Supplementary Information

Munronin V with 7/7/6 Tricarbocyclic Framework from *Munronia henryi* Harms Inhibits Tau Pathology via Activating Autophagy

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Materials and methods

General experimental procedures

Optical rotation measurements were conducted with a Jasco P-1020 automatic polarimeter. CD spectra were determined on the Applied Photophysics circular dichroism spectrometer (Applied Photophysics, Leatherhead, Surrey, UK). IR spectra were recorded on a NICOLET iS107 Mid-infrared spectrometer. High-resolution MS data were performed on an Agilent 1290 UPLC/6540 Q-TOF mass spectrometer in positive mode. ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HSQC, HMBC and ROESY spectra were collected on Bruker DRX-600 instruments (Bruker, Bremerhaven, Germany). Semi-preparative HPLC separations were performed on an Agilent 1260 liquid chromatograph (Agilent Technologies, USA) with a Waters X-bridge column (5 μm, 10 × 250 mm). Analytical TLC systems were carried out on silica gel 60 F254 plates (Qingdao Marine Chemical Inc., Qingdao, China). Column chromatography (CC) was performed by using silica gel (200-300 mesh and 60-80 mesh, Qingdao Marine Chemical, Inc., Qingdao, China) and Lichroprep RP-18 gel (40 – 63 μm; Merck, Darmstadt, Germany). Sephadex LH-20 (40 – 70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Spots were visualized by heating silica gel plates sprayed with 5% H_2SO_4 in ethanol.

Plant material

The whole plant of *Munronia henryi* was collected in August 2017 from Xingyi, Guizhou Province, China, and was identified by Prof. De-Yuan Chen of Guiyang College of Traditional Chinese Medicine. A voucher specimen (DHL 20170801) was deposited at the Laboratory of Guizhou Medical University.

Extraction and isolation

The air-dried and powdered twigs of *M. henryi* (9.0 kg) were refluxed with 95% ethanol (3 × 35 L) three times (3 × 3 h). To obtain the residue (602 g), the combined extract was concentrated under reduced pressure by a rotary evaporator. The extract

was suspended in water and then partitioned with ethyl acetate (4 × 5 L). The ethyl acetate portion (193 g) was applied to a silica gel column using PE–EtOAc (50:1–1:1, v/v) and CH_2Cl_2–CH_3OH (15:1–1:1, v/v) to obtain seven fractions (Fr. 1–Fr. 7). Fr. 6 (21.4 g) was applied to an MCI gel column and eluted with a gradient of CH_3OH/H_2O (30:70 to 95:5) to yield six fractions (Fr. 6A–Fr. 6F). Fr. 6D (27.5 g) was separated by reversed-phase column (CH_3OH–H_2O, 4:6—9:1) to get five fractions (Fr. 6D1—Fr. 6D4). Fr. 6D3 (0.6 g) was purified by Sephadex LH-20 eluting with MeOH to yield three fractions. Fr. 6D3b was further separated by semi-preparative HPLC with an X-bridge column and eluted with CH_3CN /H_2O (2.5 mL/min, CH_3OH: H_2O = 60:40, v/v) to yield compounds **1** (8 mg, t_R = 28 min).

Molecular docking method

The full-length human transcription factor EB (TFEB) structural model was established using the ab initio and hierarchical approach based on I-TASSER (https://zhanglab.ccmb.med.umich. edu/I-TASSER/)[1, 2]. The best confirmation was refined with energy minimization and molecular docking was performed by Autodock Vina with center box: x = 82.509, y = 68.741, z = 70.767 and the dimensions: 30 × 30 × 30 Å for TFEB. The docking results were analyzed and shown with Discovery Studio Visualizer (BIOVIA, San Diego, USA) and PyMOL software (Schrodinger, LLC: NY, USA)

Construction of the U251 cells with stable overexpression of the mutant MAPT (MAPTmut) and HM cells with stable overexpression of the *TFEB* gene

The U251 cells, human microglia (HM) cells, HEK293T cells were introduced from the Kunming Cell Bank, Kunming Institute of Zoology, Chinese Academy of Sciences. The U251 cells were maintained in Roswell Park Memorial Institute 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco, USA, 10099-141). The

HEK293T cells and HM cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gibco, USA, 10099-141) at 37°C incubator with 5% CO_2 and 95% humidity.

The coding region of the *MAPT* gene with flag tag was cloned into PLVX vector (PLVX-MAPT) of the Lenti-X Tet-On Advanced Inducible Expression System (Clontech). Mutant MAPT P301S was introduced into PLVX-MAPT vector by using site-directed mutagenesis PCR method. The U251 cells with stable overexpression of mutant MAPT P301S were established according to the instruction of Lenti-X Tet-On Advanced Inducible Expression System (Clontech) and following our previously reported method[3]. In brief, the response lentivirus system was composed of mutant PLVX-MAPT construct, packaging plasmid psPAX2 (Addgene, England, 12260), and envelope plasmid PMD2.G (Addgene, England, 12259), while the regulator lentivirus system was composed of PLVX-Tet-On-Advanced vector, psPAX2 and PMD2.G. The lentivirus supernatant was produced from the HEK293T cells and was used to infect U251 cells with a ratio of 4:1 for the response lentivirus and the regulator lentivirus. Infected U251 cells were selected in growth medium with 1μg/mL puromycin.

The coding region of the *TFEB* gene with flag tag was cloned into PLVX vector (PLVX-TFEB). The response lentivirus system and the regulator lentivirus system were same to the above one for making MAPT P301 overexpression, except for replacing mutant PLVX-MAPT construct with the PLVX-TFEB construct. HM cells were infected with the lentivirus systems and were selected in growth medium with and 1μg/mL puromycin.

