Chemoproteomic Profiling of Bile Acid-interacting Proteins

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Supplementary Information

Supplementary Figures

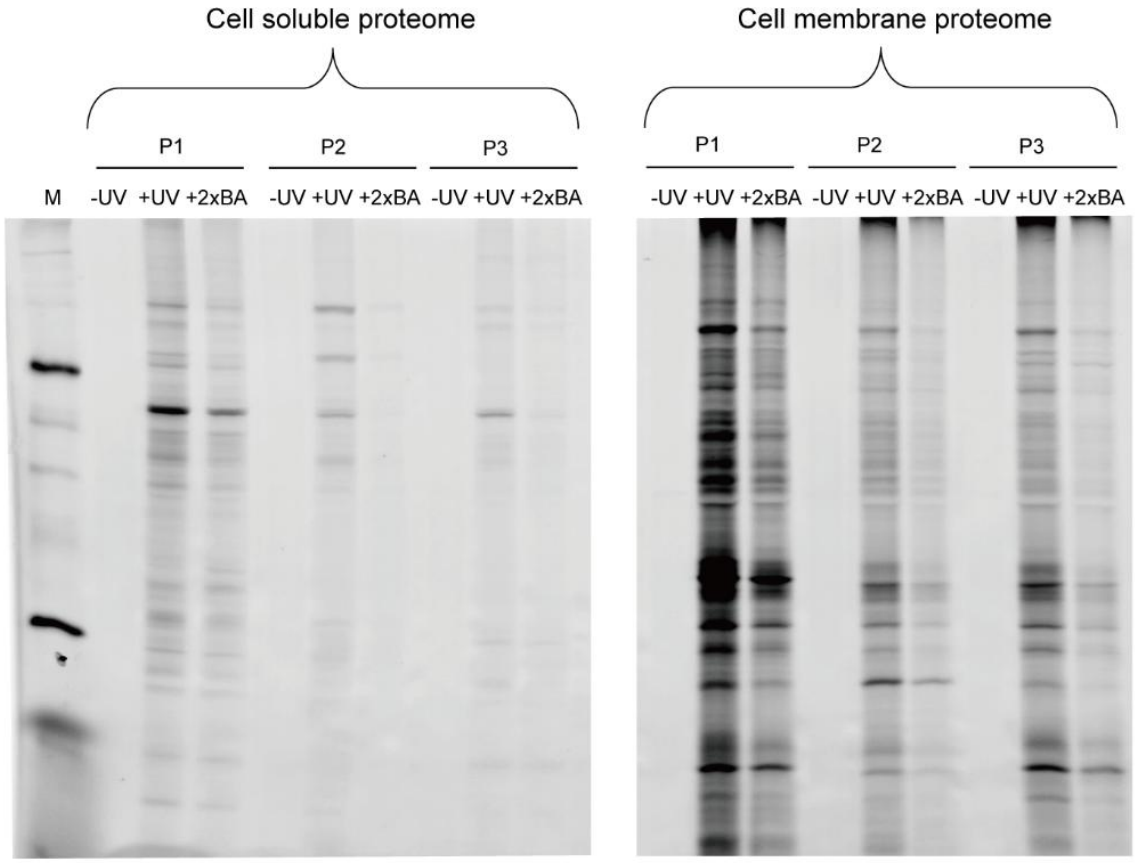


Figure S1. Gel-based ABPP profiling of BA-interacting proteins in living cells by soluble and membrane fractions.

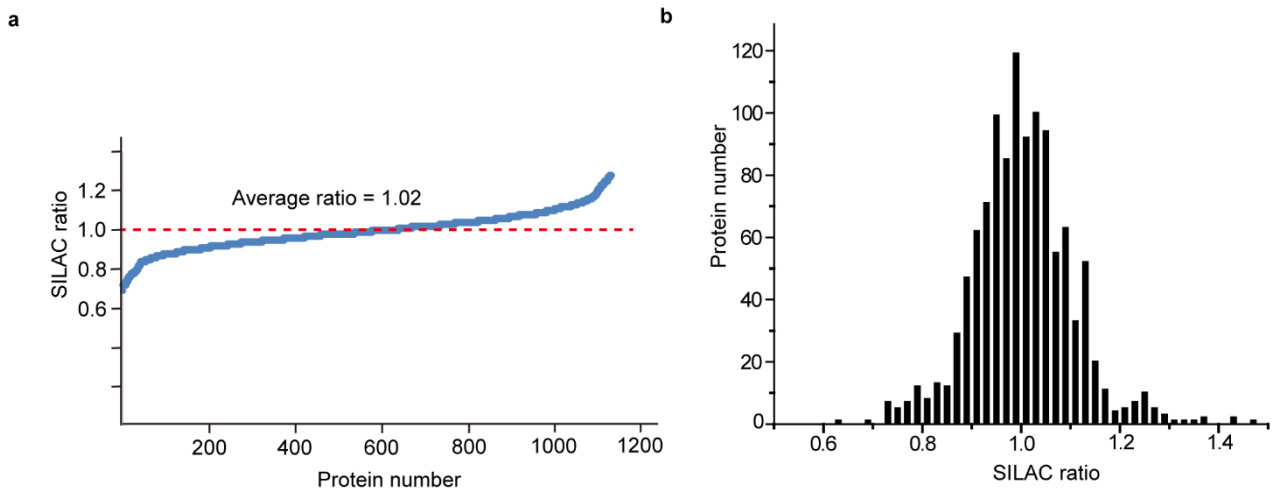


Figure S2. Verification of SILAC HeLa cell line for complete incorporation of heavy amino acids. Light and heavy cells were equally mixed. The mixed proteome was digested by trypsin and analyzed by LC-MS/MS. (a) The plot showed the linear distribution of SILAC ratios with an averaged value of 1.02. (b) The histogram plot showed the normal distribution of SILAC ratios.

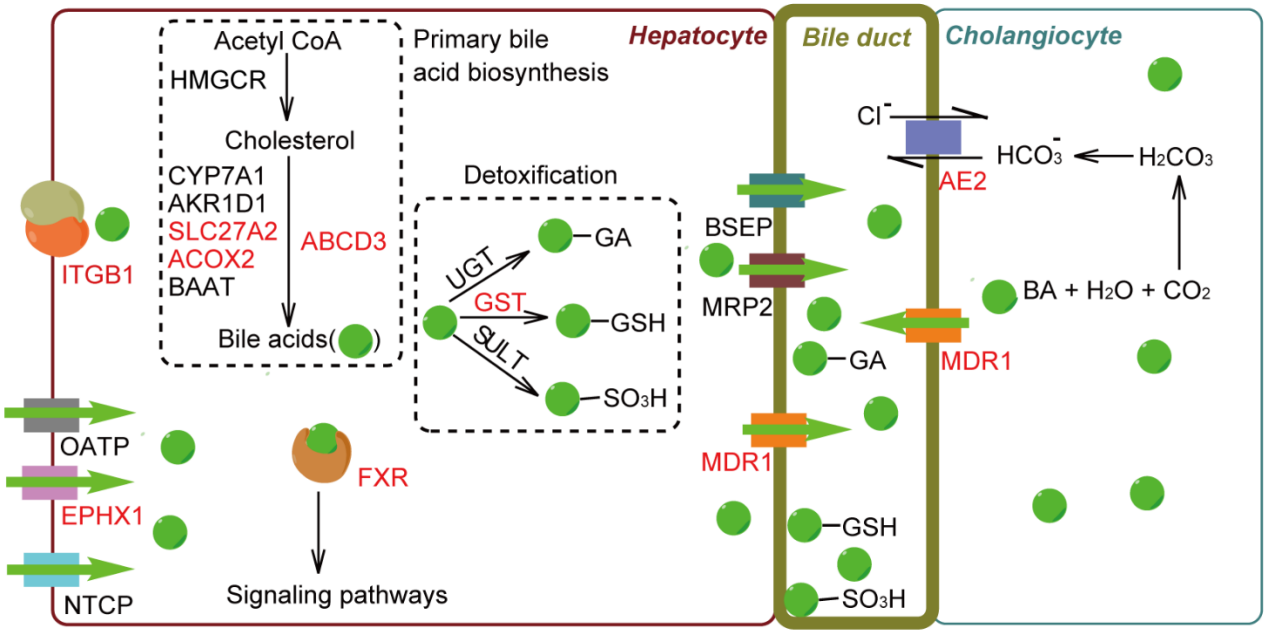


Figure S3. Scheme of the metabolic pathway of bile acid biosynthesis, transportation and detoxification. Bile acid is illustrated as a green ball and proteins that are successfully identified as BA-interacting proteins by our profiling are shown in red fonts.

