SUPPLEMENTARY MATERIAL

Diterpenoid Alkaloids from *Aconitum brevicalcaratum* as Autophagy Inducers

Haiyue Jiang[a, ǂ], Shuai Huang[a, b, ǂ], Feng Gao[a], Yongqi Zhen[a], Chunying Li[b] and Xianli Zhou[a*]

[a] *School of Life Science and Engineering, Southwest Jiaotong University, Chengdu 610031, Sichuan, P.R. China.*

[b] *Center for Molecular and Translational Medicine, Institute of Biomedical Sciences, Georgia State University, 511 Research Science Center, 157 Decatur St SE, Atlanta, GA 30303, USA*

ǂ *These authors contributed equally to this work and should be considered co-first authors.*

*Corresponding author:
Prof. Dr. Xian-li Zhou; Phone: + 862866367232; E-mail: zhouxl@swjtu.edu.cn

---

Abstract: A new C_{19} diterpenoid alkaloid, brevicanine (**1**) and six known ones (**2**–**7**) were isolated from *Aconitum brevicalcaratum* (Finet et Gagnep.) *Diels*. Their structures were elucidated on the basis of extensive spectroscopic analyses. The cytotoxicity of those compounds was investigated against HCT116 human cancer cell line, which showed none of them possessing considerable anti-proliferative activities. To evaluate the autophagy effect of compounds **1**–**7**, Western blot was used to detect the expression of autophagic marker by stimulating human cancer HCT116 cells. The results showed that compound **6** induced protective autophagy in HCT116 cells. Mechanistic insight showed that compound **6** induced protective autophagy through p53 activation, ERK1/2 and p38 MAPK signaling cascade.
Keywords: *Aconitum brevicalcaratum* (Finet et Gagnep.) *Diels*; Brevicanine; Autophagy; HCT116

Caption

	page
Experimental Section	1
Table S1. NMR spectroscopic data of compound **1** (600 MHz for ^{1}H NMR, 150 MHz for ^{13}C NMR, $CDCl_3$)	5
Figure S1. Key HMBC, NOESY and ^{1}H-^{1}H COSY correlations of compound **1**	5
Figure S2. The cell inhibitory ratio of compounds 1–7 in HCT116 cell lines was measured using an MTT assay. The cells were treated with compounds 1–7 at 0−200 μM for 24 h.	6
Figure S3. (**A**) Effect of compounds 1–7 on the induction of autophagy in HCT116 cells. HCT116 cells were cultured with 50 μM of compounds 1–7 for 24 h, and the protein expression levels of LC3-II were determined by Western blot analysis. (**B**) HCT116 cells were cultured with the indicated concentrations of compound 6 for 24 h. The protein expression levels of autophagy markers were determined by Western blot analysis.	6
Figure S4. Effect of scaconitine (**6**) on the Akt/mTOR signaling pathway and the p53-mediated mitogen-activated protein kinase (MAPK) signaling in HCT116 cells. (**A**) Cells were lysed after treatment with indicated concentrations of compound 6 for 24 h. The protein expression levels of p-Akt (Ser473), Akt, p-ERK, ERK, p-p38, p38, p-JNK and JNK were determined by Western blot analysis. (**B**) HCT116 cells were treated with 100 μM of compound 6 for **6**, 12, 24, 36, and 48 h. Western blotting was also performed for p-ERK, ERK, p-p38, p38 (B), p53, p-Akt (Ser473), and Akt. GAPDH was used as an internal standard.	7
Figure S5. Scheme representing the proposed molecular mechanism underlying the action of compound **6** in induction of protective autophagy through the activated ERK1/2, p38 of the MAPK signaling pathway and p53.	8
Figure S6. Compound **6** was docked into the binding pocket of the human p38 MAPK.	9
Figure S7. Compound **6** was docked into the binding pocket of the human ERK1/2.	9
Figure S8. IR spectrum for compound **1**	10
Figure S9. HR-ESI-MS spectrum for compound **1**	10
Figure S10. ^{1}H NMR spectrum in $CDCl_3$ for compound **1**	11
Figure S11. ^{13}C NMR spectrum in $CDCl_3$ for compound **1**	11
Figure S12. DEPT spectrum in $CDCl_3$ for compound **1**	12
Figure S13. ^{1}H-^{1}H COSY spectrum in $CDCl_3$ for compound **1**	12
Figure S14. HMQC spectrum in $CDCl_3$ for compound **1**	13
Figure S15. HMBC spectrum in $CDCl_3$ for compound **1**	13
Figure S16. Noesy spectrum in $CDCl_3$ for compound **1**	14

Experimental Section

General Experimental Procedures

Optical rotations were measured on a Perkin–Elmer 341 polarimeter. 1D and 2D NMR spectra were recorded on a Bruker AV 600 NMR spectrometer with TMS, and IR spectra on a Thermo Fisher Nicolet 6700 spectrometer (KBr discs, cm^{-1}). HR–ESI–MS were carried out on a Q–TOF micro mass spectrometer (Waters, USA). Silica gel (Qingdao Haiyang Chemical Co., Ltd., China, 200–300 mesh), and alumina (Qingdao Haiyang Chemical Co., Ltd., China, 100–200 mesh) were used for column chromatography (CC). The TLC plates were precoated with silica gel GF_{254} (Qingdao Haiyang Chemical Co., Ltd., China), the mobile phase consisted of petroleum ether: Me_2CO: Et_2N (7: 3: 0.1) or $CHCl_3$: MeOH (9: 1), and visualized under a UV lamp at 254 nm or by spraying Dragendorff's reagent or iodine. The antibodies for p-Akt (Ser473), Akt, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-p38 (Thr180/Tyr182), p38, JNK, p-JNK(Thr183/Tyr185), LC3-II, p62, beclin 1, and the secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Plant Material

Aconitum brevicalcaratum (Finet et Gagnep.) Diels were collected in Lijiang, Yunnan Province, People’s Republic of China, in August 2016, and identified by Prof. Liang-ke Song of the Institute of Life Science and Engineering, Southwest Jiaotong University. A voucher specimen (No. ZN361520160821) was deposited in the School of Life Science and Engineering, Southwest Jiaotong University, Sichuan, China.

