Electronic Supplementary Material (ESI) for RSC Advances.

Cardiac Glycosides from Digitalis lanata and Their Cytotoxic Activities

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Table S1 Antiproliferative activities of compounds **1**–**15** toward A549 cells.

	% inhibition at 5 μM for 24h against A549 cells
1	76.14±1.04
2	73.45±0.79
3	74.16±0.36
4	73.73±1.46
5	75.70±0.25
6	72.30±1.51
7	74.67±3.23
8	73.92±0.56
9	74.79±2.62
10	74.48±0.77
11	73.88±1.10
12	75.86±1.44
13	73.84±1.03
14	73.09±1.27
15	70.64±2.60

A549 cells were exposed to CGs at the 5 μM for 24h, and cell viability was determined by MTT method. Data represented the mean SD of at least three independent experiments, and each experiment was performed in triplicate.

Table S2 Primers sequences of cancer-related genes.

Gene ID	Gene	Forward primer	Reverse primer
ENSG00000143494	VASH2	GCCTTCTTGGCAAAGCCTTC	CCATTAACCCACTCAGCGGT
ENSG00000151790	TDO2	TGGGAACTACCTGCATTTGGA	CCAACTCCCAGAGGATTTGCT
ENSG00000163884	KLF15	GTGAAAAGCGTCCCCCACTA	CTCCCAAGGCCACCCTAAAG
ENSG00000160282	FTCD	TTCGACGCTGGAAGGATAGC	TCTCTGGAGACAGGAGGCTG
ENSG00000125740	FOSB	GAGCTGACCGACCGACTCC	CCCGGCAAATCTCTCACCTCC
	β-actin	CCTGGGCATGGAGTCCTGTG	TCTTCATTGTGCTGGGTGCC

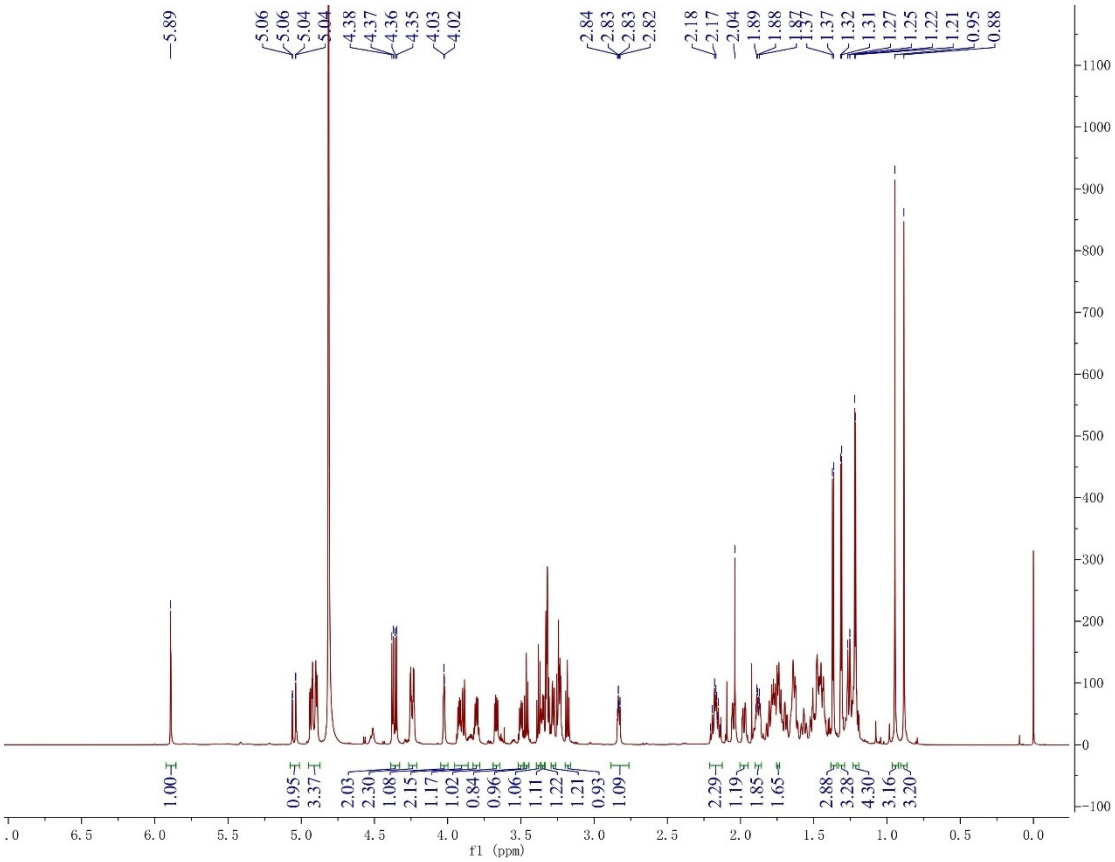


Figure S1. ^{1}H NMR spectrum of compound **1** (600 MHz, MeOD).

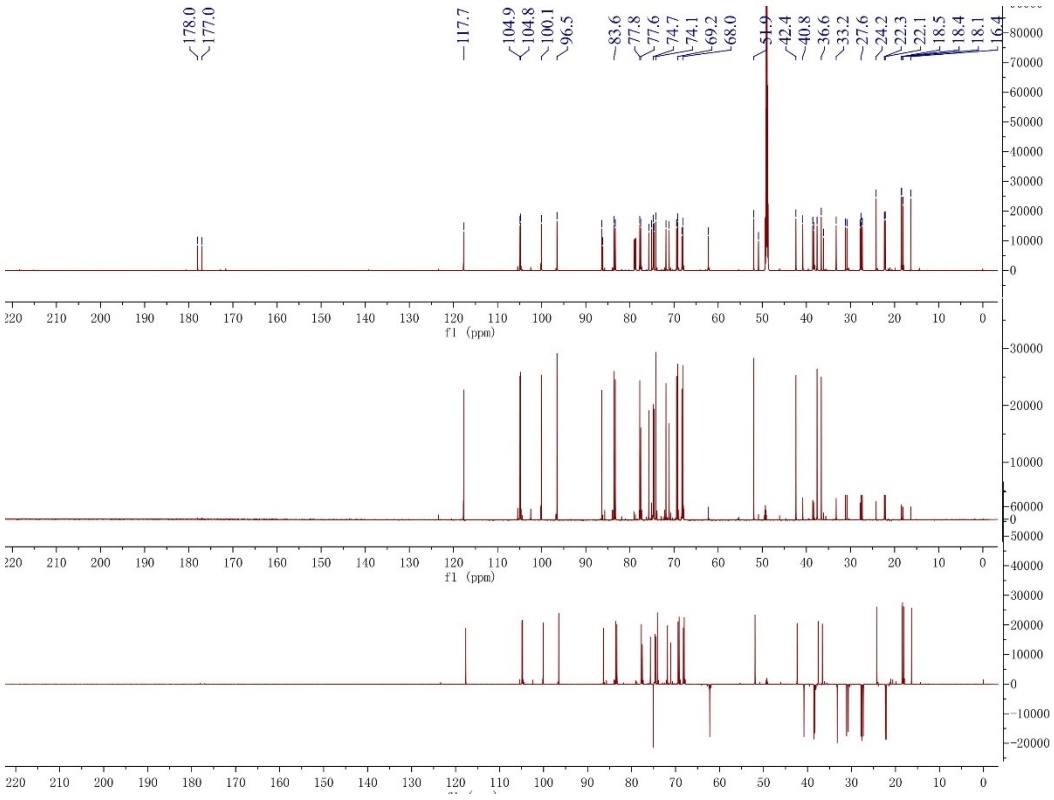


Figure S2. ^{13}C NMR spectrum of compound **1** (150 MHz, MeOD).

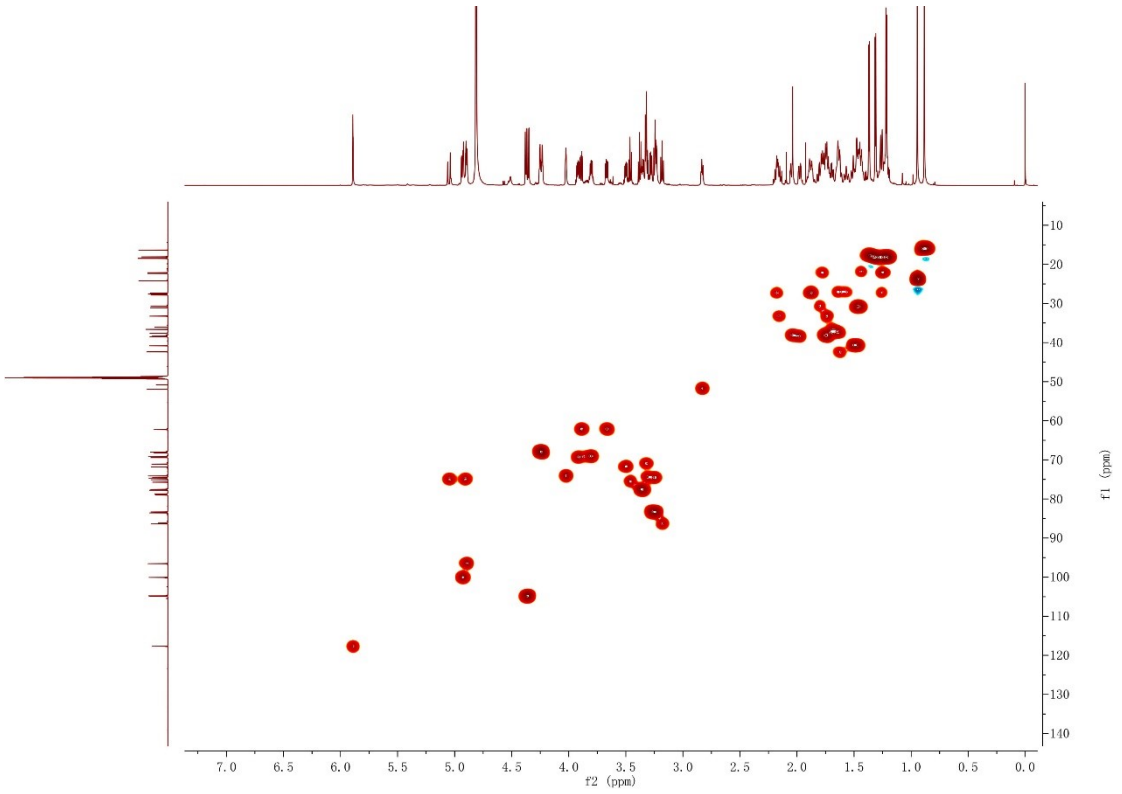


Figure S3. HSQC spectrum of compound **1** (150 MHz, MeOD).

