Supporting Information

Chemical Proteomic Profiling Reveals Prostaglandin Termination Enzyme PTGR2 as a Key Molecular Target of Natural Coumarin Fraxetin

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Supporting Information

1. Supplementary Schemes and Figures

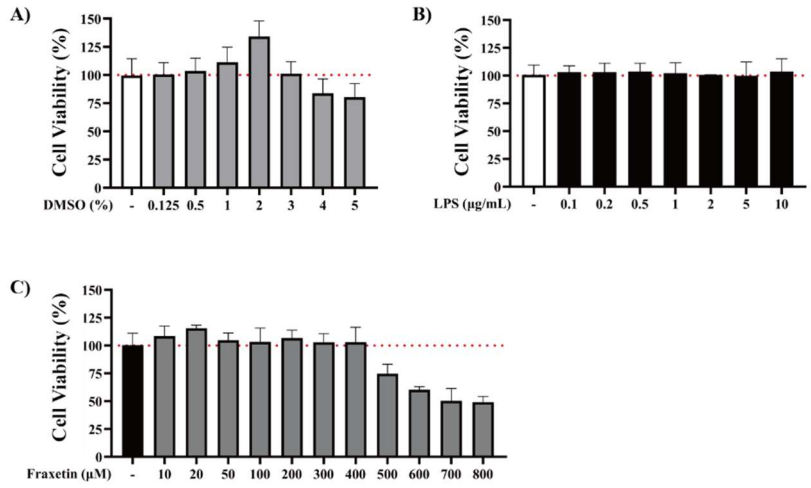


Figure S1: The impacts of DMSO (A), LPS (B) and fraxetin (C) on the viability of RAW264.7 cells.

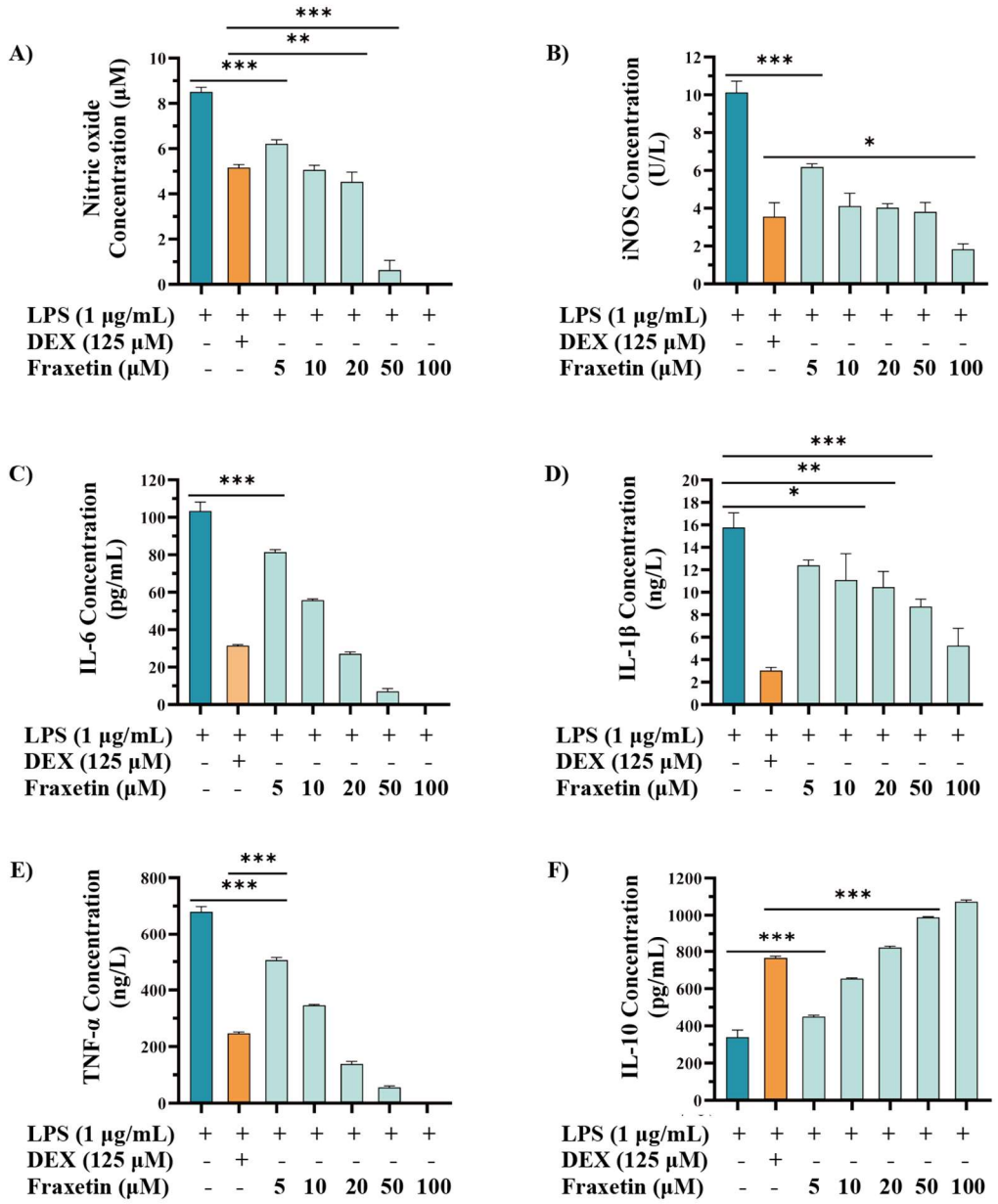


Figure S2: The modulation of LPS-stimulated inflammation in RAW264.7 cells by fraxetin. Cytokines and inflammation-associated factors, including NO (A), iNOS (B), IL-6(C), IL-1β(D), TNF-α (E) and IL-10 (F) were measured, with dexamethasone (DEX) serving as a positive control. The data were derived from two biological experiments with technical triplicates. Statistical significance was indicated as follows: ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

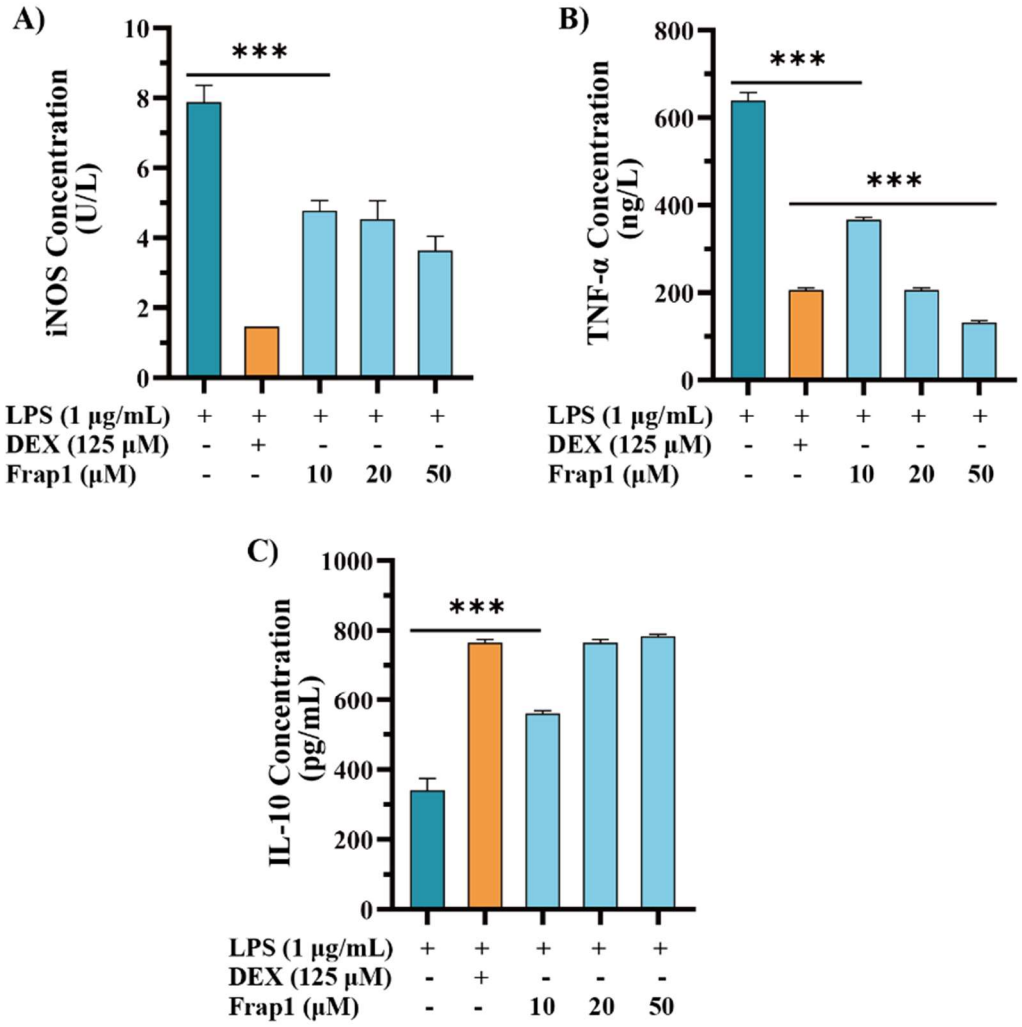


Figure S3: LPS (1 μg/mL) stimulated RAW264.7 cells were employed as the model for inflammation. Cytokines and inflammation-associated factors in RAW264.7 cells under Frap1 treatment, including iNOS (A), TNF-α (B) and IL-10 (C) were measured, with dexamethasone (DEX) serving as a positive control. The data were obtained from two biological experiments with technical triplicates. Statistical significance is indicated as follows: ***$p < 0.001$.

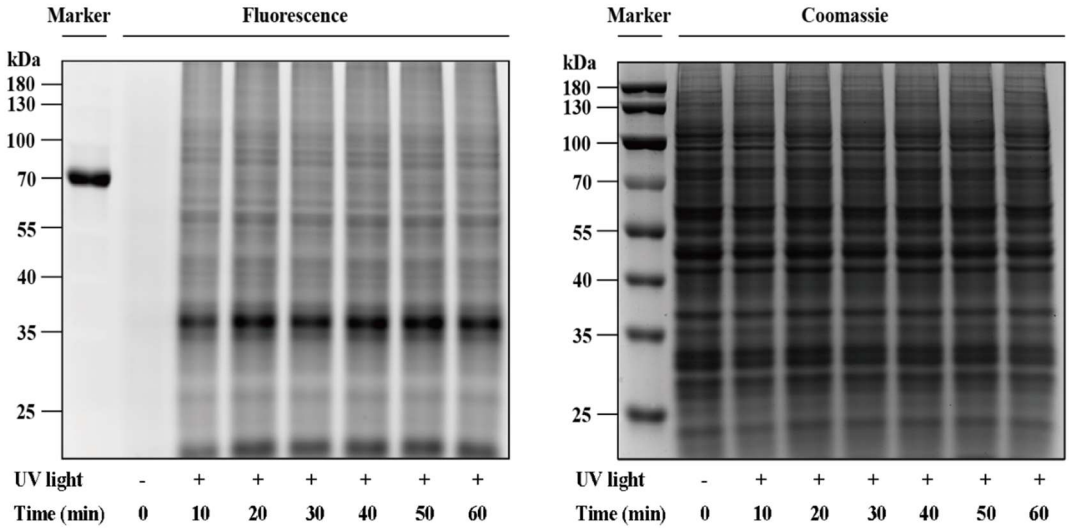


Figure S4: Experiments assessing UV light exposure duration indicate that 20 min is adequate for activating the probe and facilitating its cross-linking, with 50 μM **Frap1** employed in this context. A Coomassie-stained SDS-gel is presented alongside the fluorescent SDS-gel to facilitate comparison of protein abundance. RAW264.7 cells were employed for all the aforementioned *in situ* labeling experiments.

