Supporting Information

Euryachins A and B, a New Type of Diterpenoids from *Eurya chinensis* with Potent NO Production Inhibitory Activity

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1. Experimental section

General experimental procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded in CH_3OH on a Perkin-Elmer Lambda 35 UV-vis spectrophotometer. HR-ESI-MS spectra were recorded on a Bruker Bio TOF IIIQ mass spectrometer. IR spectra were acquired using a Bruker Vertex 33 infrared spectrophotometer (Bruker, Karlsruhe, Germany) with KBr disk. NMR spectra were recorded on a Bruker Advance-500 spectrometer using TMS as an internal standard. All chemical shifts were quoted on the δ scale in ppm using residual solvent as the internal standard ($CDCl_3$: 7.26 ppm for ^1H NMR, 77.0 ppm for ^{13}C NMR; CD_3OD: 3.30 ppm for ^1H NMR, 49.90 ppm for ^{13}C NMR). Coupling constants (*J*) are reported in Hz. ECD spectra were measured with an Anton Paar MCP 500 Chirascan spectrometer. X-ray diffraction experiment was carried out on a Gemini S Ultra (Oxford Diffraction LtD) diffractometer with a CCD area detector using Mo/CuKα X-ray source. All organic solvents used for extraction were of analytical grade (Shanghai Chemical Plant, Shanghai, People's Republic of China). Silica gel (200-300 mesh) (Qingdao Haiyang Chemical Plant, Qingdao, People's Republic of China). MCI gel (CHP20P, 75-150 μm, Mitsubishi Chemical Industries Ltd.), and Sephadex LH-20 gel (Amersham Biosciences) were used for column chromatography. TLC spots were visualized under UV light and by dipping into a solution 5% H_2SO_4 in alcohol followed by heating.

Plant material. Branches of *E. chinensis* R. Br. were collected in Jiangxi, China, in June, 2012 and authorized by Professor Fuwu Xing of South China Botanical Garden. A voucher specimen (No. 201309) has been deposited at the Laboratory of Natural Product Chemistry Biology, South China Botanical Garden (SCBG), CAS, People's Republic of China.

Extraction and isolation. Powdered air-dried branches of *E. chinensis* (50.0 kg) were extracted using 95% ethanol at room temperature and filtered. Then, the filtrate was concentrated under vacuum to afford a crude residue (4.1 kg), which was suspended in H_2O and extracted sequentially with *n*-hexane and EtOAc. The EtOAc-soluble fraction (1.2 kg) was further subjected to silica gel column chromatography (CC) eluted with a gradient solvent mixture of *n*-hexane/acetone (ratio from 100:1 to 1:1) to afford 7 sub-fractions (F-1→F-7). F-4 (237 g) was further separated by silica gel CC eluted with a gradient mixture of CH_2Cl_2/EtOAc to afford six fractions (F-4A→F-4F). F-4C (36.5 g) was again fractionated on MCI-silica gel CC using an eluting solvent mixture of CH_3OH/H_2O of decreasing polarity to give 7 fractions (F-4C1→F-4C7). Pure compound **1** (50 mg) was obtained from F-4C1 (4.9 g) using CC with Sephadex LH-20 (183 × 3 cm; $CHCl_3$/MeOH 1:1 as eluent), followed by recrystallization (CH_3OH). Similarly, F-4E (32.6 g) was fractionated on MCI-silica gel CC using a solvent mixture CH_3OH/H_2O of decreasing polarity to give five fractions (F-4E1→F-4E5). As a result, **2** (20 mg).was purified from F-4E2 (4.6 g) by silica gel CC eluted using a mixture of *n*-hexane/EtOAc (1:2) followed by recrystallization from CH_3OH.

*Euryachin A (**1**)*: Colorless crystal; $[\alpha]^{20}_D$ = + 269.2 (*c*. 1.20; CH_3OH); UV (CH_3OH) λ_{max} (log ε) 274.7 (4.03) nm; IR (KBr) ν_{max} 3464, 2953, 2868, 1740, 1690, 1633, 1389, 1195, 991, 816, 639 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Table 1 in the

manuscript; CD $\Delta\varepsilon$ (*c* 0.13, CH_3OH) + 29.3 (276.2 nm); HR-ESI-MS m/z_{obsd} 327.1612 $[M-H]^-$ (m/z_{calcd} $[C_{20}H_{24}O_4 - H]^-$ = 327.1596).

Euryachin B (2): Colorless crystal; $[\alpha]^{20}_D$ = + 91.3 (*c*. 1.15; CH_3OH); UV (CH_3OH) λ_{max} (log ε) 274.7 (4.12) nm; IR (KBr) ν_{max} 2955, 2864, 1681, 1625, 1412, 1275, 932, 817, 734 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) see Table 1 in the manuscript; CD $\Delta\varepsilon$ (*c* 0.40, CH_3OH) + 20.5 (281.3 nm); HR-ESI-MS m/z_{obsd} 329.1786 $[M-H]^-$ (m/z_{calcd} $[C_{20}H_{24}O_4 - H]^-$ = 329.1753).

Anti-inflammatory activity assay.

Extracellular NO production detection: Murine monocytic RAW264.7 Macrophages (Conservation Genetics Kunming Cell Bank, Kunming Institute of Zoology. CAS, China) were dispensed into 96-well plates (5×10^5 cells/well) containing DMEM medium (Gibco BRL Co., Ltd., Grand Island, NY, USA) with 10% FBS (Gibco BRL Co., Ltd., Grand Island, NY, USA) under a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h preincubation, cells were treated with serial dilutions of compounds **1** and **2**, the maximum concentration being 200 μM, in the presence of 1 μg/mL LPS (Sigma-Aldrich Co., Ltd., USA) for 18 h. Each compound was dissolved in DMSO (Sigma-Aldrich Co., Ltd., USA) and further diluted in medium to produce different concentrations. NO production in each well was assessed by adding 100 μL of Griess reagent (Reagent A & Reagent B, respectively, Beyotime Co., Ltd., Shanghai, China) to 100 μL of each supernatant [LPS (Sigma)-treated or LPS- and compound-treated cells] in triplicate. After 5 min incubation, the absorbance was measured at 420 nm with a Multiscan Spectrum (TECAN Genios). L-NMMA (N^G-Monomethyl-L-arginine, Monoacetate Salt, Beyotime Co., Ltd., Shanghai, China) was used as a positive control.

Table 1

IC_{50} values (μM) of **1** and **2** for NO inhibition in LPS-stimulated RAW264.7 cells.

Compounds	IC_{50} (μM) [a]
1	46.09
2	36.96
L**-NMMA**	36.21

[a] IC_{50} values of each compound were expressed as the concentration (μM), which caused 50% inhibition of NO production

Intracellular NO production detection: The intracellular NO level was measured using a NO-sensitive fluorescence probe DAF-FM DA. First, 1×10^5 Raw264.7 macrophage cells (Conservation Genetics Kunming Cell Bank, Kunming Institute of Zoology. CAS, China) per well were grown in triplicate in 96-well plates. Cells were treated with compounds 1 and 2 at different concentrations (0, 5, 10, 20,40 μ g/mL) and LPS (1 μ g/mL) (Sigma-Aldrich Co., Ltd., USA) in 1h increments for 24 h, or were untreated (negative control). Then, removed supernatant and cells were loaded with DAF-FM DA(5μM, 100 μ L/well) (Beyotime Co., Ltd., Shanghai, China) at 37 °C for 30 min. Fluorescence values (excitation 495 nm, emission 515 nm) were measured using a

Multimode Microplate Reader (Synergy ™ 2,BioTek, Winooski, Vermont, USA).