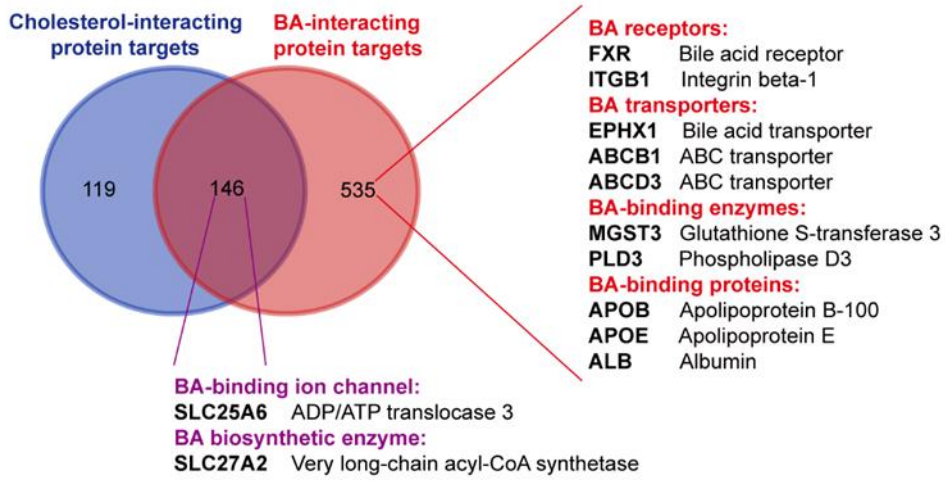


Figure S4. Comparison of BA-interacting protein targets identified in our study and cholesterol-interacting protein targets identified in a previous study[1].

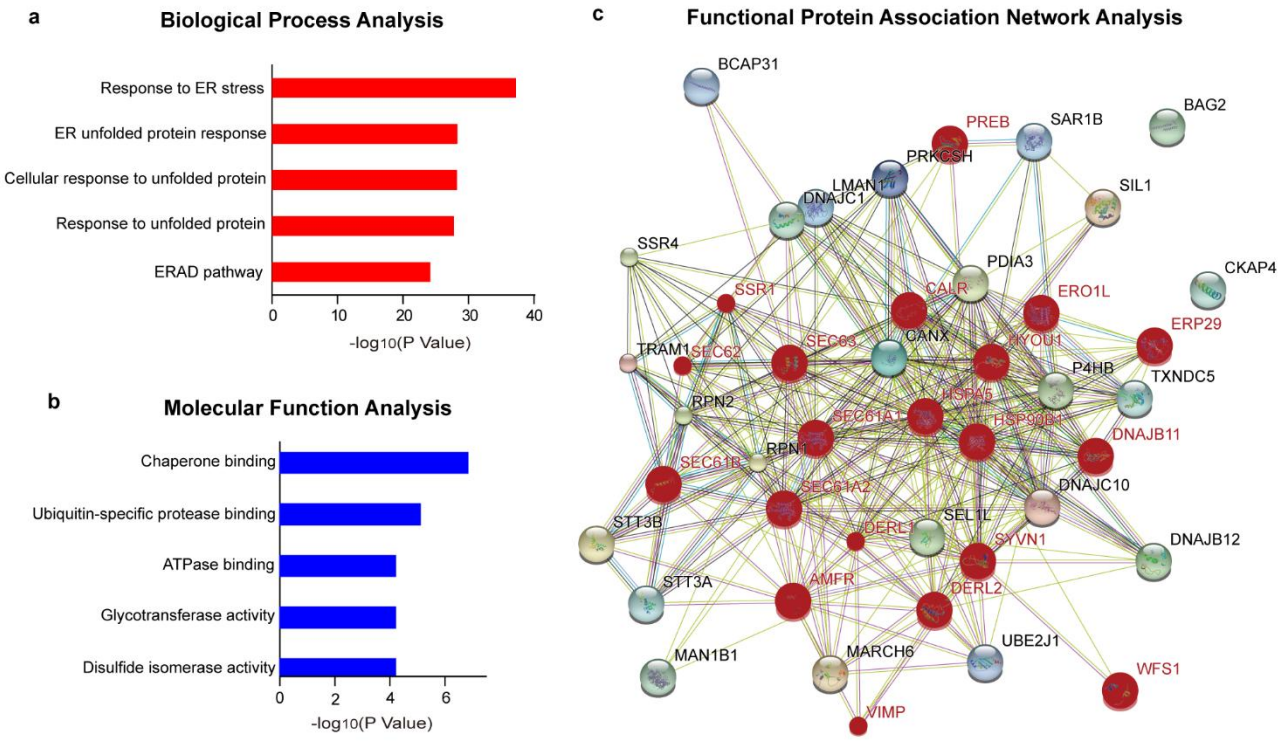


Figure S5. Analysis of BA-interacting proteins enriched in the "protein processing in ER" pathway which was performed using STRING[2]. (a) Biological process analysis of protein targets clustered in the "protein processing in ER" pathway, showing "Response to ER stress" as the top ranked process. (b) Molecular function analysis of protein targets clustered in the "protein processing in ER" pathway, showing "Chaperone binding" as the top ranked function.. (c) Functional protein association network analysis of protein targets clustered in the "protein processing in ER" pathway, with proteins that are involved in ER stress response shown in red.

Table S1. List of BA-interacting proteins identified by SILAC-ABPP. See the accompanying excel spreadsheet.

Table S2. Summary of known BA-interacting proteins identified in our MS data.

P2			R_{uv}	R_{comp}
Receptor	FXR(NR1H4)	Bile acid receptor	12.82	1.7
	ITGB1	Integrin beta-1 receptor	10.72	2.78
Transporter	EPHX1	Bile salts transporter	15	3.7
	ABCB1	ABC transporter	15	3.49
	ABCD3	ABC transporter	11.49	2.57
	SLC25A6	ADP/ATP translocase 3	11.1	2.08
Enzyme	MGST3	Glutathione S-transferase 3	13.4	2.26
	PLD3	Phospholipase D3	15	3.15
	SLC27A2	Very long-chain acyl-CoA synthetase	9.21	2.55
Plasma protein	APOB	Apolipoprotein B-100	15	15
	APOE	Apolipoprotein E	15	15
	ALB	Albumin	15	15

Table S3. Comparison of BA-interacting protein targets identified in our study and cholesterol-interacting protein targets identified in a previous study performed by Cravatt group[1]. See the accompanying excel spreadsheet.

Methods

Cell culture

HeLa cells (gifted from Prof. Chengqi Yi's lab from Peking University and authenticated by China Center for Type Culture Collection (Wuhan, China) as free from mycoplasma contamination) were cultured at 37 ℃ under a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), and 1% penicillin-streptomycin (Thermo Fisher Scientific).

Construction of a HeLa cell line with stable expression of FXR

The full-length cDNA encoding human FXR (gifted from Prof. Jianzhong Xi at Peking University) were subcloned into a pCLHCY vector with C-terminal 6xHis tag, and transfected into HeLa cells using a retrovirus system for stable expression. The expression of 6xHis-tagged FXR was verified by western blot. This cell line was subsequently used for gel-based and MS-based profiling of BA-interacting proteins.

