 82.2 (CH, C-3), 75.0 (C, C-5), 71.8 (C, C-10), 69.1 (C, C-13), 65.0 (CH_2, C-20), 42.9 (CH, C-8), 37.8 (CH, C-11), 34.9 (CH_2, C-12), 30.7 (C, C-15), 28.1 (CH, C-14), 22.7 (CH_3, C-16), 18.0 (CH_3, C-18), 16.8 (CH_3, C-17), 15.3 (CH_3, C-19), 3-OAc: 172.5, 21.1, 5-OAc: 170.7, 20.8, 13-OCO$(CH_2)_{10}CH_3$: 174.0 (C, C-1′), 34.4 (CH_2, C-2′), 31.9–29.2 (CH_2 × 8, C-3′–C-10′ overlapped), 22.7 (CH_2, C-11′), 14.1 (CH_3, C-12′); 20-OCOCH(CH_3)CH$(CH_3)_2$: 176.0 (C, C-1″), 45.9 (C, C-2″), 30.9 (C, C-4″), 19.0 (C, C-5″), 14.1 (C, C-3″), 13.4 (C, C-6″).

Alkaline hydrolysis of 1, 2, and 4

Compounds **1** (0.9 mg), **2** (1.1 mg), and **4** (8 mg) were stirred with 1 mL of 0.1 M NaOH in MeOH for 2 h at room temperature, respectively. Then each mixture was subjected to Sephadex LH-20 using MeOH as the eluent to afford the pure hydrolysis product **19**, which was identified by NMR and MS data.

Cell culture

The RAW264.7 murine macrophage cell lines purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China) were grown in DMEM supplemented 10% FBS. Cells were cultured under standard conditions (5% CO_2 in the air in a humidified environment at 37 °C). RAW264.7 cells stably transfected with NF-κB-luciferase reporter plasmid were provided by Prof. Depo Yang, School of Pharmaceutical Sciences, Sun Yat-sen University (Guangzhou, China) and were cultured under the same conditions except for the presence of G418 (500 μg/mL). DMSO was used to dissolve all compounds. The final solvent concentration in the assay was 1%.

Analysis of nitric oxide production

RAW264.7 microphages were seeded into 96-well plates at a density of 5×10^4 cells/well for 24 h and then preincubated with different concentrations of compounds for 1 h before stimulation with or without LPS (1 μg/mL) for 24 h. The NO concentration in culture medium was determined by a Griess reagent kit. The absorbance (A) at 540 nm was measured using a multifunction microplate reader (Molecular Devices, Flex Station 3). Sodium nitrite was used as a standard to calculate the nitrite concentration. Inhibition (%) = $[1 - (A_{LPS + sample} - A_{untreated})/(A_{LPS} - A_{untreated})] \times 100$. The experiments were performed in triplicate, and the IC_{50} values were calculated by nonlinear regression in GraphPad Prism 5. Quercetin was used as a positive control.

Cytotoxicity assay

The cytotoxicity of the isolated compounds toward RAW264.7 cells was determined by the MTT assay. RAW264.7 cells were seeded in 96-well plates (5×10^3/well) for 24 h. Next, the test samples dissolved in DMSO were added into the wells and diluted with 100 μL DMEM to the final concentration of 50 μM with 1% DMSO serving as the solvent control. Wells containing only 100 μL DMEM were used as a blank control. Then, 24 h later, 20 μL solution MTT (5 mg/mL) were added to each well. After incubation for 4 h, the medium was removed and 100 μL DMSO were added to each well. The absorbance (A) was recorded at 490 nm using a microplate reader. The inhibition of cell growth was calculated according to the following formula: % Inhibition = $[1 - (A_{sample} - A_{blank})/(A_{solvent} - A_{blank})] \times 100$.

Analysis of TNF-α and interleukin 6 production

RAW264.7 macrophages were pretreated with **1** and **5** for 1 h prior to stimulation with LPS (0.1 μg/mL) for 6 h. The concentrations of TNF-α and IL-6 in the culture medium were determined using commercial ELISA kits according to the instructions of each manufacturer.

Luciferase report assay

The luciferase assay was performed to analyze NF-κB-dependent reporter gene transcription as previously described [25]. RAW264.7 cell lines stably transfected with p-NF-κB-Luc reporter plasmid (4×10^4 cells/well) were plated into 96-well plates overnight. After pretreating with compounds **1, 5**, and PDTC (positive control), respectively, at indicated concentrations for 1 h, cells were stimulated with LPS (1 μg/mL) for 6 h. Then, each well was slightly washed with ice-cold PBS twice followed by lysis in lysis buffer. Luciferase activity was detected by the Promega luciferase assay system.

Supporting information

1D and 2D NMR spectra of **1** and **2**, and ^{1}H and ^{13}C NMR spectra of compounds **3–19** are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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