Flow cytometry analysis

The flow cytometry analysis was performed as described in our previous study[4]. In brief, HM mCherry-GFP-LC3 cells with stable overexpression of a triple fusion protein (red fluorescent protein (mCherry), green fluorescent protein (GFP) and the autophagosome marker LC3), was constructed as a cell line for quantifying the strength of autophagic flux. The bioactivity of compound **1** was evaluated in the HM

mCherry-GFP-LC3 cells. Briefly, HM mCherry-GFP-LC3 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco-BRL, 10099-141) at 37°C incubator with 5% CO2 and 95% humidity. The HM mCherry-GFP-LC3 cells ($2x10^5$/well) were cultured in 12-well plates overnight, then were treated with different concentrations of the compound (10 μM and 40 μM; compound was directly added into the culture medium). After 24-hour treatment of compound **1**, cells were fixed by 4% PFA (paraformaldehyde), followed by a flow cytometry test to check whether the autophagic flux was enhanced through analyzing the ratio of cells with red fluorescence which means the autophagic flux goes well as the acid-sensitive GFP was quenched by autolysosome[5]. Data were analyzed using FlowJo software (FLOWJO, LLC). This experiment was repeated at least 3 times, with 3 biological replicates for each treatment.

Confocal laser scanning assay

The HM mCherry-GFP-LC3 cells were cultured in glass-bottom cell dish (NEST, 801001). After the treatment of compound **1**, Rapamycin and Bafilomycin A1 (BAFA1) for 24 h, cells were fixed by 4% PFA and then were individually pictured under an Olympus FluoView™ 1000 confocal microscope (Olympus, Japan). The HM TFEB-GFP cells were handled the same way, with the exception of a shorter treatment with compound **1** and Torin1 for 6 h. Images were analyzed with FV10-ASW 2.1 Viewer (OlympusMicro, Japan).

Western blotting

The U251-MAPT P301S cells were cultured in 6-well plates. Western blotting for target proteins was performed using the common approach as described in our previous studies [6-8]. Briefly, a protein lysis buffer (Beyotime Institute of Biotechnology, P0013) was used for making cell lysates. After the protein concentration in cell lysate was determined by using the BCA protein assay kit (Beyotime Institute of Biotechnology, P0012), about 20 μg total proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride membrane (Bio-Rad, L1620177 Rev D).

The membrane was blocked in 5% (w:v) skim milk at room temperature for 2 hours. The membrane was incubated with primary antibody against Tau (1:1000, cell signaling technology, 46687S), TFEB (1:1000; cell signaling technology, 4240S), p-TFEB Ser122 (1:1000; cell signaling technology, 86843S), CTSB (1:1000; Affinity, AF5189), SQSTM1 (1:1000, Elabscience, E-AB-62289), LC3 (1:1000, Proteintech, 14600-1-AP), GAPDH (1:20000, Affinity, AF7021), at 4^{o}C overnight, respectively. The membrane was washed 3 times with TBST (Tris buffered saline [Servicebio, G0001] with 0.1% Tween 20 [Sangon Biotech (Shanghai) Co.,Ltd, HB09BA0007]) for 5 min each time, and incubated with either peroxidase-conjugated anti-mouse (KPL; 474-1806; 1:10000) or anti-rabbit IgG (KPL; 474-1516; 1:10000; KPL) at room temperature for 1 hour. The epitope was visualized using ECL Western Blot Detection Kit (Millipore, WBKLS0500). Western blot of GAPDH was used as an inner control for measuring the target protein level. The densitometry of target protein was evaluated by ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Statistics and reproducibility

Data analyses were carried out by using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). The one-way ANOVA (analysis of variance) was performed using the Dunnett's *post hoc* test for comparison between the treated group and control group, and the values were expressed as mean ±standard deviation (SD). The difference was considered to be statistically significant if a P value < 0.05. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Original spectroscopic data