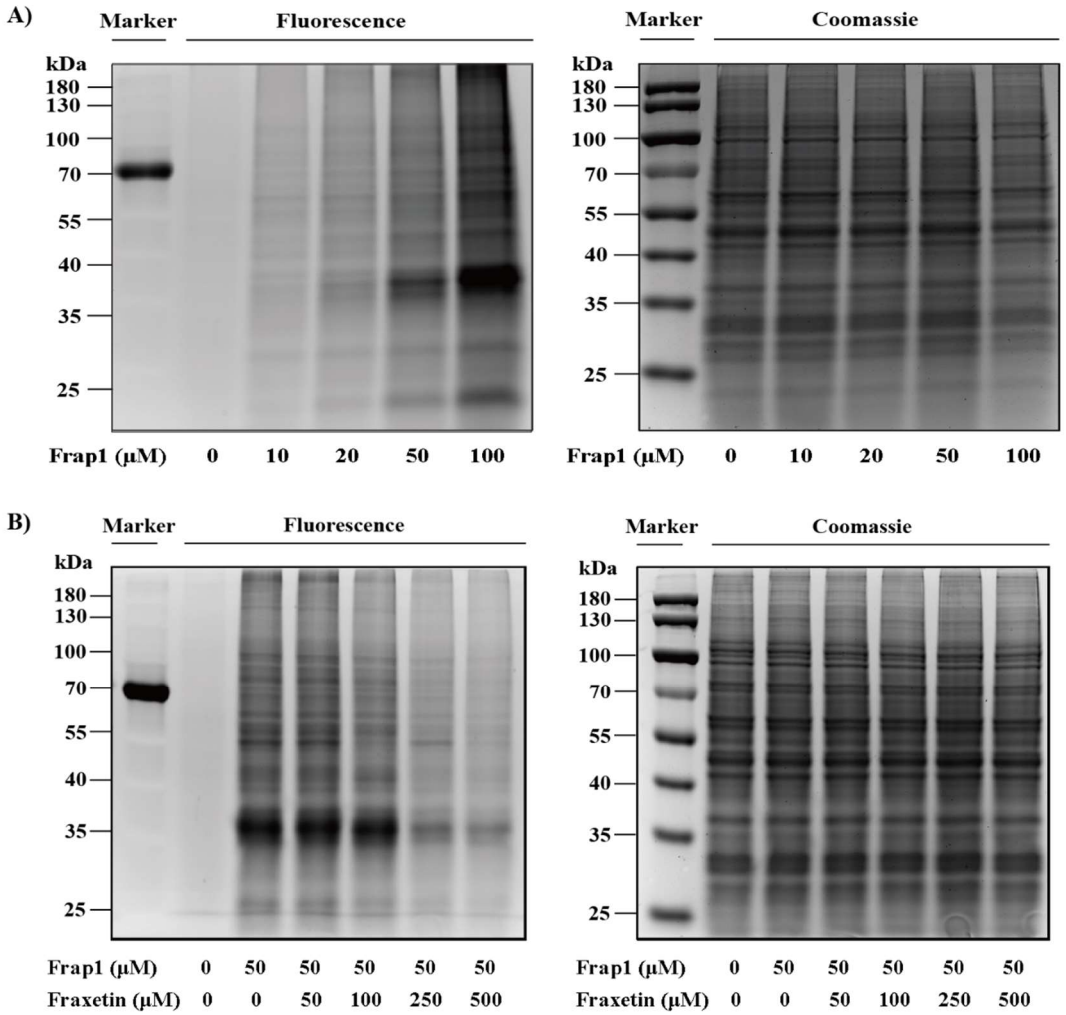


Figure S5: A) The fluorescent SDS-gel analysis of *in situ* labeled RAW264.7 cells with various concentrations of Frap1 reveals that 50 μM Frap1 can achieve satisfactory labeling results. B) The fluorescent SDS-gel analysis of competitive *in situ* labeling with 50 μM Frap1 against various folds of fraxetin indicates that a ten-fold excess of fraxetin is required for effective competition. A Coomassie-stained SDS-gel is presented alongside the fluorescent SDS-gel to facilitate comparison of protein abundance. RAW264.7 cells were employed for all the aforementioned *in situ* labeling experiments.

Protein IDs	Gene names	Protein names	Log_2-fold change	-Log (P-value)
O35459	Ech1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	1.0	2.77
Q9D338	Mrpl19	39S ribosomal protein L19, mitochondrial	1.6	2.44
Q8VDQ1	Ptgr2	Prostaglandin reductase 2	2.2	2.00
Q9JK23	Psmg1	Proteasome assembly chaperone 1	2.3	1.94
Q9QZE7	Tsnax	Translin-associated protein X	1.3	1.83
Q99MR6	Srrt	Serrate RNA effector molecule homolog	1.9	1.83
Q8BM29	Add3	Gamma-adducin	1.2	1.74
Q3UN55	Cpt2	Carnitine O-palmitoyltransferase 2, mitochondrial	1.3	1.69
Q7TPW6	Slc25a20	Mitochondrial carnitine/acylcarnitine carrier protein	2.8	1.63
Q7TQI3	Otub1	Ubiquitin thioesterase OTUB1	1.9	1.59
Q6NWW1	Ube4b	Ubiquitin conjugation factor E4 B	1.2	1.56
O88587	Comt	Catechol O-methyltransferase	1.0	1.45
Q99KK2	Cmas	N-acylneuraminate cytidylyltransferase	1.9	1.37
Q0PD56	Rab5b	Ras-related protein Rab-5B	1.2	1.36
Q91XB0	Trex1	Three-prime repair exonuclease 1	1.5	1.33
Q8VBV7	Cops8	COP9 signalosome complex subunit 8	1.4	1.30

Figure S6: List of proteins in Figure 2B and their functions (criteria: log_2-fold enrichment ≥ 1 and $-log_{10}$(p-value) ≥ 1.5).

Protein IDs	Gene names	Protein names	Log_2-fold change	-Log (P-value)
P08003	Pdia4	Protein disulfide-isomerase A4	1.2	1.96
P18242	Ctsd	Cathepsin D	1.1	1.81
G5E8V9	Arfip1	Arfaptin-1	2.5	3.38
O35459	Ech1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	1.6	3.42
P01897	H2-L	H-2 class I histocompatibility antigen, L-D alpha chain	1.1	2.21
Q3U1P2	Snx9	Sorting nexin;Sorting nexin-9	1.2	3.41
Q58E70	Tpm3	Tpm3 protein	1.8	1.59
Q7TPW6	Slc25a20	Mitochondrial carnitine/acylcarnitine carrier protein	1.6	1.78
Q8K2Q9	Kiaa1598	Shootin-1	1.3	1.67
Q8R2K3	Ssbp1	Single-stranded DNA-binding protein;Single-stranded DNA-binding protein, mitochondrial	1.2	1.60
Q8VDQ1	Ptgr2	Prostaglandin reductase 2	1.8	2.02
Q9ES97	Rtn3	Reticulon-3	1.8	1.46

Figure S7: List of proteins in Figure 2C and their functions (criteria: log_2-fold enrichment ≥ 1 and $-log_{10}$(p-value) ≥ 1.5).

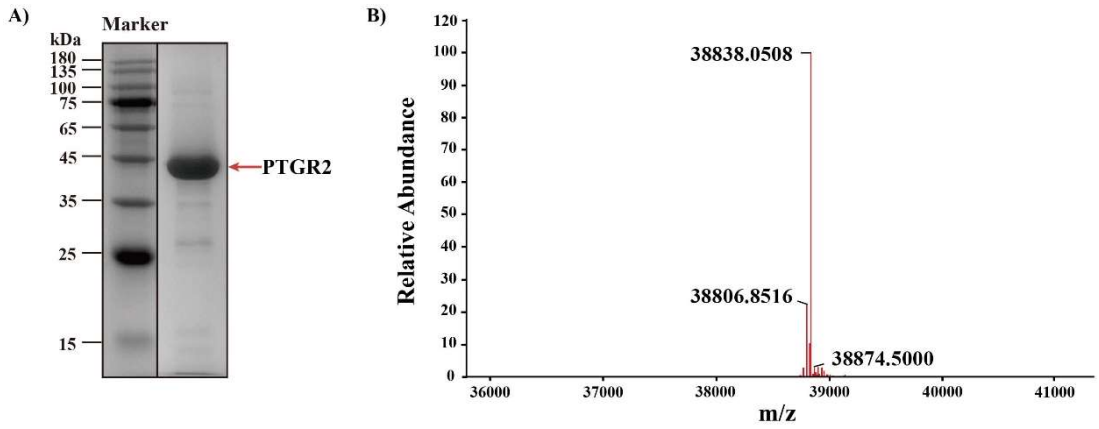


Figure S8: A) Purified His-tagged PTGR2 as visualized on a Coomassie-stained SDS gel. B) Full-length mass spectrometry measurement of intact recombinant PTGR2 confirms that its mass aligns with the expected design (theoretical molecular weight: 38838.15 Da).

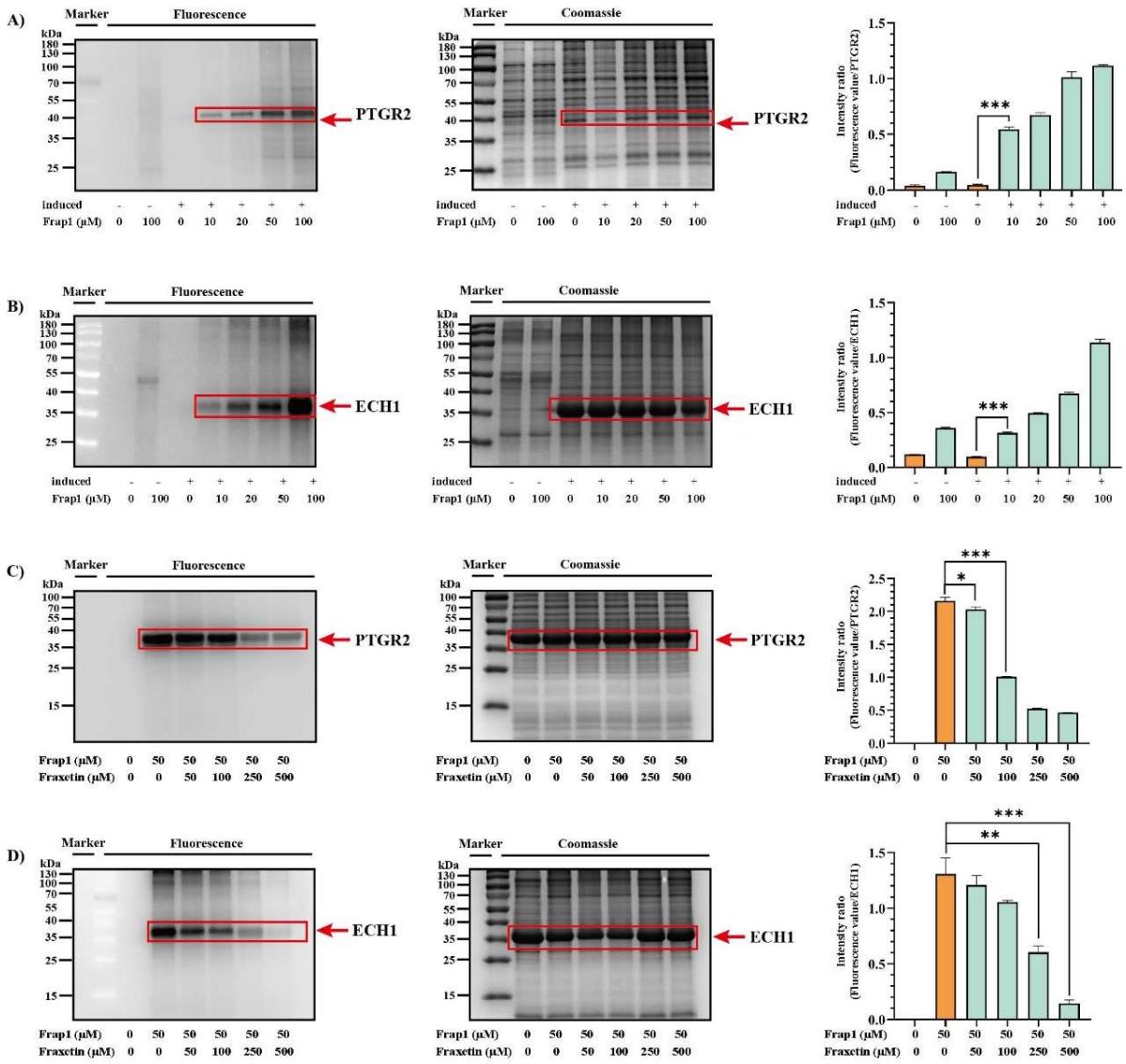


Figure S9: Concentration-dependent *in situ* labeling of recombinant PTGR2 (A) and ECH1 (B) expressed in *E. coli* with Frap1. Competitive in situ labeling of fluorescent SDS-gels with 50 μM Frap1 and different concentrations of fraxetin in recombinant PTGR2 (C) and ECH1 (D) expressed in *E. coli*. A Coomassie-stained SDS-gel is presented alongside the fluorescent SDS-gel to facilitate comparison of protein abundance and quantitative analysis of fluorescent bands using ImageJ. Induced denotes induction of protein overproduction. “0” means DMSO was added instead of Frap1.

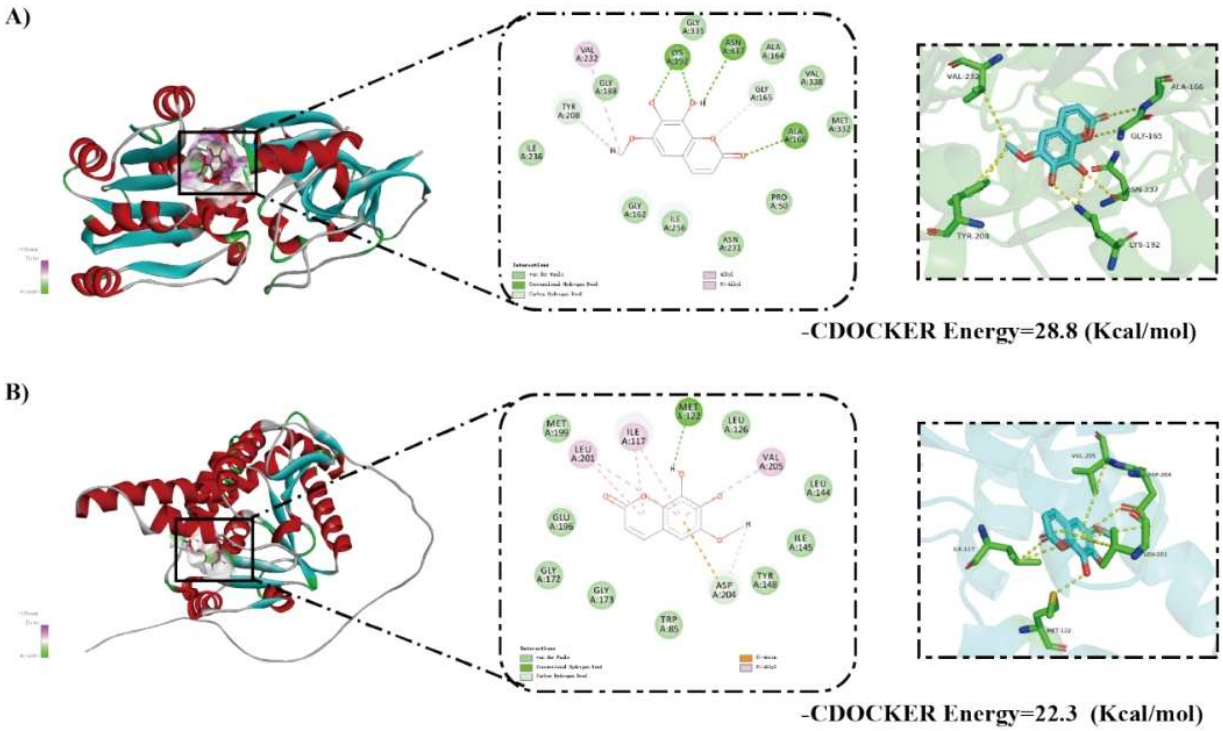


Figure S10: Molecular docking and binding mode analysis of fraxetin to PTGR2 (PDB: 2ZB3) (A) and ECH1 (Uniprot: AF-O35459) (B).