Gel-based profiling of BA-interacting proteins in living cells

For gel-based profiling, HeLa cells were seeded at a density of 5×10^5 cells/well in a six well-plate and grown for 24 h before probe labeling. The DMSO stock of BA-probes (50mM) were diluted with serum-free media by 1000 folds and cells were treated with the probe-containing media at 37 ℃ for 1h under dark conditions. For the BA competition experiment, methyl cholic acid were co-treated with the BA probe at 100 μM or 200 μM respectively. After probe labeling, cells were washed with cold PBS for 3 times and irradiated under UV light (365 nm) or ambient light for 5 min on ice. The cells were scraped off and the pallets were collected by centrifugation at 1,000 g for 3 mins at 4 ℃.

For the whole proteome profiling, cell pellets were re-suspended in PBS with 0.1% Triton X-100 (Sigma-Aldrich) and sonicated on ice. The proteome concentration was determined using the BCA protein assay

(Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) on a microplate reader (Bio-Rad) and normalized to 2 mg/mL. Each sample was conjugated with a fluorescent reporter by "click chemistry" in dark at room temperature for 1 h (100 μM rhodamine-azide, 100 μM TBTA, 1 mM $CuSO_4$ and 1 mM TCEP). The samples were then separated by 10% SDS-PAGE and the in-gel fluorescence was analyzed by a Biorad ChemiDoc imaging system. For the gel-based profiling of soluble and membrane proteomes, respectively, the cell pellets were re-suspended in PBS, sonicated on ice, and separated into soluble and membrane fractions by ultracentrifugation at 20,000 g for 45 mins at 4 ℃.

Preparation of SILAC cells

SILAC DMEM was supplemented with 10% dialyzed SILAC FBS and 1% penicillin-streptomycin, and 100 μg/mL of regular L-arginine-HCl and L-lysine-HCl (Sigma-Aldrich) or [$^{13}C_6$,$^{15}N_4$]L-arginine-HCl and [$^{13}C_6$,$^{15}N_2$]L-lysine-HCl (Cambridge Isotope Laboratory) were added to make the light or heavy media, respectively.[3] FXR-overexpressing HeLa cells were cultured in light or heavy media under identical conditions and passaged at least nine times to verify full incorporation of the isotopically labeled amino acids, 50 μg of lysates of light and heavy cells were mixed equally and denatured in 6 M urea/PBS. After reduction with 10 mM DTT at 65 ℃ for 15 mins and alkylation with 20 mM iodoacetamide at 35 ℃ for 30 mins in dark, the sample was diluted with PBS to 2 M urea/PBS and subjected to trypsin digestion at 37 ℃ with agitation overnight (2 μg of trypsin with 1 mM of calcium chloride). The digested peptide sample was acidified with 5% formic acid and analyzed by LC-MS/MS. The distribution of SILAC ratios was checked to be centered around 1.0.

Profiling of BA-interacting proteins by SILAC-ABPP

The SILAC-ABPP experiments were performed based on protocols adapted from a previous report.[4] In the UV-dependent profiling experiment, light HeLa cells were treated with 50 μM BA probe for 1h and irradiated with UV light for 5 mins, while the heavy cells were labeled with the same condition but with no UV crosslinking conducted. In the "BA competition" experiment, light cells were treated with 50 μM

BA probe and DMSO, while heavy cells were co-treated with 50 μM BA probe and 100 μM methyl cholic acid. After probe labeling, the light and heavy cells were collected separately, lysed by sonication in PBS with 0.1% Triton X-100, and the proteome concentrations were measured. The light and heavy lysates (1mg each) were mixed in a 1:1 ratio and the mixed lysates were conjugated with a biotin enrichment tag by click chemistry (100 μM biotin-azide, 100 μM TBTA, 1 mM $CuSO_4$ and 1 mM TCEP for 1 h in dark at room temperature). The precipitated proteomes were collected by centrifugation at 10,000 g for 5 min at 4 ℃, washed with cold methanol for three times and solubilized with 1.2% SDS/ PBS. The solubilized proteins were incubated with streptavidin beads (100 μl of slurry, Thermo Fisher Scientific) in 0.2% SDS/PBS at room temperature for 3 h with rotation. After washing the beads with 3x5 mL of PBS and H_2O sequentially, the enriched proteins (on beads) were denatured in 6 M urea/PBS, reduced with 10 mM DTT at 65 ℃ for 15 mins and alkylated with 20 mM IAA at 35 ℃ for 30 mins in dark. The beads were transferred into a premixed solution of 200 μl of 2 M urea/PBS, 2 μl of 100 mM calcium chloride in water and 4 μl of trypsin (20 μg reconstituted in 40 μl of the trypsin buffer, Promega) and incubated at 37 °C with agitation overnight. The digested mixture was collected through a Bio-spin filter (Bio-Rad), acidified with 5% formic acid and subject to LC-MS/MS analysis.

LC-MS/MS analysis

The SILAC peptides samples were fractionated with a fast sequencing (Fast-seq) workflow using dual reverse phase (RP) high performance liquid chromatography (HPLC).[5] The first dimension of high pH RP chromatography was performed on an Agilent 1260 infinity quaternary LC by using a durashell RP column (5 μm, 150 Å, 250 mm × 4.6 mm i.d., Agela). Mobile phases A (2% acetonitrile, adjusted pH to 10.0 using NH3·H2O) and B (98% acetonitrile, adjusted pH to 10.0 using NH3·H2O) were used to develop a gradient. The solvent gradient was set as follows: 5–8% B, 2 min; 8–18% B, 11 min; 18–32% B, 9 min; 32–95% B, 1 min; 95% B, 1 min; 95–5% B, 2 min. The tryptic peptides were separated at an eluent flow rate of 1.5 ml/min and monitored by UV at 214 nm. The temperature of column oven was set as 45 ℃. Eluent was collected every minute. The samples were dried under vacuum and reconstituted in

15 μl of 0.1% (v/v) FA, 2% (v/v) acetonitrile in water. The second dimension of low pH RP chromatography was performed on an Ultimate 3000 LC system coupled with a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) for MS/MS measurement. Each fraction from the first dimension was separated on a C18 column (75 μm inner-diameter, 360 μm outer-diameter × 10 cm, 3 μm C18). Mobile phase A consisted of 0.1% formic acid in water solution, and mobile phase B consisted of 0.1% formic acid in acetonitrile solution; according to the hydrophobicity of each fraction, an adjusted linear gradient was applied with a flow rate of 350 nL/min. The MS conditions are as the followings: Under the positive-ion mode, full-scan mass spectra were acquired over the m/z range from 400 to 1800 using the Orbitrap mass analyzer with a resolution of 70000. MS/MS fragmentation was performed in the data-dependent mode, in which the 18 most intense ions were selected from each full-scan mass spectrum for fragmentation by high-energy collision induced dissociation (HCD). MS/MS spectra were acquired with a resolution of 17500 using the Orbitrap analyzer. Some other parameters in the centroid format: isolation window, 2.0 m/z units; default charge, 2+; normalized collision energy, 28%; maximum IT, 50 ms; dynamic exclusion, 20.0 s.