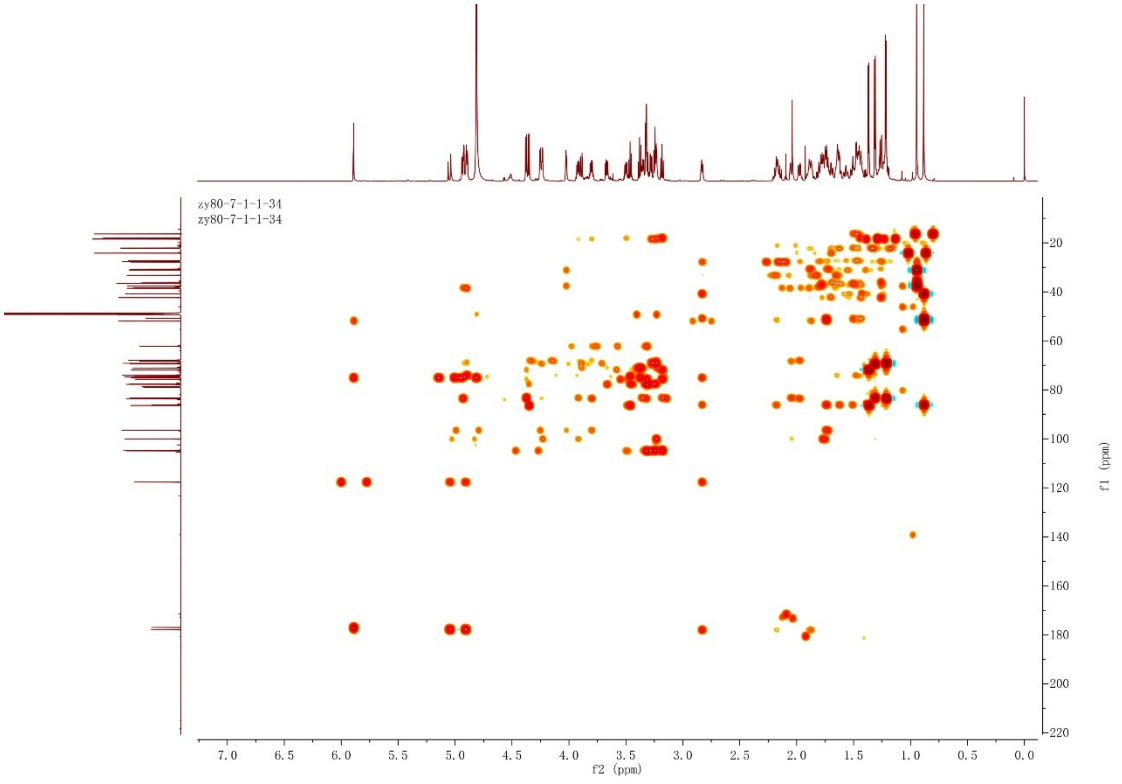


Figure S4. HMBC spectrum of compound **1** (150 MHz, MeOD).

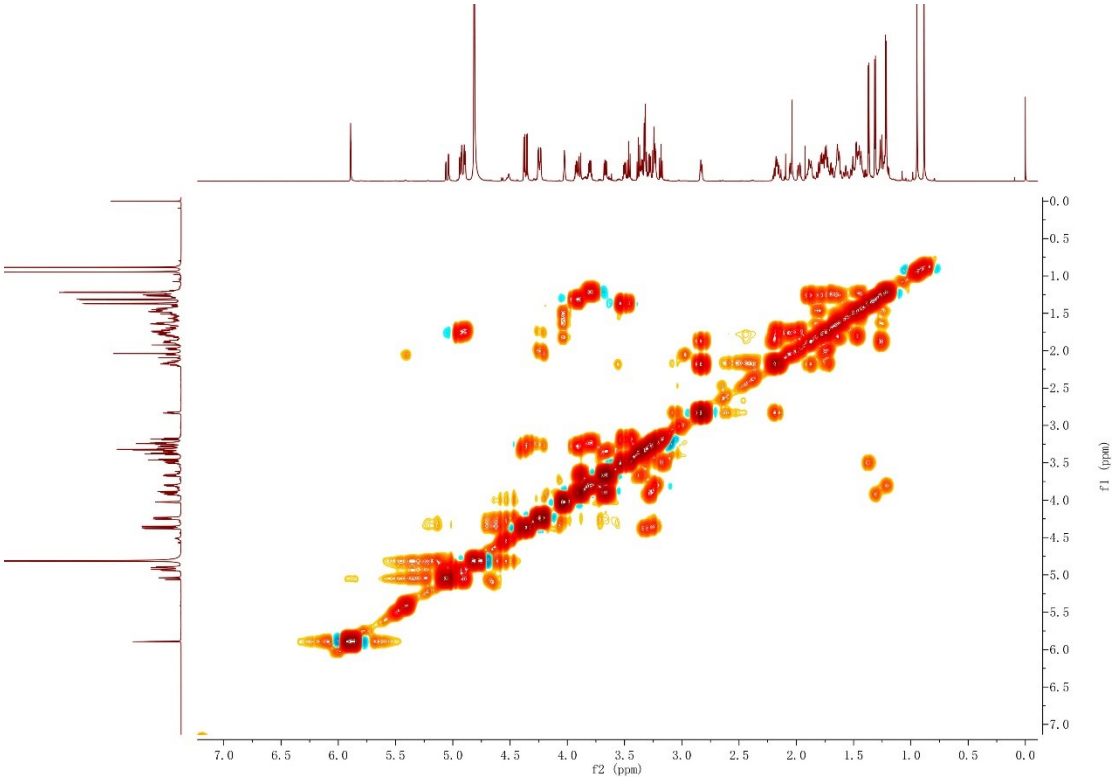


Figure S5. ^{1}H-^{1}H COSY spectrum of compound **1** (600 MHz, MeOD).

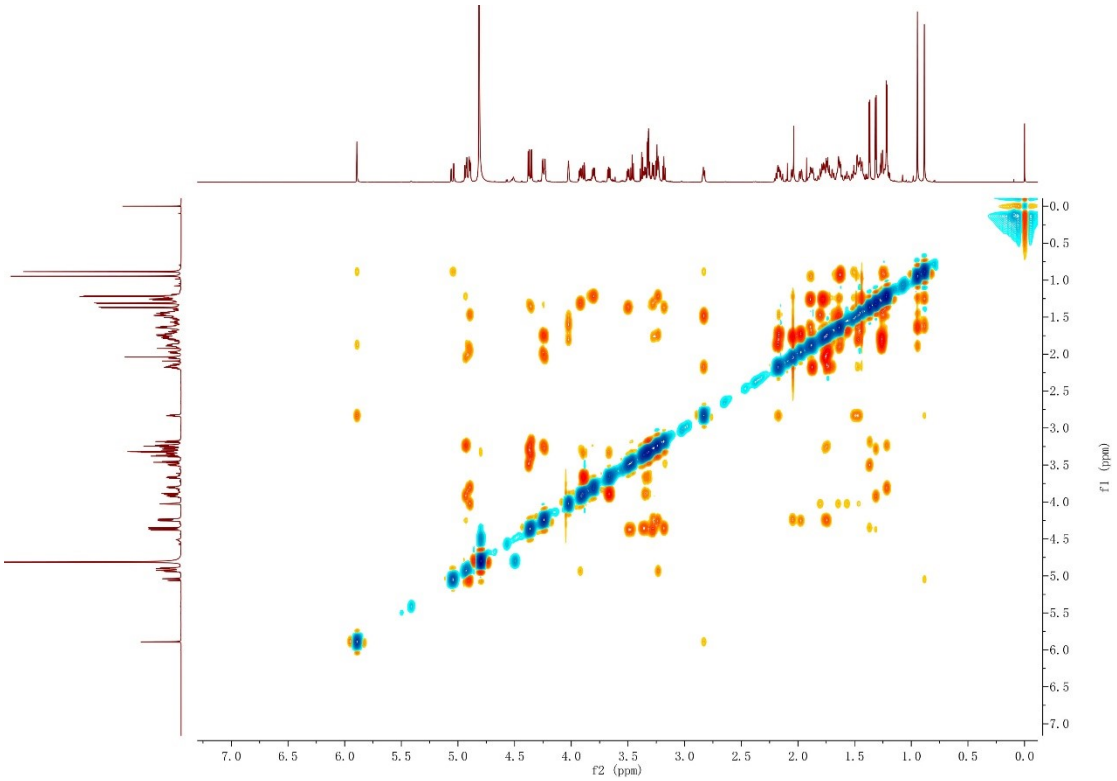


Figure S6. NOESY spectrum of compound **1** (600 MHz, MeOD).

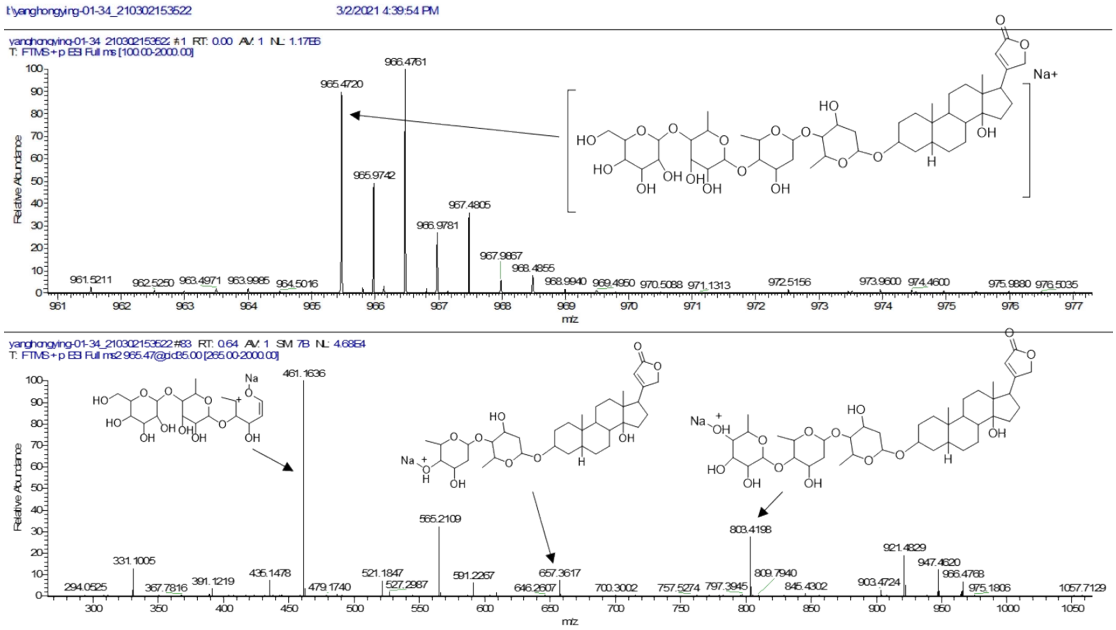


Figure S7. HR-ESIMS/MS of compound **1**.