Extraction and Isolation

The shade dried and powdered *Aconitum brevicalcaratum* (Finet et Gagnep.) Diels plant (5 kg) was soaked with 95% EtOH (6 × 20 L) at room temperature for 3 days. After removing the solvent, the ethanol extract (300 g) was recovered. The extract was then suspended water (2 L) and adjusted to pH 3.0 with hydrochloric acid solution and successively extracted with light petroleum (4 × 1 L) and ethyl acetate (4 × 1 L). The aqueous layer was adjusted to pH 10 using an aqueous ammonia solution and extracted with CH_2Cl_2 (4 × 1 L). The CH_2Cl_2 extract was concentrated to produce the crude alkaloid extract (70 g).

The crude alkaloid (70 g) was subjected to column chromatography on silica gel eluted in a gradient manner CH_2Cl_2/MeOH (100:1 to 0:1, v/v) to afford six fractions: A–F based on TLC analyses. A was subjected to CC on silica gel eluted with light petroleum: Me_2CO: Et_2N (100: 1: 0.1 to 20:1: 0.1, v/v) to afford two fractions: A_1–A_3. A_1 was subjected to CC on silica gel eluted with light petroleum: Me_2CO: Et_2N (200: 1: 0.1 to 170:1: 0.1, v/v) to obtain compounds **1** (18 mg) and **3** (30mg). A_2 were re-crystallization treated with light petroleum and Me_2CO to obtain compound **2** (300 mg). A_3 was subjected to CC on silica gel eluted with light petroleum: Me_2CO: Et_2N (150: 1: 0.1 to 60:1: 0.1, v/v) to afford three fractions: $A_{3\text{-}1}$–$A_{3\text{-}3}$. $A_{3\text{-}1}$ was subjected to CC on silica gel eluted with light petroleum: Me_2CO: Et_2N (200: 1: 0.1 to 100:1: 0.1, v/v) to afford compound **4** (30 mg); $A_{3\text{-}2}$ was subjected to CC on silica gel eluted with light petroleum: Me_2CO: Et_2N (200: 1: 0.1 to 80:1: 0.1, v/v) to obtain compound **5** (17 mg); $A_{3\text{-}3}$ was subjected to CC on silica gel eluted with light petroleum: Me_2CO: Et_2N (90: 1: 0.1 to 60:1: 0.1, v/v) to obtain compound **6** (15 mg); B was subjected to CC on silica gel eluted with CH_2Cl_2/MeOH (80:1 to 10:1, v/v) to obtain compound **7** (10 mg).

Spectroscopic data of 1

Brevicanine (1): White, amorphous solid; $[\alpha]_D^{20}$ -9.38 (*c* 0.80, $CHCl_3$); IR (KBr)ν_{max}: 3320, 2925, 1702, 1688, 1589, 1527, 1449, 1383, 1261, 1089, 757 cm^{-1}; HR–ESI–MS at *m/z*: 597.3547 $[M+H]^+$ (calcd for 597.3540 $C_{34}H_{49}N_2O_7$); ^1H and ^{13}C NMR data see Table **1**.

Cell Culture

HCT 116 human colon cancer cell line were obtained from ATCC. These cells were maintained at subconfluence in a 95% air and 5% CO_2 humidified atmosphere at 37 °C. McCoy's 5A medium supplemented with 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin was used for routine culturing.

Cytotoxicity Assay

The cytotoxicity of the compounds against HCT116 human colon cell lines was evaluated by the MTT assays described in our previous paper (Huang et al. 2013). Cells treated with DMSO (0.1% *v/v*) were used as negative controls, whereas

adriamycin (≥ 98% ; Sigma Chemical Co., Ltd., Shanghai, China) was used as the positive control.

Western Blot Analysis

After treatment with various concentrations of compounds, the cells were collected and lysed in lysis buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail, and phophatase inhibitor cocktail). A 20 μg aliquot of total protein was separated by 10% SDS–PAGE gel, transferred to nitrocellulose membranes, blocked with 5% BSA, and probed with a primary antibody (1:1000) followed by the corresponding secondary antibody (1:3000). The signal was detected with Amersham Imager 600 (GE Healthcare, England).

Molecular Docking

Molecular docking study was performed to investigate the binding mode between the compound **6** and the human p38 MAPK and ERK1/2 using Autodock vina 1.1.2 (Trott et al. 2010). The three-dimensional (3D) structure of the human p38 MAPK (PDB ID: 3S3I) and ERK1/2 (PDB ID: 5NGU) were downloaded from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The 2D structure of the compound **6** was drawn by ChemBioDraw Ultra 14.0 and converted to 3D structure using ChemBio3D Ultra 14.0 software. The AutoDockTools 1.5.6 package (Sanner 1999; Morris et al.2009) was employed to generate the docking input files. The search grid of the p38 MAPK was identified as center_x: 2.686, center_y: 29.42, and center_z: 29.194 with dimensions size_x: 15, size_y: 15, and size_z: 15, while the search grid of the ERK1/2 was identified as center_x: -14.906, center_y: 15.892, and center_z: 43.095 with dimensions size_x: 15, size_y: 15, and size_z: 15. The value of exhaustiveness was set to 20. For Vina docking, the default parameters were used if it was not mentioned. The best-scoring pose as judged by the Vina docking score was chosen and visually analyzed using PyMoL 1.7.6 software (http://www.pymol.org/).

References

Huang S, Zhou XL, Wang CJ, Wang YS, Xiao F, Shan LH, Guo ZY, Weng J. 2013. Pyrrolizidine alkaloids from *Liparis nervosa* with inhibitory activities against

LPS–induced NO production in RAW264.7 macrophages. Phytochemistry. 93:154-161.

Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. 2009. $AutoDock_4$ and $AutoDockTools_4$: Automated docking with selective receptor flexibility. J Comput Chem. 30:2785.

Sanner MF. 1999. Python: a programming language for software integration and development. J Mol Graph Model. 17:57.

Trott O, Olson AJ. 2010. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem. 31:455.

