Supporting information

A new benzaldehyde from the coral-derived fungus *Aspergillus terreus* C23-3 and its anti-inflammatory effects via suppression of MAPK signaling pathway in RAW264.7 cells

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Materials and methods

Material

The coral derived fungus *Aspergillus terreus* C23-3 which derives in the *Pectinia paeonia* was isolated from Xuwen natural reserve located at the South China Sea coastline in 2013 and identified using ITS rDNA gene sequencing (Yang et al., 2019, 2020).

General experimental procedures

1D and 2D NMR spectra were obtained on a Bruker Advance 700 MHz NMR spectrometer (Billerica, MA, USA) using tetramethylsilane as an internal standard. HR-ESI-MS spectra were recorded from a Burker maXis Q-TOF mass spectrometer (Billerica, MA, USA). The optical rotation was measured on an INESA SGW-3 polarimeter (Shanghai, China). The melting point was recorded on a WRX-4 melting point microscopic apparatus (Shanghai, China). A Büchi R-300 Rotavapor (Flawil, Switzerland) and a Büchi P-12 multivapor (Flawil, Switzerland) were used for sample preparation. Preparative purification was performed on a reversed-phased column (Elite-packed SinoChrom ODS-AP, 15 μm, 20.0×250 mm) using a HP Plus 50D HPLC separation module coupled with a UV-vis detector manufactured by Lisure Co., Ltd. (Suzhou, China). The silica gel for column chromatography was purchased from Qingdao Marine Chemistry Co. Ltd., Qingdao, China. Precoated silica gel plates (Merck, Silica gel 60 F254, Darmstadt, Germany) were used for TLC analysis.

Fermentation, extraction, and isolation

The producer strain was activated and cultured on a potato sucrose peptone agar (PSPA) petri dish at 28 ºC for seven days. The spore suspension was collected to inoculate 3 L-flasks, each containing 1 L autoclaved potato sucrose peptone broth medium containing 200 mL potato juice per L from 200 g fresh potato, sucrose 20 g/L, peptone 5 g/L, and sea salt 20 g/L, $ZnCl_2$ 10 mmol/L, with pH 7.2±0.2. The fungus was statically cultured for 28 d.

After the fermentation, the mycelia were immersed with methanol overnight, ultrasonically extracted for 30 min, filtrated to collect liquid and this process was repeated for 3 times. The fermentation broth was extracted with

ethyl acetate for 3 times. The two extracts were concentrated below 45 ºC under vacuum with a rotary evaporator and combined to give a final crude extract (191.9 g).

The above crude extract was subjected to 200–300 mesh silica gel vacuum liquid chromatography (VLC) eluted with *n*-hexane (Hex)-EtOAc (100% Hex–0% Hex) and EtOAc-methanol (100% EtOAc-0% EtOAc) to afford nine fractions (Fr1-Fr9). The Fr3 (6.43 g) was further separated by CC (200–300 mesh silica gel, CH_2Cl_2/MeOH=1:1, v/v) to provide 6 sub-fractions (Fr3-1 to Fr3-6). From sub-fraction Fr3-2 (0.41 g), compound 2 (9.7 mg) was obtained by preparative HPLC on RP-18 (methanol-H2O, 6:4). From sub-fraction Fr3-3 (0.13 g), compound 1 (1.7 mg) was also obtained by preparative HPLC on RP-18 (methanol-H_2O, 6:4).

Compound **1**: Yellowish glue. M.p. 113.2–115.3 ºC. UV λ_{max} (MeOH-H_2O, 60:40) 229 and 292 nm (Fig. S1). $[\alpha]_D^{25}$ –13.2 (*c* 0.243, $CHCl_3$). ^1H and ^{13}C NMR data: see Table 1 & Figs. S2–S6. HR-ESI$^+$-MS: *m*/*z* 383.1486 $[M+H]^+$ (calc. 383.1489 for $C_{22}H_{23}O_6^+$) (Fig. S7).