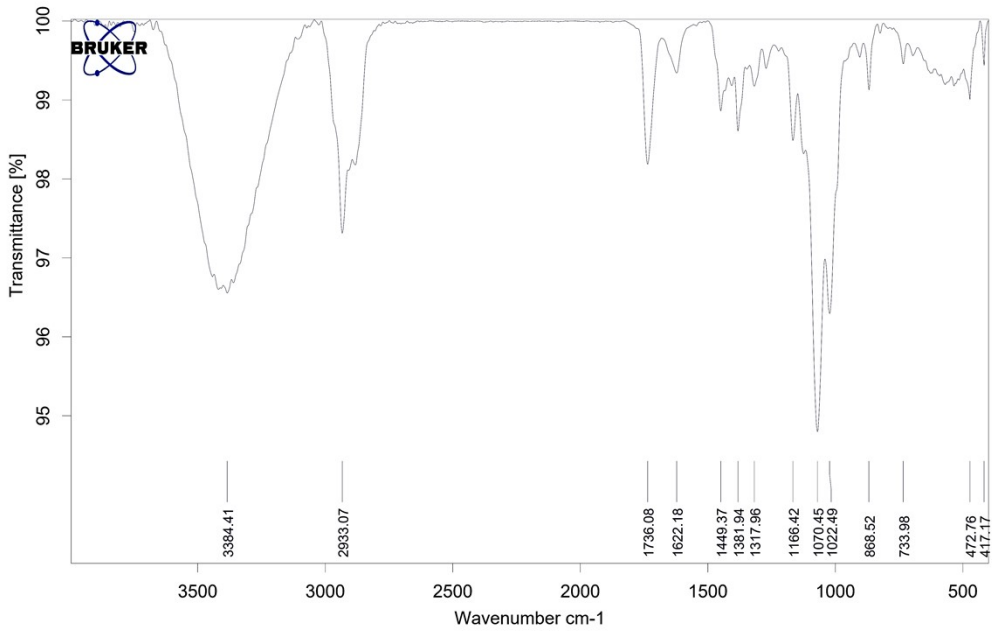


Figure S8. IR spectrum of compound **1**.

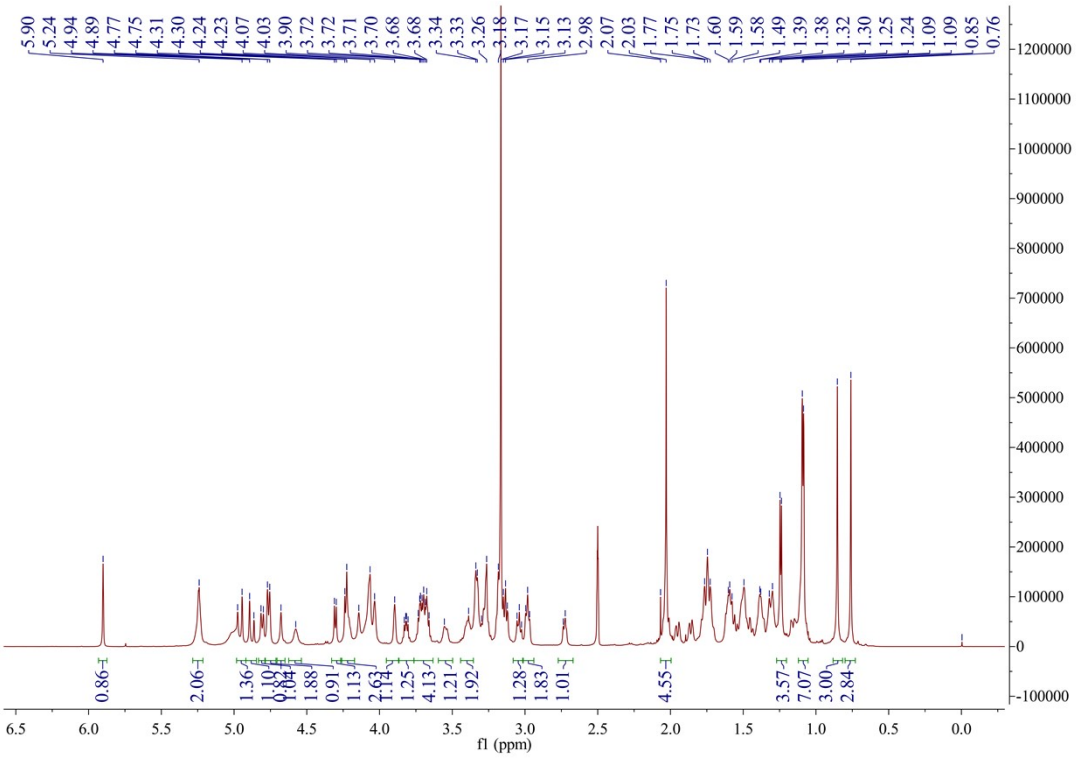


Figure S9. ^{1}H NMR spectrum of compound **2** (600 MHz, DMSO).

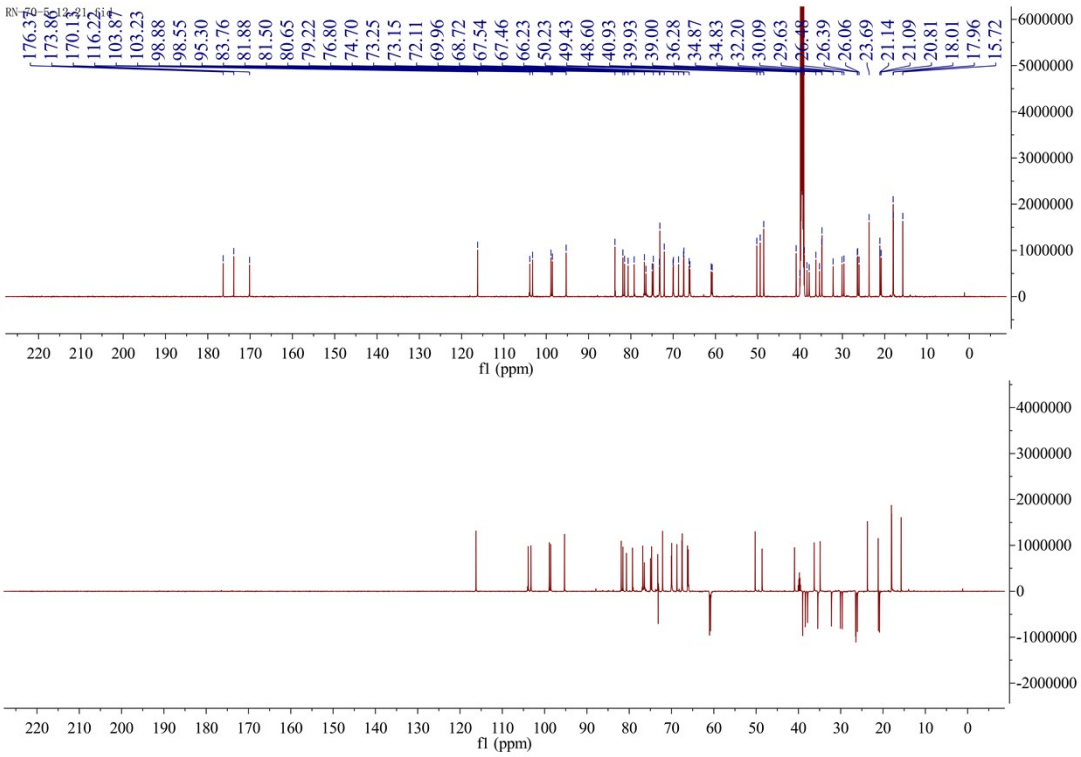


Figure S10. ^{13}C NMR spectrum of compound **2** (150 MHz, DMSO).

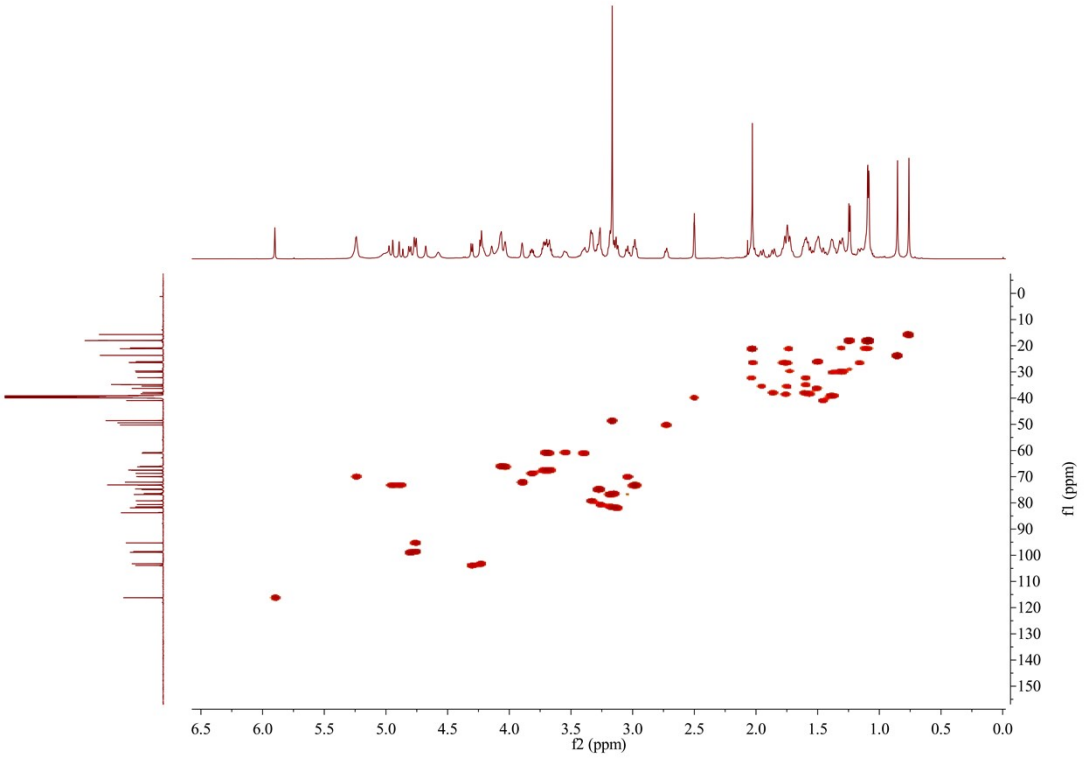


Figure S11. HSQC spectrum of compound **2** (150 MHz, DMSO).

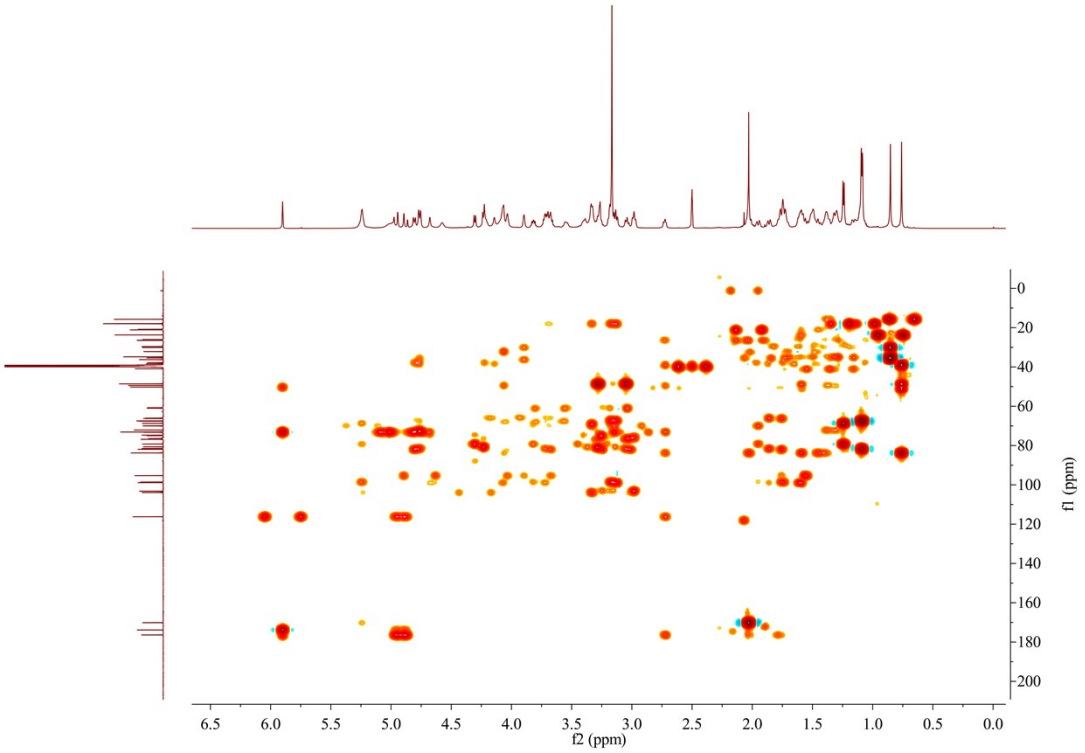


Figure S12. HMBC spectrum of compound **2** (150 MHz, DMSO).