Table S1. NMR spectroscopic data of compound **1***

NO.	^{1}H NMR	^{13}C NMR	NO.	^{1}H NMR	^{13}C NMR
1	3.14 t (7.2)	85.5 d	18	3.98 ABq (10.8)	71.1 t
2	1.85 [a]	26.5 t		4.11 ABq (10.8)	
3	1.51 m	32.9 t	19	2.15 [a]	53.0 t
	1.85 [a]			2.61 d (11.4)	
4	—	38.1 s	21	2.43 [a]	49.3 t
5	1.65 d (7.2)	45.9 d		2.52 m	
6	1.45 m	24.5 t	22	1.08 t (7.2)	13.6 q
	1.98 m		1-OCH_3	3.28 s	56.5 q
7	2.23 [a]	43.7 d	8-OCH_3	3.13 s	48.2 q
8	—	77.7 s	14-OCH_3	3.35 s	57.8 q
9	2.43 [a]	40.3 d	16-OCH_3	3.35 s	56.5 q
10	2.29 m	38.5 d	*CO*C_6H_4	—	168.3 s
11	—	49.5 s	NH*CO*CH_3	—	169.2 s
12	1.85 [a]	29.6 t		2.23 s	25.6 q
	2.36 m		1'	—	115.0 s
13	1.85 [a]	46.0 d	2'	—	141.9 s
14	3.53 t (4.8)	83.9 d	3'	8.70 d (8.4)	120.6 d
15	2.01 m	35.5 t	4'	7.54 t (8.4)	134.8 d
	2.15 [a]		5'	7.09 t (8.4)	122.5 d
16	3.22 t (8.4)	83.8 d	6'	7.97 d (7.8)	130.6 d
17	2.86 br.s	61.4 d			

[a] Means overlapped

*600 MHz for ^{1}H NMR, 150 MHz for ^{13}C NMR, $CDCl_3$

Figure S1. Key HMBC, NOESY and ^{1}H-^{1}H COSY correlations of compound **1**

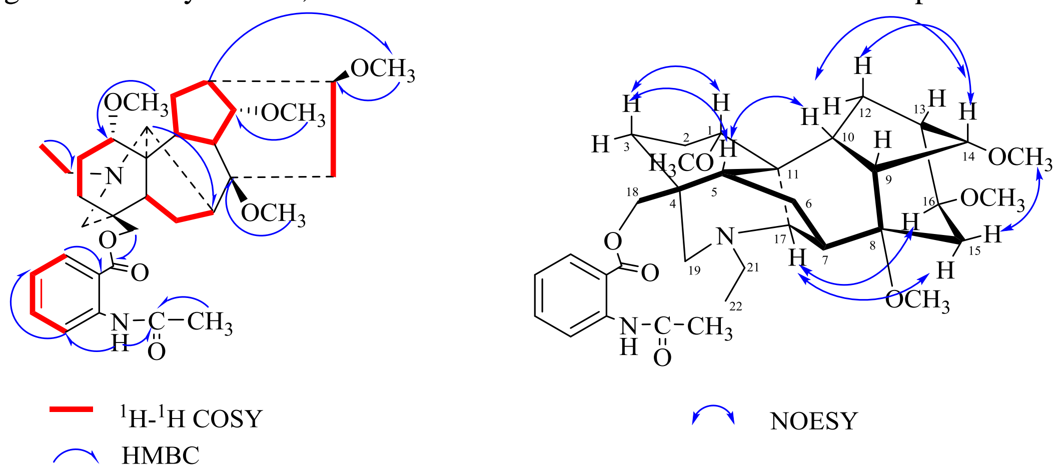


Figure S2. The cell inhibitory ratio of compounds **1**–**7** in HCT116 cell lines was measured using an MTT assay. The cells were treated with compounds **1**–**7** at 0−200 μM for 24 h.

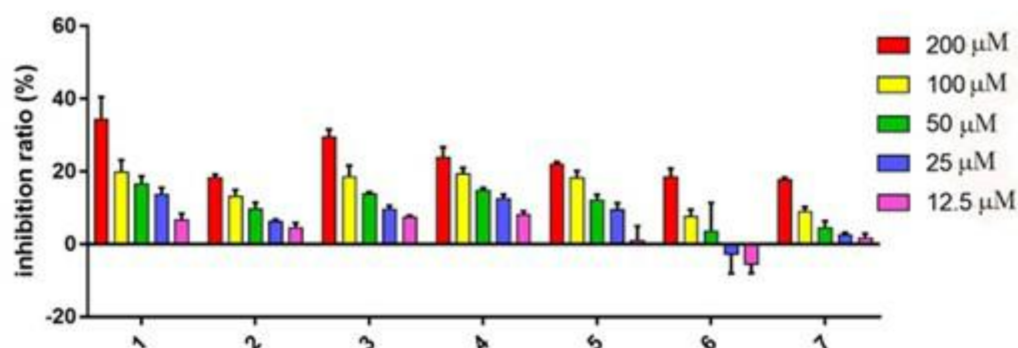
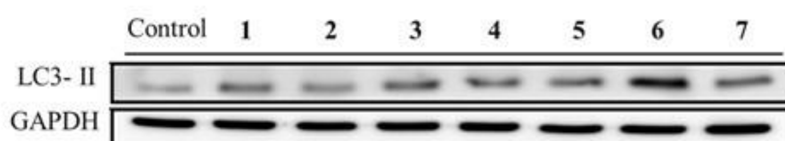


Figure S3. (**A**) Effect of compounds **1**–**7** on the induction of autophagy in HCT116 cells. HCT116 cells were cultured with 50 μM of compounds **1**–**7** for 24 h, and the protein expression levels of LC3-II were determined by Western blot analysis. (**B**) HCT116 cells were cultured with the indicated concentrations of compound **6** for 24 h. The protein expression levels of autophagy markers were determined by Western blot analysis.

(A)



(B)

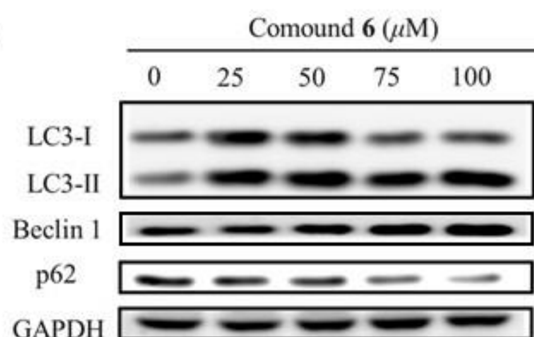


Figure S4. Effect of scaconitine (**6**) on the Akt/mTOR signaling pathway and the p53-mediated mitogen-activated protein kinase (MAPK) signaling in HCT116 cells. (**A**) Cells were lysed after treatment with indicated concentrations of compound **6** for 24 h. The protein expression levels of p-Akt (Ser473), Akt, p-ERK, ERK, p-p38, p38, p-JNK and JNK were determined by Western blot analysis. (**B**) HCT116 cells were treated with 100 μM of compound **6** for 6, 12, 24, 36, and 48 h. Western blotting was also performed for p-ERK, ERK, p-p38, p38 (B), p53, p-Akt (Ser473), and Akt. GAPDH was used as an internal standard.