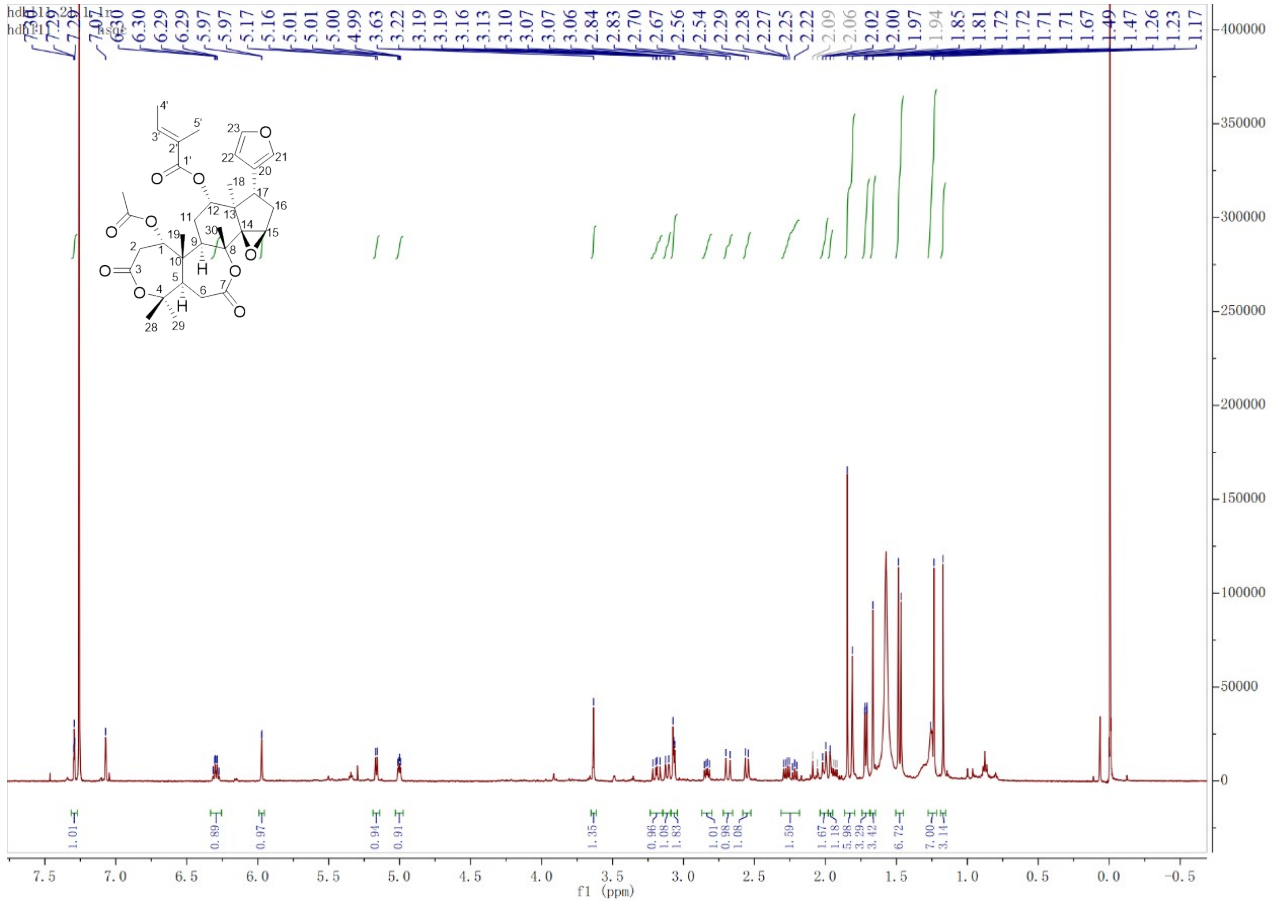


Figure S1. ^{1}H NMR (600 MHz) spectrum of munronin V (1) in $CDCl_3$.

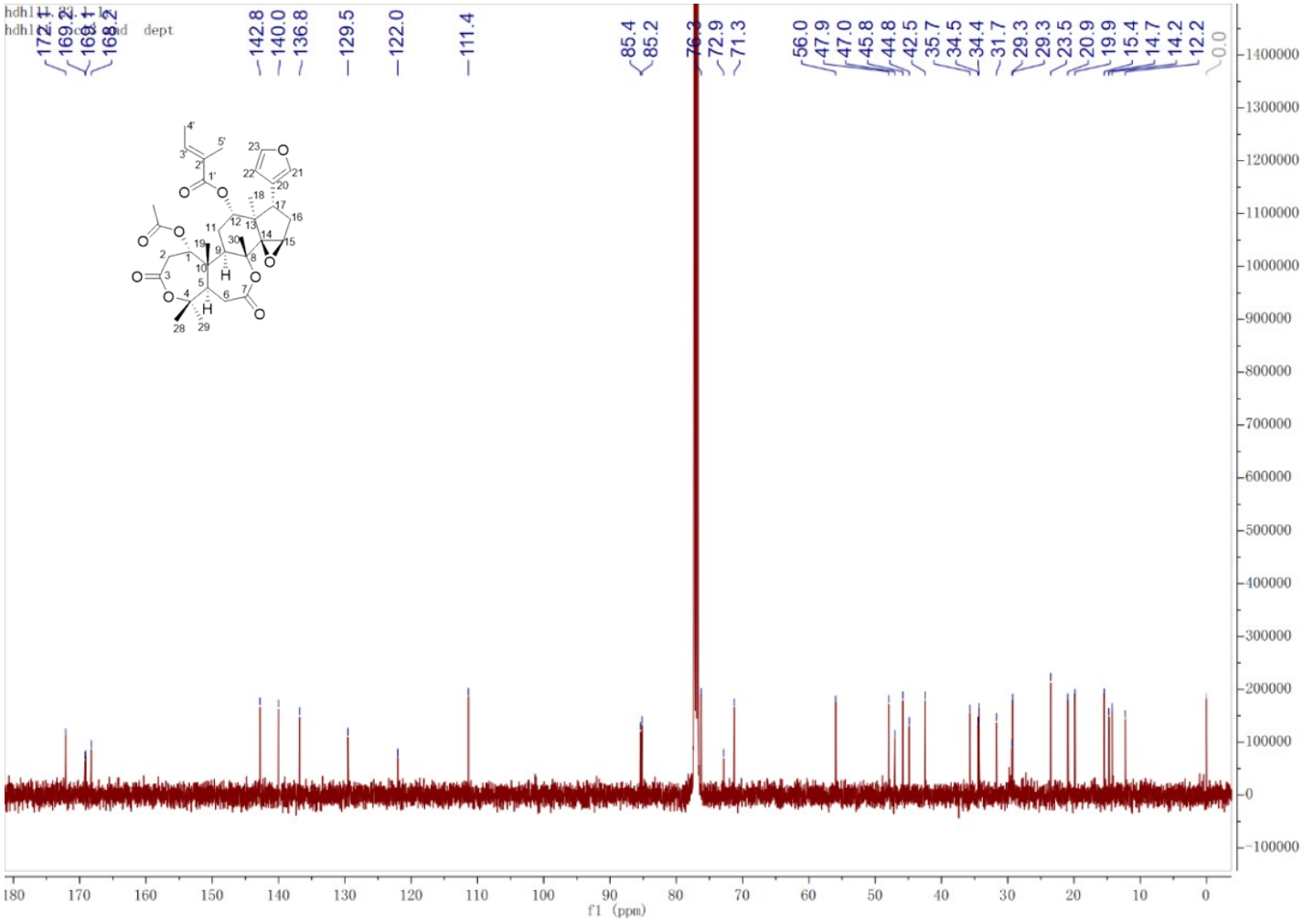


Figure S2. ^{13}C NMR (150 MHz) spectrum of munronin V (1) in $CDCl_3$.