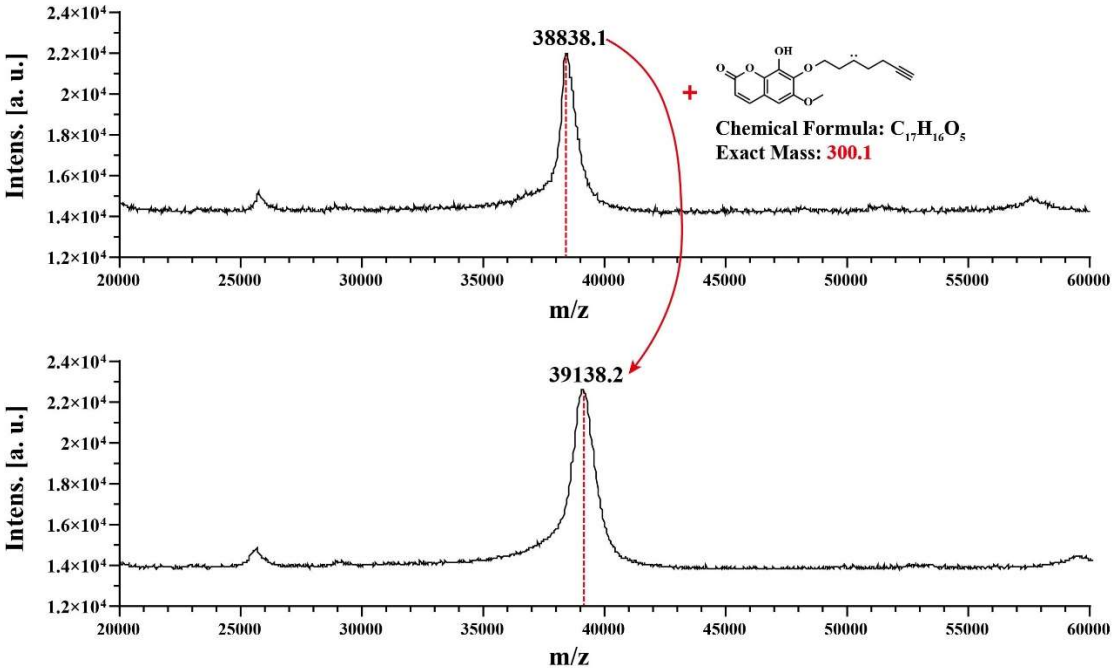


Figure S11: MALDI-TOF mass spectrometry analysis of molecular weight shift in recombinant PTGR2 before and after incubation with Frap1 under UV irradiation.

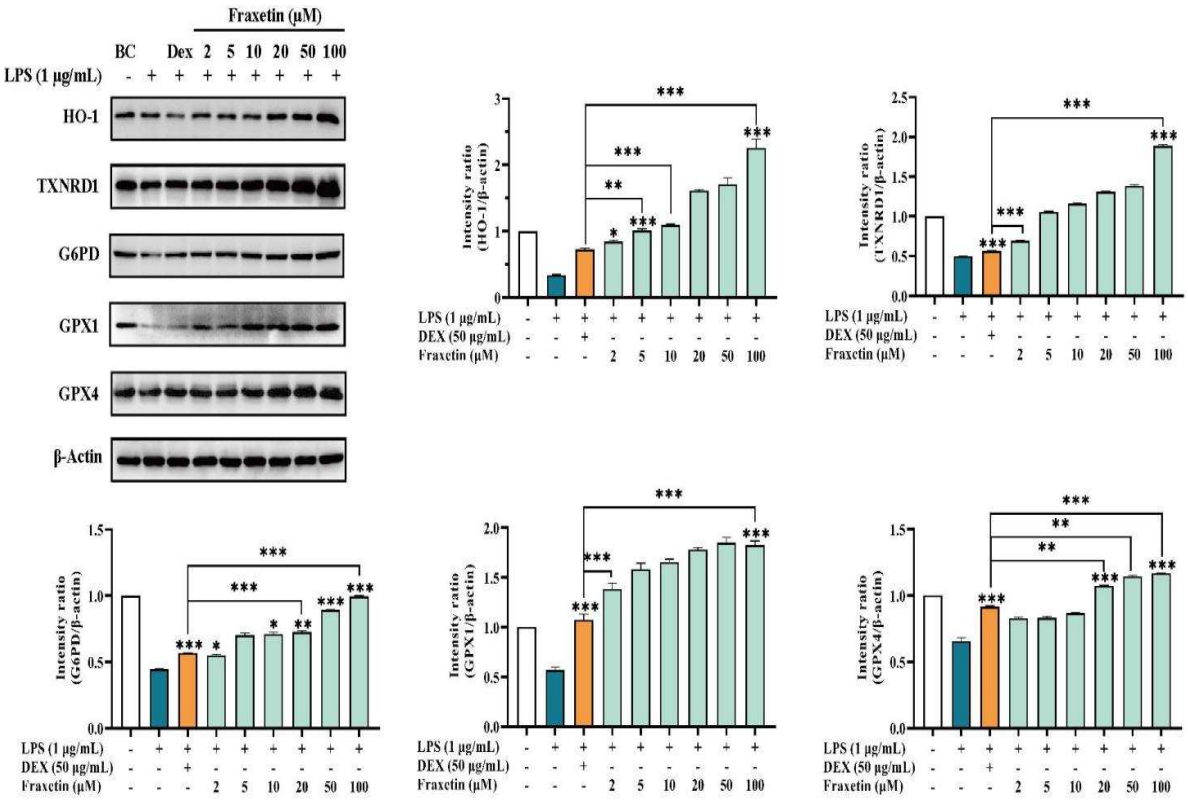


Figure S12: Western blot analysis of the expression levels of Nrf2 regulated proteins such as NRF2, HO-1, TXNRD1, G6PD, GPX1 and GPX4 in RAW264.7 cells. β-actin served as a loading control and the bands were quantitatively analyzed by ImageJ software.

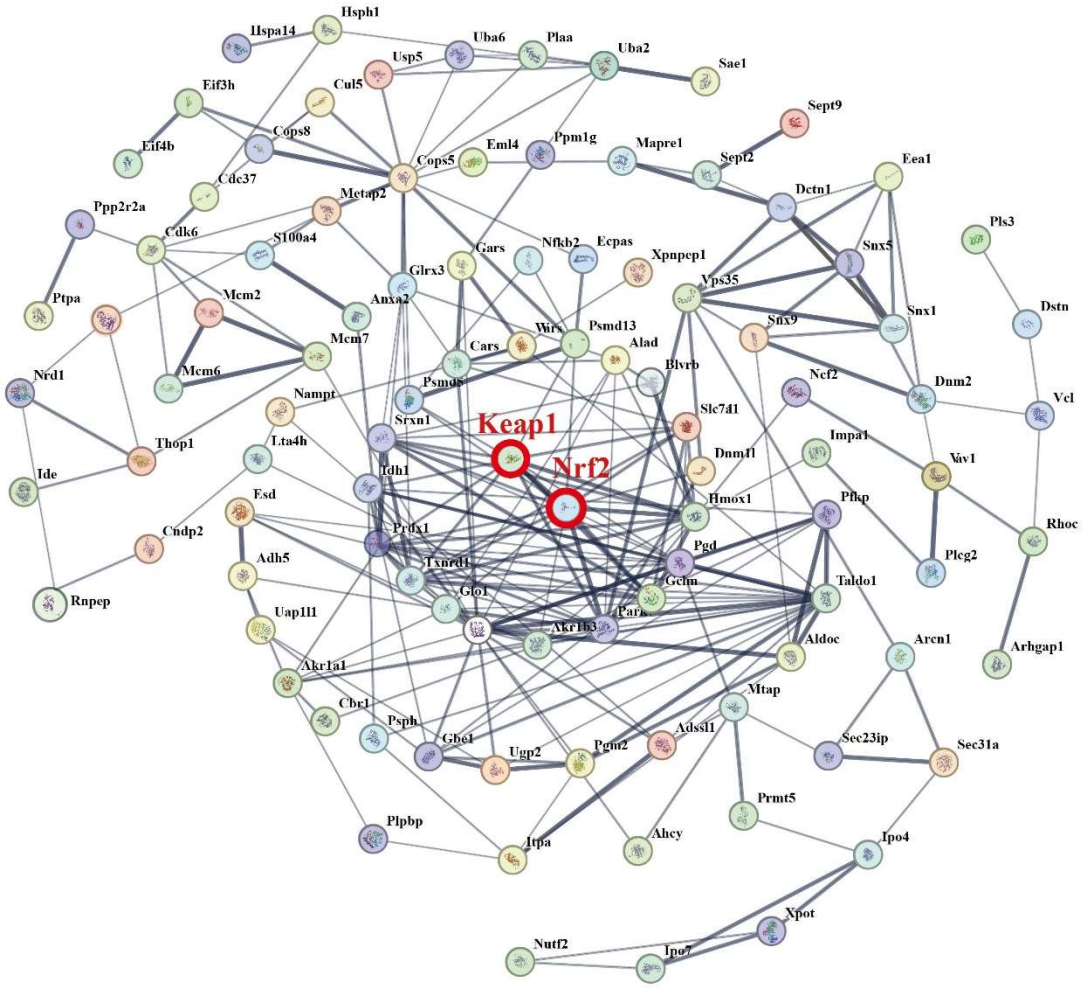
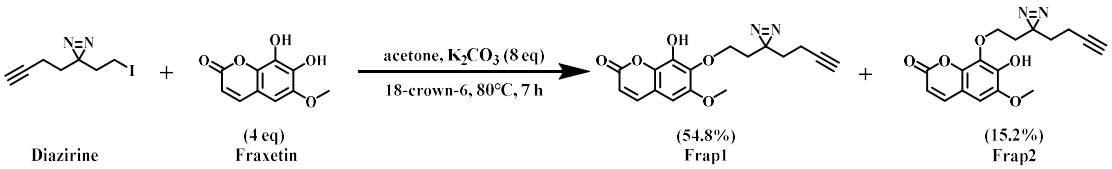


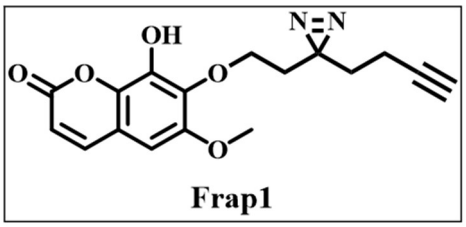
Figure S13: Network analysis of all significantly upregulated proteins after treatment of RAW264.7 cells with 100 µM fraxetin (criteria: log2-fold enrichment $\geqslant$ 1.0 and -log10(p-value) $\geqslant$ 1.3). Information about the predicted functional and physical interactions (represented by edges between nodes) is obtained from STRING database version 12.0 (https://string-db.org/) with a confidence cutoff of 0.4. The line thickness of the edge indicates the strength of the data support. The details of the relevant protein information are presented in the supplemented excel file.

2. Chemical Synthesis

To a solution of 7, 8-dihydroxy-6-methoxy-2H-chromen-2-one 161 mg, 0.76 mmol) in acetone (20 mL) was added K_2CO_3 (53 mg, 0.38 mmol), 18-crown-6 (1.0 mg) and 3-(but-3-yn-1-yl)-3-(2-iodoethyl)-3H-diazirine (48 mg, 0.19 mmol). The reaction mixture was then heated to 80°C for 7 h. The reaction was cooled to RT and diluted with EtOAc (30 mL) and washed with water[1]. The organic layer was separated, dried over sodium sulfate, filtered and concentrated. The residue was purified by silica gel chromatography (eluting with 50% EtOAc in hexane) to obtain Frap1 (34.2 mg, 54.6%) and Frap2 (10.1 mg, 15.2%) as light yellow solid. Two-dimensional correlation NMR measurements including COSY, NOESY, HMQ and HSQC experiments were conducted to confirm the structures of Frap1 and Frap2.



Scheme S1: Synthesis of AfBPP probe Frap1 and Frap2.



Frap1

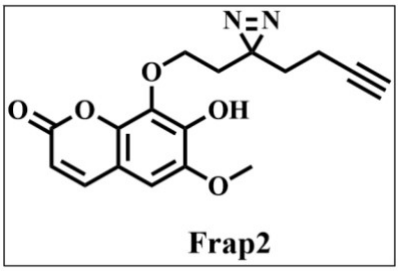
Frap1: 7-(2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethoxy)-8-hydroxy-6-methoxy-2H-chromen-2-one

Frap1 (34.2 mg, 54.6%) was obtained as a light yellow solid.