Protein identification and quantification

LC-MS/MS data was analyzed by ProLuCID with static modification of cysteine (+57.0215 Da) and variable oxidation of methionine (+15.9949 Da). The precursor ions mass tolerance was set as 50 p.p.m. The data was searched using a human non-redundant (gene-centric) FASTA-formatted database with reverse concatenation. Each raw data was additionally searched with a heavy parameter file, in which static modification on lysine (8.0142 Da) and arginine (10.0082 Da) were set. The searching results were filtered by DTASelect with restriction to fully tryptic peptides and a defined spectrum false positive rate of 1%. SILAC ratios were quantified by an in-house CIMAGE software as described before. The MS1 ion chromatograms (+/−10 p.p.m.) from light and heavy peptide masses were extracted when one peptide ion was selected for MS/MS fragmentation and identified. The Light/Heavy peptide ratios were calculated according to the peak areas of the light and heavy chromatographs with some computational filters,

including co-elution correlation score filter and envelope correlation score filter. Peak pairs that have poor co-elution scores (R^2 correlation value less than 0.7), or poor envelope correlation score (R^2 correlation value less than 0.7) are eliminated from consideration. For example, isotopic peptides that have the incorrect monoisotopic mass or charge, or whose isotopic envelopes are not well correlated with the predicted envelope are excluded. After ratios for unique peptide entries are calculated from each LC fractions, the median ratio of peptides from a specific protein was reported as the final protein SILAC ratio. In the"singleton" cases where only light peaks exist due to UV-dependent labeling or complete BA competition, the singleton MS1 chromatographic peaks were aligned with the corresponding peptide sequence to validate the charge state and monoisotopic mass using the envelope correlation score filter (R^2 correlation value $\geq$ 0.7). Then the candidate peak was cross-checked to ensure there was no other peaks co-eluting around the same retention time window. Only after all these criteria are met, the "singleton" peptide was assigned with an artificial threshold ratio of 15.

Analysis of BA-interacting proteins in human proteome

Gene ontology and KEGG pathway analyses were performed using DAVID bioinformatics resources (https://david.ncifcrf.gov/).[6,7] The analysis of functional protein association network was performed using STRING (http://string-db.org/).[2]

Validation of BA-Protein interactions

Full-length cDNAs encoding each protein of interest (gifted from Prof. Jianzhong Xi at Peking University) were sub-cloned into a pCLHCY vector with C-terminal 6xHis-tag, and transiently overexpressed in Hela cells using Lipo2000 (Thermo Fisher) according to vendors' instruction. After 48 hours, cells were treated *in situ* with 50 μM BA probes, with or without 100 μM methyl cholic acid for 1h followed by extensive washing with cold PBS and UV irradiation (365nm, 5mins) on ice. The cells were collected, lysed and adjusted to 2 mg/mL. A small aliquot of each sample was run on SDS-PAGE and equal expression of the protein of interest among different samples was verified by western blot using anti-

6xHis antibody. The remaining cell lysates were conjugated to an azide-biotin tag by CuAAC and enriched with streptavidin for 3h. After enrichment, the beads were washed with 3x5 ml PBS and 3x5 ml water, and boiled with gel loading buffer to elute off the biotinylated proteins. The samples were separated on SDS-PAGE and immunoblotted using anti-6xHis antibody.

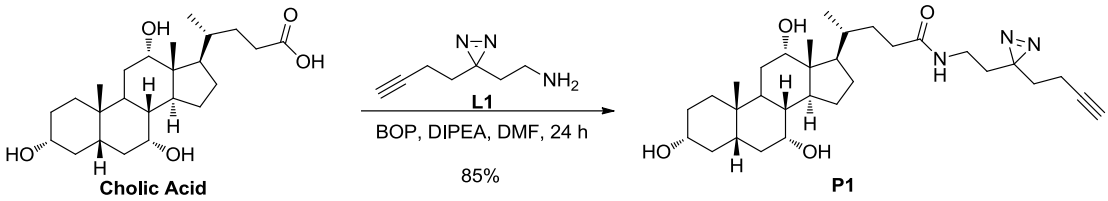
Preparation of chemical probes

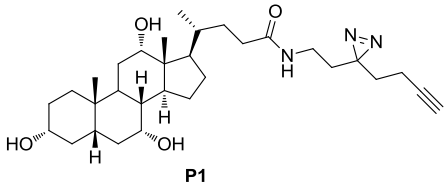
General information

Nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR) were recorded with Varian Gemini (400 MHz, ^{1}H NMR at 400 MHz, ^{13}C NMR at 100 MHz) at ambient temperature. For $CDCl_3$ solutions the chemical shifts are reported as parts per million (ppm) referenced to residual protium or carbon of the solvents; $CHCl_3$ δ H (7.26 ppm) and $CDCl_3$ δ C (77.00 ppm). Coupling constants are reported in Hertz (Hz). Data for ^{1}H NMR are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration and coupling constants. High- resolution mass spectra were obtained at Peking University Mass Spectrometry Laboratory using a Bruker APEX Flash chromatography and were reported as m/z (relative intensity). Accurate masses were reported for the molecular ion $[M+H]^+$ or $[M+Na]^+$. The samples were analyzed by HPLC/MS on a Waters Auto Purification LC/MS system (3100 Mass Detector, 2545 Binary Gradient Module, 2767 Sample Manager, and 2998 Photodiode Array (PDA) Detector). The system was equipped with a Waters C18 5μm SunFire separation column (150*4.6 mm), equilibrated with HPLC grade water (solvent A) and HPLC grade acetonitrile (solvent B) with a flow rate of 0.3 mL/min. Flash chromatography was performed using 200-400 mesh silica gel. Yields refer to chromatographically and spectroscopically pure materials, unless otherwise stated.

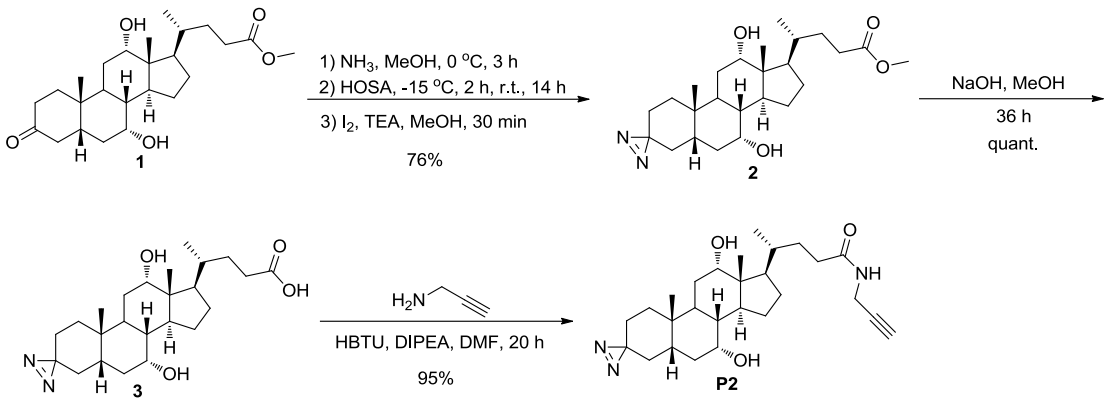
All chemical reagents were used as supplied by Sigma-Aldrich, J&K and Alfa Aesar Chemicals. DMF was distilled from calcium hydride prior to use. Minimalist linkers **L1** and **L2**,[8] compound **1**,[9] compound **4**[10] were prepared according to the literature reported procedures. All reactions were carried out in oven-dried glassware under an argon atmosphere unless otherwise noted.

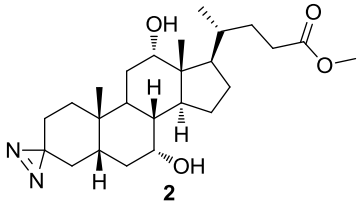
Synthetic procedures



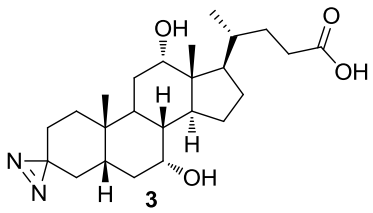


P1: To a solution of cholic acid (137 mg, 0.34 mmol) and compound **L1** (48.3 mg, 0.35 mmol) in anhydrous DMF (2.2 mL) was added BOP (186 mg, 0.42 mmol). Anhydrous DIPEA (167 μL, 1.01 mmol) was added subsequently. The reaction mixture was stirred at room temperature for 24 h. After that, H_2O (5 mL) was added and the mixture was extracted with ethyl acetate (10 mL×3). The combined organic layers were washed with brine (5 mL). After dried over Na_2SO_4, the solution was concentrated *in vacuo* and purified by flash chromatography (silica gel, 4~9% methanol in methylene chloride) to afford **P1** as a white solid (150 mg, 85%): ^{1}H NMR (400 MHz $CDCl_3$): δ 5.90 (s, 1H), 3.98 (s, 1H), 3.85 (d, *J* = 2.4 Hz, 1H), 3.46 (m, 1H), 3.10 (q, *J* = 6.0 Hz, 2H), 2.25-1.10 (m, 34H), 0.99 (d, *J* = 6.0 Hz, 3H), 0.89 (s, 3H), 0.69 (s, 3H); ^{13}C NMR (100 MHz $CDCl_3$): δ 174.19, 82.71, 77.20, 73.07, 71.85, 69.36, 68.44, 46.52, 46.36, 41.61, 41.45, 39.53, 39.38, 35.33, 34.73, 34.30, 33.08, 32.49, 32.09, 31.59, 30.41, 29.63, 28.13, 27.52, 26.86, 26.31, 23.24, 22.41, 17.44, 13.21, 12.43. HRMS (ESI): $[M+H]^+$ calculated for $C_{31}H_{50}N_3O_4$: 528.3796, found: 528.3798.