Compound **2**: Yellowish glue. ^1H and ^{13}C NMR data (in CD_3OD): δ_H 7.61 (1H, d, *J*=2.0 Hz, H-2), 6.87 (1H,d, *J*=8.2 Hz, H-5), 7.59 (1H, dd, *J*=8.2, 2.1 Hz, H-6), 9.70 (1H, s, H-7), 3.32 (2H, d, *J*=7.4 Hz, H_2-1'), 5.32 (1H, m, H-2'), 1.75 (3H, s, H_3-4'), 1.71 (3H, s, H_3-5'); δ_C 131.54 (C-1), 129.94 (C-2), 130.50 (C-3), 163.55 (C-4), 116.08 (C-5), 134.01 (C-6), 193.12 (C-7), 28.94 (C-1'), 122.94 (C-2'), 132.26 (C-3'), 25.94 (C-4'), 17.82 (C-5').

Cell culture and viability assay

RAW264.7 murine macrophage cell line acquired from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). They were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator. The potential cytotoxicity of the two benzaldehydes (**1** and **2**) on cells was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Briefly, RAW264.7 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well before treated with the two benzaldehydes (**1** and **2**) in dose of 0.1, 1, and 10 μmol/L for 24 h, respectively. Then, 100 μL of MTT was added to each well. After 4 h, 100 μL DMSO was added to every well to dissolve the formazan crystals. Ultimately, the absorbance was measured at 540 nm.

NO determination

The levels of NO of RAW264.7 cells, treated with and without lipopolysaccharide (LPS) and the two benzaldehydes (**1** and **2**), were assessed via the Griess Reagent System according to specification (Promega, Madison, USA).

RAW264.7 cells were seeded in a 24-well plate at a density of 5×10^4 cells/well. In all test groups, cells were treated with the two benzaldehydes (**1** and **2**) in the dose of 0.1, 1, and 10 μmol/L for 1 h, respectively. Subsequently, activating them with LPS (1 mg/mL) for 24 h, except for the blank group. Finally, NO production was measured estimated photometrically at 540 nm.

ROS determination

The accumulation of ROS was assessed by DCFH-DA assay kit. RAW264.7 cells cultured in 24-well plates of 1×10^4 cells/well for 24 h before the experiment, were treated with either of the two benzaldehydes (**1** and **2**) in the dose of 0.1, 1, and 10 μmol/L for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Then cells were washed with phosphate-buffered saline (PBS) three times before incubated with DCFH-DA (10 μmol/L) for 30 min. Ultimately, the fluorescence intensity was visualized by an inverted fluorescence microscope (Olympus Opticals, Tokyo, Japan).

Western blot analysis

RAW264.7 cells seeded in 6-well plates at a density of 5×10^6 cells/well were pre-treated with the two benzaldehydes (**1** and **2**) in the dose of 0.1 and 10 μmol/L for 1 h, respectively, before being stimulated by LPS (1 μg/mL) for 24 h. Cell lysates were acquired using RIPA buffer with a protease and phosphatase inhibitor cocktail behind rinsed thrice with cold PBS.

The concentrations of protein were determined using Pierce BCA Protein Assay Kit. Isometric proteins (20–40 μg) were separated electrophoretically using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). And it transferred onto nitrocellulose (NC) filter membranes (Amersham, USA). Then the membranes were blocked at room temperature with 5% skim milk dissolved by TBST (Tris-buffered saline with 0.1% Tween-20) for 2 h. Subsequently, washing with TBST thrice before the membranes were incubated with primary antibodies at 4 °C overnight. Then wash the membranes in the same way, following incubated with HRP-conjugated secondary antibodies (goat anti-mouse) diluted by TBST at 1:2000 at room temperature for 2 h. Images obtained with Blots were quantified by chemiluminescence apparatus and analyzed by imageJ.