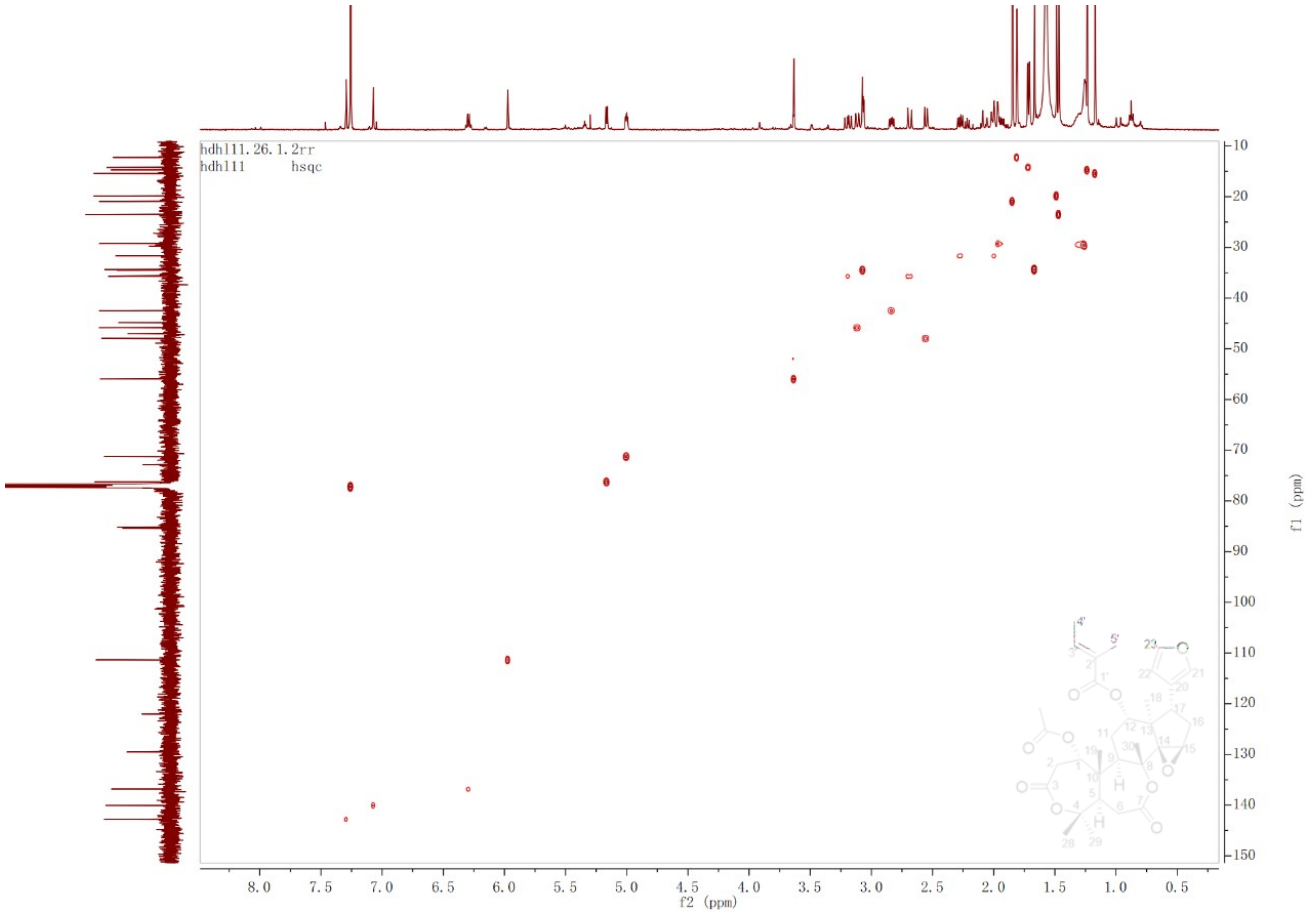


Figure S3. HSQC (600 MHz) spectrum of munronin V (1) in $CDCl_3$.

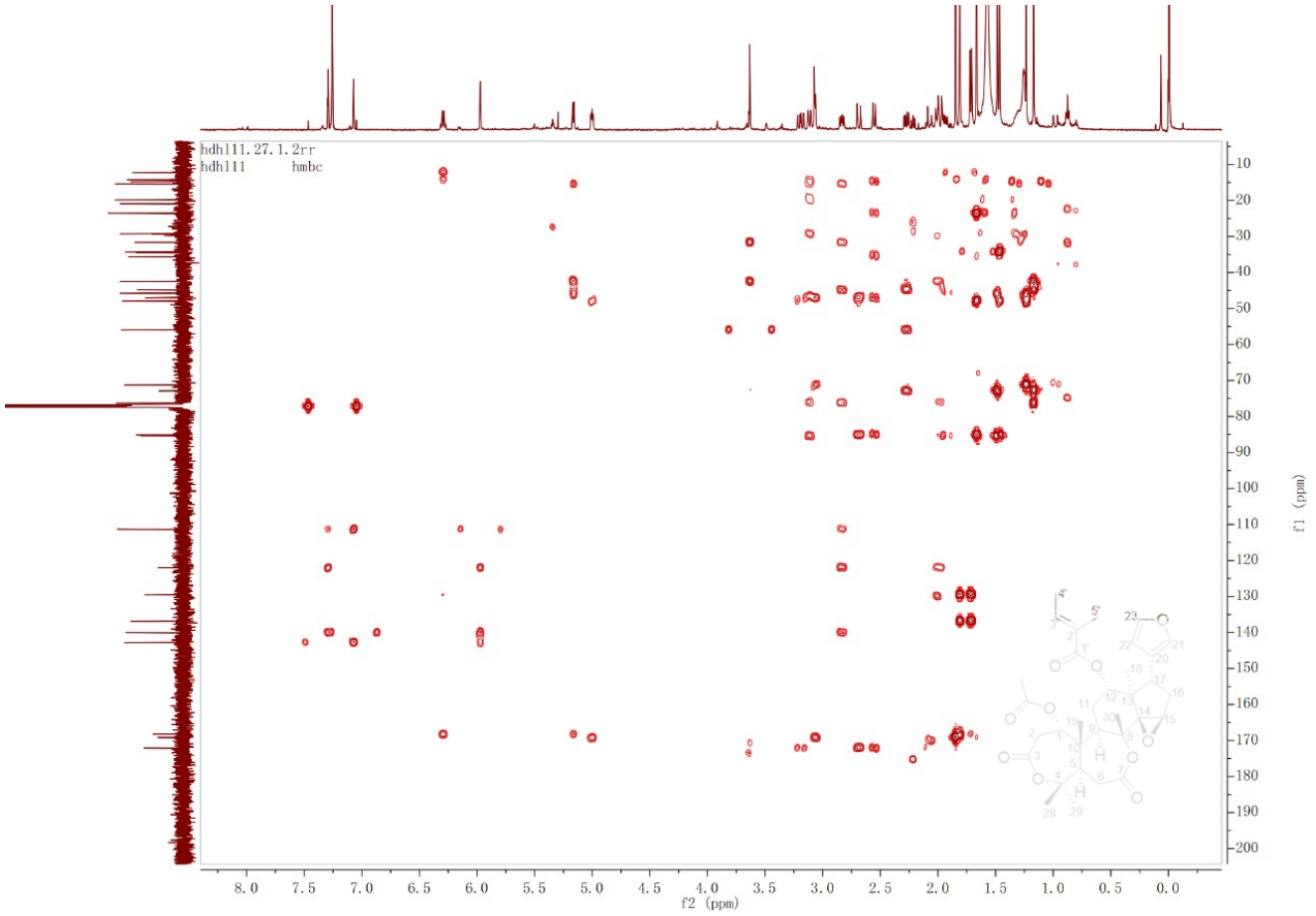


Figure S4. HMBC (600 MHz) spectrum of munronin V (1) in $CDCl_3$.