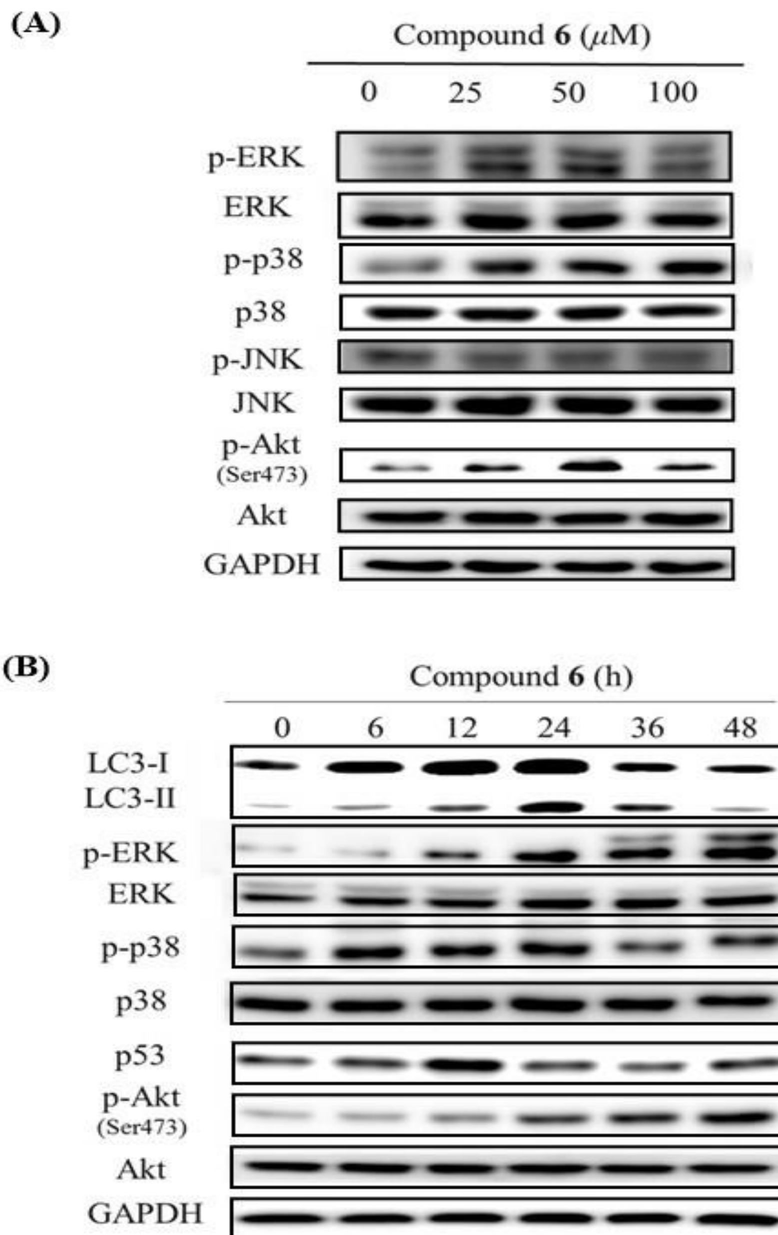


Figure S5. Scheme representing the proposed molecular mechanism underlying the action of compound **6** in induction of protective autophagy through the activated ERK1/2, p38 of the MAPK signaling pathway and p53.