IL-6 determination

The levels of IL-6 in the supernatant of cultured cells were detected by using enzyme-linked immunosorbent assay (ELISA). Briefly, RAW264.7 cells were seeded at 5×10^5 cells/well in 24-well plates for 24 h. The cells were treated with either of the two benzaldehydes (**1** and **2**) in the dose of 0.1 and 10 μmol/L for 1 h and then activated by LPS (1 μg/mL) for 24 h. The culture supernatant fluid was collected and centrifuged at 1000×*g* for 20 min. The level of IL-6 in the culture medium was quantified using ELISA kits according to the manufacturer's instruction book (Elabscience, China). Ultimately, the optical density (OD) at 450 nm was detected in a microplate reader.

Molecular docking

The molecular docking was taken to explore the interaction of the two benzaldehydes (**1** and **2**), iNOS and COX-2 at the molecular level. First of all, ChemBioDraw Ultra 14.0 and ChemBio3D Ultra 14.0 were used to draw the structure of the two benzaldehydes (**1** and **2**) shown in Fig. 1 and make the transformation of the

three-dimensional structure. Then, the outcome was optimized by the force field of MMFF94. In addition, the three-dimensional structure of iNOS and COX-2 was acquired from RCSB Protein Data Bank (www.rcsb.org). The three-dimensional crystal structure of COX-2 and its inhibitor celecoxib (PDB ID: 3LN1) was used as the protein which was described to conduct the molecular docking with the two benzaldehydes (**1** and **2**) in this study. The two benzaldehydes (**1** and **2**), iNOS and its inhibitor SAR (PDB ID: 3NW2) were conducted the molecular docking in the same way. Furthermore, the two benzaldehydes (**1** and **2**), iNOS and COX-2 were converted to PDBQT format using Autodock Tools 1.5.6 (Morris and Huey, 2009; Sanner, 1999). Autodock vina 1.1.2 (Trott and Olson, 2009) was adopted in molecular docking in this study. According to location of ligand celecoxib, the coordinates of COX-2 active site were determined as following: center_x=25.812, center_y=−30.399, center_z=−3.788; size_x=40, size_y=40, size_z=40, while the coordinates of the active site of iNOS were determined as: center_x=120.828, center_y=110.64, center_z=30.084; size_x=40, size_y=40, size_z=40, according to position of SAR. To increase the accuracy of the calculation, we set the parameter Exhaustion to 100. Default values are used for all parameters unless otherwise stated. Finally, the conformation with the highest scoring value was selected to analyze the results with Pymol 1.7.6.

Statistical analysis

All experiment results were performed in at least triplicate and data were expressed as the means±SD (n=3). Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA) and t-test. A value of $P<0.05$ was considered statistically significant.

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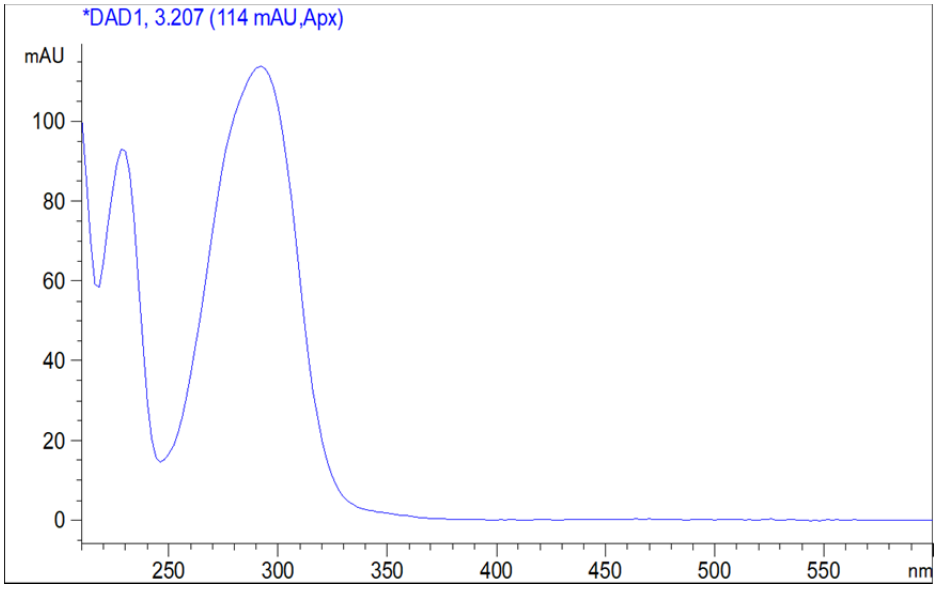