The inhibitory effects of compounds 1 and 2 on NO overproduction in LPS-stimulated RAW264.7 macrophages were examined to evaluate the anti-inflammatory effect of PNFS. Extracellular NO concentrations were assessed using the Griess method, and intracellular NO concentrations were examined using a DAF-FM DA fluorescence assay. Compounds 1 and 2 (0, 5, 10, 20, 40 μ g/mL) markedly decreased intracellular NO concentrations ($P < 0.001$, $P < 0.001$, $P < 0.001$) (Fig.1). In summary, our experiments showed that compounds 1 and 2 suppressed LPS-stimulated NO overproduction.

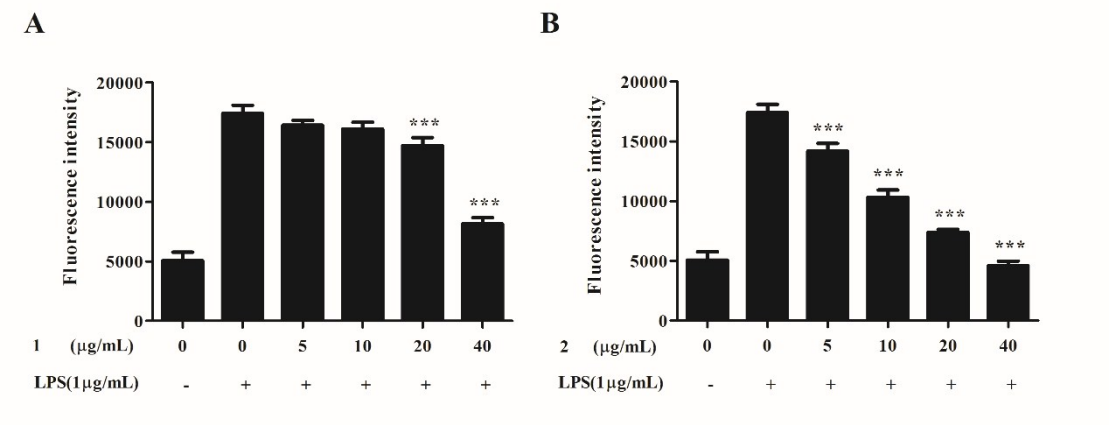
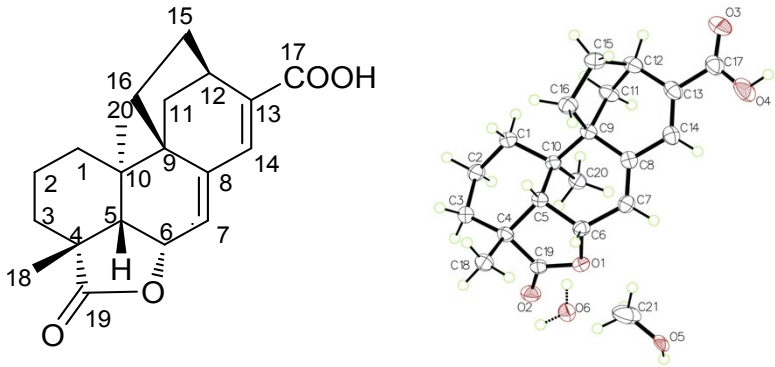


Fig. 1 compounds 1 and 2 suppressed NO overproduction in LPS-stimulated RAW264.7 macrophages. A compound 1 decreased LPS-stimulated NO overproduction. B compound 2 reduced LPS-stimulated intracellular NO levels. The first two bars at "0" in Fig. A, B are negative control (without this two compounds and LPS treated) and positive control (without Compounds 1 and 2 but with LPS treated). Data in Fig. A, B were all expressed as the mean (SD) of 3 independent experiments. One-Way ANOVA test was used to analyzed the data and the results were $F = 121$; $P < 0.001$, $F = 274$; $P < 0.001$; respectively. Then, data in Fig. A, B were all subjected to Dunnett's Multiple Comparison Test to determine the statistical difference between groups. The P values represented the statistical differences between each group and the corresponding positive control (without Compounds 1 and 2 with LPS treated). *** means $P < 0.001$

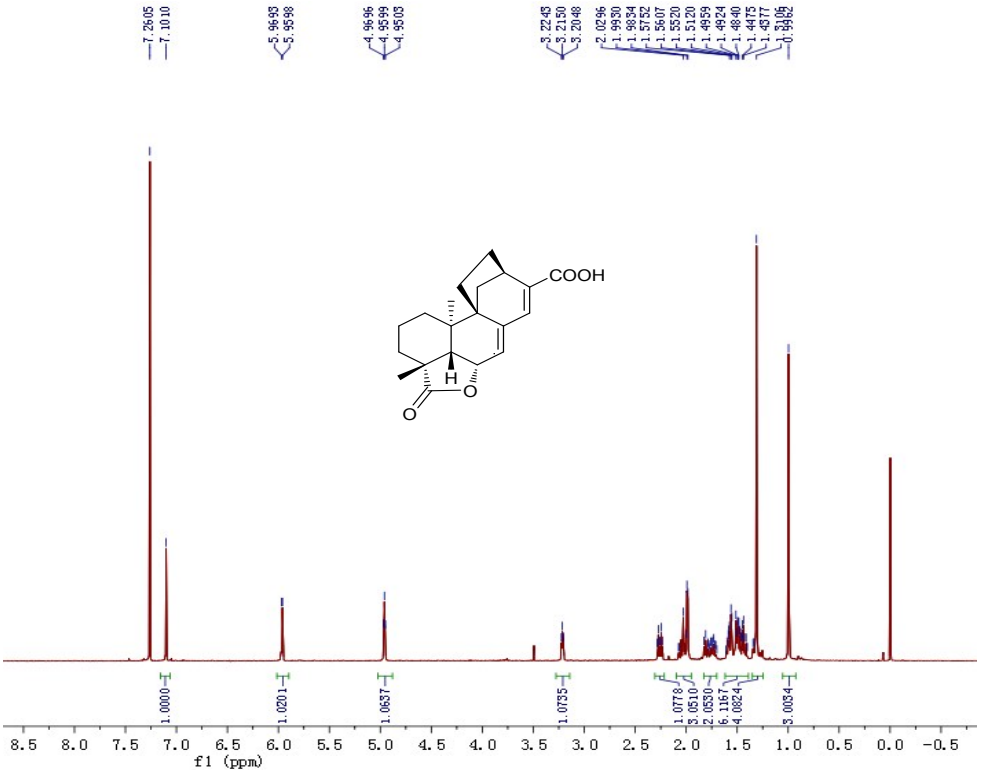
2. X-ray crystal data for euryachin A (1)



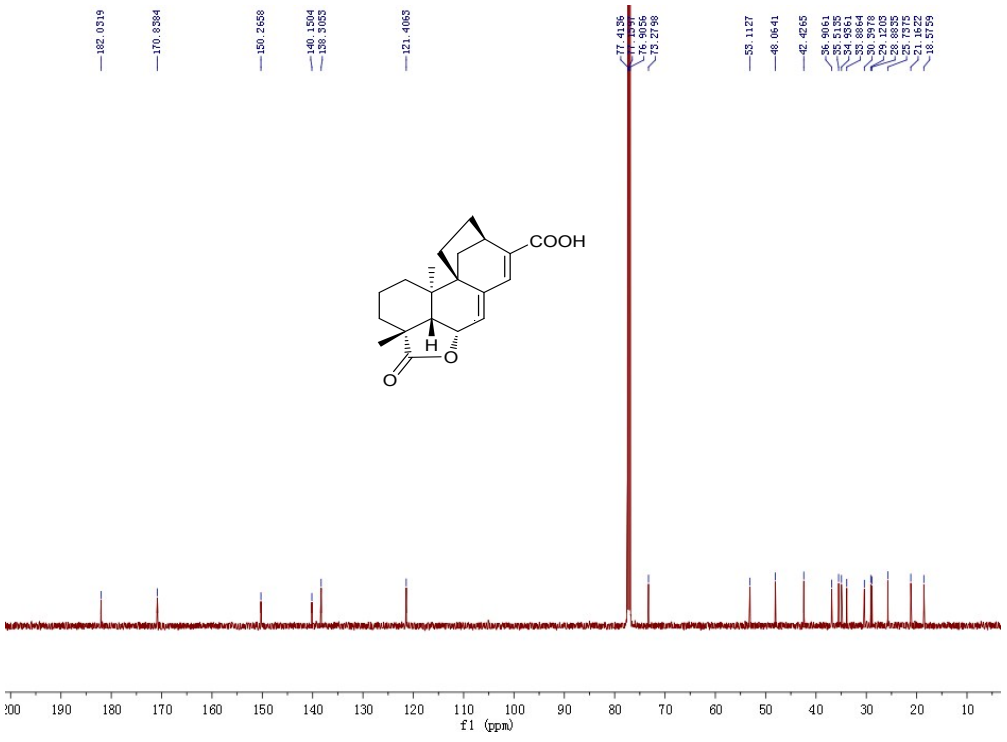
Crystal data and structure refinement for euryachin A