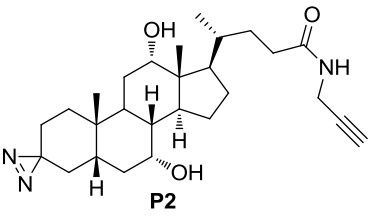




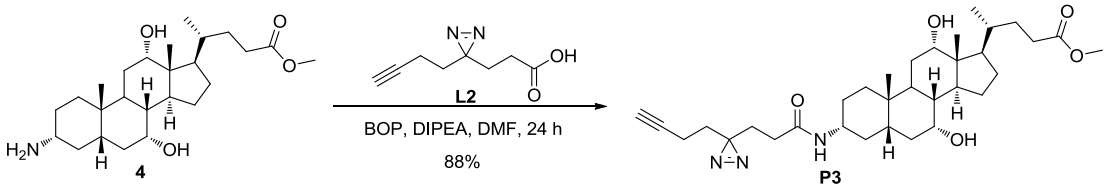
Compound **2**: Compound **1** (993 mg, 2.36 mmol) was dissolved in a 7 M solution of NH_3 in MeOH (12.5 mL, 7 M). The reaction was stirred at -20 ^oC for 1 h. The temperature then was raised to room temperature and the reaction mixture was stirred for another 2 h. After that, the temperature was lowered to -15 ^oC and hydroxylamine-O-sulfonic acid (65 mg × 5 times) was added every 30 mins. After completing the addition, the mixture was stirred at room temperature for 14 h. The solvent and NH_3 were removed under reduced pressure, and the residue was dissolved in MeOH (12.5 mL). Then TEA (0.56 mL, 4.01 mmol) was added at 0 ^oC and subsequently I_2 (1080 mg, 4.25 mmol) was added portion-wise. The mixture was stirred at room temperature for 30 min. Sat. $Na_2S_2O_3$ solution (8 mL) was added to reduce the excess of I_2. Most MeOH was removed under reduced pressure and the mixture was extracted with ethyl acetate (30 mL ×3). The combined organic layers were washed with brine (10 mL). After dried over Na_2SO_4, the solution was concentrated in vacuo and purified by flash chromatography (silica gel, EtOAc/in petrol ether/DCM = 15:80:5) to afford the desired product **3** as a white solid (776 mg, 76%): ^1H NMR (400 MHz $CDCl_3$): δ 4.01 (t, *J* = 2.8 Hz, 1H), 3.86 (m, 1H), 3.67 (s, 3H), 2.93 (t, *J* = 13.6 Hz, 1H), 2.38 (m, 1H), 2.25 (m, 2H), 1.93 (m, 4H), 1.77 (m, 4H), 1.59 (m, 6H), 1.38 (m, 5H), 1.17 (m, 1H), 1.00 (s, 3H), 0.99 (d, *J* = 6.4 Hz, 3H), 0.72 (s, 3H), 0.23 (m, 2H); ^{13}C NMR (100 MHz $CDCl_3$): δ 174.74, 72.94, 68.27, 51.48, 47.23, 46.55, 41.74, 40.62, 39.41, 35.19, 35.04, 34.75, 34.47, 33.85, 31.06, 30.83, 29.12, 28.43, 27.40, 26.44, 23.18, 22.42, 17.32, 12.50, -0.05. HRMS (ESI): $[M+Na]^+$ calculated for $C_{25}H_{40}N_2NaO_4$: 455.2880, found: 455.2884.

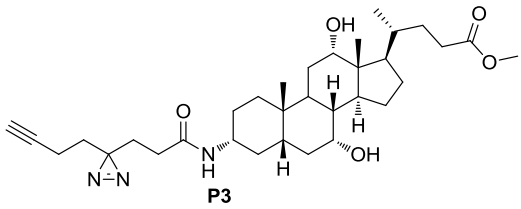


Compound **3**: A solution of compound **2** (405 mg, 0.94) in MeOH (10.5 mL) was added NaOH (375 mg, 9.4 mmol). The reaction mixture was stirred at room temperature for 36 h. After that, the mixture was acidified with HCl (1 M) and extracted with ethyl acetate (30 mL×3). The combined organic layers were dried over Na_2SO_4, the solvent was removed in vacuo to afford the desired product **3** as a white solid without further purification (391 mg, quant.).



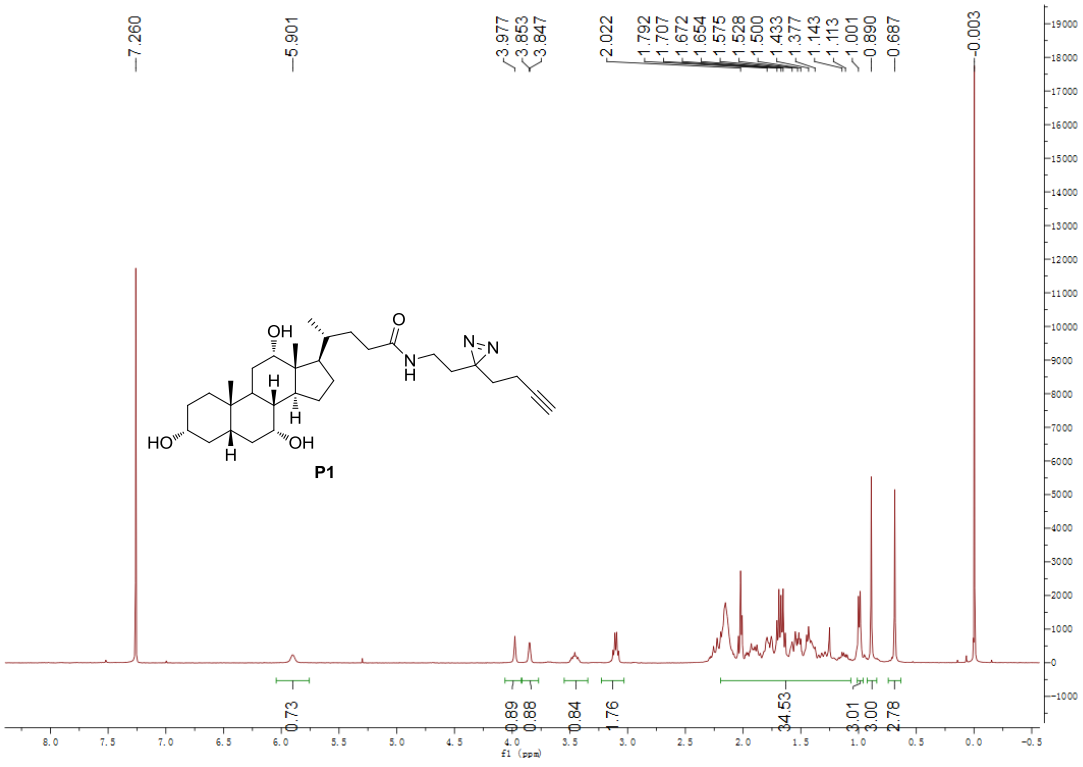
P2: A solution of compound **3** (391 mg, 0.94 mmol) in anhydrous DMF (11 mL) was added HBTU (535 mg, 1.41 mmol). 2-propynylamine (180 μL, 2.82 mmol) and anhydrous DIPEA (0.47 mL, 2.82 mmol) were added subsequently. The reaction mixture was stirred at room temperature for 20 h. After that, H_2O (8 mL) was added and the mixture was extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with brine (10 mL). After dried over Na_2SO_4, the solution was concentrated in vacuo and purified by flash chromatography (silica gel, 2~3% methanol in methylene chloride) to afford **P2** as a white solid (405 mg, 95%): ^{1}H NMR (400 MHz $CDCl_3$): δ 5.65 (s, 1H), 4.05 (q, *J* = 2.4 Hz, 2H), 4.01 (s, 1H), 3.86 (s, 1H), 2.93 (t, *J* = 13.6 Hz, 1H), 2.31-2.09 (m, 4H), 1.98-1.87 (m, 4H), 1.85-1.59 (m, 8H), 1.45-1.12 (m, 8H), 1.00 (s, 3H), 0.99 (d, *J* = 6.4 Hz, 3H), 0.72 (s, 3H), 0.23 (m, 2H); ^{13}C NMR (100 MHz $CDCl_3$): δ 174.14, 79.62, 73.12, 71.25, 68.20, 50.42, 46.49, 46.39, 41.65, 40.56, 39.12, 35.34, 35.00, 34.67, 34.34, 33.87, 32.56, 31.35, 29.15, 28.99, 28.21, 27.51, 26.36, 26.19, 23.20, 22.27, 17.23, 12.35. HRMS (ESI): $[M+H]^+$ calculated for $C_{27}H_{42}N_3O_3$: 456.3220, found: 456.3216.