Fig. S1 The UV spectrum of compound 1 (obtained on a HPLC-DAD system eluted by MeOH-H_2O=60:40).

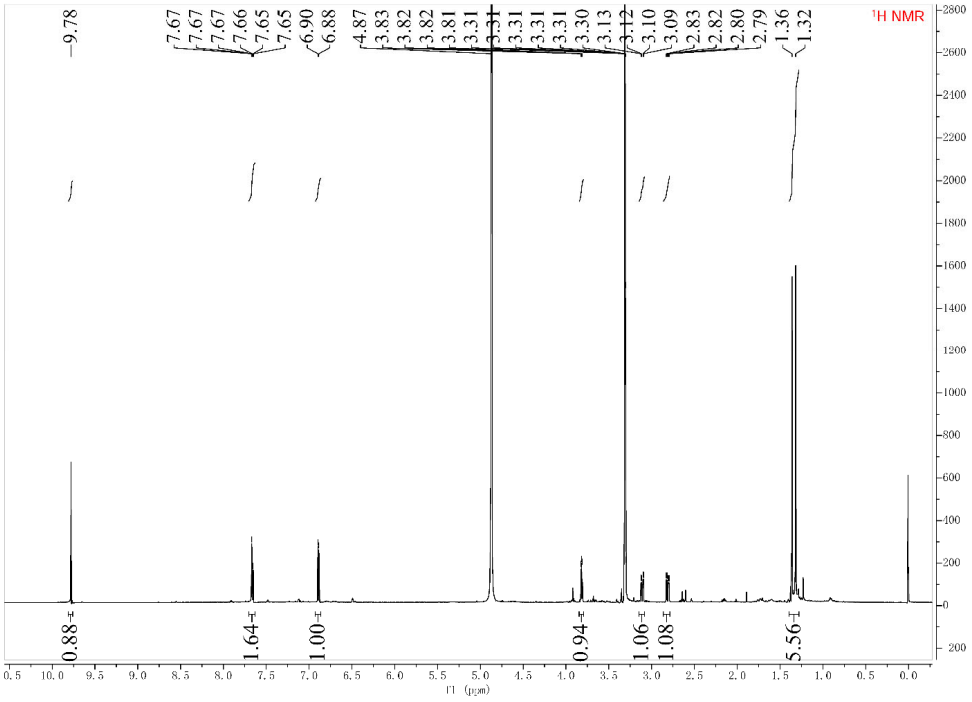

Fig. S2 The ^{1}H NMR spectrum of compound 1 (in CD_3OD).