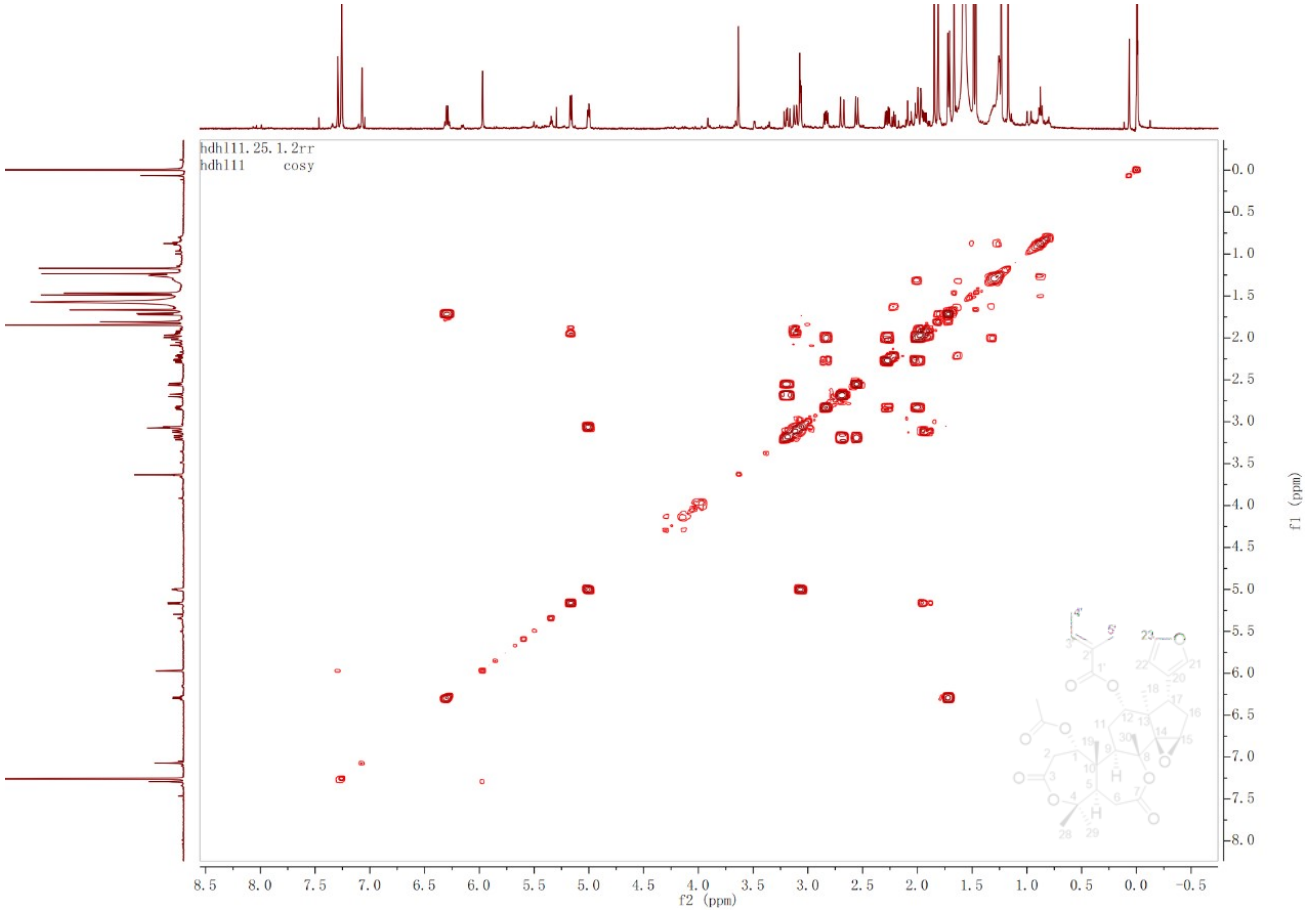


Figure S5. ^{1}H-^{1}H COSY (600 MHz) spectrum of munronin V (1) in $CDCl_3$.