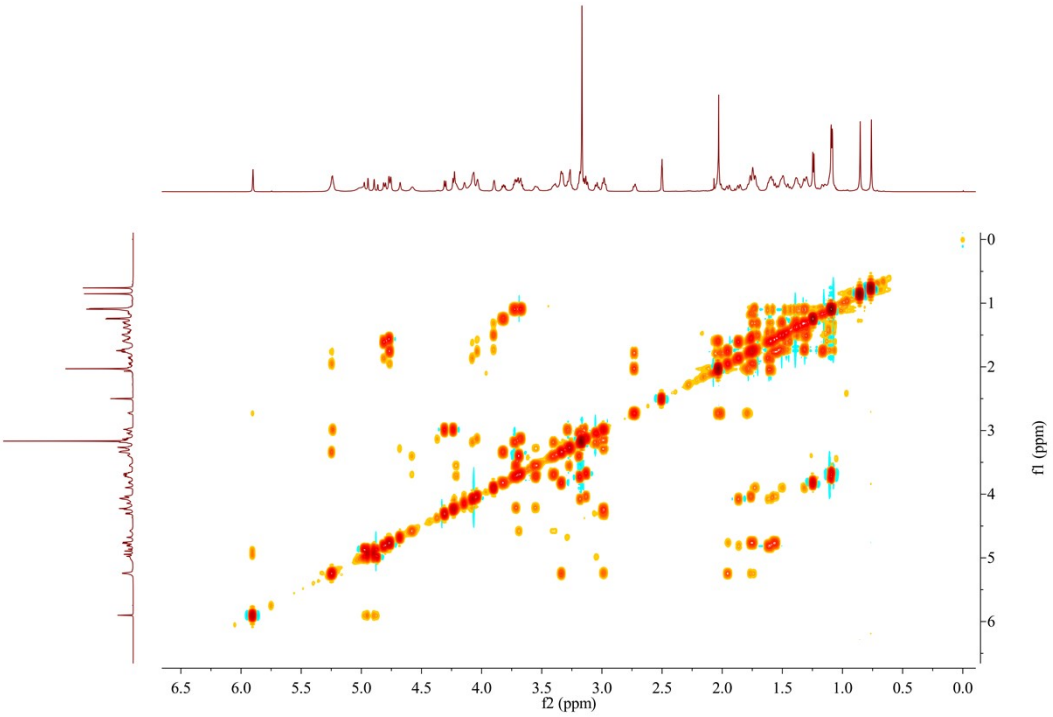


Figure S13. ^{1}H-^{1}H COSY spectrum of compound **2** (600 MHz, DMSO).

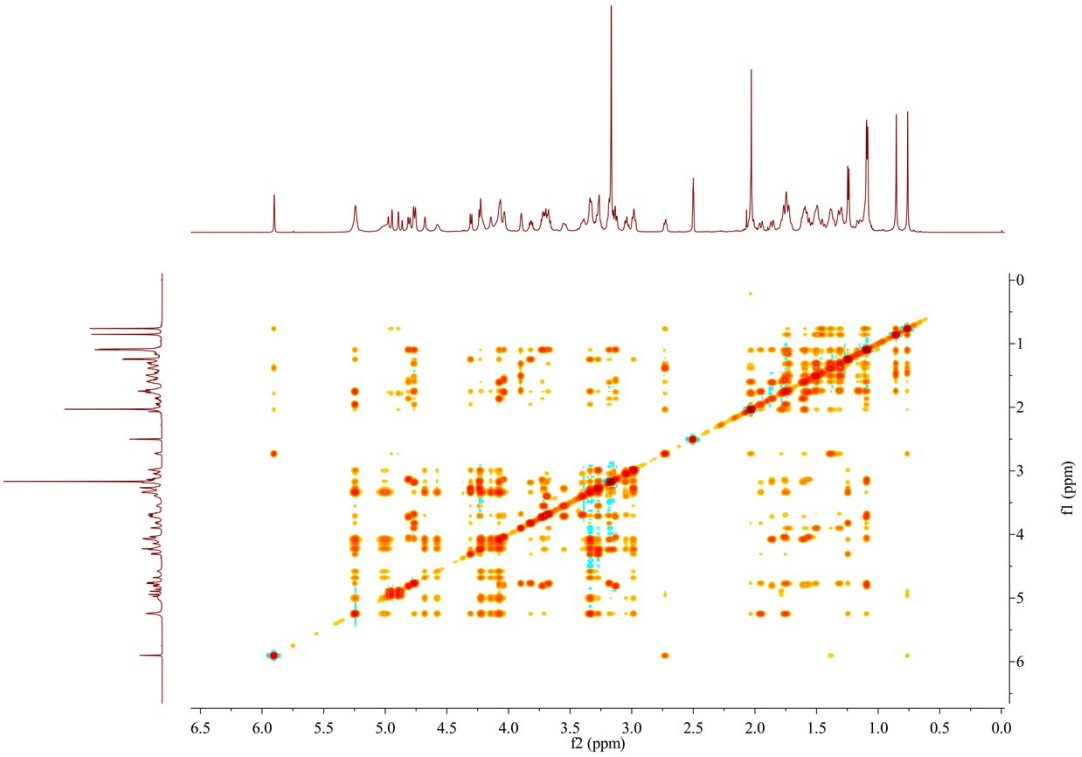


Figure S14. NOESY spectrum of compound **2** (600 MHz, DMSO).

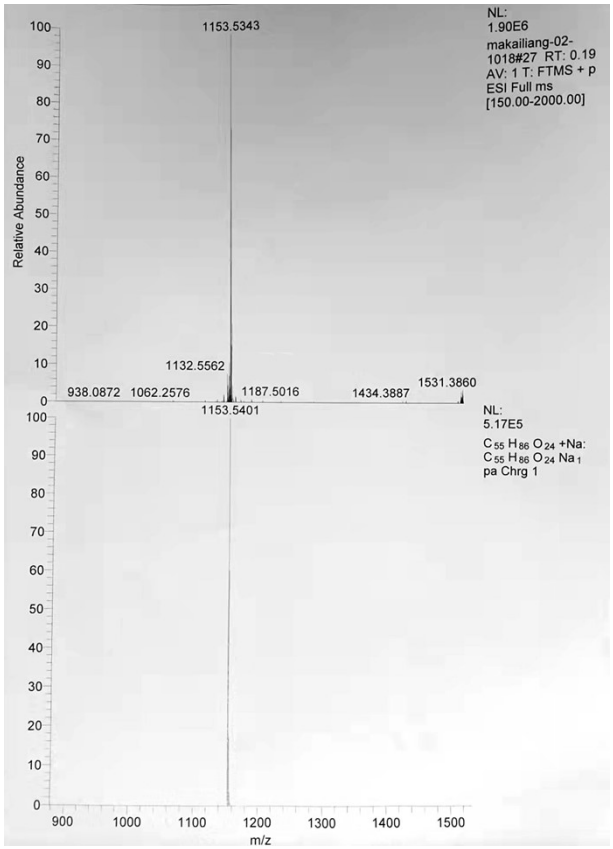


Figure S15. HR-ESIMS of compound **2**.

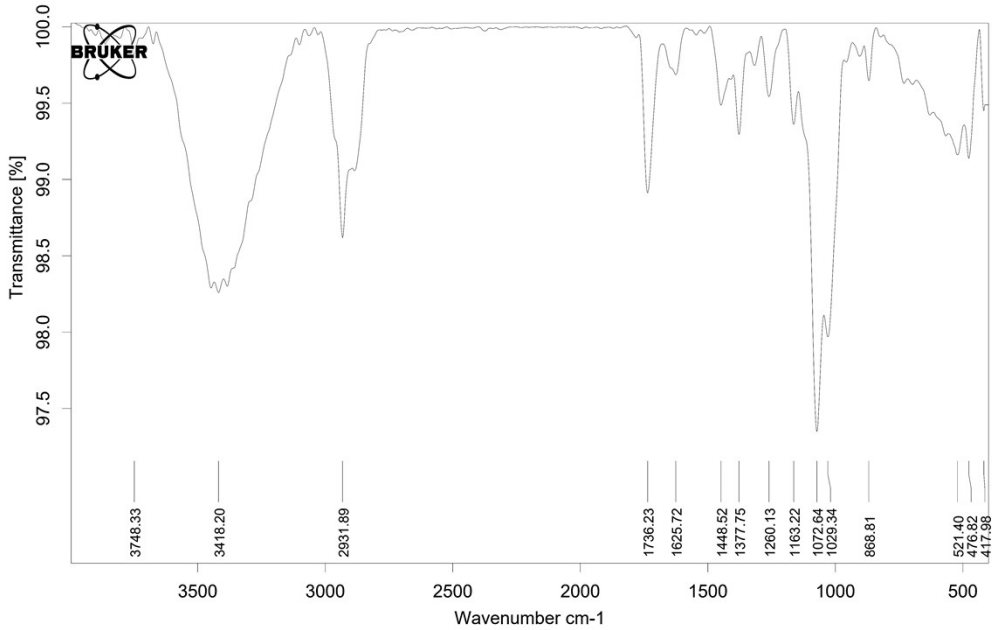


Figure S16. IR spectrum of compound **2**.