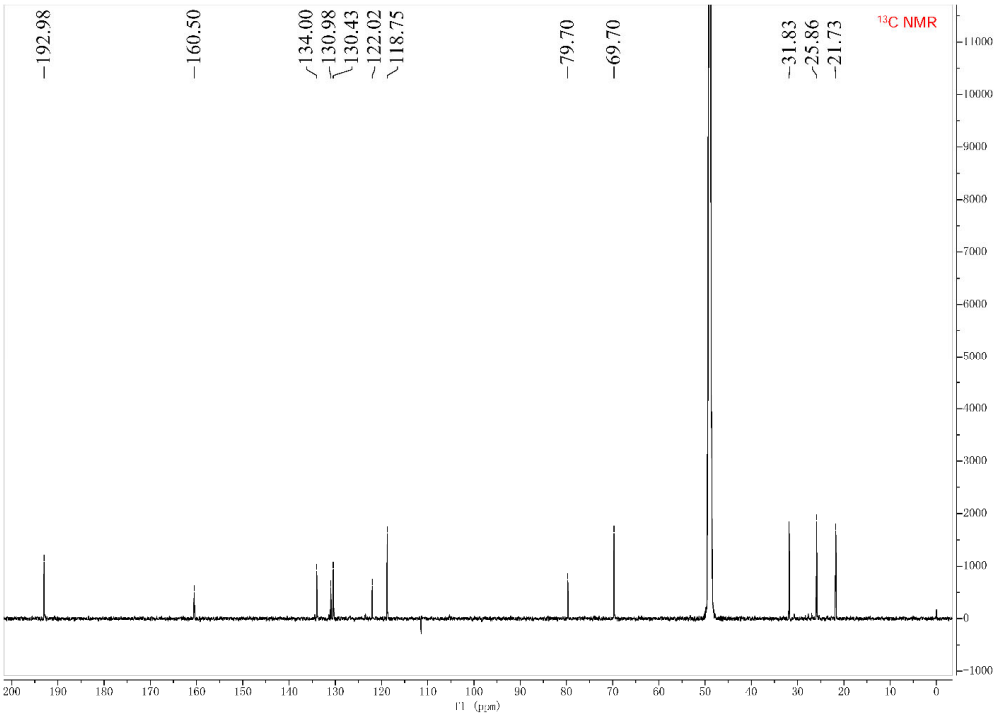

Fig. S3 The ^{13}C NMR spectrum of compound 1 (in CD_3OD).

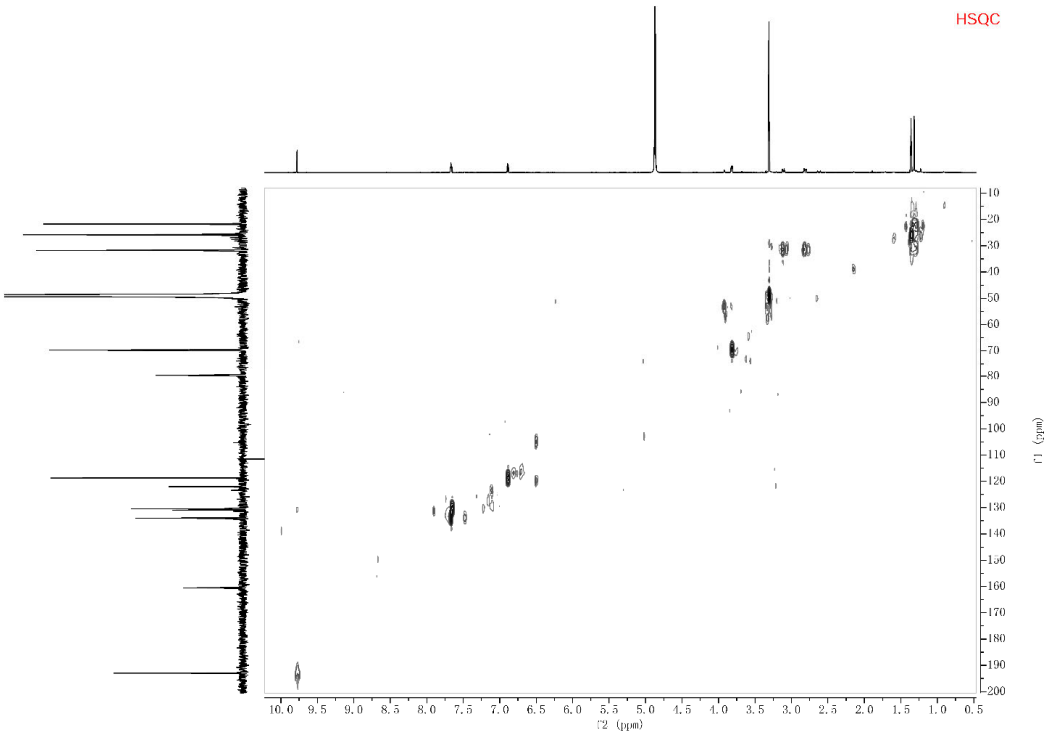

Fig. S4 The HSQC spectrum of compound 1 (in CD_3OD).