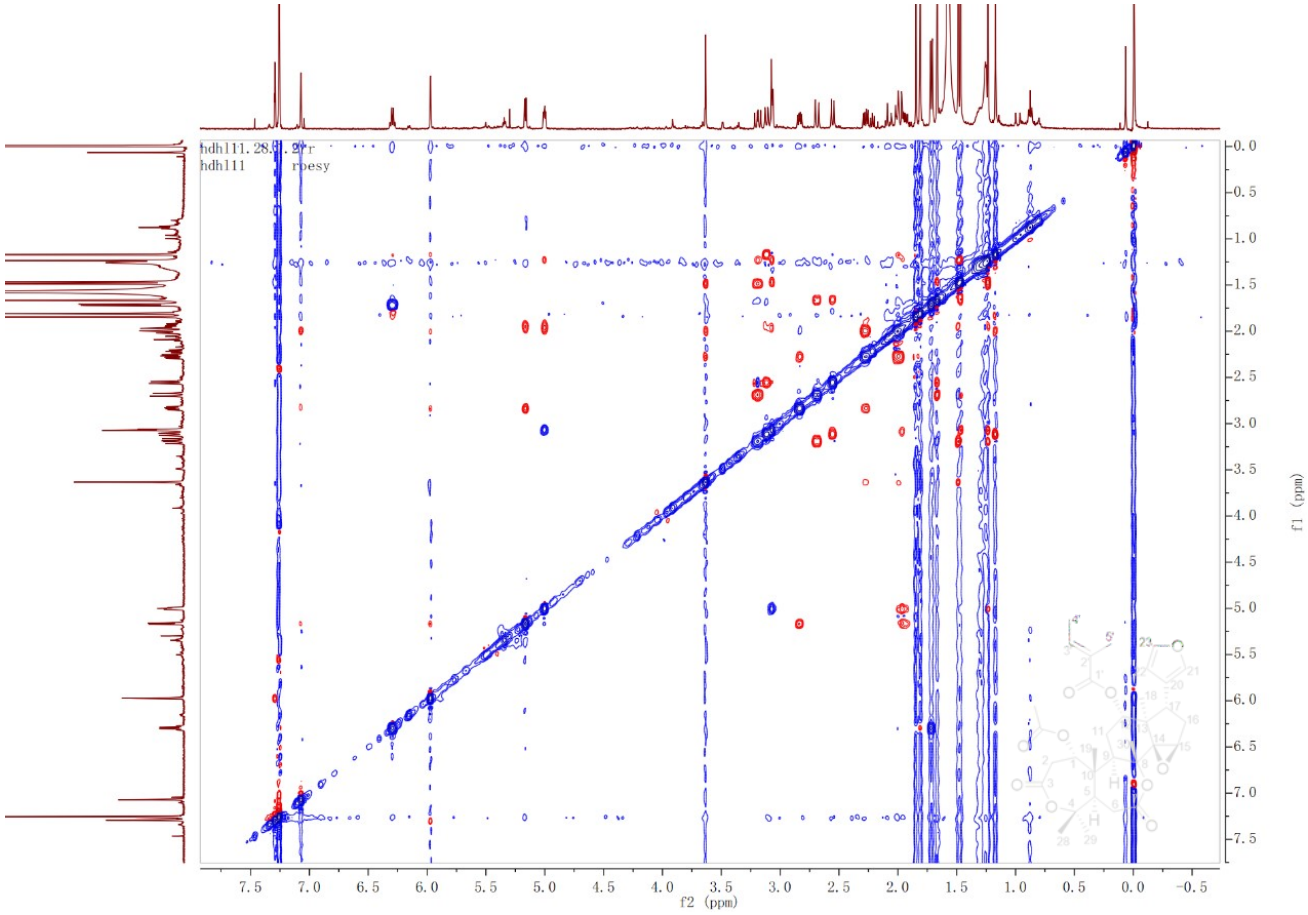


Figure S6. ROESY (600 MHz) spectrum of munronin V (1) in $CDCl_3$.

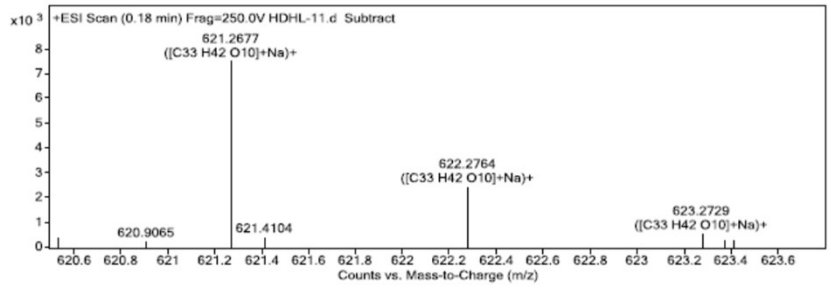
Qualitative Analysis Report

Data Filename	HDHL-11.d	**Sample Name**	HDHL-11
Sample Type	Sample	**Position**	P1-A3
Instrument Name	Instrument 1	**User Name**	
Acq Method	s.m	**Acquired Time**	9/1/2020 2:47:52 PM
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group	**Info.**
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125.2)

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
250	0	ESI



Peak List

m/z	z	Abund
256.2626	1	67156.39
274.2736	1	60743.55
300.2894	1	17320.86
318.2999	1	75757.48
340.2812	1	18160.7
353.2658	1	19327.06
362.3257	1	36945.31
437.1926	1	82691.19
438.1965	1	22311.29
653.2917	1	17143.64

Formula Calculator Element Limits

Element	Min	Max
C	3	60
H	0	120
O	0	30

Formula Calculator Results

Formula	CalculatedMass	CalculatedMz	Mz	Diff. (mDa)	Diff. (ppm)	DBE
C33 H42 O10	598.2778	621.2670	621.2677	-0.70	-1.13	13.0000

Figure S7. HRESIMS spectrum of munronin V (1).