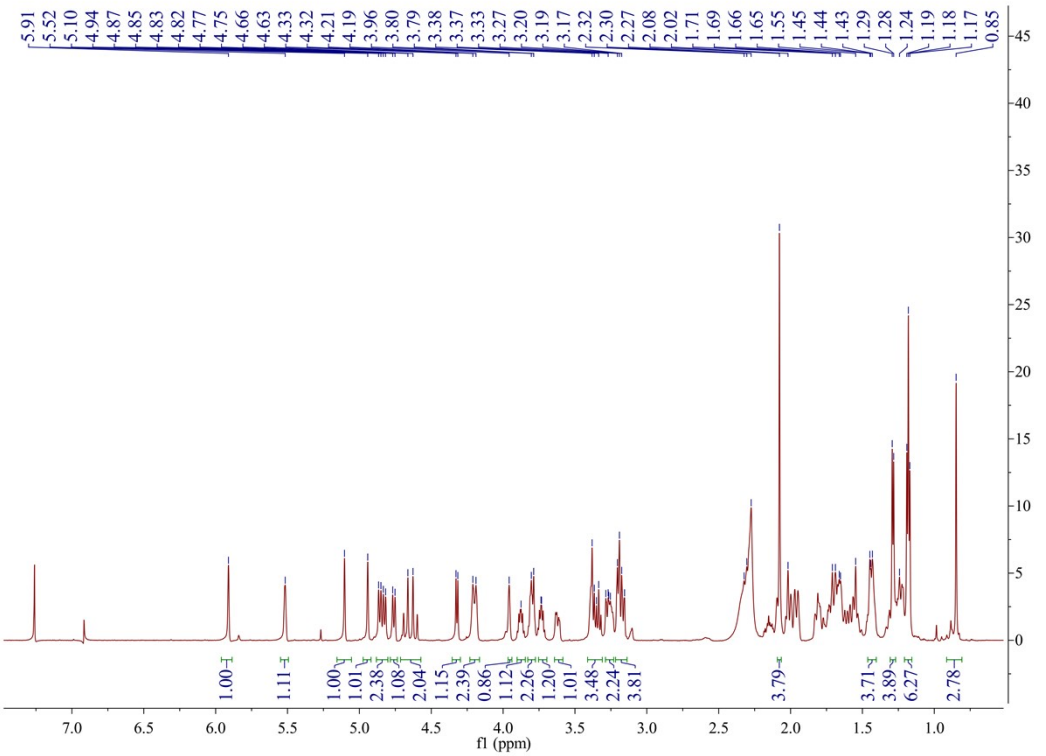


Figure S17. ^{1}H NMR spectrum of compound **3** (600 MHz, $CDCl_3$).

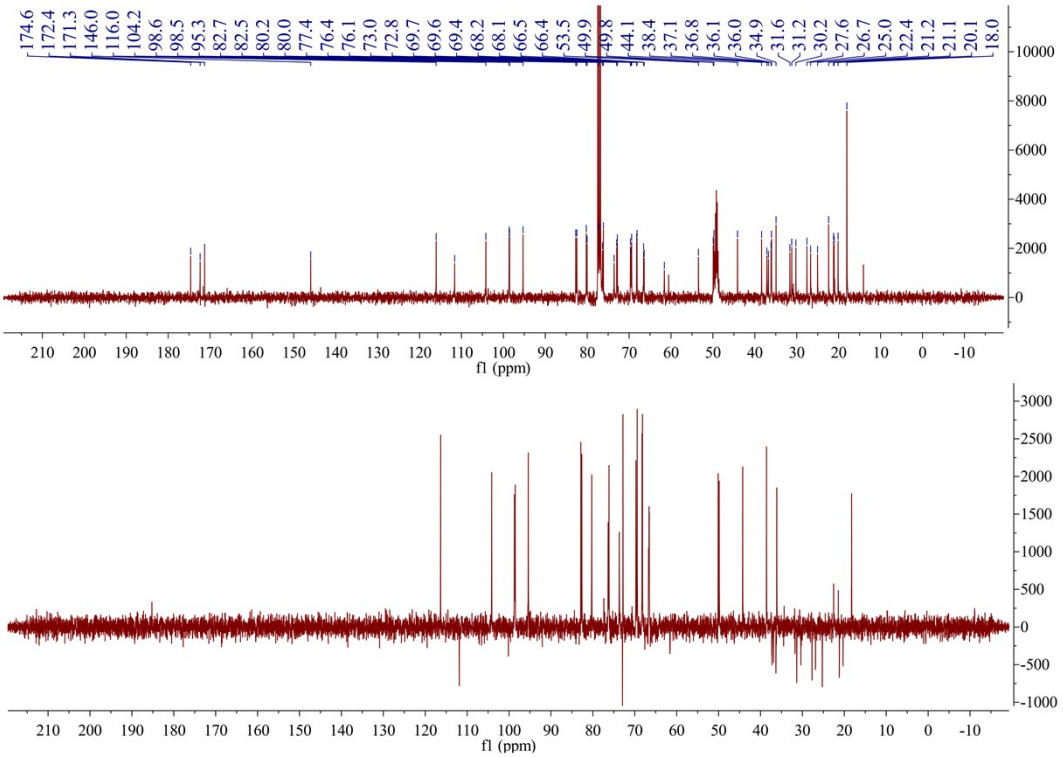


Figure S18. ^{13}C NMR spectrum of compound **3** (150 MHz, $CDCl_3$).

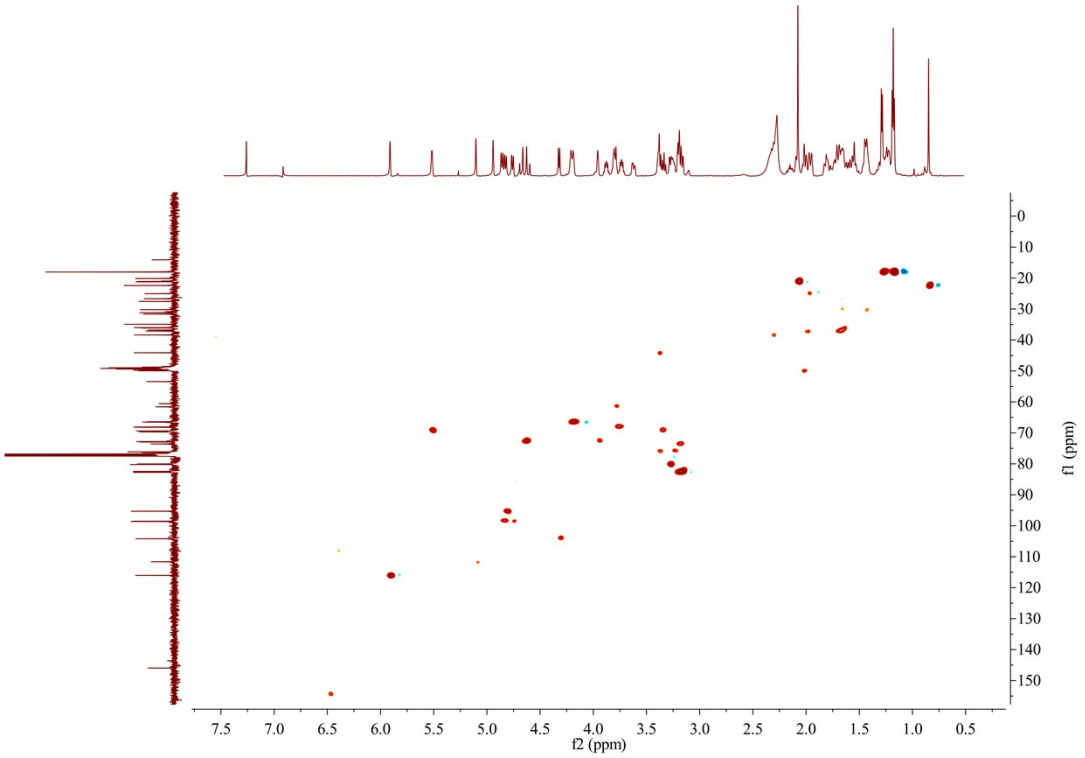


Figure S19. HSQC spectrum of compound **3** (150 MHz, $CDCl_3$).

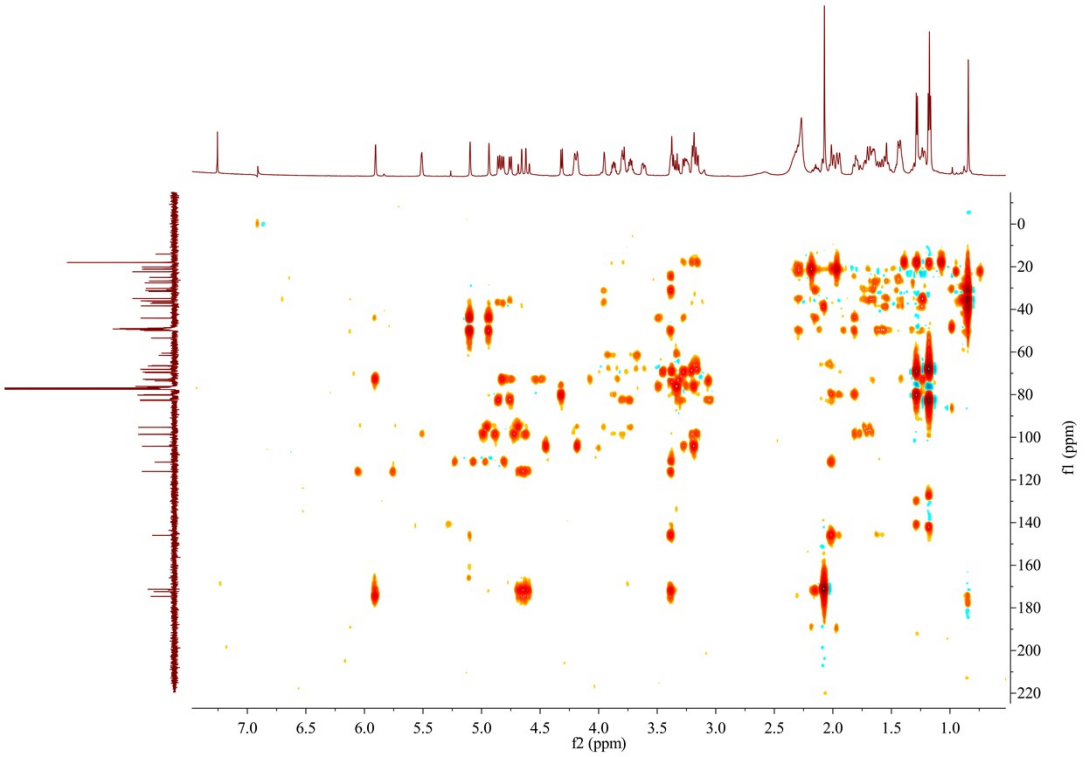


Figure S20. HMBC spectrum of compound **3** (150 MHz, $CDCl_3$).