Identification code	SONGJIALING_43-3B
Empirical formula	$C_{83}H_{110}O_{20}$
Formula weight	1427.71
Temperature/K	173.00(10)
Crystal system	orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions	a = 6.27813(14) Å, α = 90.00°
	b = 10.3446(2) Å, β = 90.00°
	c = 27.8319(6) Å, γ = 90.00°
	90.00
	90.00
	90.00
Volume/Å^3	1807.55(7)
Z	1
ρ_{calc}g/cm^3	1.312
μ/mm^{-1}	0.753
F(000)	768.0
Crystal size/mm^3	0.28 × 0.26 × 0.2
Radiation	CuKα (λ = 1.54184)
θ range for data collection/°	9.12 to 125.4
Index ranges	-7 ≤ h ≤ 4, -11 ≤ k ≤ 11, -32 ≤ l ≤ 32
Reflections collected	14188
Independent reflections	2873 [R_{int} = 0.0305, R_{sigma} = 0.0177]
Data/restraints/parameters	2873/0/252
Goodness-of-fit on F^2	1.093
Final R indexes [I>=2σ (I)]	R_1 = 0.0365, wR_2 = 0.0984
Final R indexes [all data]	R_1 = 0.0369, wR_2 = 0.0987
Largest diff. peak/hole / e Å^{-3}	0.49/-0.17
Flack parameter	0.1(2)

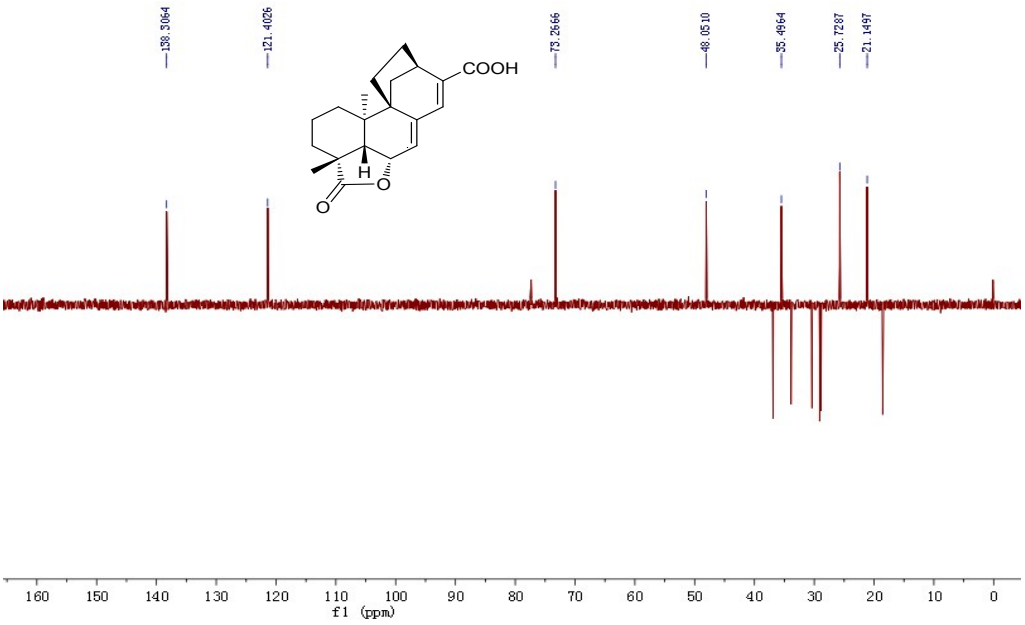
3. NMR, IR, HRESIMS, UV and CD spectral data:



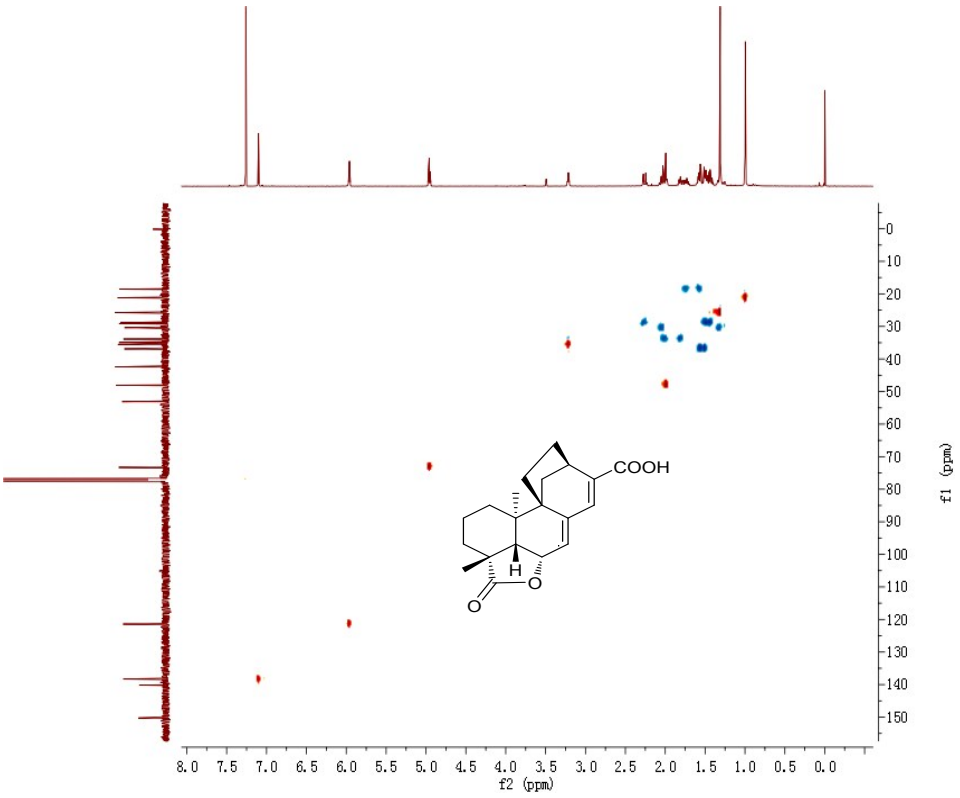
S1. ^{1}H NMR (500 MHz; $CDCl_3$) spectrum of euryachin A (**1**)