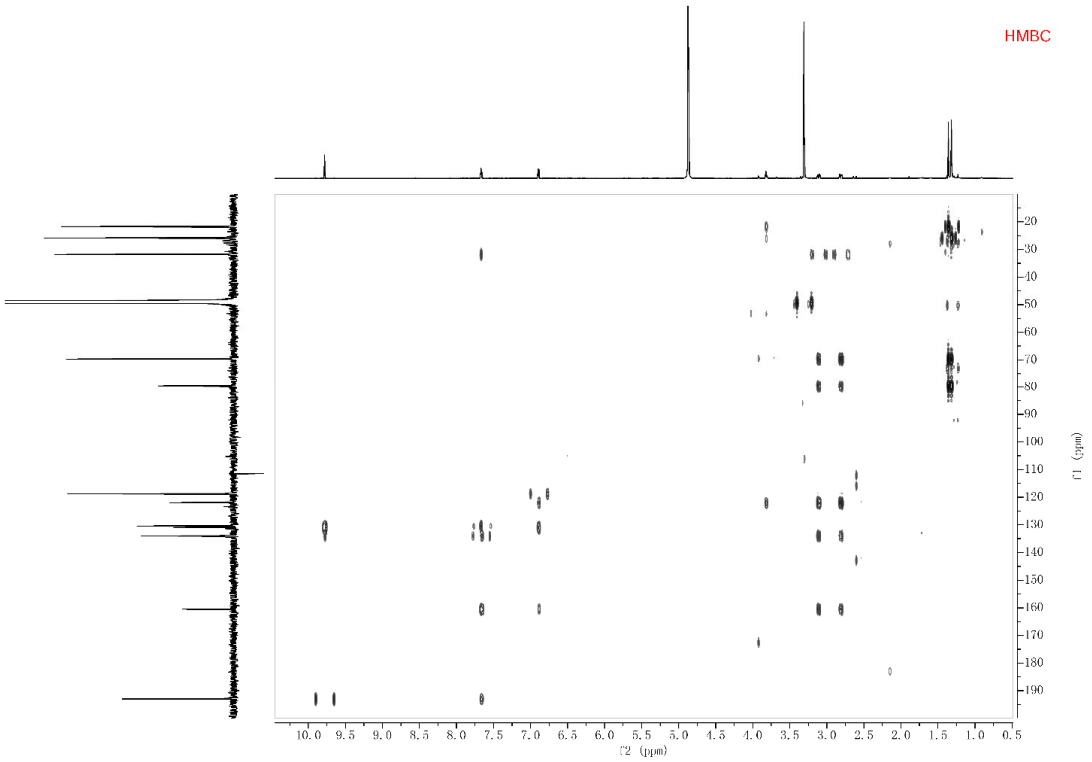

Fig. S5 The HMBC spectrum of compound 1 (in CD_3OD).