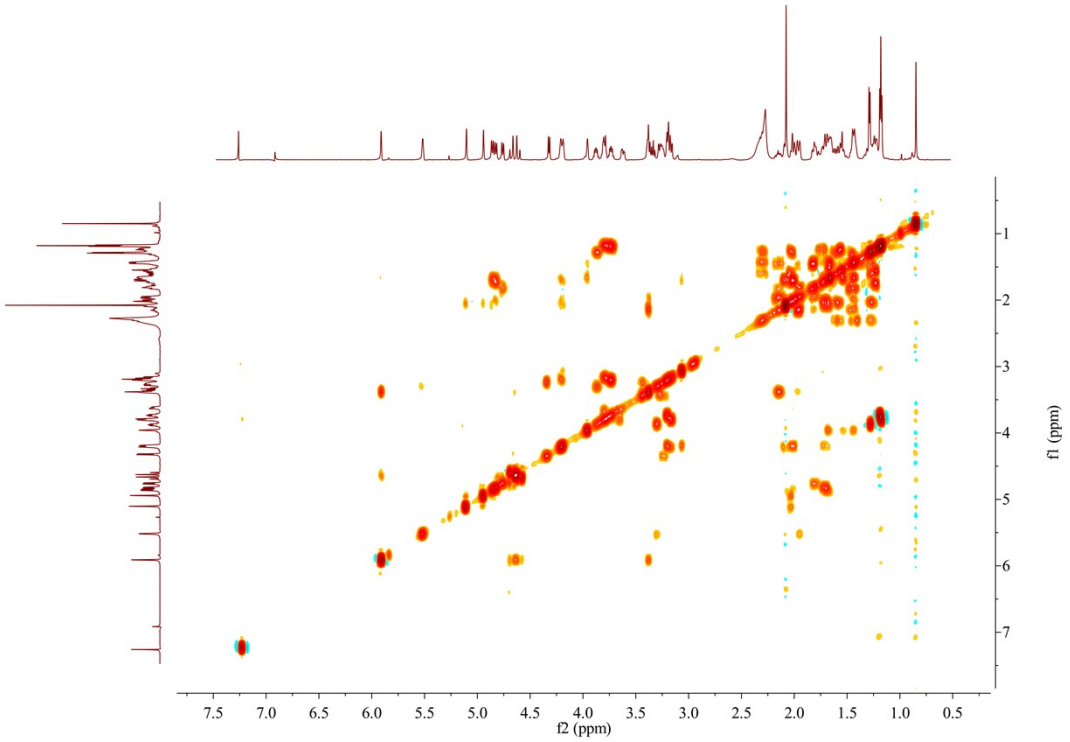


Figure S21. ^{1}H-^{1}H COSY spectrum of compound **3** (600 MHz, $CDCl_3$).

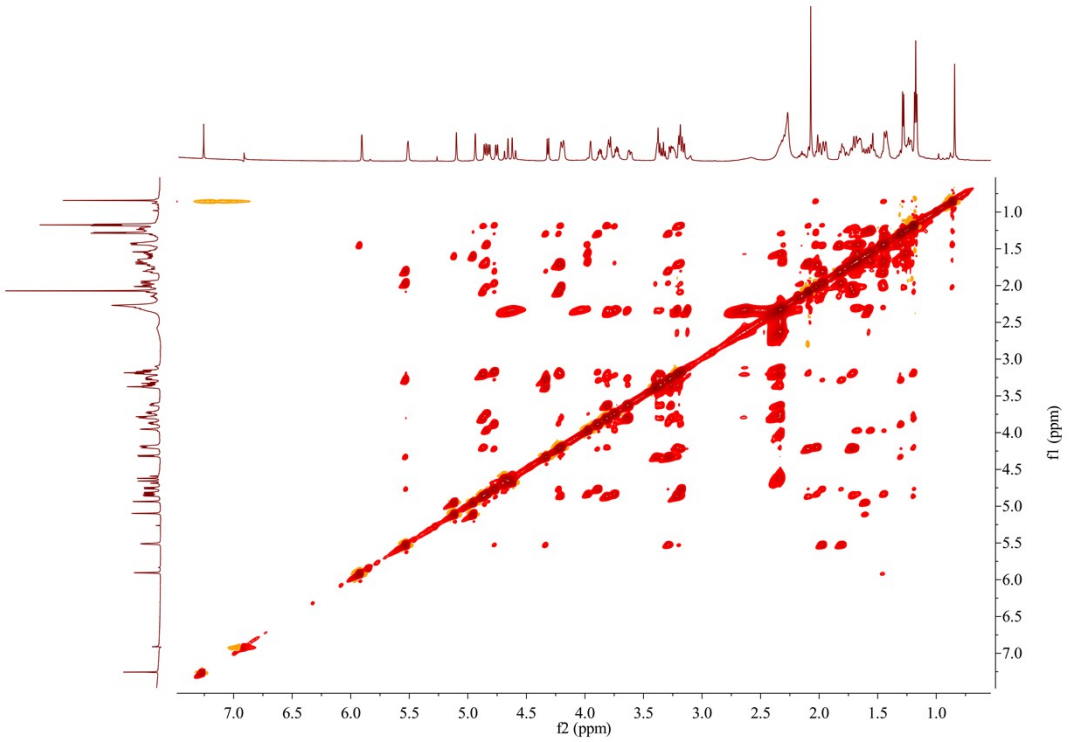


Figure S22. NOESY spectrum of compound **3** (600 MHz, $CDCl_3$).

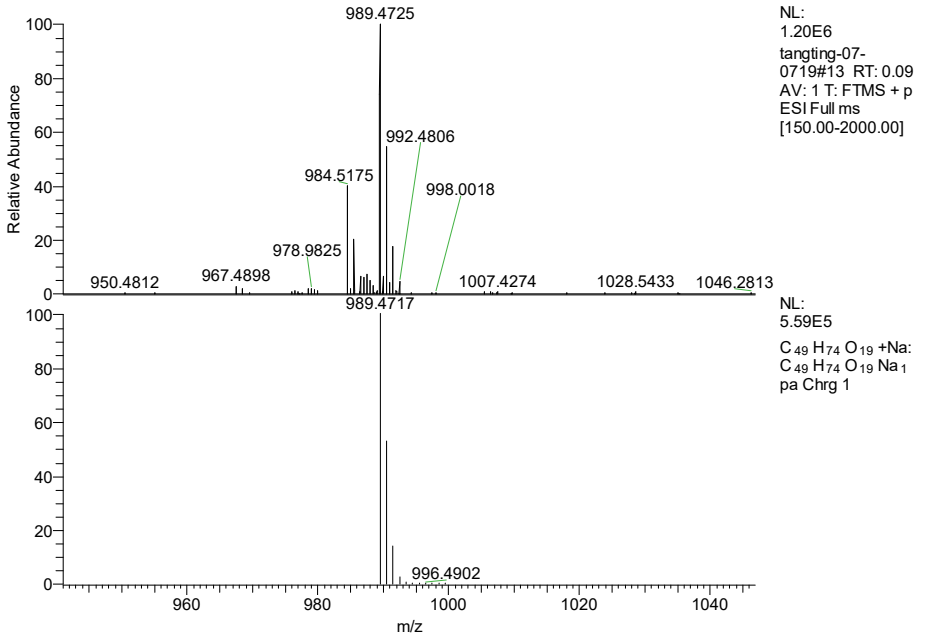


Figure S23. HR-ESIMS of compound **3**.

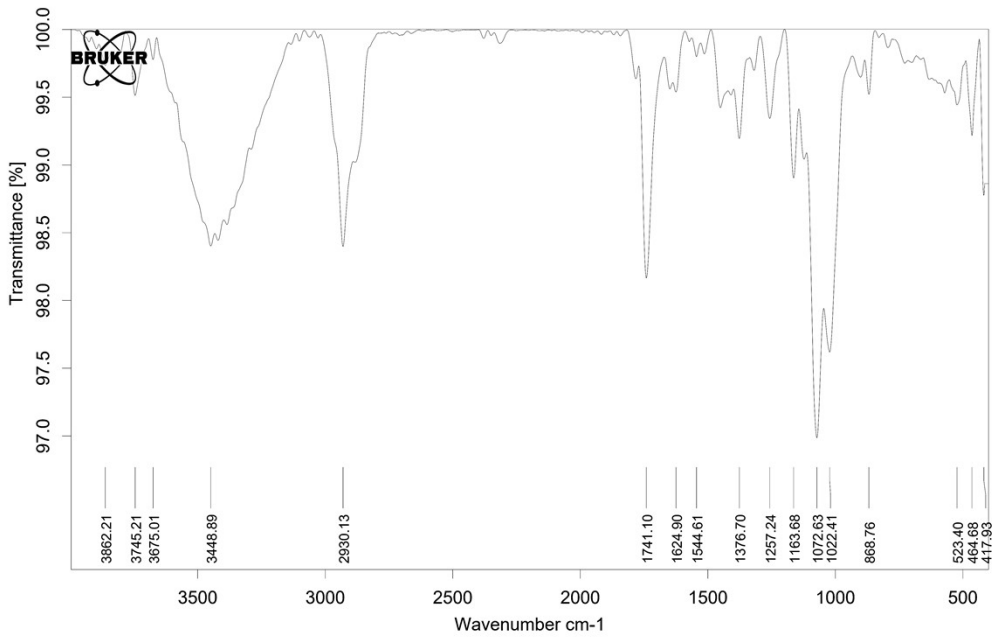


Figure S24. IR spectrum of compound **3**.

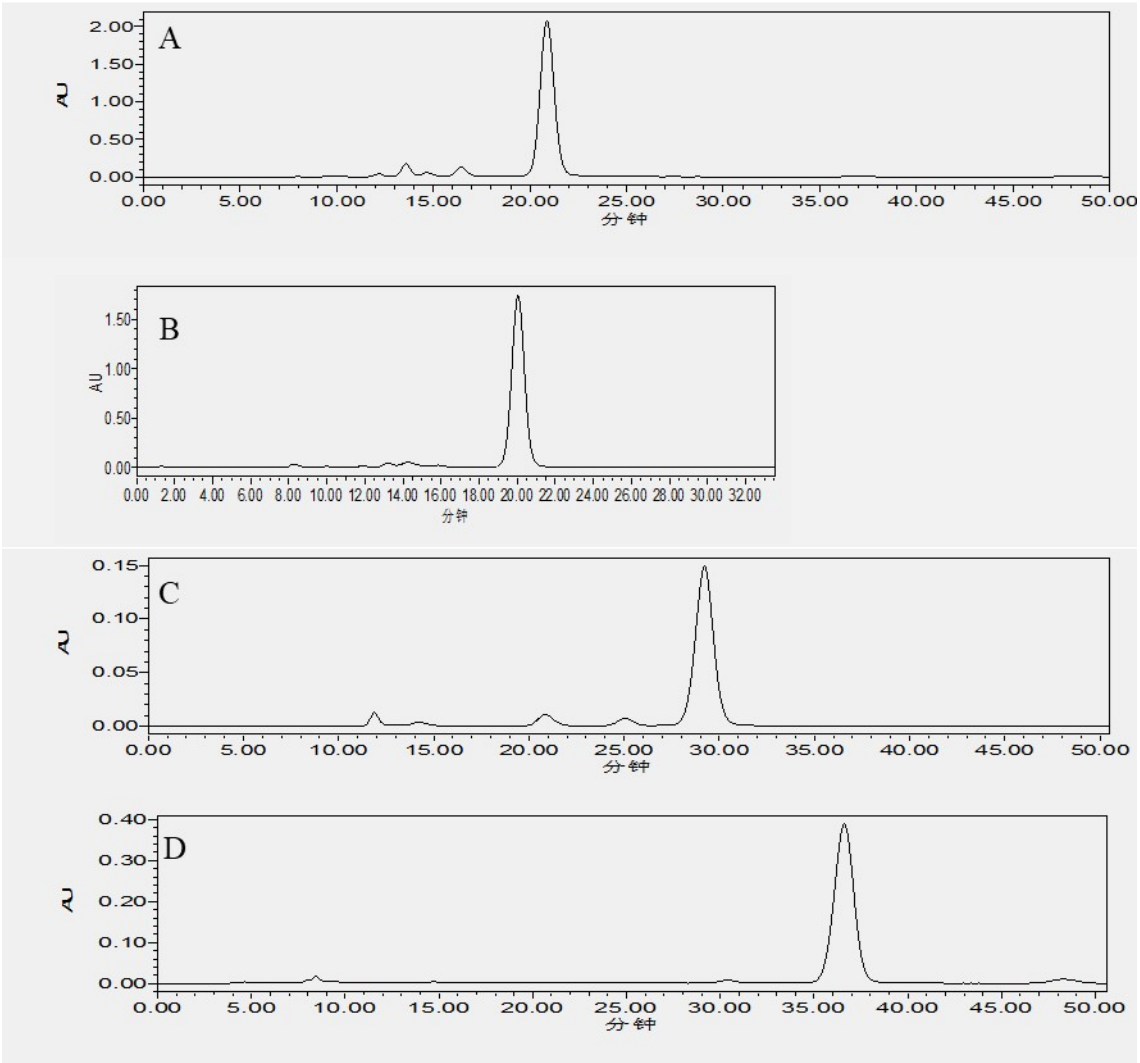


Figure S25 HPLC analyses of derivatives of the authentic samples (A) D-glucose, (B) and L-glucose, (C) D-quinovose and (D) D-digitoxose.