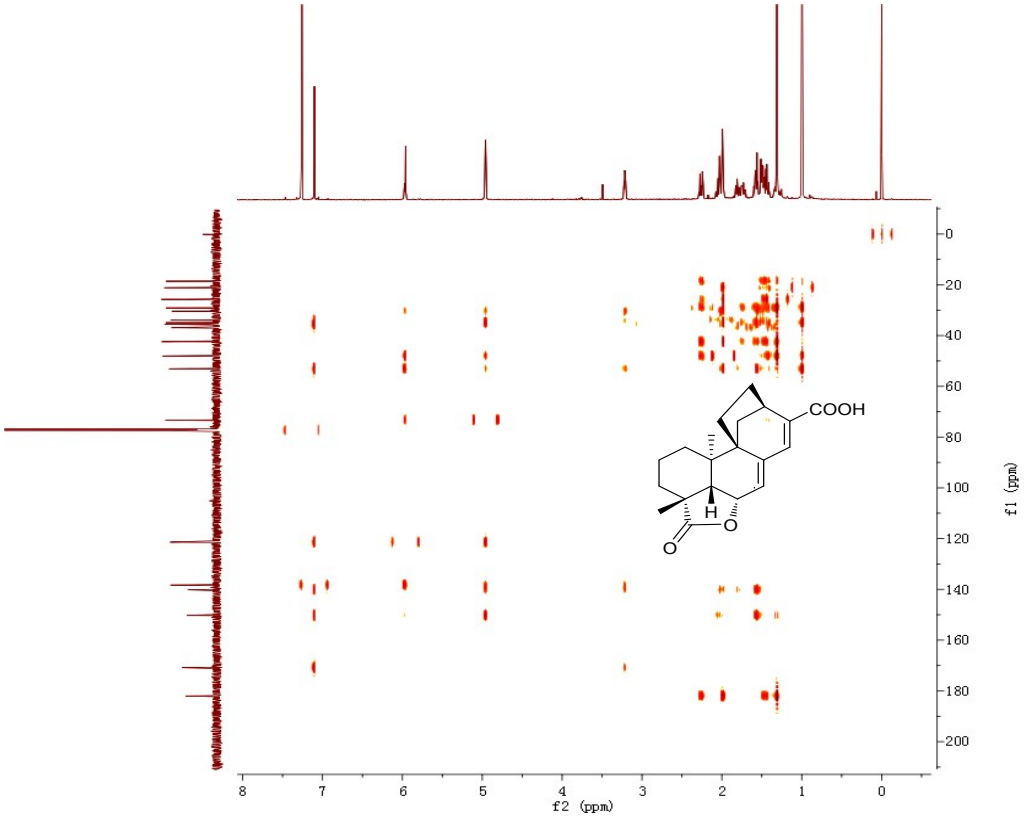
S2. ^{13}C NMR (125 MHz; $CDCl_3$) spectrum of euryachin A (**1**)



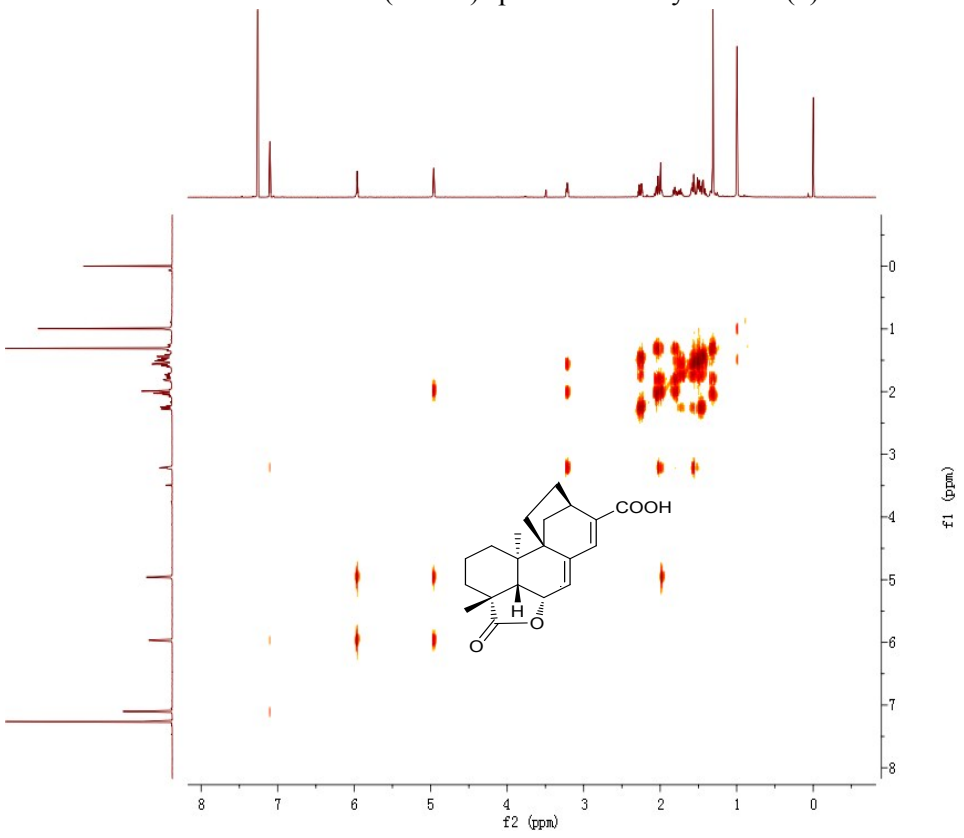
S3. DEPT-135 (125 MHz; $CDCl_3$) spectrum of euryachin A (**1**)



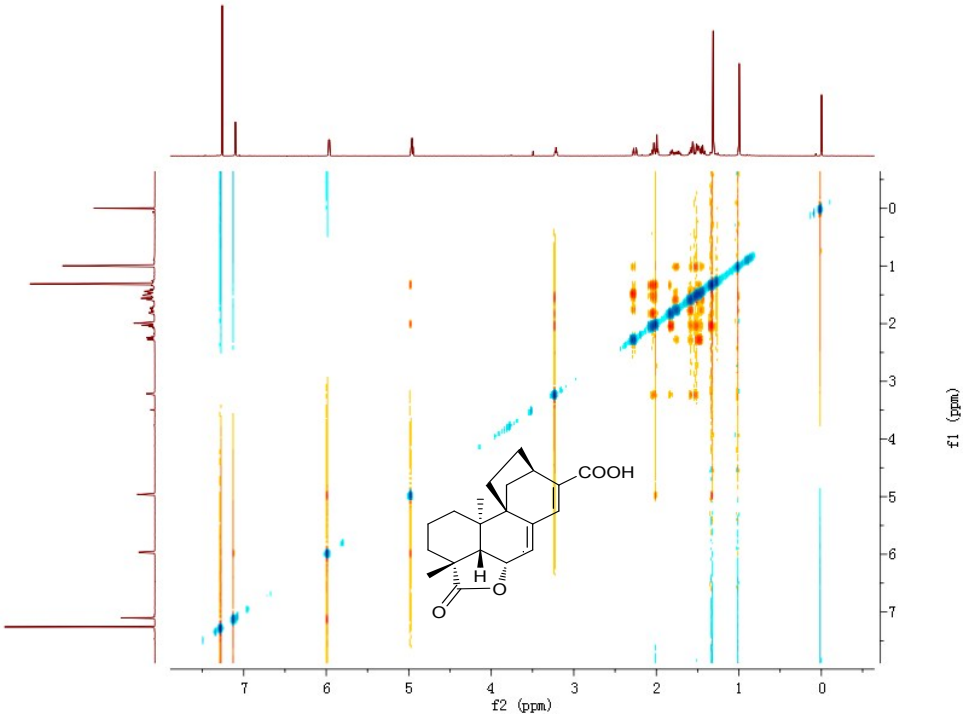
S4. HSQC ($CDCl_3$) spectrum of euryachin A (**1**)