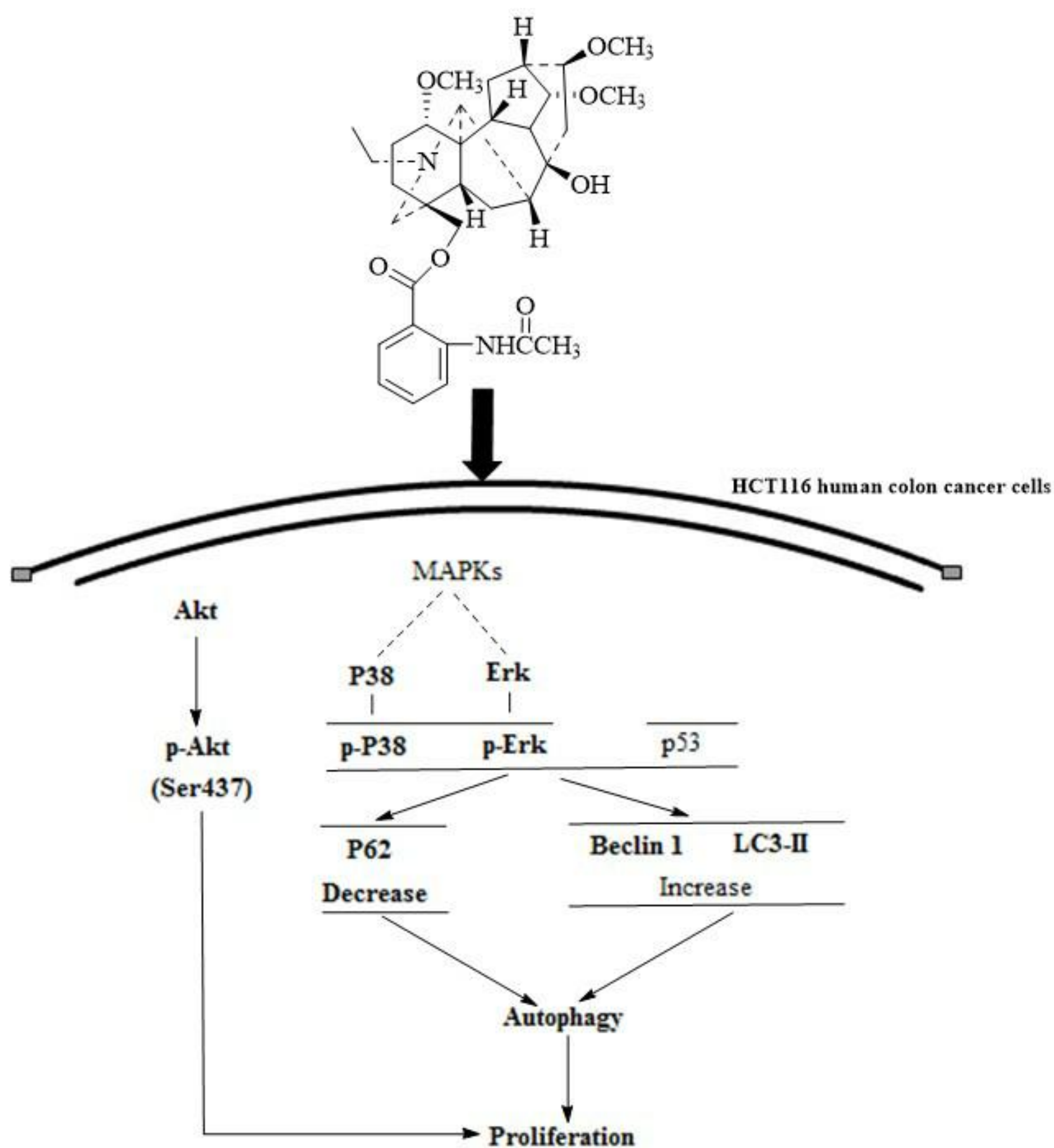


Figure S6. Compound 6 was docked into the binding pocket of the human p38 MAPK.

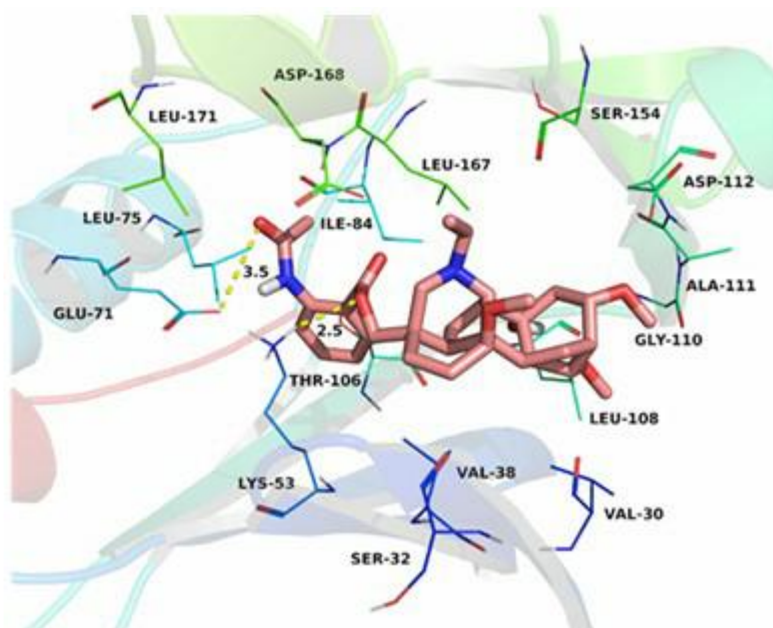


Figure S7. Compound 6 was docked into the binding pocket of the human p38 MAPK.