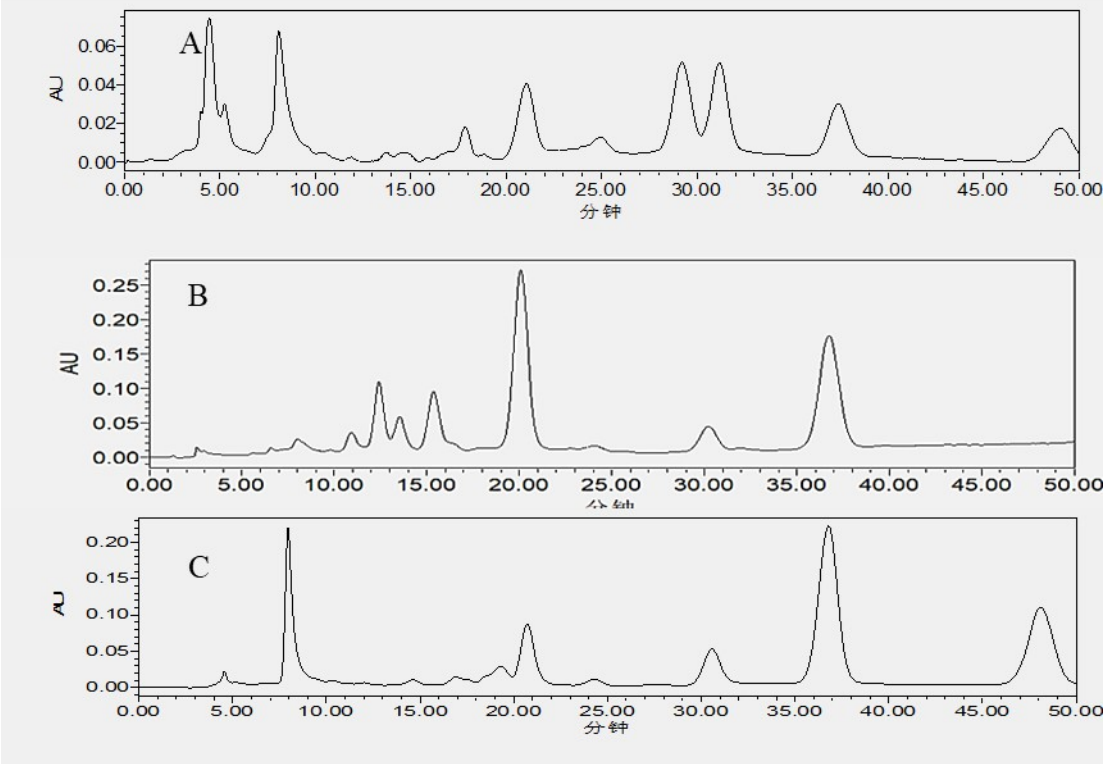


Figure S26 HPLC analyses of hydrolysates and derivatives of compounds (A) **1**, (B) **2** and (C) **3**.

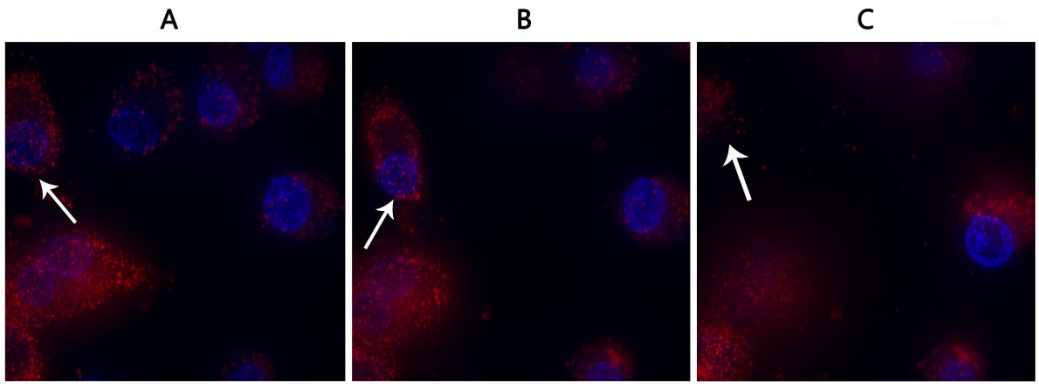


Figure S27. Detection of apoptotic morphological changes in A549 cells treated with compound **1**. A549 cells were labeled by Dil (red), and nuclei were stained by Hoechst 33342 solution (blue) after treatment with **1** (5 μM) for 24 h. Cells were continuously monitored with Delta Vision Elite (General Electric Company, GE). White arrow shows (A) Intact cell structure (B) the cell membrane was broken and the nucl-ei released from intact cells. (C) The nuclear membrane and nucleolus both dis-appear.

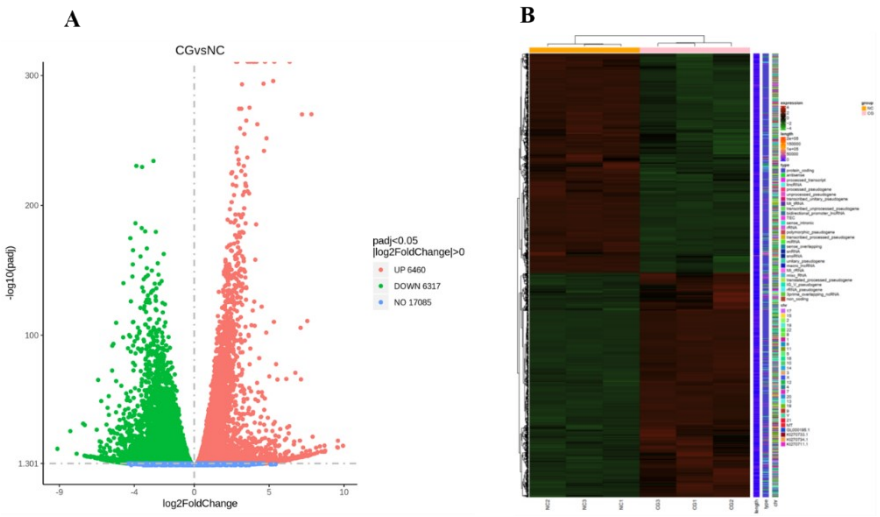


Figure S28 DEGs of A549 cells exposed to compound **1**. (A) volcano map indicated DEGs between groups. (B) Heat map represented the expression of DEGs.

Experimental section

General

Semipreparative HPLC was carried out on a Waters 1525 with a reversed-phase C18 (150 × 10 mm, 10 μm) column and Waters 2998 photodiode array detector. NMR spectra were recorded on a Bruker AVANCE NEO-600 spectrometer. Bruker TENSOR27 spectrometer was used to acquire the IR spectra. RNase A (BS109-25mg, CAS9001-99-4) and propidium iodide staining solution (BL708A, Lot: 71015604) were purchased from bioship. Fast start essential DNA green master (06402712001) was purchased from Roche (China). VASH2 primary polyclonal antibodies were purchased from Invitrogen (lot number:3364EA21, catalog PA5-113065), *β*-actin (GB11001), and HRP-conjugated goat anti-rabbit secondary antibodies (G1213-100UL) was purchased from servicebio. Annexin VFITC apoptosis analysis kit (C1062S) was purchased from beyotime.

Plant Material.

The leaves of *D. Lanata* were obtained from Wuhu City, Anhui Province, P. R. China, in September 2016, and were authenticated by Dr. Jianyin Li from the School of pharmacy, Lanzhou University. A voucher specimen (accession number: 20160912) has been deposited at the School of State Key Laboratory of Applied Organic Chemistry, College of Chemistry and Chemical Engineering, Lanzhou University.

Extraction and isolation.

Air-dried leaves of *D. lanata* (15 kg) were macerated in MeOH at room temperature and followed concentrated by rotary evaporation at 45℃. Completely dissolved MeOH crude extract (3.5 kg) in warm water was partitioned to three fractions with petroleum ether, EtOAc and *n*-BuOH. According to the monitored results of Kedde's reagent on thin-layer chromatography, the EtOAc-soluble fraction shows that it contains more CGs. The EtOAc fraction (420 g) was separated with a D101 macroporous resin column chromatography (CC) and gradiently eluted with proportionally mixture of MeOH/H_2O (0:100, 20:80, 50:50, 70:30, 100:0, v/v) to give five fractions. The Fr. 80% (Fr. D) MeOH/H_2O was chromatographed on a silica gel CC and eluted with CH_2Cl_2/MeOH (20:1, v/v) gradient system to get Fr. D1–Fr. D4 fractions. Fr. D1 to Fr. D4 were respectively purified with sephadex LH-20 CC and eluted with MeOH/CH_2Cl_2 1:1, v/v or MeOH) or suffered from semi-preparative HPLC CC (C18, MeOH /H_2O). Fr. 50% (Fr. C) was sustained an MCI CC (eluting with gradient MeOH/H_2O 50:50, 60:40, 70:20, 80:10 v/v) to obtain four fractions (Fr. C1–Fr. C4). Fr. C1 to Fr. C4 was firstly severally suffering from silica gel CC and eluted with CH_2Cl_2/MeOH at with different volume ratios and Sephadex LH-20 CC put forward next, and finally purified using semipreparative HPLC CC. Fr. 30% (Fr. B) was chromatographed by Sephadex LH-20 (MeOH or MeOH/CH_2Cl_2 1:1 v/v), and RP-C18 semipreparative HPLC. Finally, compounds **1**, **2**, **3**, **4**, **8**, **14**, **15** were obtained in Fr. 80%, **5**, **9**, **10**, **12**, **13** in Fr. 50% and **6**, **7**, **11** in Fr. 30%.