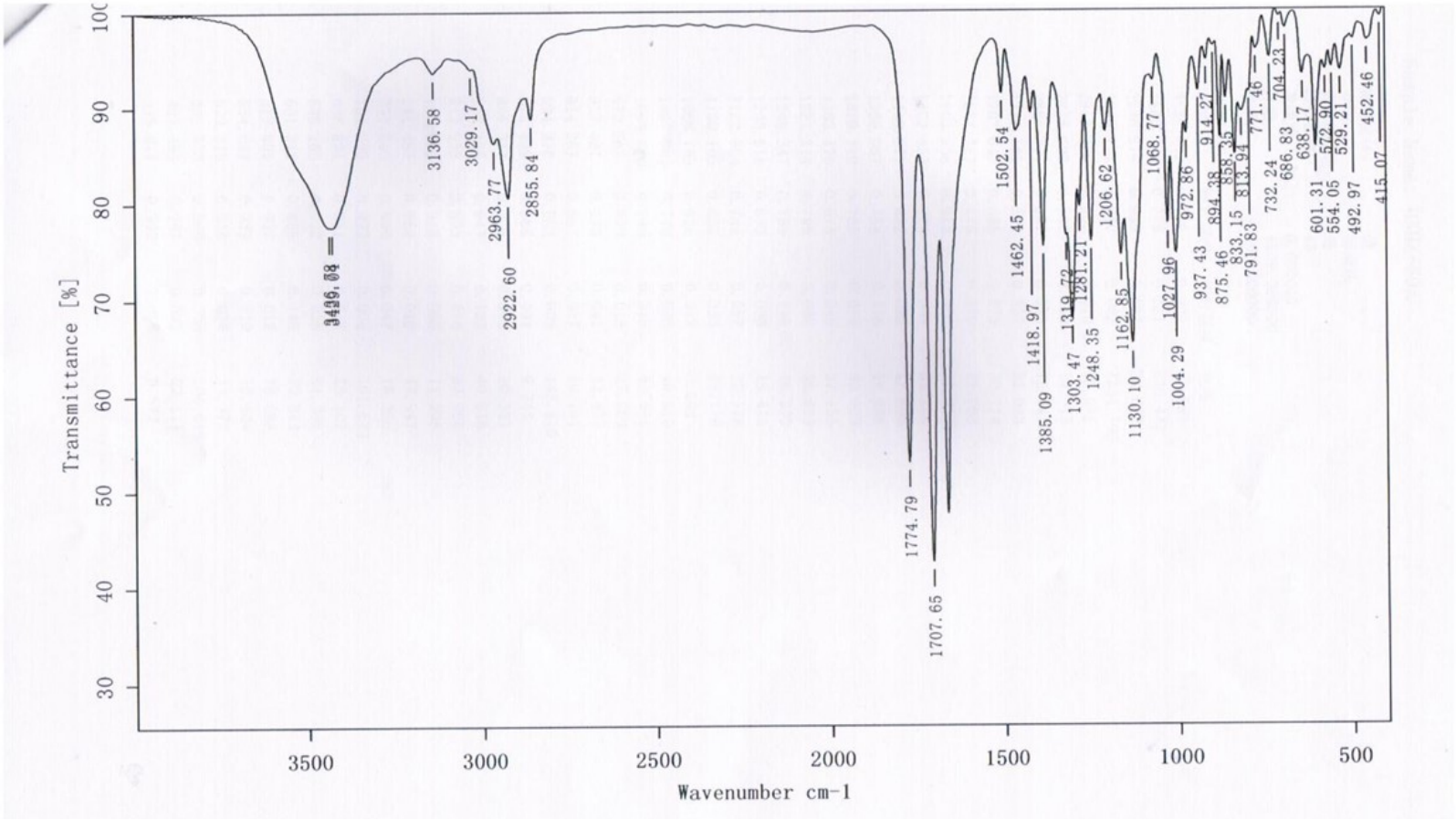

Transmittance [%]
100
90
80
70
60
50
40
30
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1
3136.58
3029.17
2963.77
2922.60
2855.84
1774.79
1707.65
1502.54
1462.45
1418.97
1385.09
1319.72
1303.47
1281.21
1248.35
1206.62
1162.85
1130.10
1068.77
1027.96
1004.29
972.86
937.43
914.27
894.38
875.46
858.33
833.15
813.94
791.83
771.46
732.24
704.23
686.83
638.17
601.31
572.90
554.05
529.21
492.97
452.46
415.07

Figure S8. IR spectrum of munronin V (1).

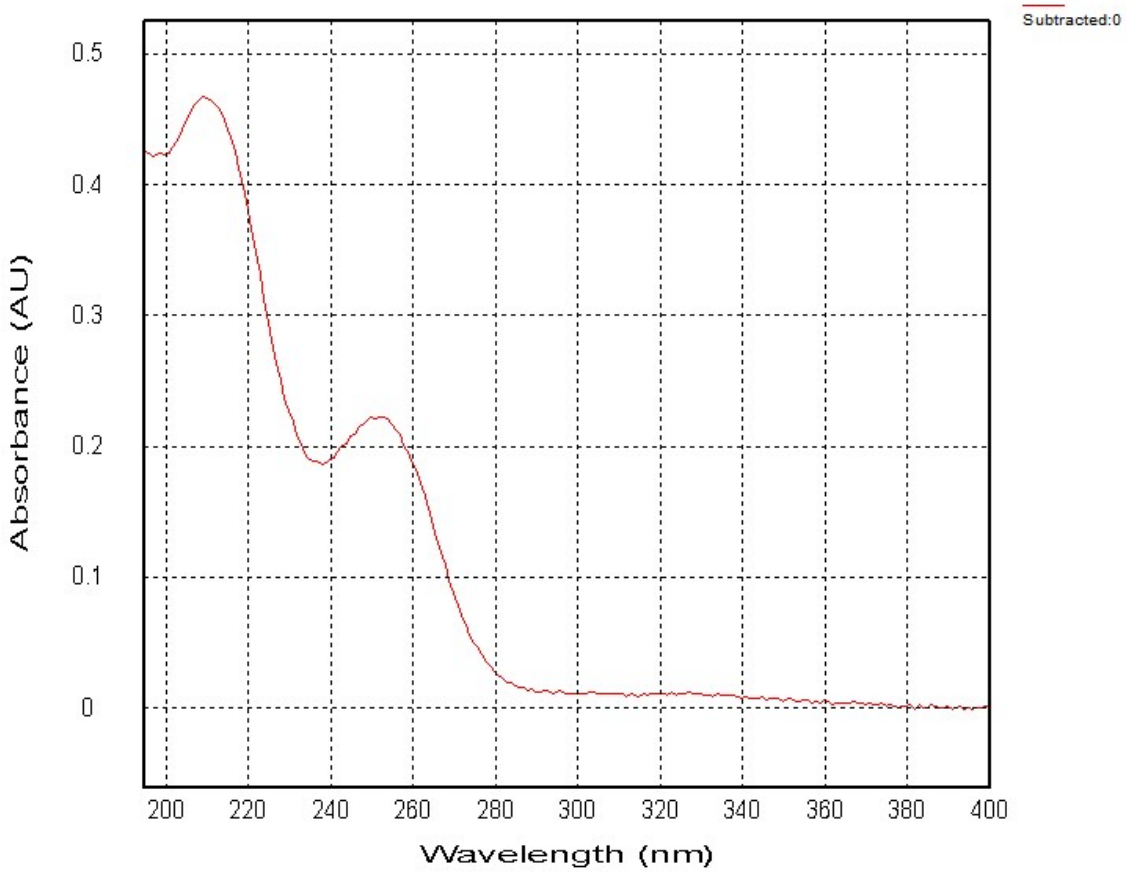


Figure S9. UV spectrum of munronin V (1).