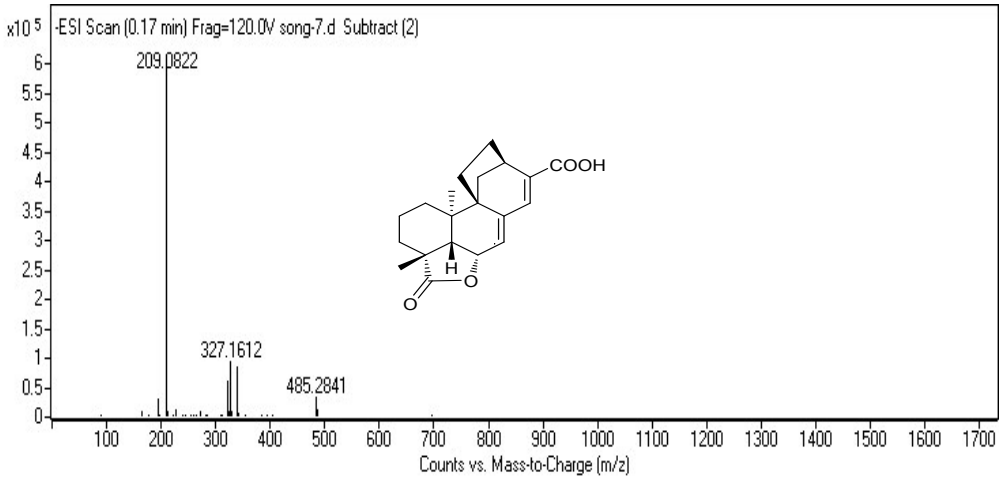
S5. HMBC ($CDCl_3$) spectrum of euryachin A (**1**)



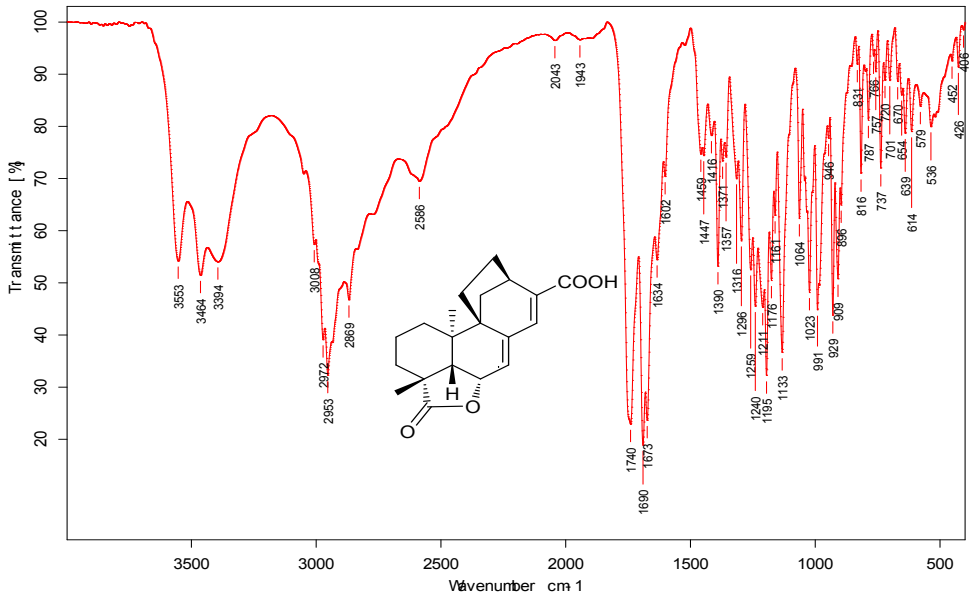
S6. ^{1}H-^{1}H COSY ($CDCl_3$) spectrum of euryachin A (**1**)



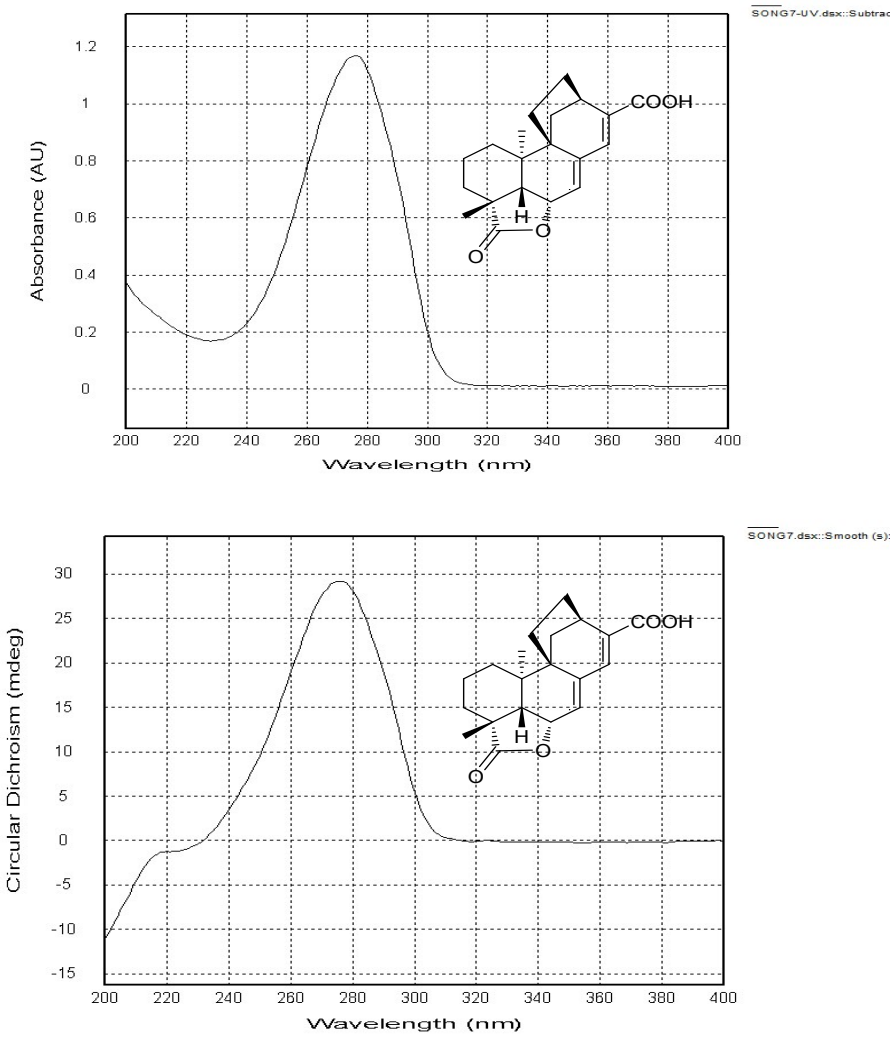
S7. NOESY ($CDCl_3$) spectrum of euryachin A (**1**)



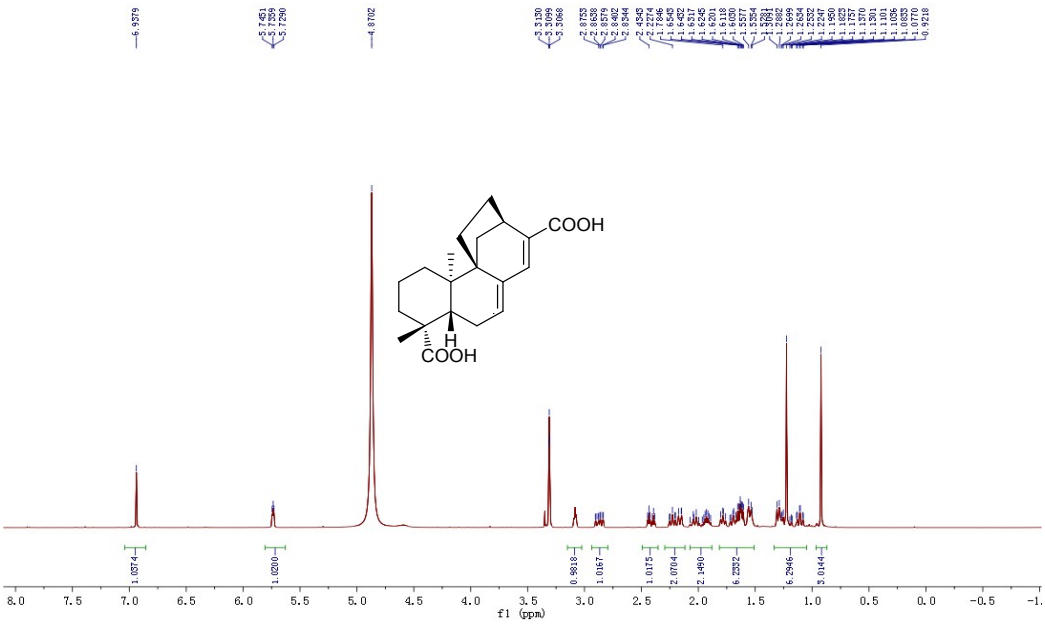
S8. HRESIMS of euryachin A (**1**)