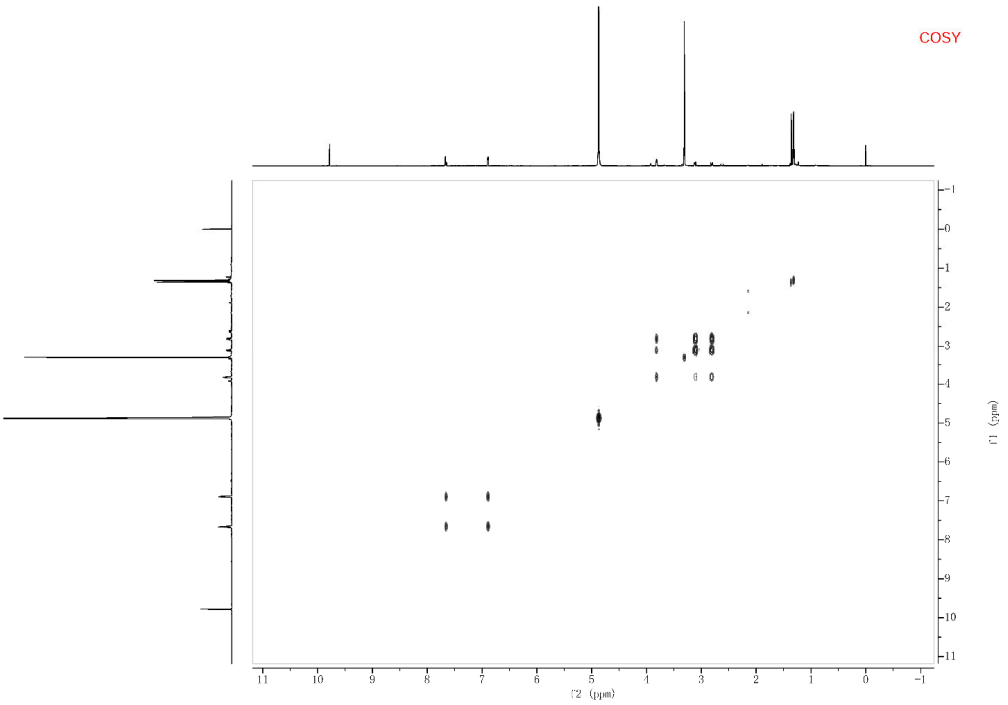

Fig. S6 The COSY spectrum of compound 1 (in CD_3OD).

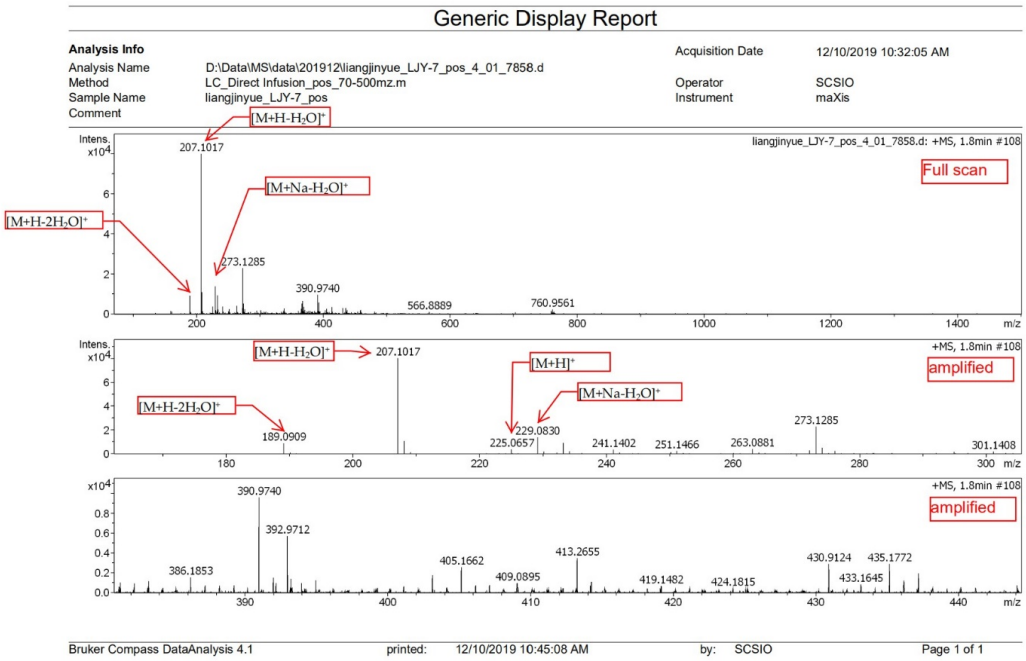

Fig. S7 HR-ESI(+) mass spectra of compound 1.