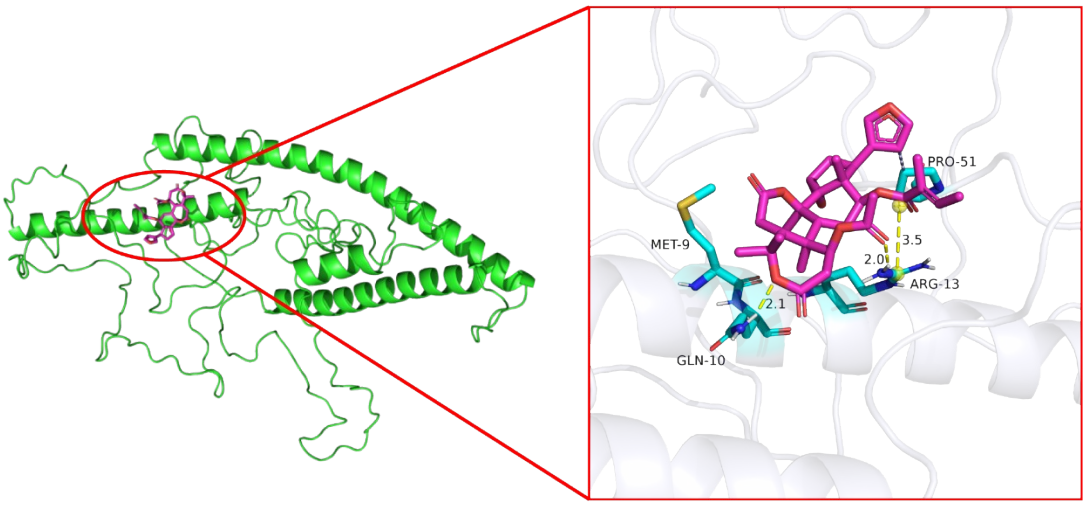


Figure S10. The molecular docking mode of munronin V (1) with TFEB.

The molecular docking experiment displayed that compound **1** could bind to the active site of TFEB in a proposed pose illustrated with predicted binding energy of -6.47 kcal/mol. Compound **1** forms hydrogen bonds with TFEB at GLN10 and ARG13, and forms hydrophobic interaction at MET9 and PRO51. These interactions would stabilize the binding of compound **1** with TFEB and potentially affect the activity of TFEB.

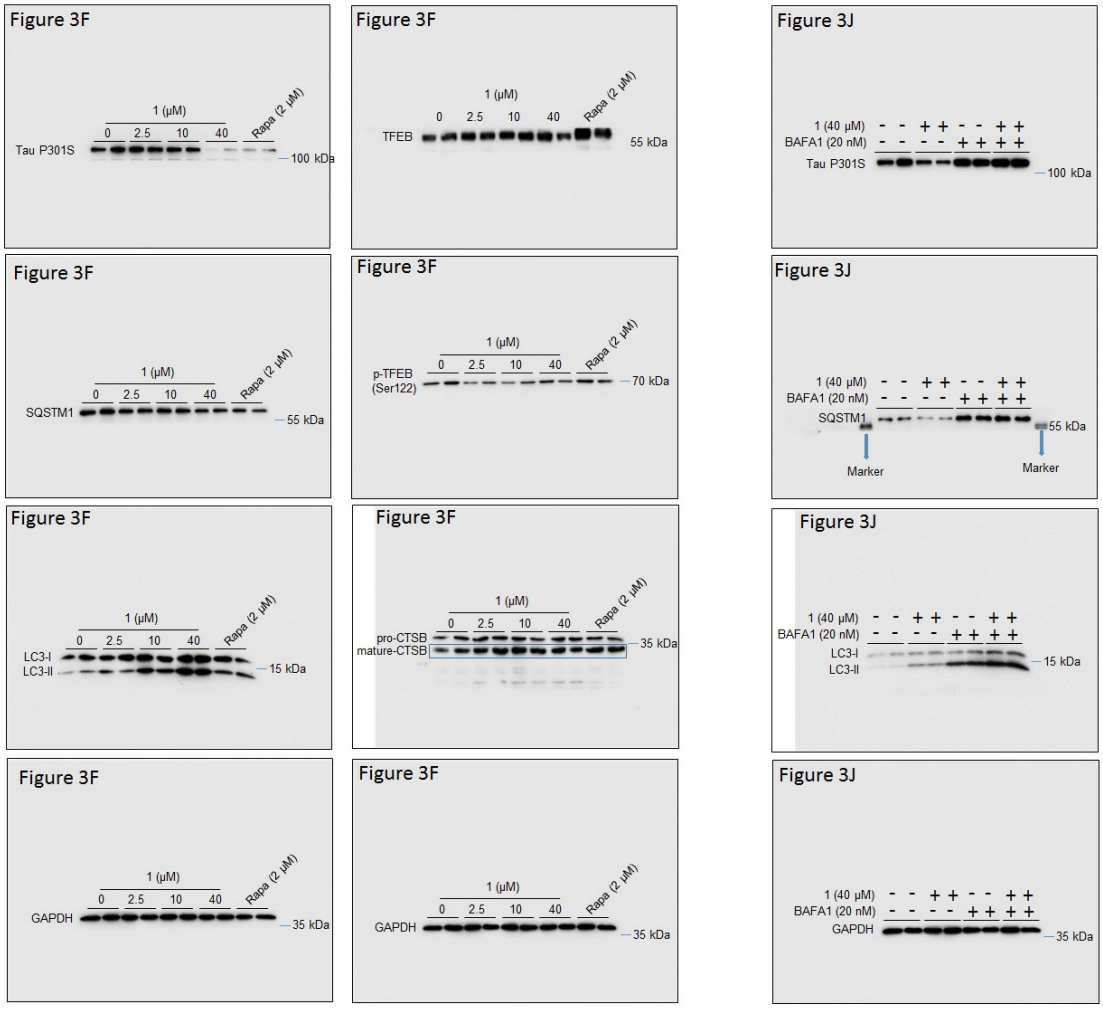


Figure S11. The raw images of the Western blot in Figure 3.

Reference

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Lett, 2021, **23**, 262-267.

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