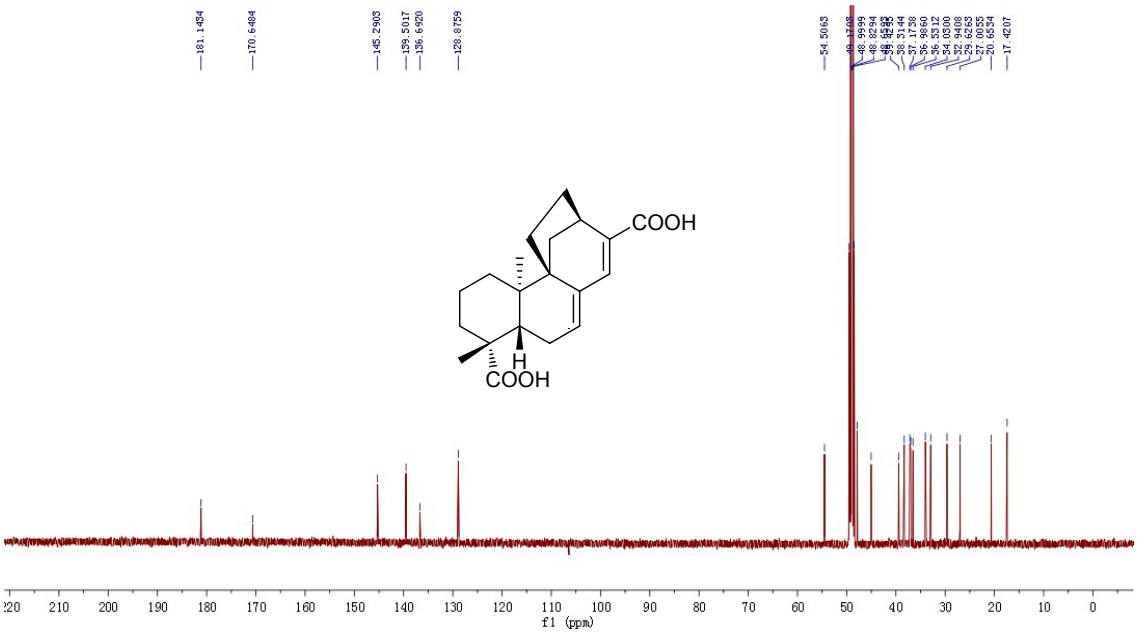
S9. IR spectrum of euryachin A (**1**)



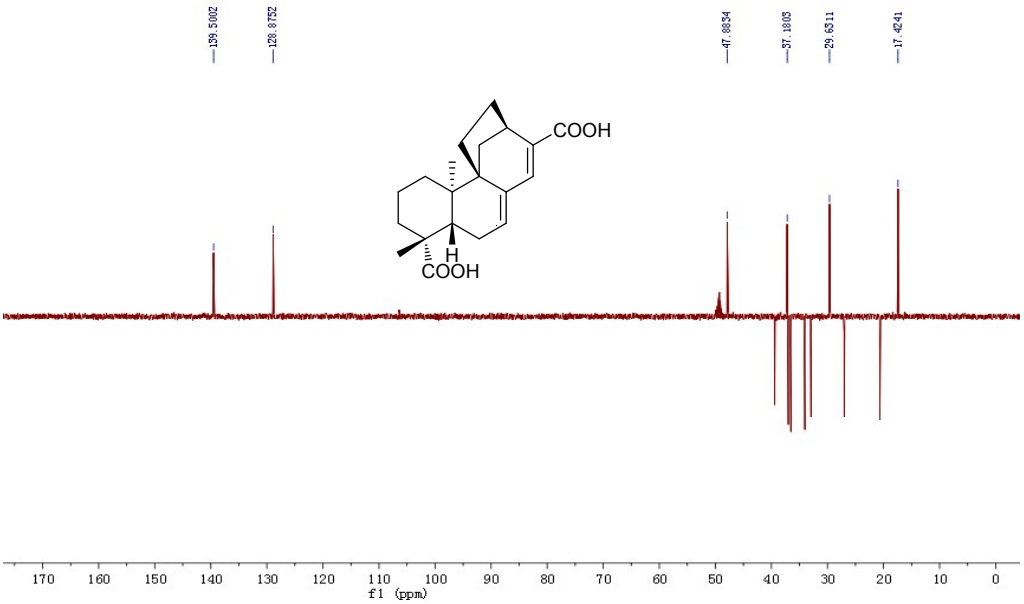
S10. UV (CH_3OH) spectra and experimental ECD (in CH_3OH) of euryachin B (**1**)



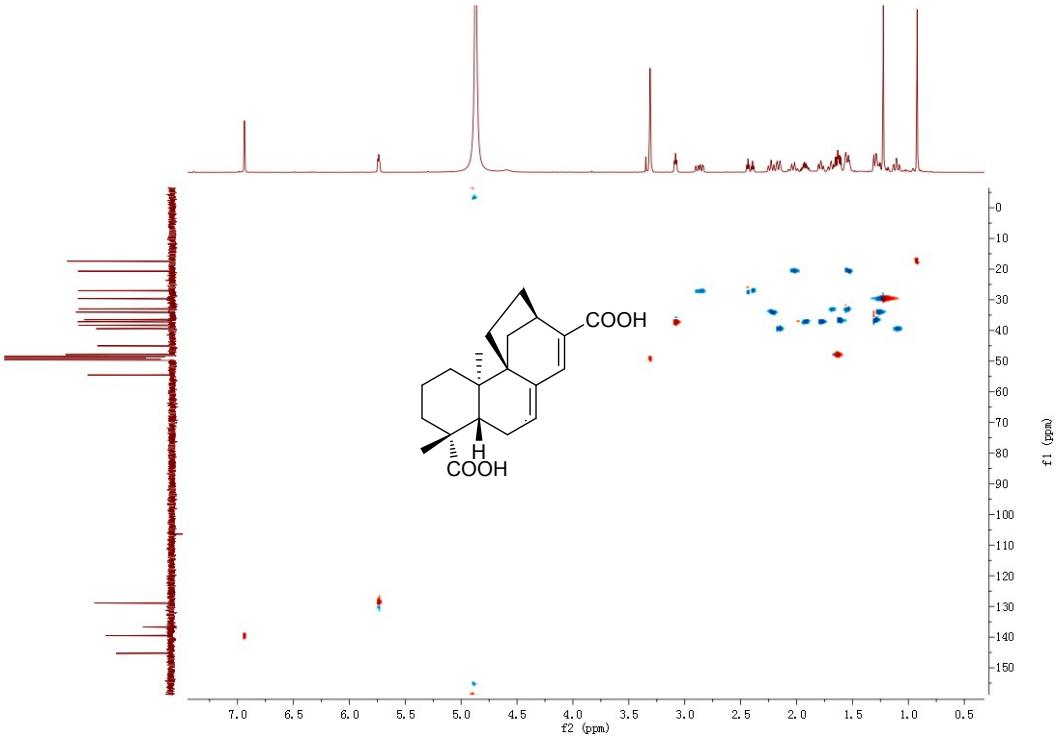
S11. ^{1}H NMR (500 MHz; CD_3OD) spectrum of euryachin B (**2**)