^{1}H NMR (600 MHz, Methanol-d_4): δ 7.88 (d, *J* = 9.5 Hz, 1H), 6.75 (s, 1H), 6.35 (d, *J* = 9.5 Hz, 1H), 4.02 (t, *J* = 6.5 Hz, 2H), 3.92 (s, 3H), 2.28 (t, *J* = 2.7 Hz, 1H), 2.09 (td, *J* = 7.5, 2.7 Hz, 2H), 1.85 (t, *J* = 6.5 Hz, 2H), 1.77 (t, *J* = 7.5 Hz, 2H).

^{13}C NMR (600 MHz, Methanol-d_4): δ 161.68, 150.39, 144.86, 139.17, 138.78, 138.74, 114.70, 113.93, 99.62, 82.00, 68.82, 67.81, 55.25, 33.35, 32.08, 26.38, 12.49.

HRMS (pos. ESI) calcd for $C_{17}H_{16}N_2O$ $(M+H)^+$: 329.1132, found 329.1137.



Frap2

Frap2: 8-(2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethoxy)-7-hydroxy-6-methoxy-2H-chromen-2-one

Frap2 was obtained with the same procedure as Frap1 as a light yellow solid (10.1 mg, 15.2%).

^{1}H NMR (600 MHz, Chloroform-*d*): *δ* 7.61 (d, *J* = 9.5 Hz, 1H), 6.69 (s, 1H), 6.28 (d, *J* = 9.5 Hz, 1H), 4.08 (t, *J* = 5.9 Hz, 2H), 3.96 (s, 3H), 2.12 (td, *J* = 7.3, 2.6 Hz, 2H), 2.06 (t, *J* = 5.9 Hz, 2H), 2.03 (t, *J* = 2.6 Hz, 1H), 1.81 (t, *J* = 7.4 Hz, 2H).

^{13}C NMR (600 MHz, Chloroform-*d*): *δ* 160.54, 144.93, 143.91, 143.02, 142.67, 132.95, 113.41, 111.11, 103.76, 82.69, 69.44, 68.21, 56.54, 32.96, 32.45, 27.05, 13.35.

HRMS (pos. ESI) calcd for $C_{17}H_{16}N_2O$ $(M+H)^+$: 329.1132, found 329.1126.

3. Biochemical Procedures

3.1 Cell culture

RAW264.7 cells were purchased from Wuhan Punosai Life Technology Co., Ltd (Wuhan, China). Following the manufacturer's instructions, a complete medium consisting of 90% DMEM, 10% fetal bovine serum and 1% penicillin/streptomycin was prepared. The cells were maintained in a humidified incubator at 37°C with 5% CO_2 (Thermo, USA).

3.2 Cell viability assay

RAW264.7 cells ($1x10^5$ cells/mL) were seeded into 96-well plates and treated with 11 concentrations of fraxetin (10, 20, 50, 100, 200, 300, 400, 500, 600, 700 and 800 μM) for a duration of 24 hours. Then cell viability was evaluated using the MTT assay.

3.3 LPS induced inflammation in RAW264.7 cells

Cells in the logarithmic growth phase were utilized for the experiments and inoculated at a density of 1×10^5 cells/mL in 96-well plates, with 100 μL per well, or at a density of 1×10^6 cells/mL in 6-well plates, with 2 mL per well. The experiments were conducted with three groups: a control group, an LPS group (1 μg/mL) and a Fraxetin group (5, 10, 20, 50 and 100 μM). Fraxetin was added for pre-activation for 2 h, followed by the addition of 1 μg/mL of LPS solution to each well for co-incubation over a period of 24 h. Then the cell supernatant was

used to detect cytokines and inflammation-associated factors (described in 3.4 and 3.5).

3.4 Nitric oxide assay

Nitrite concentration serves as an indicator of nitric oxide (NO) production and was quantified using the Griess reaction. A 50 μL aliquot of cell supernatant (its compound treatment is described in related experiments) was mixed with Griess reagent in a 1:1 ratio (solution A, S0021S-2; solution B, S0021S-3; Beyotime Biotechnology Co., Ltd., Shanghai, China) and the absorbance was measured at 540 nm using an ELISA plate reader.

3.5 Cytokine level Measurement by Enzyme-linked immunosorbent assay (ELISA)

The ELISA kits obtained from Jiangsu Jingmei Biotechnology Co., Ltd. (Jiangsu, China) were employed to assess the alterations in inflammatory cytokines during the cell experiments. Following the collection of culture supernatants, the concentrations of the inflammatory cytokines IL-10, IL-6, IL-1β, TNF-α and iNOS were measured in accordance with the manufacturer's instructions.

3.6 Affinity-based protein profiling (AfBPP) experiments

3.6.1 *In situ* analytical labeling

(1) Cells in the logarithmic growth phase were inoculated into 6-well plates at a density of 1 x 10^6 cells/mL, with 2 mL of medium added per well. After 24 h, the cell density reached 80% to 90%.

(2) The treatment of different experimental samples is as follows:

a. Lysis method comparison: Frap1 was added to the cells at concentrations of 0, 50 and 100 μM, and the cells were cultured in a CO_2 incubator at 37°C for 1 hour. Subsequently, the *in situ* labeling samples were placed on ice and cross-linked for 20 min under 360 nm ultraviolet light. The samples were lysed either by physical ultrasound or by using RIPA lysis buffer. The resulting lysates were then utilized for the subsequent *in situ* labeling steps.

b. Experiments were conducted to evaluate the effects of varying crosslinking energies. Frap1 was introduced at a final concentration of 50 μM and the samples were cultured in a CO_2 incubator at 37°C for 1 h. Subsequently, the samples were placed on ice and subjected to crosslinking for different durations (10, 20, 30, 40, 50 and 60 mins) under 360 nm ultraviolet light. For the DMSO group, the crosslinking duration was set at 60 min.

c. *In situ* labeling experiments with different probe concentrations: The cells treated with different final concentrations of Frap1 (0, 10, 20, 50 and 100 μM) were cultured in CO_2 incubator at 37°C for 1 h. The samples were then placed on ice and crosslinked under 360 nm ultraviolet light for 20 min.

d. *In situ* competitive labeling experiment: The cells of the competing group were first cultured in a CO_2 incubator at 37°C for 1 h with fraxetin (0, 50, 100,

250 and 500 μM). Then, Frap1 (50 μM) was added to each sample in the competing group and the samples were cultured in a CO_2 incubator at 37°C for another hour. A control sample was treated with DMSO in both incubation steps. The samples were then placed on ice and crosslinked under 360 nm ultraviolet light for 20 min.

(3) After ultraviolet irradiation, the cells were collected using a cell scraper and washed twice with 2 mL PBS to remove residual compounds or probes. The precipitates were suspended in 120 μL PBS (PMSF, 1 mM) and then lysed on ice with 3 pulses of 15 s (70% of maximum power) using an ultrasonic cell crusher. Subsequently, the samples were then centrifuged at 13,000 rpm for 30 min at 4°C.

(4) Click reaction step: Transfer the 100 μL supernatant to the new EP tube, and then the click chemistry was performed.

For each 100 μL sample, add the following reagents:

a. 2 μL RhN_3/TAMRA-azide (5 mM in DMSO).

b. 2 μL TCEP (52 mM, 15 mg/mL dissolved in ddH_2O, ready to use).

c. 6 μL 1 x TBTA ligand (1.667 mM TBTA, t-BuOH/DMSO = 4 : 1).

d. Gently swirl the sample, then add 2 μL $CuSO_4$ (50 mM $CuSO_4$ solution, dissolved in deionized water).

(5) Following the addition of the click reagents, the total reaction volume was adjusted to 112 μL. The samples were incubated at 25°C with shaking at 300 rpm in darkness for 1 hour. Subsequently, an equal volume of 2 x SDS

loading buffer was added. After thorough mixing, 10 μL of each sample was loaded into a 10% PAGE analysis gel alongside 10 μL of a pre-dyed protein marker (10-180 kD). The fluorescence intensity was measured at an excitation wavelength of 546 nm using a BIO-RAD ChemiDoc imaging system.

3.6.2 Gel free AfBPP

RAW264.7 cells treatment groups	Frap1: The cells were cultured in 6 cm plates, grew to 90% confluency and then incubated with DMSO for 1 hour and then Frap1 (final concentration: 50 μM) at 37°C for another hour. Frap1+10 x fraxetin: The cells were cultured in 6 cm plates, grew to 90% confluency, then incubated with Fraxetin (Final concentration: 500 μM) at 37°C for 1 h, then added Frap1 with a final concentration of 50 μM, incubated at 37°C for another hour. DMSO: The cells were cultured in 6 cm plates, grew to 90% confluency and then incubated with DMSO at 37°C for 2 h.

The cells were cultured in 6 cm plates and allowed to grow to 90% confluency before being incubated with the corresponding compounds or DMSO, as described in the table above. The samples were then placed on ice and crosslinked under 360 nm ultraviolet light for 20 min. Following irradiation, the samples were pelletized and washed twice with 1 mL of PBS to remove any residual probe. The pellet was then resuspended in 500 μL of PBS and lysed by sonication, applying 3 pulses of 15 seconds each at 70% maximum power

on ice. After centrifugation at 13,000 rpm at 4°C for 30 min to separate the soluble and insoluble fractions, the supernatant was transferred to a new Protein LoBind Eppendorf tube.

The samples were then used to append a reporter tag via click chemistry. For each 500 µL sample, add 5 µL Azide-PEG_3-Biotin Conjugate (10 mM in DMSO), 10 µL freshly made TCEP (52 mM tris (2-carboxyethyl) phosphine in ddH_2O), 30 µL TBTA ligand (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine, 1.667 mM in t-BuOH/DMSO = 4 : 1). The cycloaddition was initiated by 10 µL $CuSO_4$ (50 mM $CuSO_4$ in dd H_2O) and incubated at RT for 1 h. After click chemistry., proteins were precipitated with two-fold volumes of pre-chilled (-80°C) acetone. The samples were stored at -20°C overnight to get sufficient precipitation of the proteins. Then they were pelletized at 13,000 rpm for 30 min at 4°C. The supernatant was discarded and the pellet was washed twice with prechilled (-80°C) methanol. Subsequently, the pellet was dissolved in 500 µL PBS with 0.4% (w/v) SDS by sonication at RT and the protein concentration was measured with BCA assay. All samples were adjusted to same protein amount accordingly. They were incubated under gentle mixing with 50 µL of avidin-agarose beads from Sigma-Aldrich (avidin-agarose beads were pre-washed with 1 mL PBS with 0.4% (w/v) SDS three times) overnight at RT. After that, the beads were washed three times with 3 mL of 0.4% (w/v) SDS in PBS, twice with 1 mL of 6 M urea and three times with 1 mL PBS (collect at 1,500 rpm, 2 min, RT after each washing step). The beads were resuspended with

200 µL denaturation buffer (7 M urea, 2 M thiourea in 20 mM pH 7.5 HEPES buffer). Dithiothreitol (DTT, 100 mM, 2 µL) was added and the tubes were mixed by vortexing shortly and incubated in a thermomixer (450 rpm, 45 min, RT). Then 2-Iodoacetamide (IAA, 550 mM, 2 µL) was added. The tubes were mixed again by vortexing shortly and incubated in the darkness (450 rpm, 30 min, RT). The excess of IAA was quenched by the addition of dithiothreitol (DTT, 100 mM, 8 µL). The tubes were shortly mixed by vortexing and incubated in a thermomixer (450 rpm, 30 min, RT). 600 µL TEAB solution (tetraethylammonium bromide, 50 mM in ddH_2O) and 1.5 µL trypsin (0.5 µg/µL in 50 mM acetic acid) were added to the tubes. The microcentrifuge tubes were incubated in a thermomixer overnight (450 rpm, 13-15 h, 37°C). The digestion was stopped by adding 6 µL formic acid (FA) and the solution was collected by centrifugation (3,000 rpm, 2 min, RT). The trypsin digest solution was transferred to a new Protein LoBind Eppendorf tube. Further extraction was done with 50 µL 0.1% (v/v) FA (aqueous solution) twice (3,000 rpm, 2 min, RT). Finally, the tubes were centrifuged for 3 min at 13,000 rpm to collect the residual supernatant. All the supernatant was combined to the trypsin digest solution. The pH values of the samples were checked and more 1% (v/v) FA was added if the pH was above 3. 50 mg SepPak C18 columns were pre-equilibrated by gravity flow with 2 mL acetonitrile, 1 mL elution buffer (80% ACN, 0.5% FA) and 3 x 1 mL 0.5% FA aqueous solution. Subsequently the samples were loaded and washed with 5 x 1 mL 0.5% FA aqueous solution. Add 250 µL elution buffer

(80% ACN, 0.5% FA) and let them run through the column without vacuum for 3 times, with the last under reduced pressure until no liquid came out from the SepPak C18 columns. Hereby, the peptides were eluted into 2 mL Protein LoBind tubes and lyophilized to get the dried peptides which can be stored at -80°C.

Before MS measurement, the samples were dissolved in 35 µL 1% FA (formic acid) in ddH_2O by pipetting up and down, vortexing and sonication for 15 min. Then, the supernatants were collected by centrifugation. 0.45 µm centrifugal filters were pre-equilibrated with 500 µL ddH_2O twice, 500 µL 0.05 N NaOH once and 500 µL 1% FA twice (centrifugation of the filters: 13,000 rpm, 1 min, RT). The peptide solutions were filtered through the equilibrated filters (centrifugation: 13,000 rpm, 2 min, RT).

The peptides were analyzed by on-line nanoflow liquid chromatography tandem mass spectrometry, which was performed by connecting a nano-electrospray ion source to the Orbitrap Exploris™ 480 system. The peptide was detected by mass spectrometry on C18 column (75 µM x 25 cm, Thermo, USA). The separation time was 120 min. Volume flow rate 300 nL/min. EASY-nLC liquid phase gradient elution, mobile phase: A phase: 2% acetonitrile (containing 0.1% formic acid), B phase: 80% acetonitrile (containing 0.1% formic acid). Elution process: 0-2 min: 0-3% B; 2-92 min: 5%-25% B; 92-102 min: 25%-45% B; 102-105 min; 45%-100% B; 105-120 min: 100% B.