Compounds characterization

digitoxigenin 3-O-*β*-D-glucopyranosyl-(1→4)-*β*-D-methylglucopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4) -*β*-D-digitoxopyranoside (**3**). White solid, m.p. 250-253℃. IR (KBr) ν_{max} 3384, 2933, 1736, 1070, 1022 cm^{-1}; UV (MeOH)

λ_{max} (log ε) 216 (4.19) nm; [α]26 D +2.97 (c 0.3, MeOH). ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) see Table 1. HRESIMS: *m/z*: 965.4720 $[M + Na]^+$ (calcd for $C_{47}H_{74}O_{19}Na$, 965.4717).

digitoxigenin 3-O-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-digitoxopyranosyl- (1→4)-*β*-D-digitoxopyranoside (**4**). White solid, m.p. 263-267 ℃. IR (KBr) ν_{max} 3418, 2931, 1736, 1072, 1029 cm^{-1}; UV (MeOH) λ_{max} (log ε) 215 (4.24) nm; [α]26 D +27.6 (c 0.3, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) see Table 2. HRESIMS: *m/z* 1153.5343 $[M + Na]^+$ (calcd for $C_{55}H_{86}O_{24}Na$, 1153.5401).

thevetiogenin 3-O-*β*-D-glucopyranosyl-(1→4)-3'-O-acetyl-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-digitoxopyranoside (**15**) White solid, m.p. 201-203 ℃. IR (KBr) ν_{max} 3448, 2930, 1741, 1072, 1022 cm^{-1}; UV (MeOH) λ_{max} (log ε) 210 (4.21) nm; [α]26 D +9.0 (c 0.3, MeOH). ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) as shown Table 3. HRESIMS: *m/z*: 989.4725 $[M + Na]^+$, (calcd for $C_{49}H_{74}O_{19}Na$, 989.4717).

Cell culture

A549, Hela, MCF-7 and BEAS-2B (obtained from Shanghai Cell Bank) cells were cultured in RPMI 1640 medium (cytiva, USA). MCF-7 cells were maintained in DMEM medium (cytiva, USA). cells were cultured in medium supplemented 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin solution (Gibco, USA) at 37°C and 5% CO_2-filled humidified atmosphere.

Cell viability assay

Antiproliferative activity of CGs was evaluated using MTT assay. Cells were seeded in 96-well plates (5000 cells/well). After adhering overnight, cells were treated with concentration gradient compounds. Control groups received an equal volume of DMSO (DMSO is replaced by RPMI-1640 medium when the percentage of DMSO is less than 0.01 ‰). After incubation for 48 hours. The medium was removed and 20 μL MTT (5 mg/mL) reagents in 100 μL culture medium were added to each well. After 4h incubating, 100 μL trigeminy reagent (containing 95% SDS, 5% isobutanol and 0.1% hydrochloric acid) was added to each well until the crystal formazan was dissolved. Subsequently, the optical density (OD) was measured at 570 nm. The cell viability was calculated as following equation:

$$\text{cell viability (\%)} = \frac{\text{the OD of the treated group} - \text{the OD of the blank group}}{\text{the OD of the control group} - \text{the OD of the blank group}} * 100\%.$$

Wound healing assay

Cells were collected after treated with compound **3** (0, 40, 80, 120 nM) for 48 h and centrifuged to remove dead cells. Living cells were seeded in 6-well plates and attached overnight. the completely covered cell monolayer was scratched with a 100 μL pipette tip to create a cleared area. The wounded cells were gently rinsed with PBS. Cells were then cultured in 1% FBS medium and photographed at 0 h, 24 h, 48 h at same wound position. The cell migration ratio was calculated as equation

$$\text{Cell migration ratio (\%)} = \frac{\text{Gap distance at t(0h)} - \text{Gap distance at t (24h) or t(48h)}}{\text{Gap distance at t(0h)}} * 100\%$$

Soft agar cloning assay

Soft agar colony formation assay was performed as previous description, with minor modifications.[1] Briefly, A549 cells treated with compound **3** (0, 80, 120 nM) for 48 h and collected and centrifuged. Living cells were suspended in RPMI 1640 medium which containing 10% FBS and mixed with 0.7% of agar by volume ratio 1:1. Then cells seeded in 60 mm culture dish over a base layer which was aforehand prepared with isopyknic 1% agarose and medium. after 20 days. Colonies were counted.

Cell apoptosis analysis with Annexin V-FITC/PI

Apoptosis of A549 and BEAS-2B cells were examined with Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime Shanghai China) and followed manufacturer's recommendations. Cells were treated with compound **3** (80 nM) for 24 h or 48 h and then harvested and washed with ice-cold PBS. 1×binding buffer (100 μL) was used to resuspended cells which were then incubated in lucifuge condition and stained with Annexin V-FITC (5 μL) and PI staining solution (10 μL) for 10 to 15 min. 1×binding buffer (400 μL) were finally added and analyzed using a flow cytometer. Statistical analysis was analyzed with the flowJo-V10 software program.

Flow cytometric analysis of cell cycle

The distribution of the cell cycle was evaluated using propidium iodide (PI) solution with flow cytometric

analysis. Experimental procedure was performed as previous description.[2] A549 cells were treated with concentration gradient (0, 40, 80, 120 nM) compound **3** for 24 h. cells were harvested and fixed with 70% ice-cold ethanol at -20 °C for 12h. Cells were then stained with propidium iodide (PI) solution for 15 min after reacted with RNAse for 30 min at 37 ℃. The cell cycle distribution was analyzed using flow cytometric analysis.

Transcriptome analysis sample preparation and cDNA library construction

A549 Cells were harvested after being treated with **3** for 24 h, and total RNA was extracted with TRIzol reagent. RNA purity was assessed using the NanoPhotometer ® spectrophotometer (IMPLEN, CA, USA). cDNA libraries were constructed using NEBNext® UltraTM RNA Library Prep Kit.

Transcriptome analysis

Differential expression analysis was performed using the DESeq2 package (1.16.1).[3] P value < 0.05 and | $\log_2$ (Fold Change) | > 0 were used to assess differentially expressed genes. All the differential genes were ordered according to the absolute value of $\log_2$FC. The genes with the greatest differences associated with the cancer process were further validated by RT-PCR.

Real-time PCR analysis

Real-time PCR experiment was performed as previous study.[4] In simple terms, total RNA samples were extracted from CG-treated A549 and BEAS-2B cells using TRIzol reagent, following by extracted with chloroform, and precipitated by isopropanol, and washed with 75% alcohol. Then RNA was reverse-transcribed into cDNA. cDNA was synthesized with the Transcriptor first strand cDNA synthesis Kit (Roche, Switzerland). RT-PCR was performed to examine the mRNA expression using Faststart essential DNA green master kit using aglilent technologies stratagene M×3005P following the recommended protocol, The PCR amplification were carried out at 95 ℃ for 10 min (pre-incubation) and 40 cycles at 95 ℃ for 10 s, 55 ℃ for 10 s and 72 ℃ for 10 s. The relative expression levels of RNA were normalized by using *β*-actin as the reference gene and calculated using the $2^{-\Delta\Delta Ct}$ method. Primer pairs were designed and validated at web of https://www.ncbi.nlm.nih.gov/.

Western blot

The expression levels of VASH2 protein were analyzed with western blot. Briefly, Cells were treated with compound **3** (0, 40, 80, 120 nM) for 24 h. Then, cells were washed with PBS and lysed with ice-cold RIPA lysing buffer mixed with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Protein samples were separated with 10% SDS-PAGE after quantified with Bradford Protein Assay Kit (servicebio, Wuhan, China). Protein was transferred onto PVDF membranes (0.22 μM) (solarbio, Beijing, China) and then blocked with 5% nonfat milk and incubated with specific first antibodies (Invitrogen PA5-113065). After washing with TBS (Tris-buffered saline), the membranes were incubated with specific secondary antibodies. Protein bands were visualized with detection system.

Molecular docking

Binding sites of VASH2 (PDB ID 6JZE) with compound **3** were examined using Glide module in Schrödinger.

Chick embryo chorioallantoic membrane (CAM) assay

The CAM assay was performed as literature described.[5] Briefly, the fertilized egg shells were cleaned using 0.1% bromogeramine and then incubated for 8 days at 37.8℃ and 40–50% humidity and then windowed. After removal of the shell membrane with tweezers, a polyethylene resin ring was placed and 0 μg, 0.3 μg, 0.6 μg of compound **3** in saline solution (20 μL) was added onto the CAM. The window was sealed with sterile sealing film and incubated for another 48h at 37.8 °C and 60% humidity. Subsequently, the blood vessels network of the CAM was photographed. Two parallel experiments were performed with 10 eggs in each group. Vascular plexuses were quantitatively analyzed using an Image J.

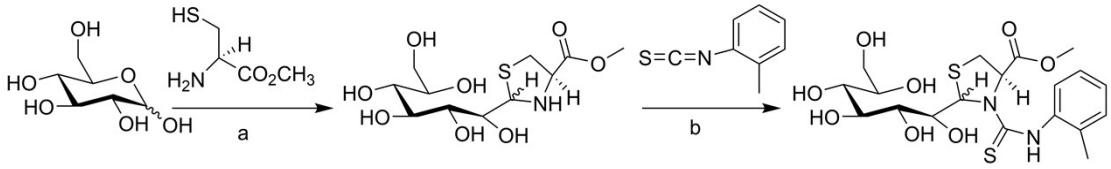
Statistical analysis

Data were shown as mean value ± standard deviation (SD) from at least three experiments. Statistical significance of differences between groups was determined using student's t test by origin 2018. Results were considered statistically significant at *p<0.05, **P < 0.01, and ***P < 0.001.

Reaction process of hydrolysis of compounds **1–3**

A solution of each compound (1.0 mg) in 0.5 mol/L HCl (0.2 ml) was stirred at 80 °C for 2 h, and then neutralized with 0.5 mol/L NaOH. After cooling, the solution was evaporated in vacuo. the residue was dissolved in anhydrous pyridine (0.2 ml) and added L-cysteine methyl ester hydrochloride (1 mg). The mixture was heated at 60 °C for 1 h. A 0.2 ml solution of o-torylisothiocyanate (1 mg) in pyridine was added and heated at 60 °C for

another 1 h. After drying the solution, the residue was partitioned between H_2O and cyclohexane. The cyclohexane layer was concentrated, then dissolved in methanol and analyzed by semipreparative HPLC with 45% methanol. The wavelength was set to 250nm. The peaks at 19.90, 20.87, 29.20, and 36.60 min were coincided with derivatives of L-glucose, D-glucose, D-quinovose and digtoxose. The peaks at 20.89, 29.24 and 36.60 min were coincided with derivatives of D-glucose D-quinovose and D-digtoxose in compound **1.** The peaks at 20.21



and 36.62 min were coincided with derivatives of D-glucose and D-digtoxose in compound **2**. The peaks at 20.72 and 36.72 min were coincided with derivatives of D-glucose and D-digtoxose in compound **3.**

Scheme 1 Reaction of aldoses with L-cysteine methyl ester and *o*-tolyl lisothiocyanate. Reaction conditions: a 60 °C for 1 h, b 60 °C for 1 h.

Notes and references

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