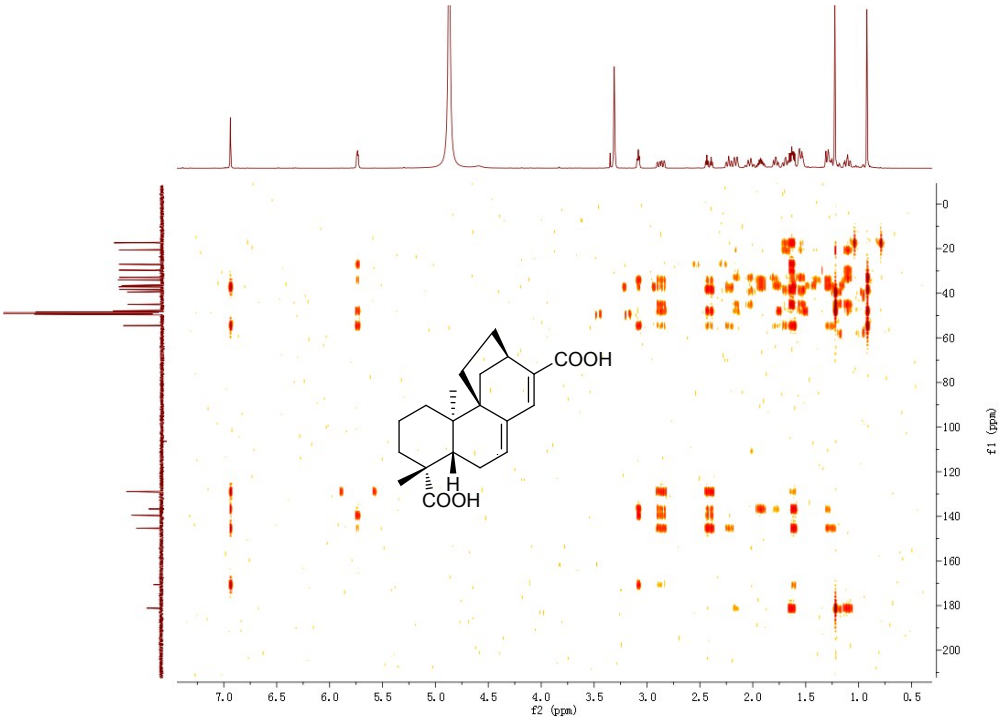
S12. ^{13}C NMR (125 MHz; CD_3OD) spectrum of euryachin B (**2**)



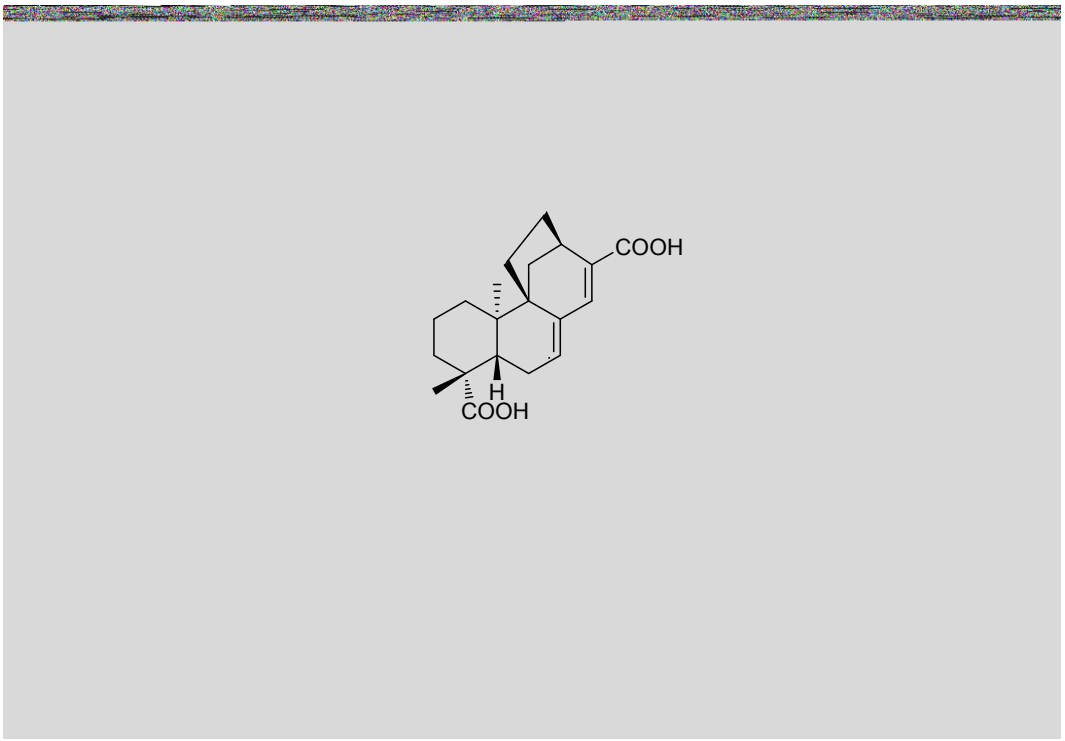
S13. DEPT-135 (125 MHz; CD_3OD) spectrum of euryachin B (**2**)



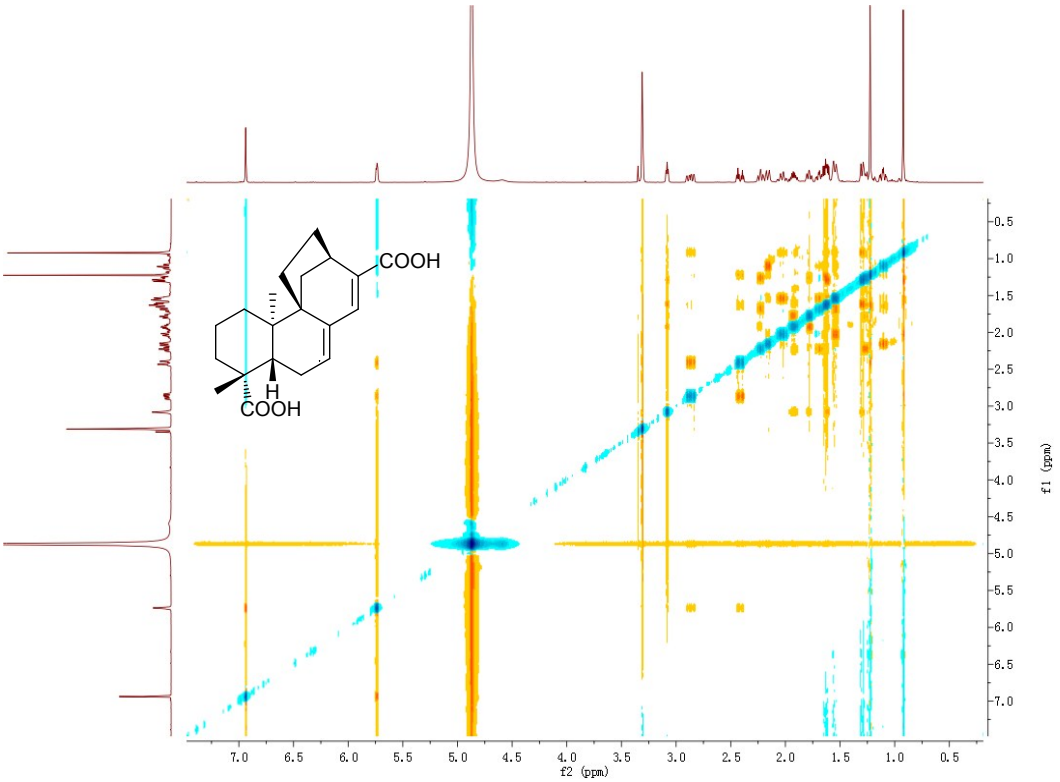
S14. HSQC (CD_3OD) spectrum of euryachin B (**2**)