The Orbitrap Exploris™ 480 operates in DDA data acquisition mode and

automatically switches between full-scan MS and MS/MS acquisition with mass spectral resolutions of 70 K and 35 K, respectively. MS performs a full scan (m/z 350-1, 500) with an AGC target of $3e^6$ and a maximum fill time of 20 ms. The parent ion top 20 was selected for secondary fragmentation, the MS/MS resolution was set to 35,000 (m/z 100), the AGC target was $3e^5$, the maximum filling time was 50 ms, and the dynamic exclusion time was 30 s.

Peptide and protein identifications were performed using MaxQuant 2.0.1.0 software with Andromeda as search engine using following parameters: trypsin/P as the proteolytic enzyme, 4.5 ppm for precursor mass tolerance (main search ppm) and 0.5 Da for fragment mass tolerance (ITMS MS/MS tolerance). Searches were done against the Uniprot database sequence for *Mus musculus* (downloaded on 11.07.2022). Identification was done with at least 2 unique peptides and quantification only with unique peptides. Statistical analysis was performed with Perseus 1.6.3.0. Putative contaminants, reverse peptides and peptides only identified by site were omitted from further processing. The data obtained from MaxQuant 2.0.1.0 were first transformed with $\log_2(x)$ and then normalized using z-score. $-\log_{10}$(p-value) were obtained by a two-sided two sample t-test over 12 gel-free ABPP experiment results (four biological replicates for each group).

3.6.3 Gel-based ABPP labeling of recombinant PTGR2 and ECH1

Protein PTGR2 and ECH1 were cloned into PET28a(+) plasmid by Sangon Biotech (Shanghai) Co., Ltd. They were recombinantly expressed in *E. coli*

BL21DE(3) plysS. The bacteria were grown at 37°C until OD_{600} ≈ 0.5 with 50 μg/mL kanamycin sulfate and then induced with 1mM IPTG (Isopropyl β-D-Thiogalactoside) at 25°C for 4 h. The bacteria were harvested and washed with PBS. After pelletizing, the bacterial pellets were used for labeling.

1,000 μL aliquots of bacterial suspension in PBS (OD_{600} ≈ 4) were treated with Frap1 (0, 10, 20, 50 100 μM) at 37°C for 1 h on 6 well plates (both IPTG induced and not induced *E. coli* BL21DE(3) plysS containing PET28a(+) plasmid harboring PTGR2 or ECH1 gene were used). After incubation, the samples were then placed on ice and crosslinked under 360 nm ultraviolet light for 20 min. They were then pelletized and washed twice with 1 mL PBS to remove the residual compounds or probes. The pellet was suspended in 120 μL PBS and then lysed by sonication with a ultrasonic cell crusher (XM-650T, China) with 3 x 15 sec. pulsed at 70% max. power on ice. After that, the samples were centrifuged (13,000 rpm, 4°C, 30 min). 88 μL supernatant was transferred to a new Eppendorf tube while the pellet was washed twice with 500 μL PBS and resuspended in 88 μL PBS. The samples were then used to append a reporter tag via click chemistry (CC). For each 88 μL sample, add 2 μL TAMRA azide (5 mM in DMSO), 2 μL freshly made TCEP (52 mM tris(2-carboxyethyl) phosphine in ddH_2O), 6 μL TBTA ligand (tris[(1benzyl-1H-1,2,3-triazol-4-yl)methyl]amine, 1.667 mM in t-BuOH/DMSO = 4 : 1). Samples were gently vortexed and 2 μL $CuSO_4$ (50 mM $CuSO_4$ in ddH_2O) was added to initiate the cycloaddition reaction. After the addition of CC reagents, the total reaction

volume was 100 µL. The samples were incubated at RT for 1 h and then 100 µL 2 x SDS loading buffer were added. The samples were mixed and stored at -20°C after 1 hour incubation at RT. 10 µL of the samples was applied on the analytical gel and 10 µL marker (Vazyme, China) was used as ladder in parallel. Fluorescence was detected using a BIO-RAD imaging system (BIO-RAD, Hercules, CA, United States) with 546 nm EPI excitation wavelength.

3.7 PTGR2 enzymatic assay

3.7.1 Expression of recombinant PTGR2

(1) The bacteria solution of *E coli* BL21 (DE3) pLySs containing recombinant plasmid (PTGR2 protein) was stored in the refrigerator at -80°C. It was defrosted on ice. Inoculation was performed by adding 5µL stored bacterial solution into 5 mL LB broth (with 50 µg/mL Kana). Then the culture was incubated at 37°C and 200 rpm overnight.

Recombinant PTGR2 amino acid sequence:

MIIQRVVLNSRPGKNGNPVAENFRVEEFSLPDALNEGQVQVRTLYLSVDPYM
RCKMNEDTGTDYLAPWQLAQVADGGGIGVVEESKHQKLTKGDFVTSFYWP
WQTKAILDGNGLEKVDPQLVDGHLSYFLGAIGMPGLTSLIGVQEKGHISAGS
NQTMVVSGAAGACGSLAGQIGHLLGCSRVVGICGTQEKCLFLTSELGFDAA
VNYKTGNVAEQLREACPGGVDVYFDNVGGDISNAVISQMNENSHIILCGQIS
QYSNDVPYPPPLPPAVEAIRKERNITRERFTVLNYKDKFEPGILQLSQWFKE
GKLKVKETMAKGLENMGVAFQSMMTGGNVGKQIVCISEDSSLHHHHHH

Recombinant ECH1 amino acid sequence:

MATAMTVSSKLRGLLMQQLRGTSQLYFNISLRSLSSSAQEASKRAPEEVSD
HNYESIQVTSAQKHVLHVQLNRPEKRNAMNRAFWRELVECFQKISKDSDCR
AVVVSGAGKMFTSGIDLMDMASELMQPSGDDAARIAWYLRDLISKYQKTFT
VIEKCPKPVIAAIHGGCIGGGVDLVSACDIRYCTQDAFFQIKEVDMGLAADVG
TLQRLPKVIGNQSLVNELTFSARKMMADEALDSGLVSRVFQDKDAMLNAAFA
LAADISSKSPVAVQGSKINLIYSRDHSVDESLDYMATWNMSMLQTQDIIKSVQ
AAMEKRDTKSITFSKLHHHHHH

(2) Transfer the cultured 1 mL bacterial solution to 500 mL LB (50 μg/mL Kana) and incubate it in a shaker at 37°C and 200 rpm.

(3) When the OD_{600} value of the bacterial solution reached about 0.5, add IPTG to a final concentration of 0.25 mM.

(4) After induction for 4 hours, pelletize the bacteria in two 50 mL centrifuge tubes at 4°C and 6,000 rpm.

(5) Add 10 mL lysis buffer solution (pH 8.0, 20 mM Trizma, 150 mM NaCl, 0.2% Triton X-100) and mix well with a pipette to disperse the lysis solution evenly.

(6) Use ultrasonic crusher to lyse cells; Crushing parameters (60% maximum power, 2 s ultrasonic gap: 4 s); Crushing interval of 3 min and then crushing, a total of 15 min.

(7) After homogenization, centrifuge the samples at 12,000 rpm, 4°C for 30 min.

(8) Transfer the supernatant into new centrifugal tubes, filter it with a 0.45 mm filter membrane. The filtrate was used for further protein purification.

3.7.2 Purification of His-tagged PTGR2

Biorad NGC was used to separate and purify PTGR2 in the following ways:

(1) Buffer preparation: all buffers were degassed prior to use.

a. Equilibrium buffer: 2.420 g of Trizma and 8.766 g of NaCl were dissolved in 1 L ddH2O, and the pH was subsequently adjusted to 8.0 using an aqueous NaOH solution.

b. Elution buffer: Dissolve 17.020 g imidazol in 500 mL equilibrium buffer.

(2) First balance the column (EconoFit Profinity IMAC Columns, Ni-charged) with equilibrium buffer, then eluate with different concentrations of imidazole, the concentration from low to high (40, 100, 200 and 500 mM) until no peak occurs, then clean the column with 20% ethanol, then rinse the column with ddH_2O, and finally store the column in 20% ethanol.

(3) The eluents were analyzed on SDS-gel and the gels were stained with Coomassie brilliant blue to confirm protein purity

(4) The desired protein eluent was concentrated to 1 mL with 10 kD ultrafiltration tube, and then replaced with 100 mM Tris·HCl (pH 7.5) twice. The protein concentration was determined with BioTek Synergy H1. PTGR2 was packaged and stored in the refrigerator at -80°C for further use. The purified His-tagged PTGR2 was further confirmed via full-length mass spectrometry measurement (SYNAPT XS High Resolution Mass Spectrometer equipped with

an ACQUITY UPLC I-Class PLUS system (Waters)).

3.7.3 PTGR2 enzymatic activity

The *in vitro* enzymatic activity of PTGR2 was determined by a reported chromogenic assay[2], 15-keto-PEG2 forms an unstable red pigment in alkaline solutions, and this property was used to determine the activity of PTGR2. The reaction system consisted of 0.1 M Tris-HCl (pH 7.5), 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM NADPH, 1 mM DTT, 60 μM 15-keto-PEG2 and 1 μM PTGR2. The reaction system was 100 μL in size and was incubated at 37°C with shaking at 350 rpm for 30 min. The reaction was terminated by the addition of 20 μL of 2 M NaOH solution. The concentration of remaining 15-keto-PEG2 was quantified by measuring the absorbance at 500 nm. The rate of the enzymatic reaction of PTGR2 and the effects of compounds on it were assessed.

a. Standard curve: A standard curve was established using 15-keto-PEG2 concentrations of 10, 20, 40, 60, 80, 100 and 200 μM, with a PTGR2 enzyme concentration fixed at 2 μM. All other experimental conditions were kept constant, and the absorbance was measured at 500 nm to construct the standard curve.

b. The determination of the inhibitory effect of fraxetin on PTGR2 enzyme: The inhibitory effect of fraxetin on the PTGR2 enzyme was determined by incubating various concentrations of fraxetin (0, 1, 2, 5, 10, 20, 50 and 100 μM) with 1 μM of the PTGR2 enzyme at 37°C with shaking at 350 rpm for 30 min.

Subsequently, the other substrates and reactants were added, and the reaction was allowed to continue for an additional 30 min before measuring the absorbance at 500 nm.

3.8 Western blot analysis

Information list of antibodies:

Antibodies	Dilution ratio	Article Number	manufacturer
Anti-Heme Oxygenase 1	1: 1,000	ab52947	Abcam, Britain
Anti-Keap1	1: 1,000	ab227828	Abcam, Britain
Anti-G6PD antibody	1: 1,000	ab210702	Abcam, Britain
Anti-TXNRD1 Antibody	1: 1,000	BM5241	BOSTER, China
Anti-GPX4 Antibody	1: 1,000	A02059-1	BOSTER, China
β-actin	1: 1,000	4967	Cell Signaling, America

RAW264.7 cells were harvested and centrifuged (2,000 rpm, 10 min). Cells were washed with PBS and lysed with RIPA lysis buffer (Beyotime, China). The protein abundance was quantified using the Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime, China). The proteins were denatured by adding protein loading buffer and boiling at 98°C for 10 min. An equal amount of total protein (25 μg) was separated by SDS-PAGE and transferred to PVDF (polyvinylidene fluoride) membrane (Millipore, USA). After blocking with 5% nonfat milk in 1 x TBST at room temperature (RT) for 1 h, membranes were incubated with the primary antibody (Sources and Dilutions of primary antibodies are shown in

Supplementary Table 2) overnight at 4°C. The membranes were washed with TBST and incubated with a secondary antibody for 1 h at RT. Protein bands were visualized using an enhanced chemiluminescence kit (Millipore, USA) and detected using a Tanon imaging system (Tanon, China). ImageJ 18.0 software was used to analyze the protein bands for grey-scale values.

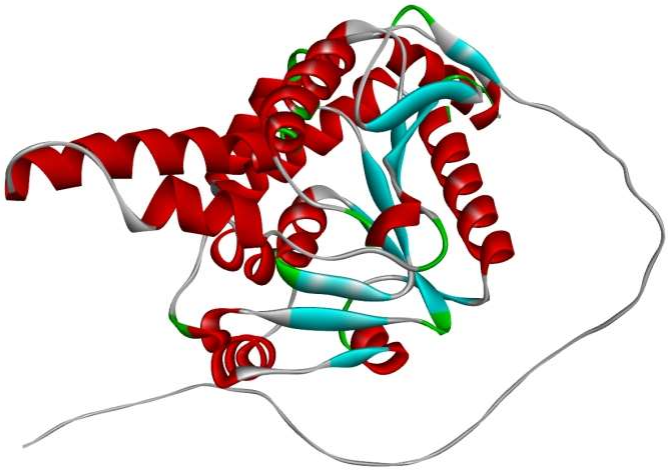
3.9 Molecular docking

(1) Preparation of small molecules：The 2D structure of fraxetin was obtained from the PubChem database and saved in SDF format. The energy of the small molecule compounds was minimized and calculated using the Calculate Molecular Properties program within the “Small Molecules” module of Discovery Studio (DS) software (Biovia Inc., San Diego, CA, USA, 2020).

(2) Preparation of receptor protein: The structure of the fraxetin target protein PTGR2 was obtained from the PDB database, and the protein crystal 2ZB3 resolution was 2.00 Å. Preprocess the receptor protein with protein 2ZB3 in the Prepare Protein program of the “Macromolecules” module of DS software to supplement missing amino acid residues.