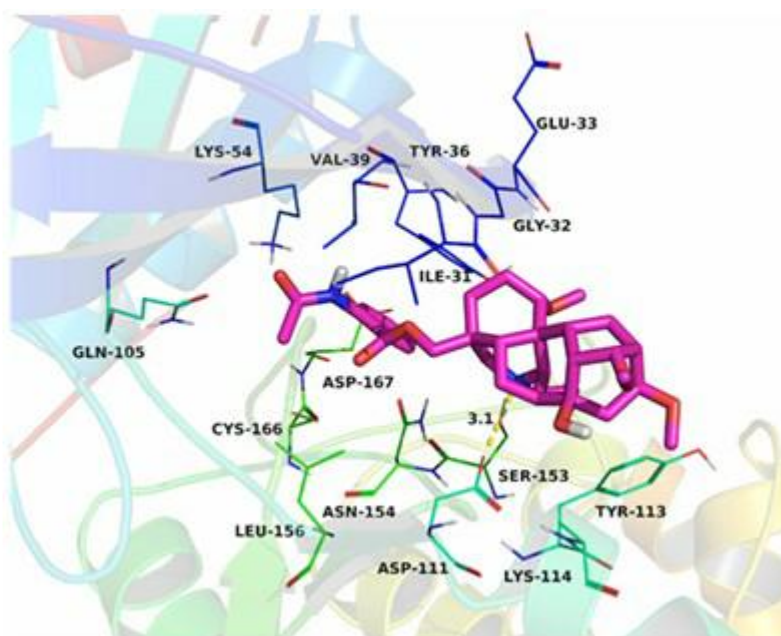


Figure S8. IR spectrum for compound **1**

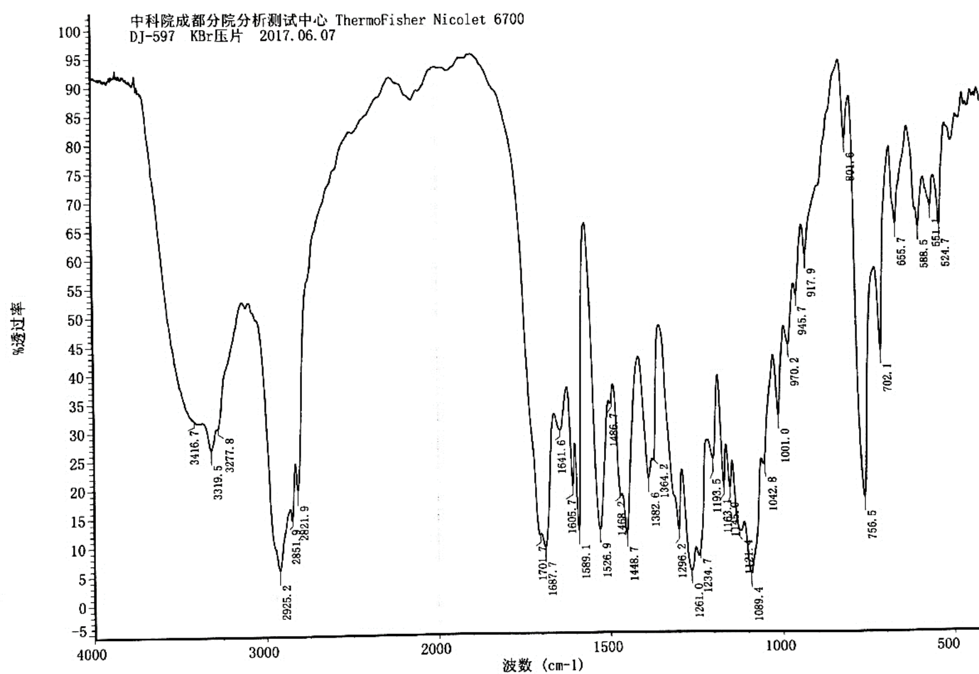


Figure S9. HR-ESI-MS spectrum for compound **1**

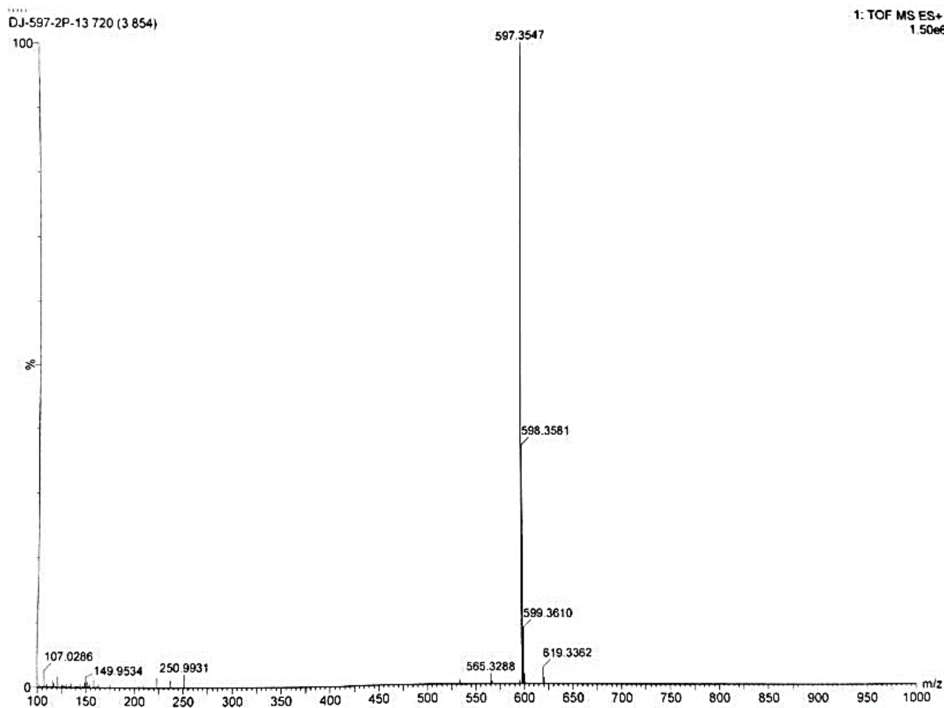


Figure S10. ^{1}H NMR spectrum in $CDCl_3$ for compound **1**