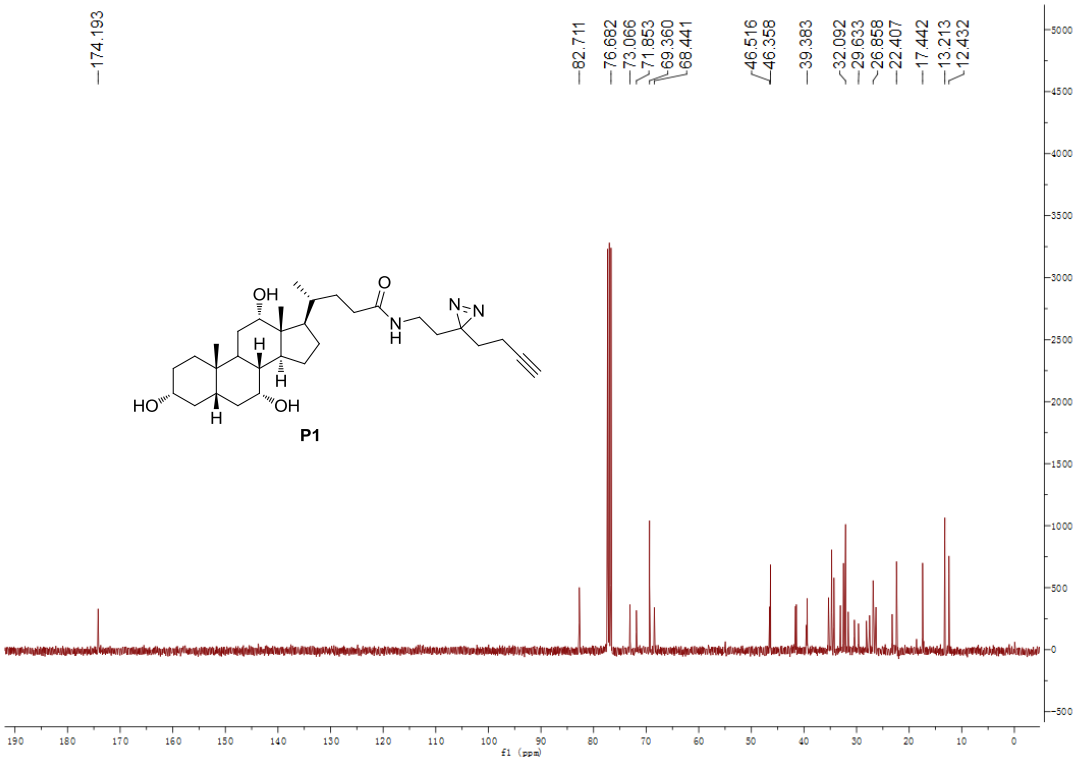


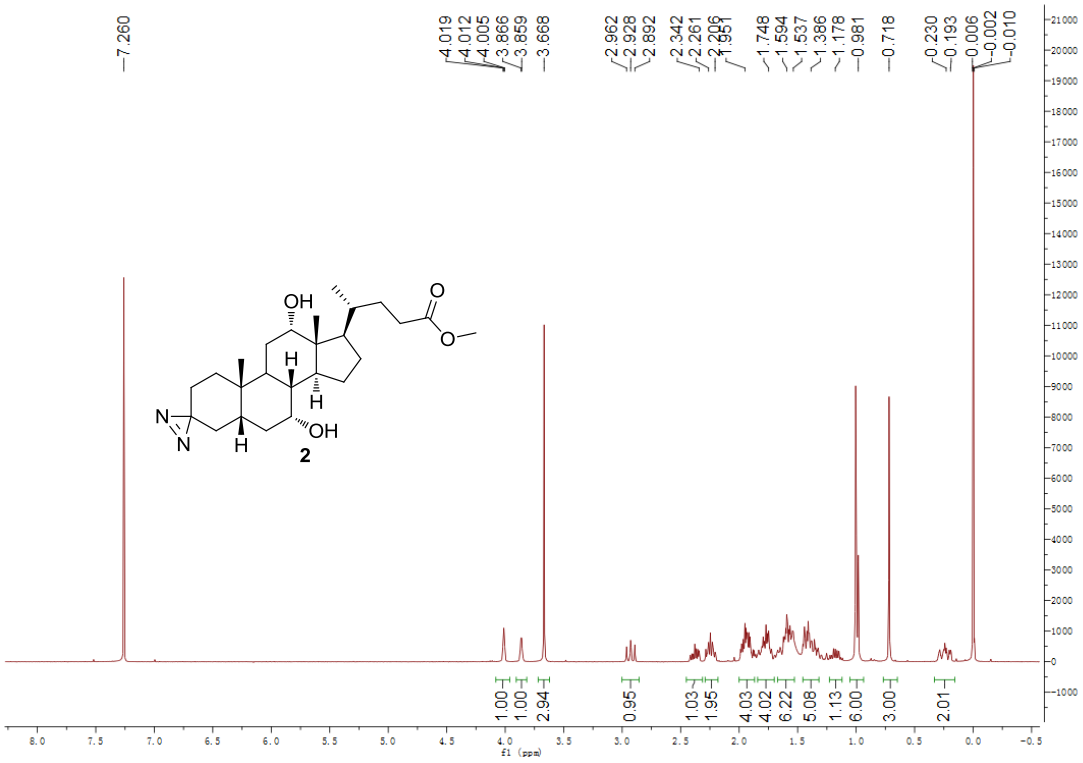


P3: To a solution of compound **4** (222 mg, 0.53 mmol) and compound **7** (105 mg, 0.63 mmol) in anhydrous DMF (4 mL) was added BOP (280 mg, 0.63 mmol). Anhydrous DIPEA (260 μL, 1.58 mmol) was added subsequently. The reaction mixture was stirred at room temperature for 24 h. After that, H_2O (8 mL) was added and the mixture was extracted with ethyl acetate (20 mL×3). The combined organic layers were washed with brine (8 mL). After dried over Na_2SO_4, the solution was concentrated in vacuo and purified by flash chromatography (silica gel, 2~3% methanol in methylene chloride) to afford **P3** as a white solid (264 mg, 88%):^{1}H NMR (400 MHz $CDCl_3$): δ 5.35 (d, *J* = 7.6 Hz, 1H), 3.99 (s, 1H), 3.85 (s, 1H), 3.67 (m, 5H), 3.18 (m, 1H), 2.40-1.10 (m, 33H), 0.98 (d, *J* = 6.0 Hz, 3H), 0.88 (s, 3H), 0.70 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.69, 170.29, 82.73, 72.83, 69.23, 68.24, 55.55, 51.45, 49.53, 47.07, 46.40, 41.87, 41.67, 39.36, 36.30, 35.61, 35.08, 34.53, 34.41, 32.19, 30.96, 30.77, 30.47, 28.51, 28.20, 27.88, 27.61, 27.37, 26.54, 23.05, 22.58, 17.24, 13.19, 12.43. HRMS (ESI): $[M+H]^+$ calculated for $C_{33}H_{52}N_3O_5$: 570.3901, found: 570.3908.