For ECH1，using SWISS-MODEL tool（https://swissmodel.expasy.org）Perform homology modeling on ECH1. The modeling process of SWISS-MODEL tool mainly includes four steps: identifying structural templates, aligning target sequences and template structures, model construction, and model quality evaluation. These steps can be repeated until satisfactory

modeling results are obtained. Then the ECH1 structure was used in the following steps.



(3) Active ingredient-target protein molecular docking: Molecular docking technology can be used to predict the strength of binding affinity of small compound molecules in biological macromolecules to judge the stability of their binding.

Molecular docking is carried out by the CDOCKER program of the “Receiver ligand Interactions” module of DS software. Firstly, the “Define and Edit Binding Site” protocol is used to define the binding sites of 2ZB3 (with a size of 13 Å) or ECH1 around the original ligand. All heteroatoms and water molecules were removed from the protein file. By default, the negative score of -CDOCKER Energy is set to rank, saving the top 10 best docking positions for each molecule. The small molecules were docked to the protein to determine the interaction with the key residues at the active site. After docking, manually check the binding posture of the ligand and remove the molecules that fail to produce reasonable conformation. Finally, by analyzing the interaction between

ligands and target enzymes, and -CDOCKER Energy value was employed to output structures. The matching degree between the active ingredient and the target protein was judged according to the docking Score. It is generally believed that the -CDOCKER Energy is more than 0 kcal/mol has a good binding activity. PyMOL software (Ver 2.5.4) was used to visualize the results.

3.10 Full Length MS of PTGR2 with Frap1

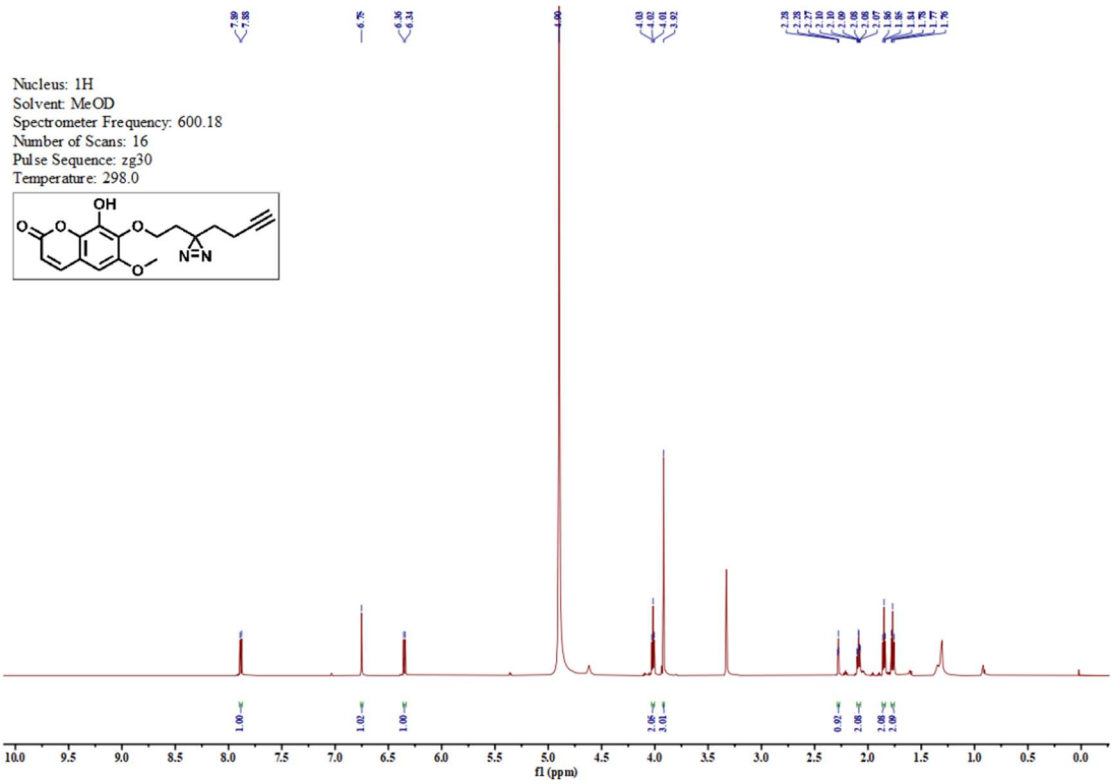
The conditions for incubating the proteins with the corresponding compounds were as follows: 5 μM PTGR2 was incubated with either 0 μM or 100 μM Frap1 in 100 mM Tris·HCl (pH 7.5) at room temperature for 20 minutes. Subsequently, the samples were placed on ice and crosslinked under 360 nm ultraviolet light for an additional 20 minutes. Following this, the samples were collected and freeze-dried; the molecular weight of the protein was determined using a Bruker UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonics, Inc., Billerica, MA).

3.11 Whole proteome analysis of RAW264.7 treated with fraxetin or not

Logarithmic growth phase cells were taken for experiments and inoculated at a density of 1 x 10^7 cells/mL in 96-well plates at cell culture dish (10 cm). After incubating 12 h, two groups of samples each with four replicates were prepared. One group is treated with 100 μM fraxetin while the other not. Then, 1 μg/mL of LPS solution was added to each well to induce inflammatory response for 24 h.

Cells were detached using a cell scraper, washed with PBS (10 mL) and lysed using pre-cooled lysis buffer (Beyotime, China). Cell debris was removed by centrifugation (21,000 g, 30 min, 4°C), resulting supernatants transferred into new falcons. The protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime, China) and then 100 µg protein was transferred to protein LoBind tubes. Proteins were precipitated in 500 µL ice-cold acetone at -20°C overnight, pelleted (21,000 x g,15 min, 4°C), and washed twice with 500 µL ice-cold methanol. Pellets were air-dried and dissolved in 200 µL denaturation buffer (7 M urea, 2 M thiourea in 20 mM HEPES pH 7.5). Sample preparation and LC-MS/MS measurements were carried out as described in 3.6.2 Gel free AfBPP. MS raw files were processed with MaxQuant version 2.0.1.0 as described above and peptides were identified from the MS/MS spectra searched against the UniProt *Mus musculus* reference proteome (downloaded on 11.07.2022). Statistical analysis was performed as described above using Perseus version 1.6.3.0 LFQ intensities were $\log_2$ transformed and a two-sample two-sided *t*-test was applied (FDR 0.05).

