Supporting Information

Structure Elucidation and Inhibitory Effects on NO Production of Clerodane Diterpenes from *Ajuga decumbens*

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General experimental procedures

Melting points were determined with an XT-4 microscopic thermometer. The optical rotations were measured in CH₂Cl₂ using an Autopal IV automatic polarimeter made by Autopal Industries Limited Company. The IR spectra were taken on a Bio-Rad FTS 6000 Fourier transform infrared (FTIR) spectrometer with KBr discs. The ESIMS spectra were obtained on a LCQ-Advantage mass spectrometer (Finnigan). HR-ESIMS spectra were taken by an Ionspec 7.0 T FTICR MS. 1D and 2D NMR spectra were recorded on a Bruker AV 400 instrument (400 MHz for ¹H and 100 MHz for ¹³C) with TMS as an internal standard. HPLC separations were performed on a CXTH system (Beijing Chuangxintongheng Instrument Co. Ltd.), equipped with a UV3000 detector at 210 nm, and a YMC-pack ODS- M80 column (20 × 250 mm, i.d.). Silica gel (200-300 meshes; Qingdao Marine Chemical Group Co. Ltd.) was used for column chromatography. Chemical reagents for isolation were analytical grade and purchased from Tianjin Yuanli Chemical Co. Ltd. Biological reagents were from Sigma Company. The murine microglial BV-2 cell line was from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (China).

Plant material

The whole plants of *A. decumbens* were collected from Zhejiang Province, China, in Aug. 2008. The botanical identification was made by Dr Yuanqiang Guo (College of Pharmacy, Nankai University, China), and a voucher specimen (No. 20080810) was deposited at the laboratory of the Research Department of Natural Medicine, College of Pharmacy, Nankai University, China.

Extraction and isolation

The air-dried and powdered whole plants of *A. decumbens* (20 kg) were extracted with methanol three times (3 × 50 L) under reflux. The organic solvent was evaporated to afford a crude extract. The extract was suspended in H₂O (1.5 L) and then partitioned successively with petroleum ether and ethyl acetate. The ethyl acetate soluble part (382.0 g) was subjected to silica gel column (⌀ 9 × 70
chromatography, using a gradient solvent system from 1–40% acetone in petroleum ether, to give 10 fractions (F₁–F₁₀) based on TLC analyses. F₆ was separated by MPLC over C-18 eluting with a step gradient from 55% to 85% MeOH in H₂O to give four subfractions (F₆₋₁ to F₆₋₄). F₆₋₃ was purified by HPLC (YMC-pack J'Sphere ODS-M80, 20 × 250 mm, 75% MeOH in H₂O) to afford 1 (24.2 mg). F₇, F₈, and F₁₀ were also separated by the same MPLC over C-18, eluting with a step gradient from 55% to 85% MeOH in H₂O, to give subfractions F₇₋₁–F₇₋₄, F₈₋₁–F₈₋₄, and F₁₀₋₁–F₁₀₋₄, respectively. Compounds 2 (25.0 mg) and 3 (29.1 mg) were obtained from subfraction F₁₀₋₃ using the above HPLC system (73% MeOH in H₂O). The further purification of F₇₋₃ with the same HPLC system resulted in the isolation of compounds 4 (15.1 mg) and 6 (17.2 mg) (76% MeOH in H₂O). With the same protocols, compound 5 (20.0 mg) was isolated from subfractions F₁₀₋₂ (65% MeOH in H₂O), and compound 7 (35.7 mg) was obtained from F₈₋₂ (66% MeOH in H₂O). The purification of subfraction F₈₋₁ (73% MeOH in H₂O) with the same HPLC system led to produce compound 8 (28.9 mg).

**Bioassay for NO production**

Murine microglial BV-2 cells were cultured at 37°C in DMEM supplemented with 10% (v/v) inactivated fetal bovine serum and 100 U/mL penicillin/streptomycin under a water-saturated atmosphere of 95% air and 5% CO₂. The cells were seeded in 96-well culture plates (5 × 10⁴ cells/well) and allowed to adhere for 24 h at 37°C. The cells were incubated for 20 h with or without 0.5 μg/mL of LPS (Sigma Chemical Co.) in the absence or presence of the test compounds. 2-Methyl-2-thiopseudourea sulfate (SMT; Sigma) was used as a positive control. As a parameter of NO synthesis, the nitrite concentration was measured by the Griess reaction using the supernatant of the BV-2 cells. Briefly, 50 μL of the cell culture supernatant were reacted with 50 μL of Griess reagent [1:1 mixture of 0.1% N-(1-naphtyl)ethylenediamine in H₂O and 1% sulfanilamide in 5% phosphoric acid] in a 96-well plate, and the absorbance was read with a microplate reader (Thermo Fisher Scientific Inc.) at 550 nm. The experiment was performed three times, and the IC₅₀ values for
the inhibition of NO production were determined on the basis of linear or nonlinear regression analysis of the concentration-response data curves.

The 1D, 2D NMR and HRMS spectra of compounds 1–3

1S $^1$H NMR spectrum for compound 1.
2S $^{13}$C NMR spectrum for compound 1.
3S HMQC spectrum for compound 1.
4S HMBC spectrum for compound 1.
5S $^1$H-$^1$H COSY spectrum for compound 1.
6S NOESY spectrum for compound 1.
7S HR-ESIMS spectrum for compound 1.
8S $^1$H NMR spectrum for compound 2.
9S $^{13}$C NMR spectrum for compound 2.
10S HMQC spectrum for compound 2.
11S HMBC spectrum for compound 2.
12S $^1$H-$^1$H COSY spectrum for compound 2.
13S NOESY spectrum for compound 2.
14S HR-ESIMS spectrum for compound 2.
15S $^1$H NMR spectrum for compound 3.
16S $^{13}$C NMR spectrum for compound 3.
17S HMQC spectrum for compound 3.
18S HMBC spectrum for compound 3.
19S $^1$H-$^1$H COSY spectrum for compound 3.
20S NOESY spectrum for compound 3.
21S HR-ESIMS spectrum for compound 3.
1H NMR spectrum for compound 1.

13C NMR spectrum for compound 1.
3S HMQC spectrum for compound 1.

4S HMBC spectrum for compound 1.
$5\text{S}^1\text{H}^1\text{H}$ COSY spectrum for compound 1.

$6\text{S}$ NOESY spectrum for compound 1.
7S HR-ESIMS spectrum for compound 1.

8S $^1$H NMR spectrum for compound 2.
$9S^{13}C$ NMR spectrum for compound 2.

$10S$ HMQC spectrum for compound 2.
11S HMBC spectrum for compound 2.

12S \(^1\)H-\(^1\)H COSY spectrum for compound 2.
13S NOESY spectrum for compound 2.

14S HR-ESIMS spectrum for compound 2.
**15S** $^1$H NMR spectrum for compound 3.

**16S** $^{13}$C NMR spectrum for compound 3.
17S HMQC spectrum for compound 3.

18S HMBC spectrum for compound 3.
19S $^1$H-$^1$H COSY spectrum for compound 3.

20S NOESY spectrum for compound 3.
21S HR-ESIMS spectrum for compound 3.