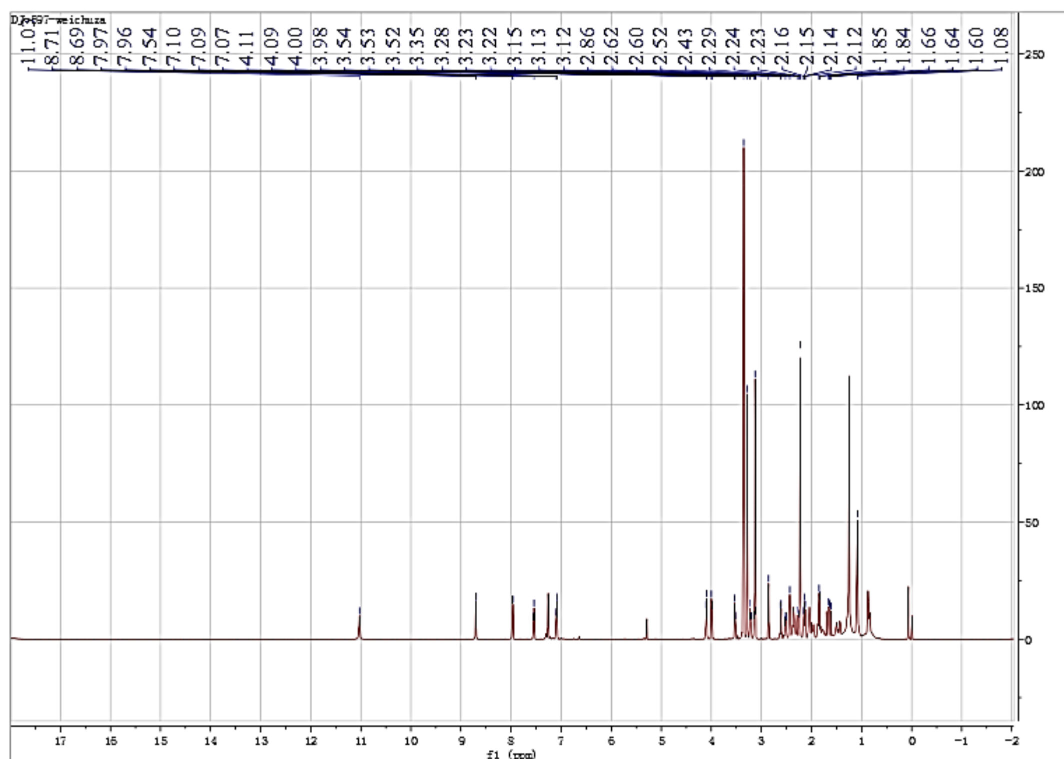


Figure S11. ^{13}C NMR spectrum in $CDCl_3$ for compound **1**

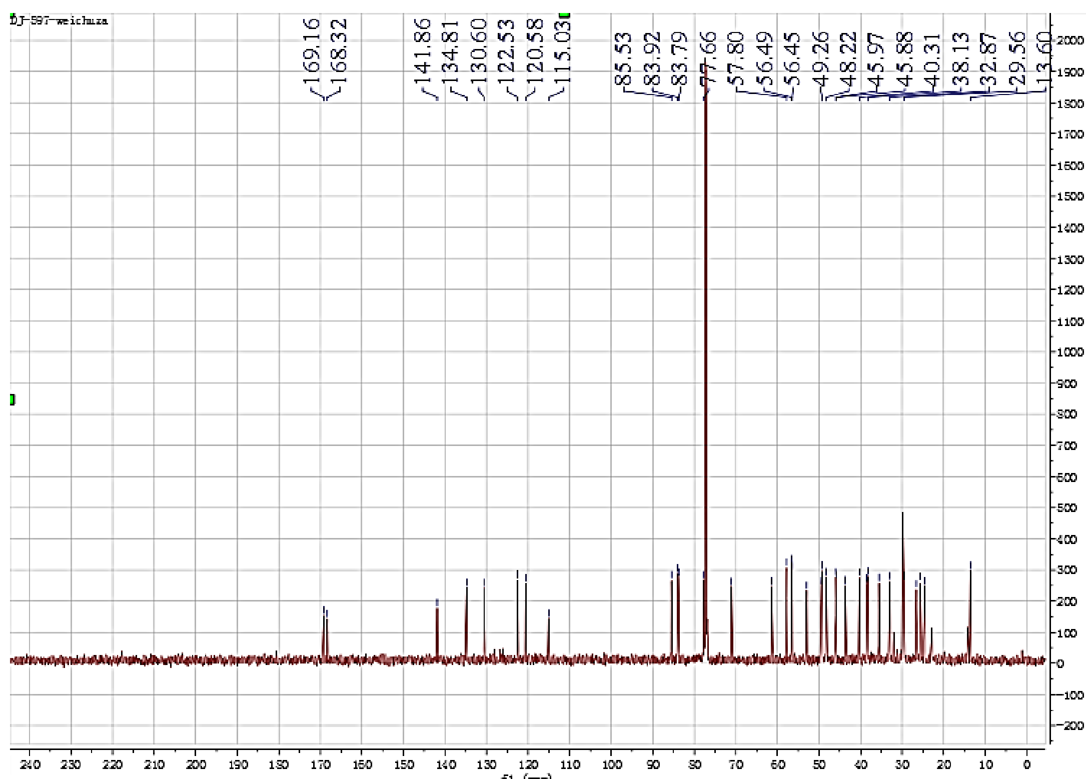


Figure S12. DEPT spectrum in $CDCl_3$ for compound **1**

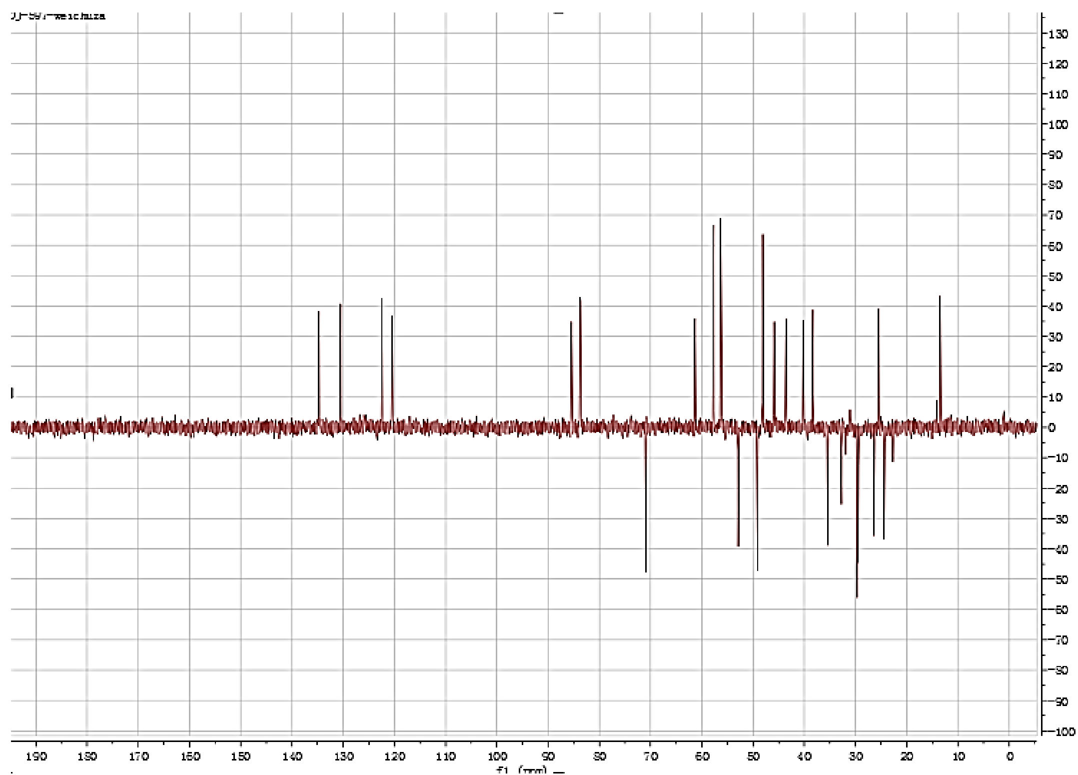


Figure S13. ^{1}H-^{1}H COSY spectrum in $CDCl_3$ for compound **1**