4. Appendix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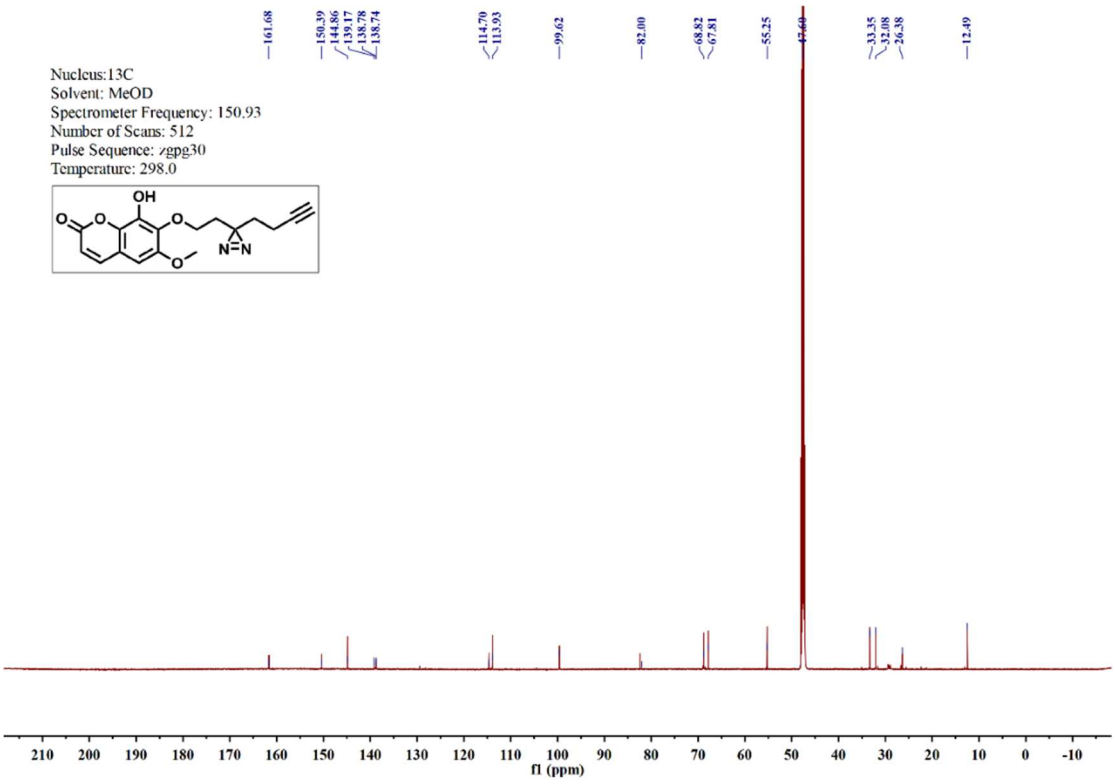
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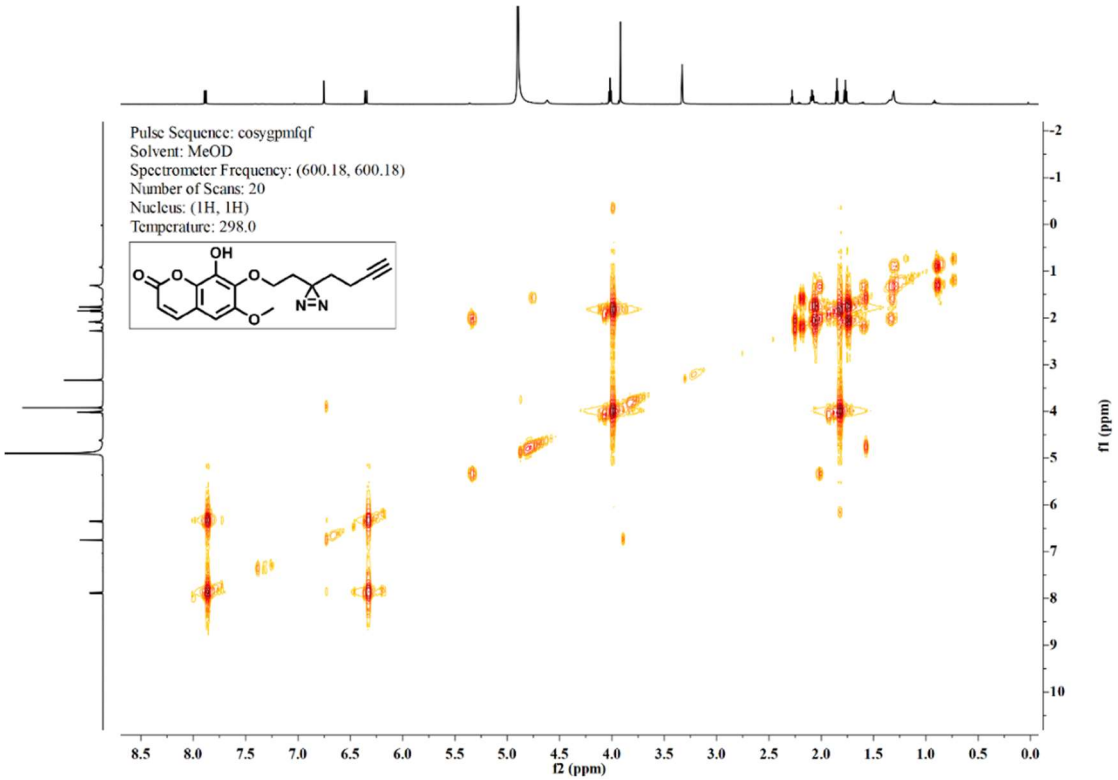
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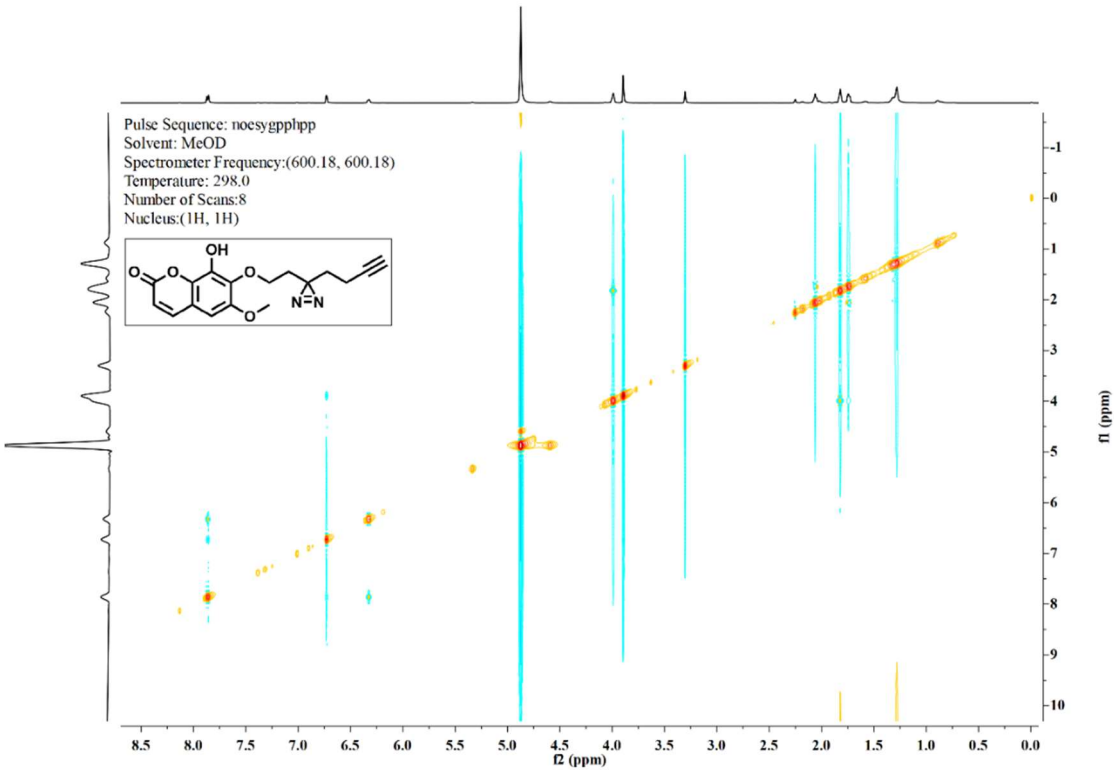
f1 (ppm)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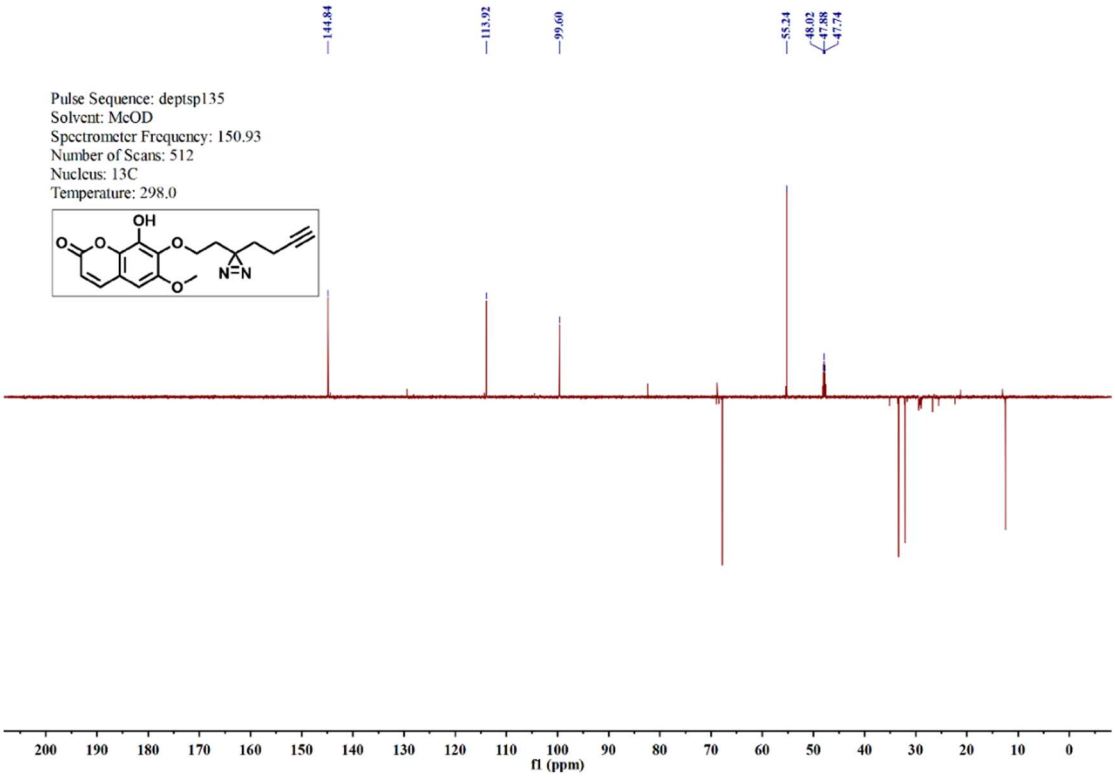
f2 (ppm)
f1 (ppm)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O
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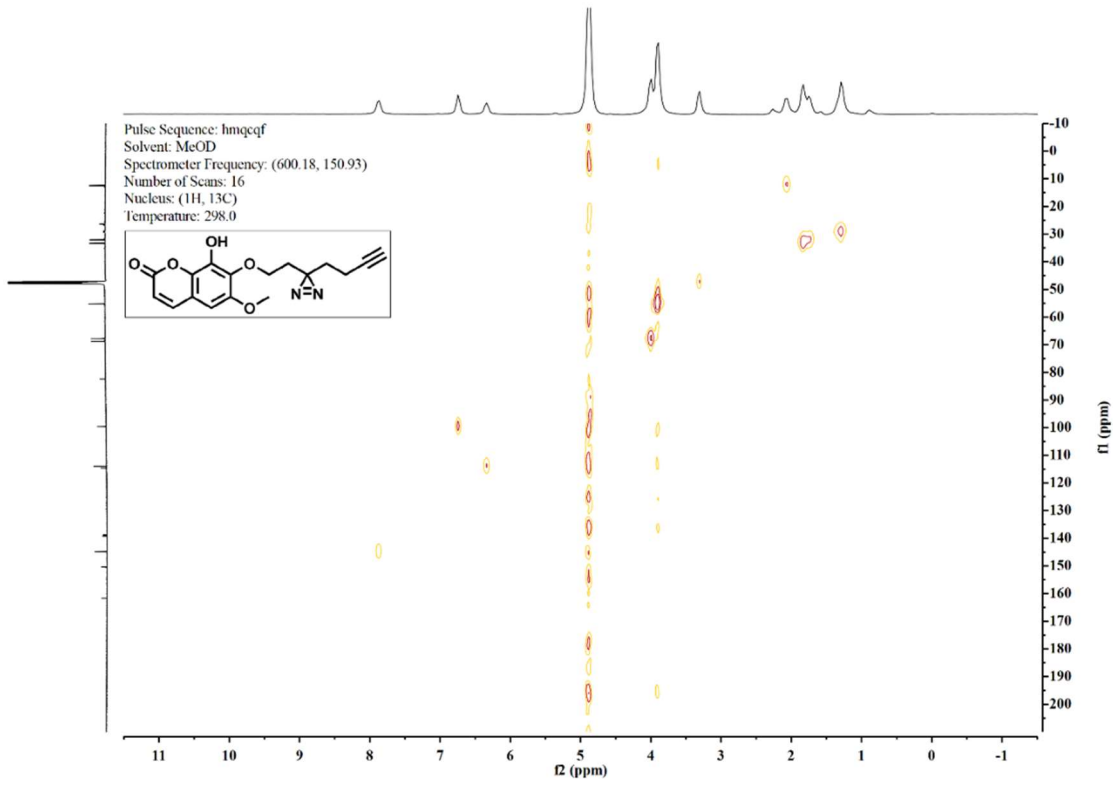
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O
O
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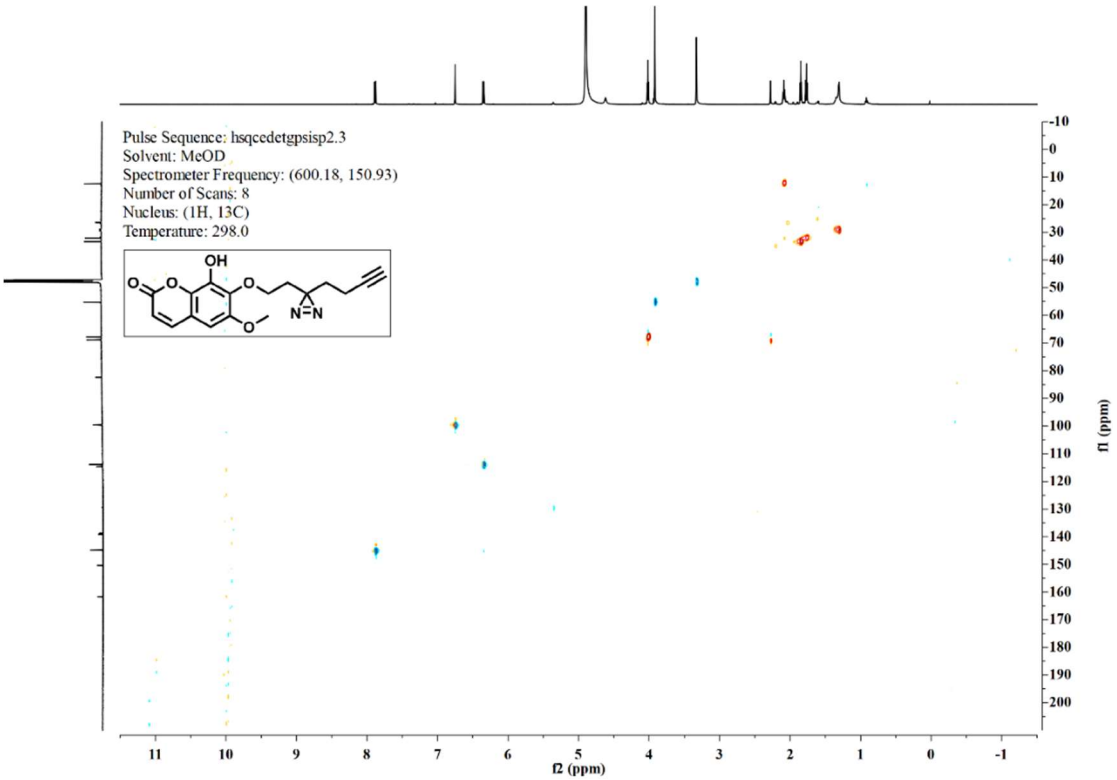

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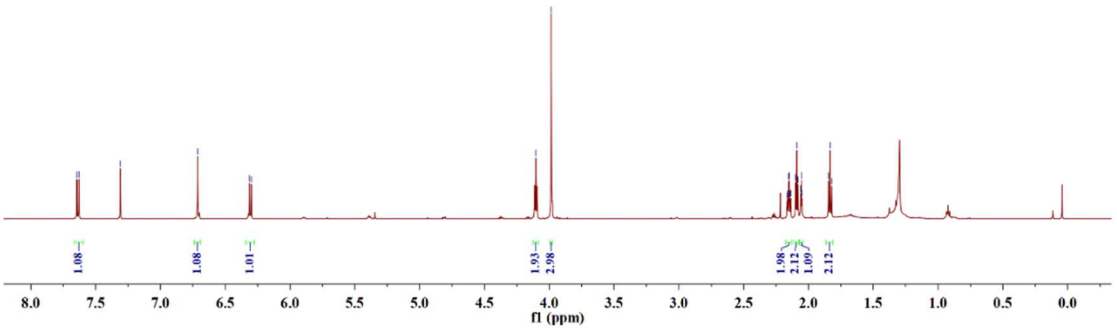