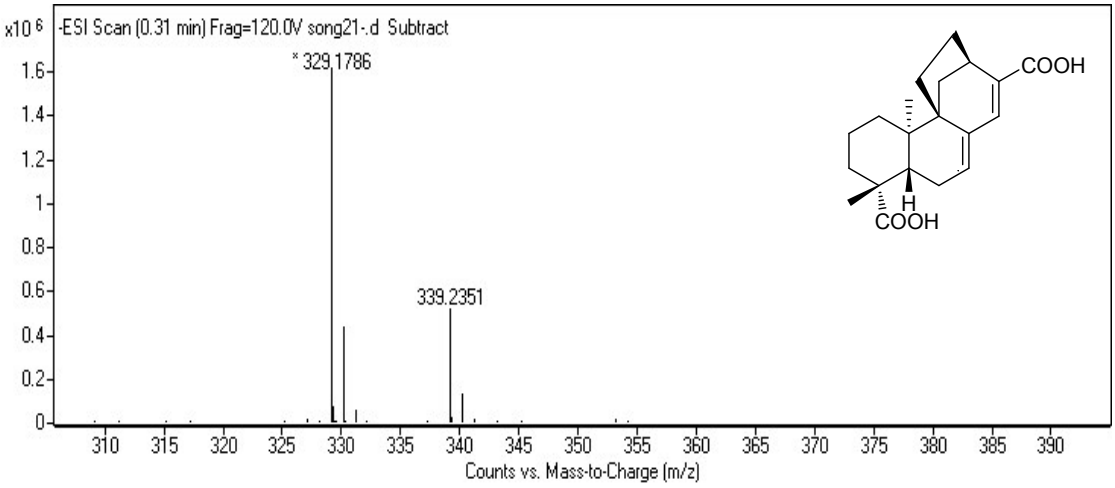
S15. HMBC (CD_3OD) spectrum of euryachin B (**2**)



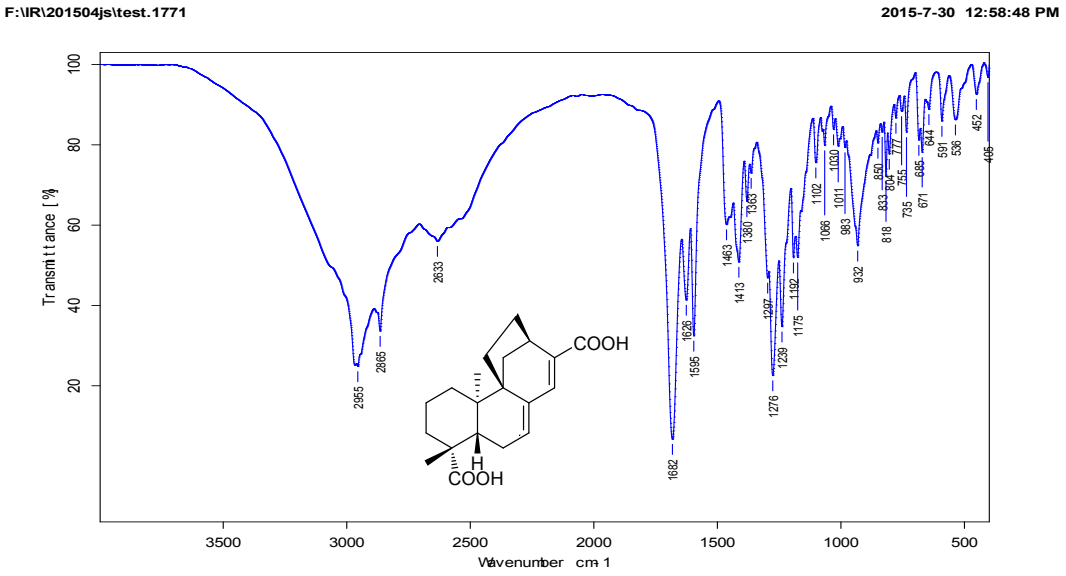
S16. ^1H-^1H COSY (CD_3OD) spectrum of euryachin B (**2**)



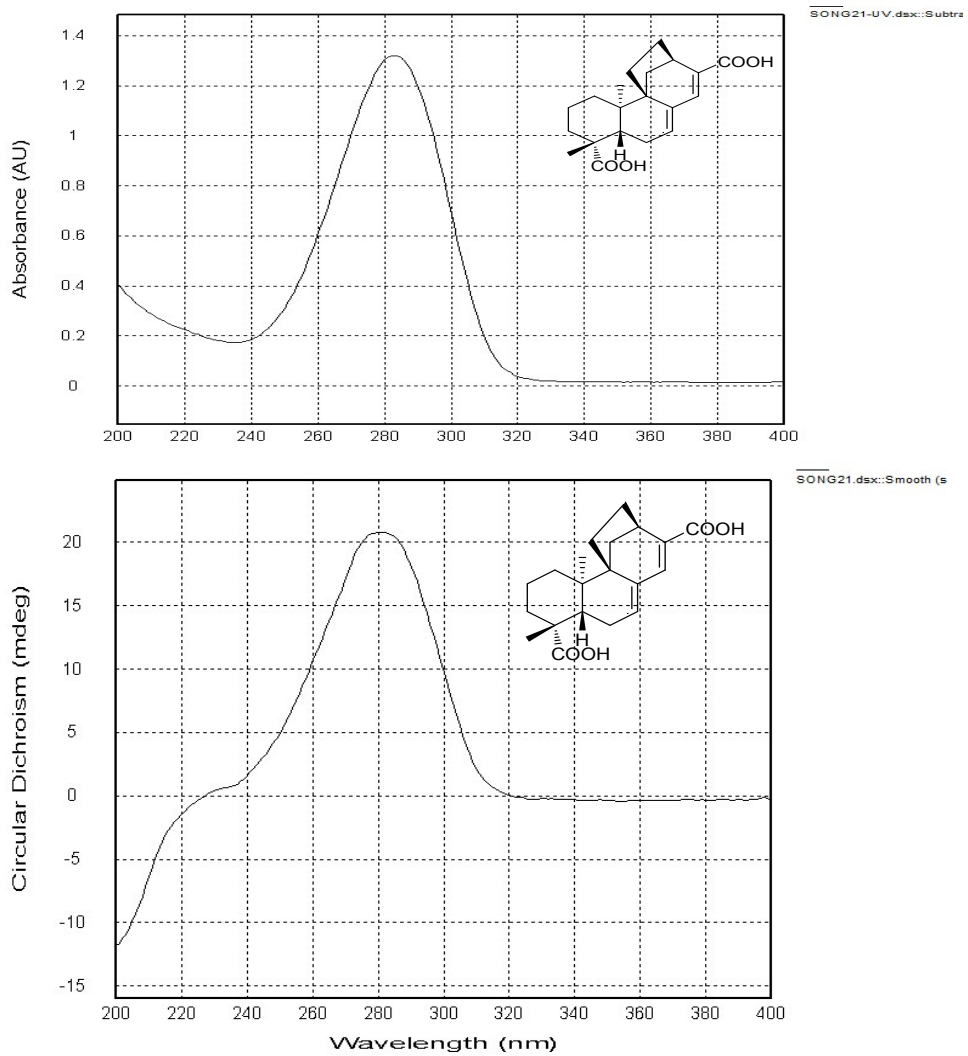
S17. NOESY (CD_3OD) spectrum of euryachin B (**2**)



S18. HRESIMS of euryachin B (**2**)



S19. IR spectrum of euryachin B (**2**)



S20. UV (CH_3OH) spectra and experimental ECD (in CH_3OH) of euryachin B (**2**)