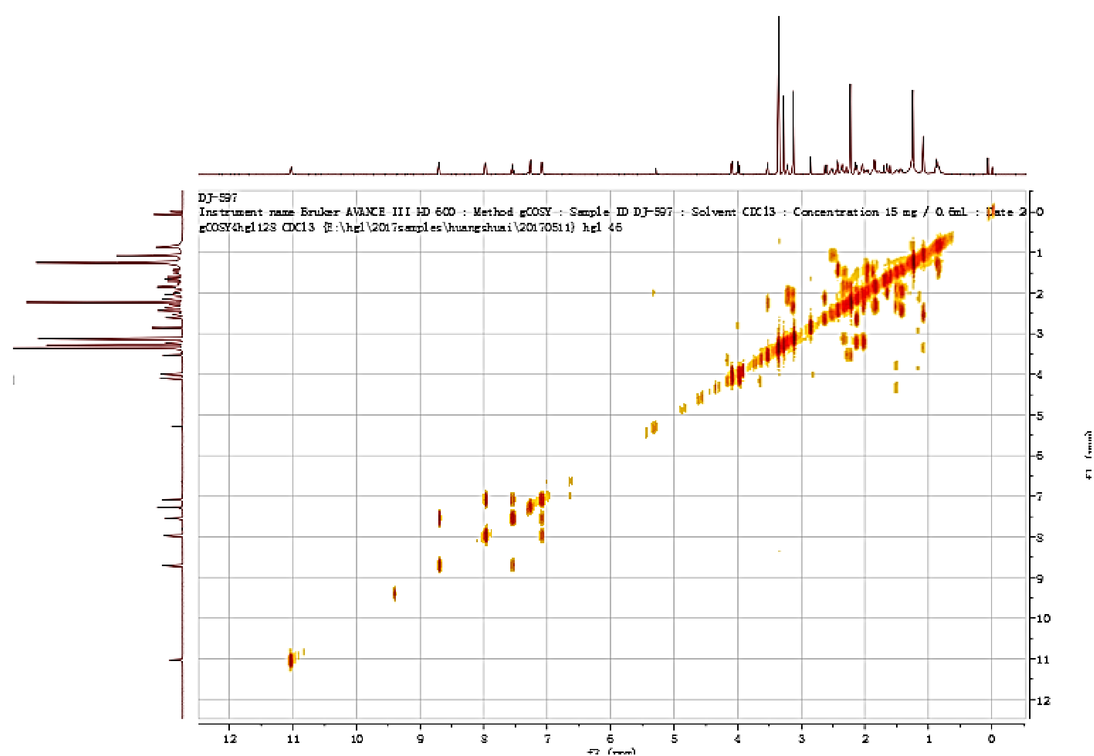


Figure S14. HMQC spectrum in $CDCl_3$ for compound **1**

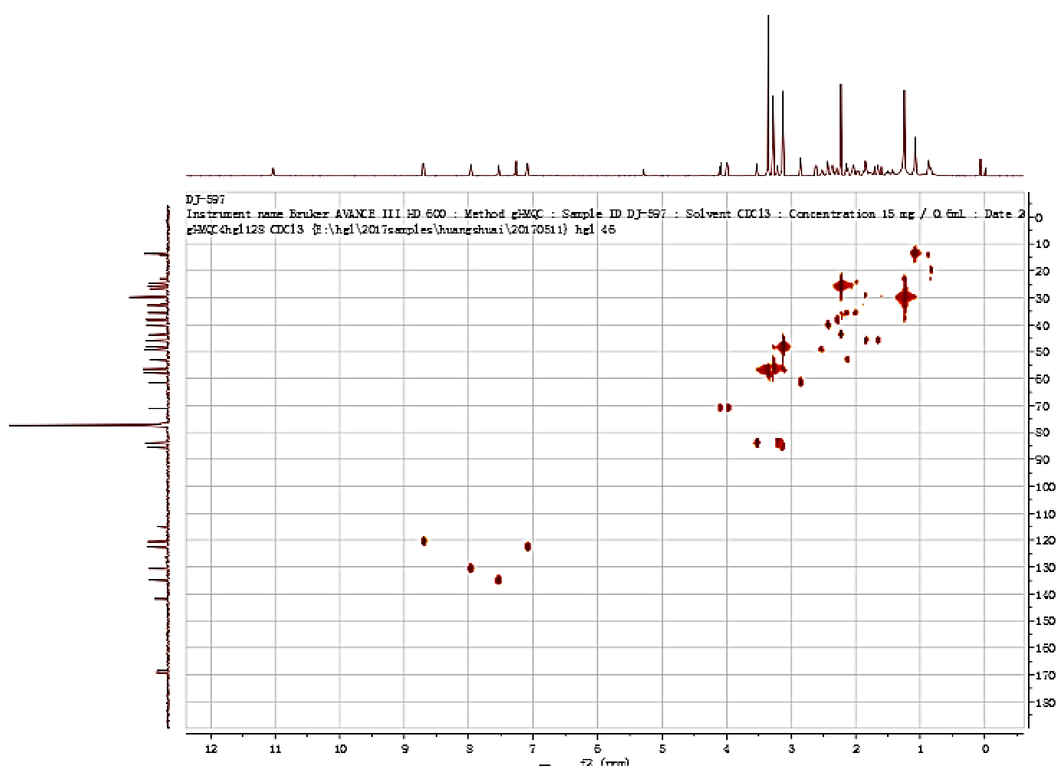


Figure S15. HMBC spectrum in $CDCl_3$ for compound **1**

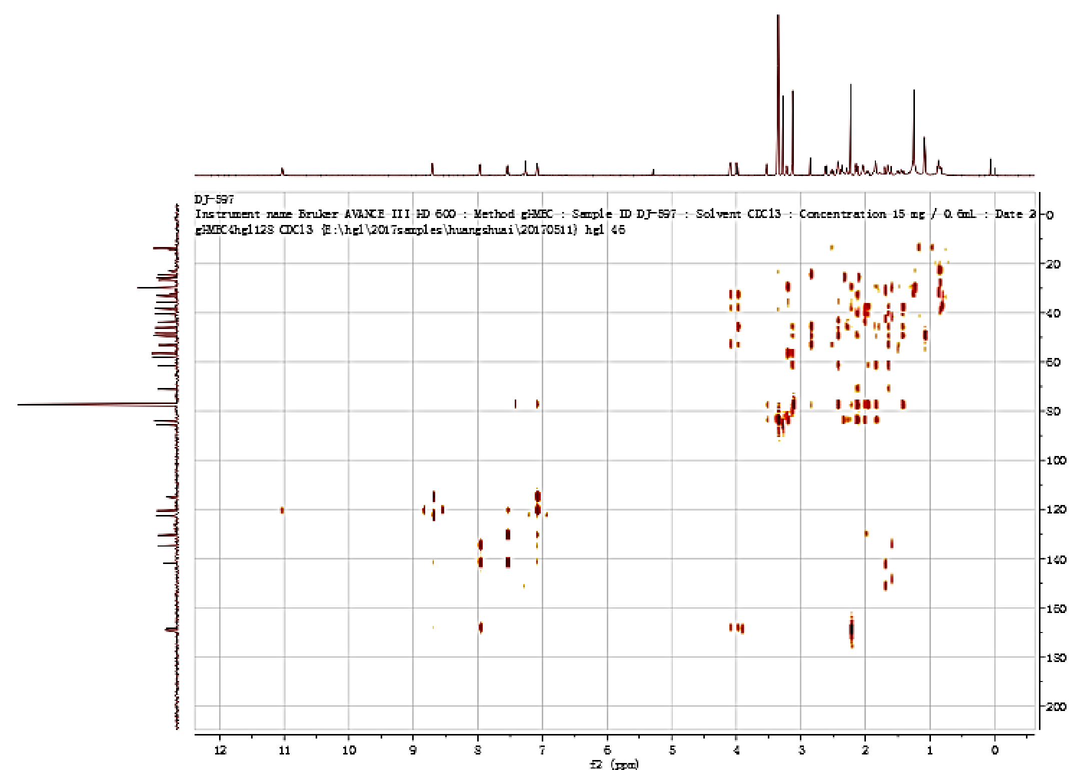


Figure S16. Noesy spectrum in $CDCl_3$ for compound **1**