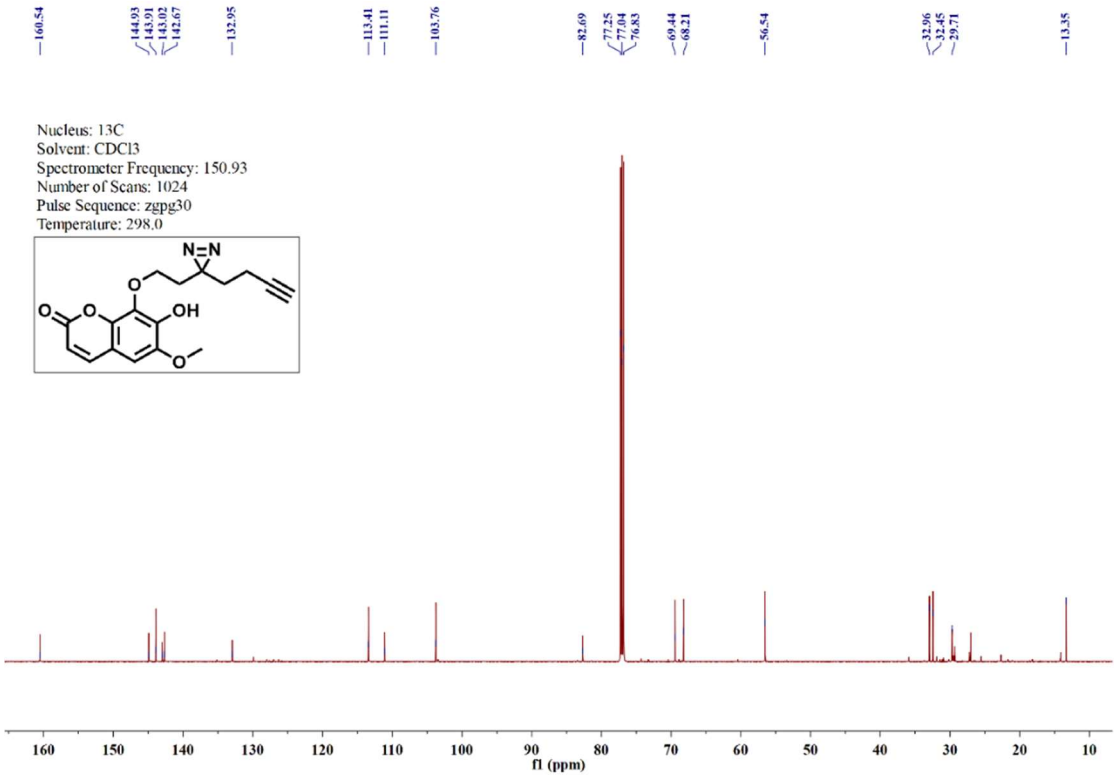
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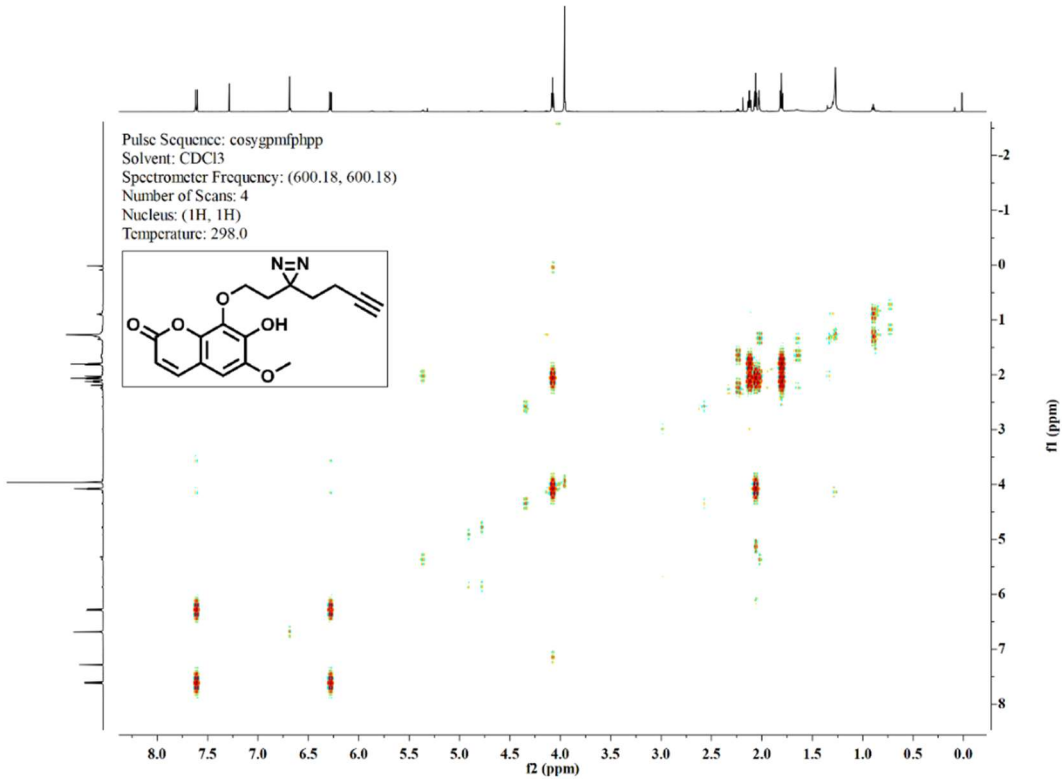
50
40
30
20
10
f1 (ppm)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6.0
5.5
5.0
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4.0
3.5
3.0
2.5
2.0
1.5
1.0
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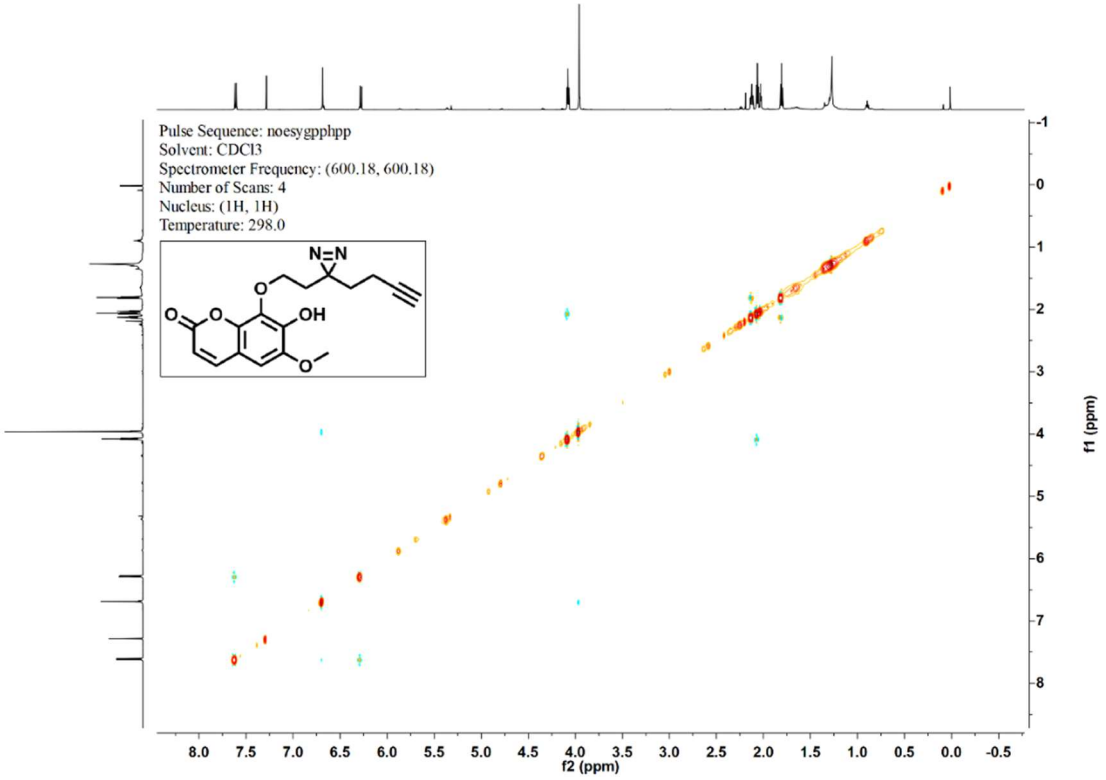
0
1
2
3
4
5
6
7
8
f1 (ppm)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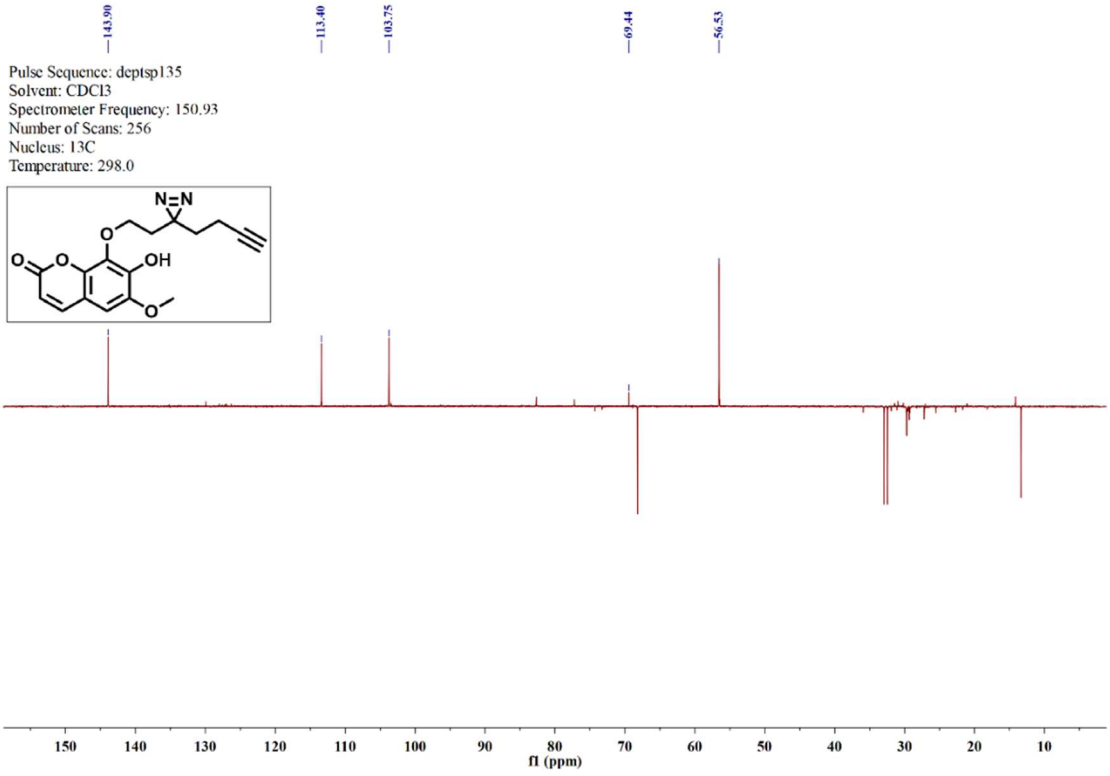
f2 (ppm)
f1 (ppm)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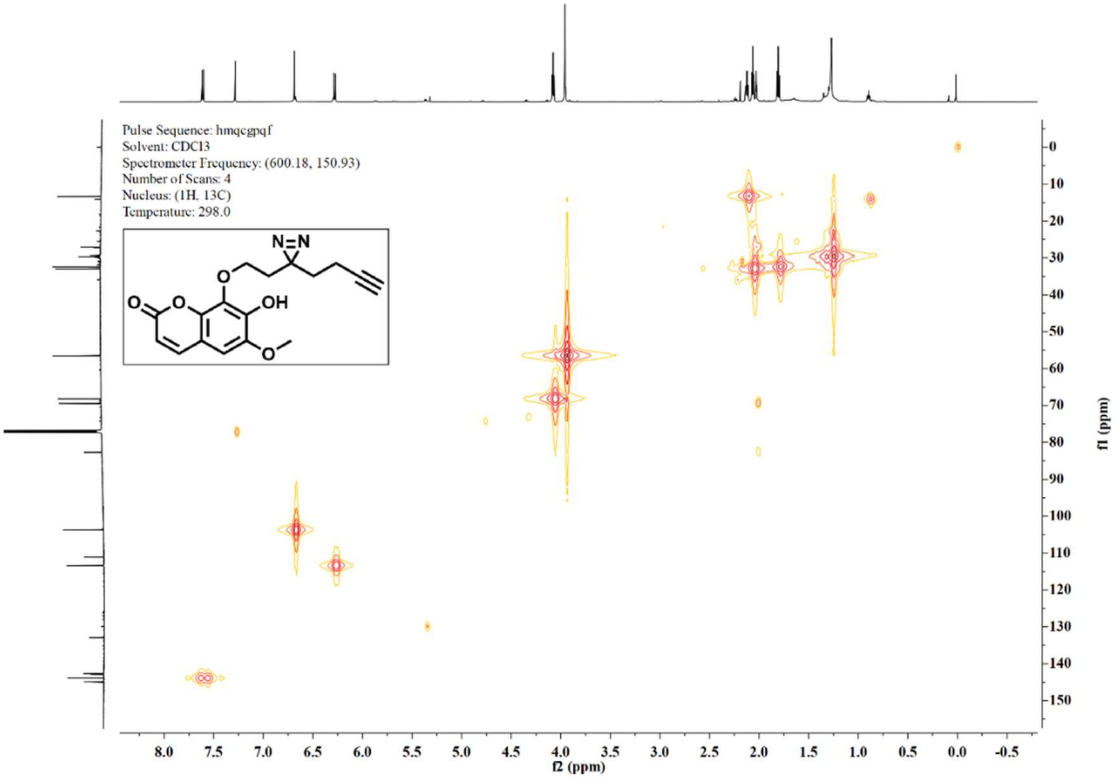
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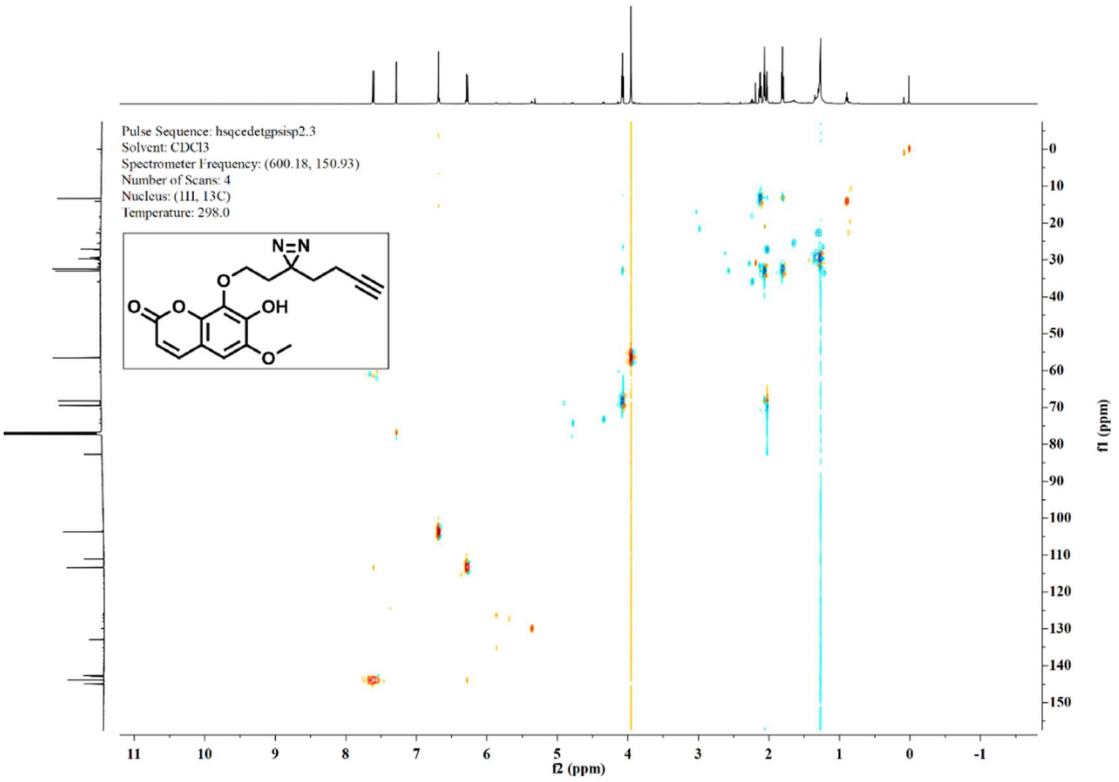
f1 (ppm)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O
OH
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f2 (ppm)
f1 (ppm)