^{1}H and ^{13}C NMR spectra



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3.668
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2.892
2.342
2.261
2.206
1.951
1.748
1.594
1.537
1.386
1.178
0.981
0.718
0.230
0.193
0.006
0.002
0.010
1.00
1.00
2.94
0.95
1.03
1.95
4.03
4.02
6.22
5.08
1.13
6.00
3.00
2.01
f1 (ppm)
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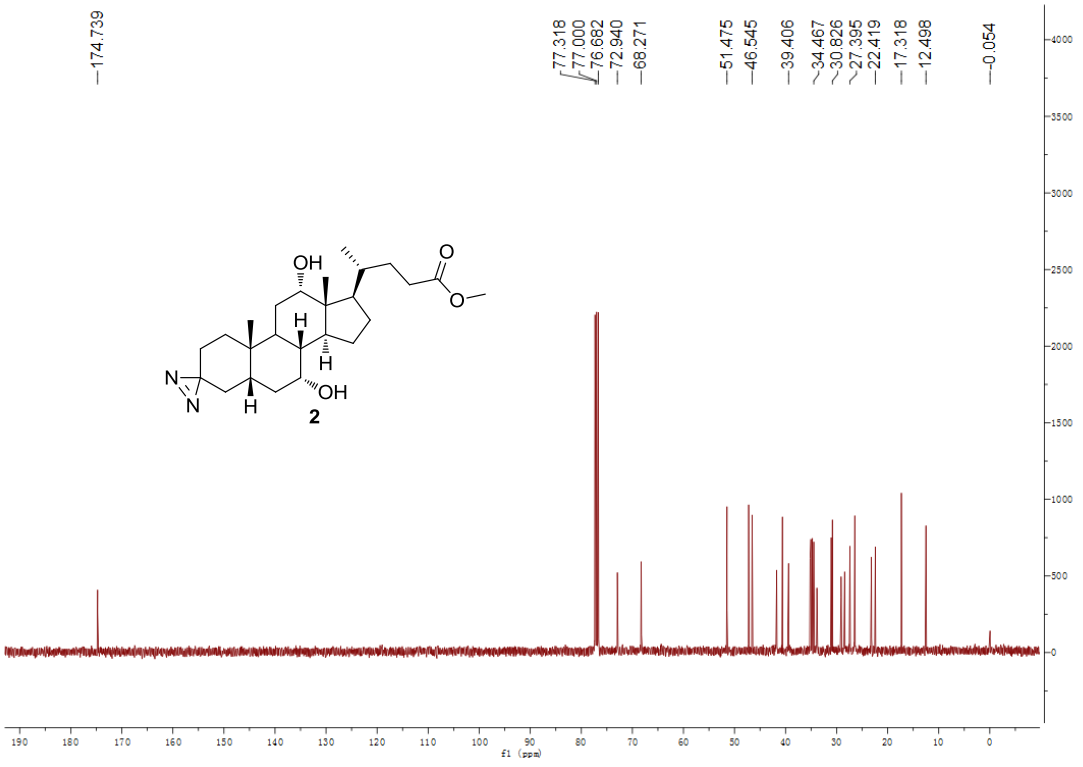
O
O
H
H
N
N
H
OH
2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76.682
72.940
68.271
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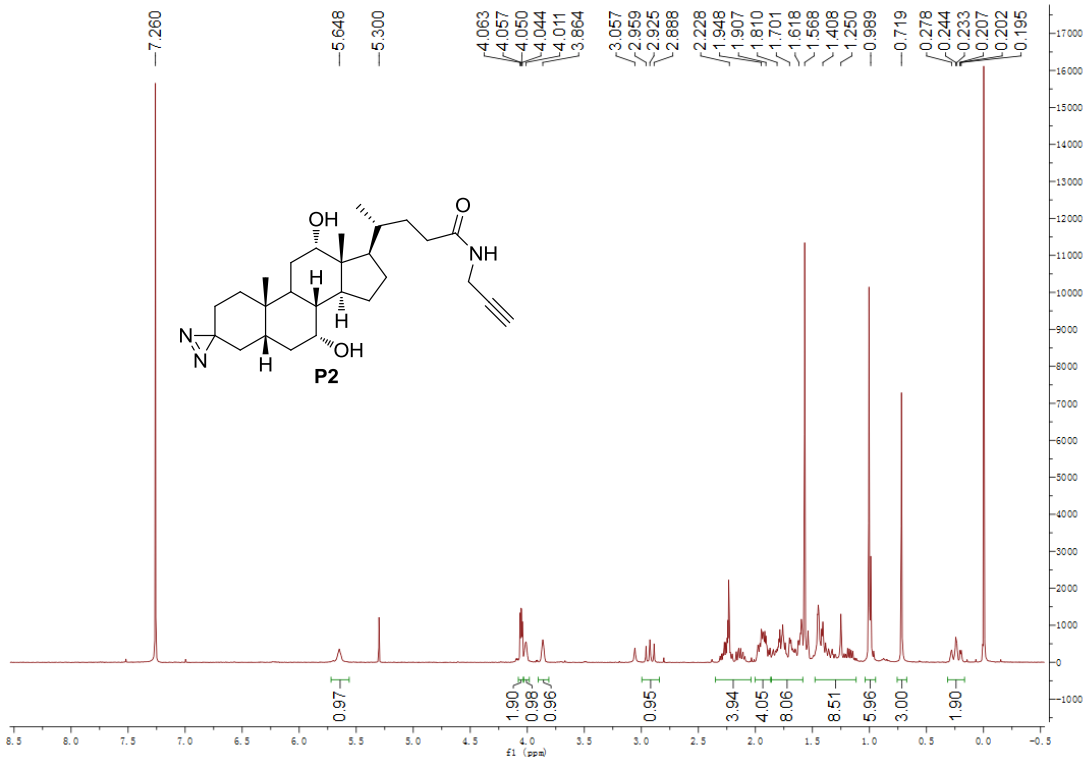
27.395
22.419
17.318
12.498
0.054
f1 (ppm)
OH
O
O
H
H
N
N
H
OH
2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4.050
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3.057
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2.888
2.228
1.948
1.907
1.810
1.701
1.618
1.568
1.408
1.250
0.989
0.719
0.278
0.244
0.233
0.207
0.202
0.195
0.97
1.90
0.98
0.96
0.95
3.94
4.05
8.06
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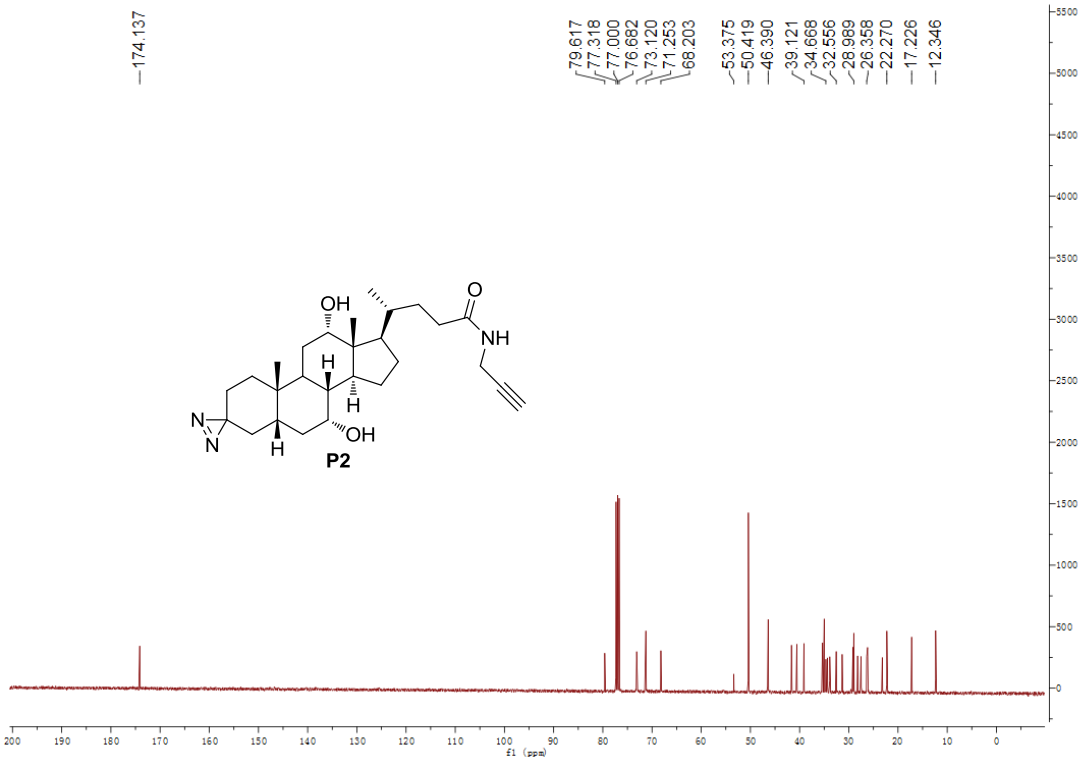
3.00
1.90
OH
O
NH
H
H
N
N
H
OH
P2
f1 (ppm)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34.668
32.556
28.989
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17.226
12.346
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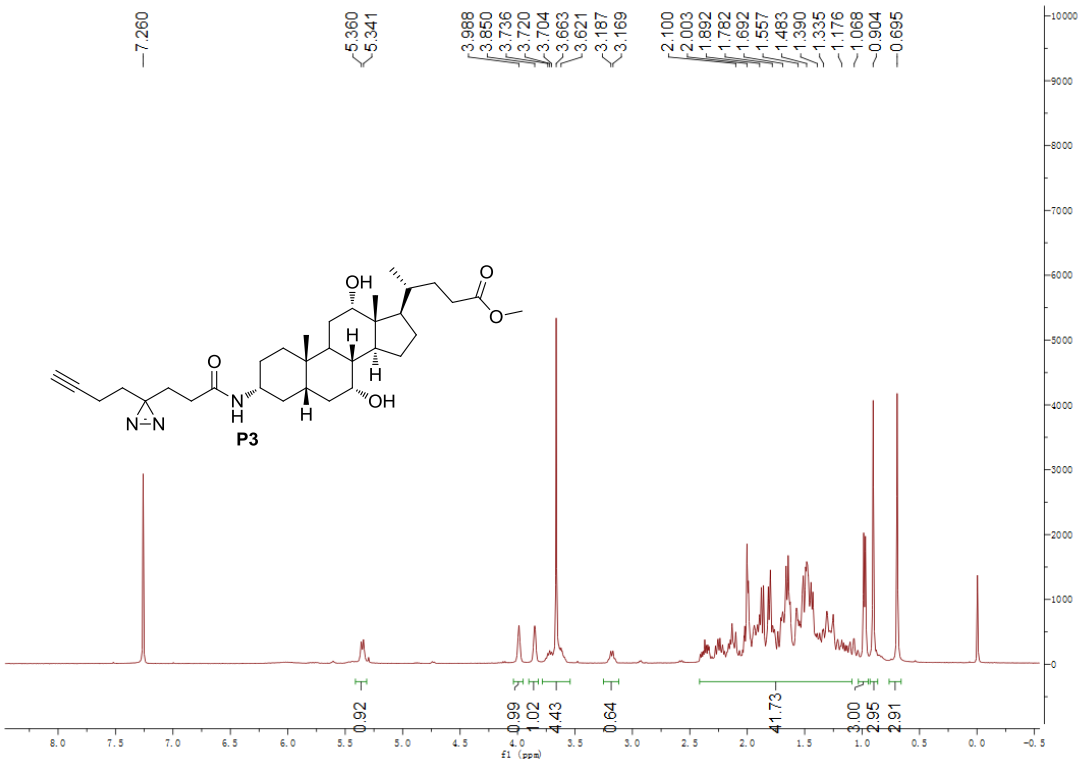
O
NH
H
H
N
N
H
OH
P2
f1 (ppm)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1.557
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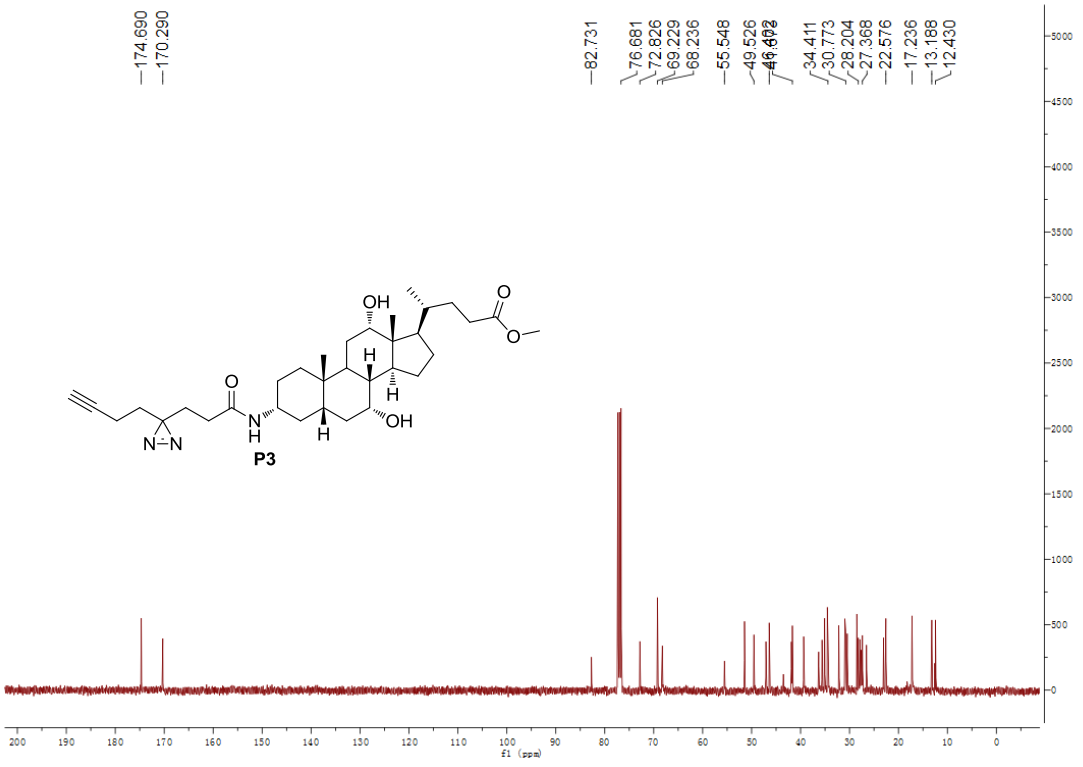
f1 (ppm)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22.576
17.236
13.188
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OH
O
N
H
N
N
H
P3
f1 (ppm)

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